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**Psychopathologies characterised by social deficits:
Neurobiological mechanisms and treatment
implications**

by Fani Pantouli

Thesis submitted for examination in the degree of Doctor of Philosophy

Institute of Medical and Biomedical Education, Faculty of Health, Social Care and
Education

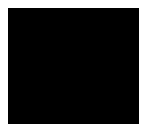
St. George's, University of London

October 2017

Declaration of Originality

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Name: Fani Pantouli

Abstract

Autism spectrum disorder (ASD) is characterised by impairment in the social domain and is often comorbid with depression and social anxiety. Several neuronal systems have been implicated in the neuropathology of ASD, yet the underlining neurobiology remains unclear, with no effective treatment being identified. Therefore, we utilised a well-validated mouse neurobiological candidate systems and we detected dysregulation of opioid, oxytocin, vasopressin, glutamate and dopamine in this model. Current ASD pharmacotherapy is only symptomatic and does not target core symptomatology. Intranasal oxytocin is a promising target for the treatment of ASD with known acute effects on related ASD-like symptomatology, but little currently is known about the effect of chronic exposure to the drug. Therefore, we performed a battery of behavioural experiments to assess the efficacy and neurobiological consequences of chronic oxytocin administration in the *Fmr1* KO and wild-type mice. We observed restoration of sociability but not stereotypy upon acute and chronic oxytocin treatment in the mutants and prevention of comorbid anxiety following chronic oxytocin administration. Opposingly, detrimental effects in sociability, stereotypy and emotionality following chronic oxytocin treatment were detected in wild-type mice. Differential regulation of several transcripts was detected in *Fmr1* KO and wild-type mice. Lastly, we investigated the effect of chronic exposure to social-defeat stress (CSS), a common psychopathology characterised by impairment in sociability and depression on central nicotinic acetylcholine receptor (nAChR) subtype and D₂ dopamine receptor density. While CSS did not affect α_7^* and $\alpha_4\beta_2^*$ nAChR binding, it downregulated D₂. These findings offer better understanding of the neurobiology underpinning psychopathologies characterised by social deficit and highlight chronic OXT as an effective pharmacotherapy for their treatment. Nonetheless, our findings reveal potential safety concerns of its chronic use in healthy subjects with intact oxytocinergic system.

Publications

Papers

- ♦ Morel, C., Fernandez, SP., **Pantouli, F.**, Meye, FZ., Marti, F., Tolu, S., Parnaudeau, S., Marie, H., Tronche, F., Maskos, U., Moretti, M., Gotti, C., Han, MH., Bailey, A., Mameli, M., Barik, J., Faure, Ph. (2017). Nicotinic receptors mediate stress-nicotine detrimental interplay via dopamine cells' activity. *Molecular Psychiatry*. DOI: 10.1038/mp.2017.145. (IF: 13.204)
- ♦ **Pantouli, F.**, Pellissier, LP., Becker, JAJ., Le Merrer, J., Bailey, A. Chronic intranasal administration of oxytocin reverses social impairment in the *Fmr1* KO animal model of Autistic Spectrum Disorders. *Neuropsychopharmacology* (IF: 6.403): (In preparation).
- ♦ **Pantouli, F.**, Pellissier, LP., Becker, JAJ., Le Merrer, J., Bailey, A. Morphine-induced behavioural and transcriptomic changes in the *Fmr1* KO mouse model. *Neuropsychopharmacology* (IF: 6.403): (In preparation).
- ♦ Georgiou, P., Zanos, P., Sklirou, M., **Pantouli, F.**, Wells, L., Bailey, A. Alcohol administration and withdrawal induces brain region-specific alternations of the oxytocin and mu-opioid receptor binding. *European Journal of Neuroscience* (IF: 2.941) (Under Review).

Conference presentations

Poster Presentations

- ♦ μ and δ opioid receptor autoradiographic binding in brains of a mouse model of autism (Jul 2016) *International Narcotics Research Conference (INRC) Meeting, Bath*
- ♦ μ and δ opioid receptor autoradiographic binding in brains of a mouse model of autism (Nov 2015) *British Society of Neuroendocrinology Conference, Lille, France*

- ◆ μ and δ opioid receptor autoradiographic binding in brains of a mouse model of autism (Dec 2015) *British Pharmacological Society Conference, London, UK*
- ◆ $\alpha_4\beta_2^*$, α_7^* nicotinic acetylcholine and D_2 dopamine receptor autoradiographic binding in brains of mice subjected to a social defeat paradigm (Dec 2015) *British Pharmacological Society Conference, London, UK*
- ◆ μ and δ opioid receptor autoradiographic binding in brains of a mouse model of autism (Nov 2016) *St. George's University Conference Research Day, London, UK*

Oral Presentation

- ◆ Experimental models of autism for the development of new targets for drug therapy (Jun 2015) *Faculty of Health and Medical Sciences, Festival of Research Conference, Guildford, University of Surrey*
- ◆ Mu and delta opioid receptor autoradiographic binding in brains of a mouse model of autism (Jul 2015). *Faculty of Health and Medical Sciences, Postgraduate Research meetings, University of Surrey, Guildford, Surrey, UK*

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Contents

Declaration of Originality	2
Abstract.....	3
Publications	4
Acknowledgements	6
List of Figures	13
List of Tables	16
List of Abbreviations.....	17
1. Chapter 1: General Introduction	27
1.1. Evolutionary implications of sociability	28
1.2. Autistic spectrum disorders	30
1.2.1. Autism: Historical approach	30
1.2.2. Epidemiological data of ASD.....	31
1.2.3. Clinical symptoms of ASD.....	32
1.2.4. Aetiology of ASD.....	34
1.2.5. Neurobiology of ASD	39
1.2.6. Preclinical models of ASD.....	46
1.2.7. Treatment of ASD	54
1.2.8. ASD comorbidity with anxiety and depression.....	58
1.3. Other psychopathologies characterised by social deficits: Anxiety and depression	59
1.3.1. Symptomatology and prevalence	60

1.3.2.	Aetiology of other psychopathologies characterised by social deficit.....	62
1.3.3.	Neurobiology of other neuropathologies characterised by social deficit: anxiety and depression	67
1.3.4.	Anxiety and depression association with social defeat consequences	72
1.4.	Focus of the thesis.....	75
1.5.	Aims and Hypotheses	78
2.	Chapter 2: The involvement of the endogenous opioid system in the neurobiology of autism	81
2.1.	Introduction	82
2.1.1.	Pharmacology of the opioids.....	82
2.1.2.	The concept of the ‘opioid theory’ in autism	83
2.1.3.	Role of the endogenous opioid system in social reward.....	85
2.1.4.	Fmr1 null mice as a model of autism spectrum disorders.....	90
2.1.5.	The opioid system in the Fmr1 KO mouse	99
2.2.	Hypotheses and Objectives	100
2.2.1.	Hypotheses.....	100
2.2.2.	Objectives.....	100
2.3.	Materials and Methods.....	102
2.3.1.	Methods.....	102
2.3.2.	Materials	107
2.4.	Results.....	108
2.4.1.	Autoradiographic analysis by receptor binding	108

2.4.2.	Real-time Quantitative PCR analysis	115
2.5.	Discussion	122
2.5.1.	Regulation of the endogenous opioid system in the Fmr1 KO	122
2.5.2.	Regulation of oxytocin and vasopressin in the Fmr1 KO	124
2.5.3.	Regulation of the dopamine and metabotropic glutamate receptors.....	126
2.6.	Conclusion	129
3.	Chapter 3: Effects of chronic intranasal oxytocin treatment on social behaviour, gene expression and receptor binding in an animal model of autism	130
3.1.	Introduction	131
3.1.1.	Neurobiology of oxytocin: structure, pharmacology, anatomy and physiology 132	
3.1.2.	Routes of oxytocin administration	136
3.1.3.	Oxytocin interactions with motivational reward circuitry.....	140
3.1.4.	The relationship between oxytocin and autism.....	142
3.2.	Hypothesis and objectives	151
3.2.1.	Hypotheses.....	151
3.2.2.	Objectives.....	151
3.3.	Materials and Methods.....	153
3.3.1.	Methods.....	153
3.3.2.	Materials	172
3.4.	Results.....	173

3.4.1. Effect of chronic intranasal OXT administration in WT and Fmr1 KO on core symptoms of ASD	173
3.4.2. Receptor binding of [125I] OVTA, [3H] MPEP and [3H] MK801 by quantitative autoradiography in WT and Fmr1 KO mice brains treated chronically with OXT or saline	177
3.4.3. Battery of behavioural paradigms assessing core symptoms of autism and comorbidity symptoms in the Fmr1 KO and WT mice treated with chronic OXT or saline for 39 days	193
3.4.4. Discussion	200
3.4.5. Chronic and acute intranasal OXT administration in the Fmr1 KO mice reverses social impairment and motor stereotypies in an Fmr1 KO model of ASD	200
3.4.6. Chronic and acute intranasal OXT administration in the Fmr1 KO mice has beneficial effects on comorbid symptoms of autism	203
3.4.7. Receptor binding by quantitative autoradiography in brains of Fmr1 KO mice after chronic and acute intranasal OXT administration	206
3.4.8. Altered transcription of several candidate genes of autism in the Fmr1 KO mice after chronic intranasal OXT administration.....	210
3.5. Concluding remarks	215
4. Chapter 4: Effect of social defeat stress on nAChRs and D ₂ receptors: An autoradiography study.....	217
4.1. Introduction	218
4.1.1. Depression	218
4.1.2. Nicotinic receptors: pharmacology, localisation and function.....	219

4.1.3.	Nicotinic receptors in depression	223
4.1.4.	'Social defeat' as a model for depression	226
4.2.	Hypothesis and aims.....	229
4.2.1.	Hypothesis	229
4.2.2.	Aims	229
4.3.	Materials and Methods.....	230
4.3.1.	Methods.....	230
4.3.2.	Materials	236
4.4.	Results.....	237
4.4.1.	Chronic social defeated and non-defeated mice assessed for social interaction 237	
4.4.2.	Effect of chronic social defeat on $\alpha_4\beta_2^*$ nAChRs binding in mice	238
4.4.3.	Effect of chronic social defeat on α_7^* nAChRs binding in wild-type mice	242
4.4.4.	Effect of chronic social defeat on D ₂ R binding in wild-type mice.....	245
4.5.	Discussion	247
5.	Chapter 5: General discussion	251
5.1.	General discussion	252
5.1.1.	Efficacy of long-term oxytocin administration	253
5.1.2.	Oxytocin's safety for general consumption.....	255
5.1.3.	Progresses in ASD neurobiology	257
5.1.4.	Progresses in neurobiology of social defeat consequences	259
5.1.5.	Future directions	259

5.1.6.	Limitations	261
5.1.7.	Concluding remarks	262
6.	References	263
7.	Appendix.....	326

List of Figures

Figure 1.1: Core symptoms, frequent associated behavioural dysfunctions and co-occurring conditions in individuals with ASD. Modified from Lai et al. (2014).	33
Figure 1.2: Major brain regions associated with core diagnostic symptoms of ASD. These form putative neural systems and are involved in the functions most impacted in ASD. Modified from Amaral et al. (2008).	42
Figure 2.1: Schematic representation of the FMR1 gene illustrating the location of the CGG triplet repeats within the 5'-untranslated region of exon 1 of chromosome 27. Adapted from Robertson and Wolffe (2000).	91
Figure 2.2: [³ H] DAMGO MOR binding in WT and Fmr1 KO mice.	111
Figure 2.3: [³ H] Deltorphan DOR binding in WT and Fmr1 KO mice.	113
Figure 2.4: [³ H] U69593 KOR binding in WT and Fmr1 KO mice	114
Figure 2.5: Fmr1 KO mice display transcriptional alterations within the endogenous opioid system.	118
Figure 2.6: qRT-PCR data analysis of candidate genes across five regions; the nucleus accumbens(NAc), caudate putamen (CPu), prefrontal cortex (PFC), central amygdala (CeA) and the ventral tegmental area (VTA) in the Fmr1 KO mice.	121
Figure 3.1: Representation of the oxytocinergic projections circuitry in the brain of rodents.	133
Figure 3.2: Representation of the A) OXT gene, B) mRNA and C) the neuropeptide itself.	135
Figure 3.3: Intranasal oxytocin administration potential routes for therapeutic regulation of brain function in the ASD.	139

Figure 3.4: Platform consisting of four arenas, in which direct social interaction sessions were performed.....	157
Figure 3.5: Cohort #1 timeline of chronic oxytocin administration or saline in Fmr1 KO and wild-type mice for the social interactions (SI) assessments.	158
Figure 3.6: Cohort #2 timeline of chronic oxytocin administration or saline in Fmr1 KO and wild-type mice and a battery of behavioral tests assessed.	159
Figure 3.7: Illustration of habituation and social interaction phases of the three-chamber sociability test.....	160
Figure 3.8: Representation of the three phases of the novel object recognition paradigm; habituation session, displaced object and novel object sessions.....	162
Figure 3.9: Illustration of marble-burying paradigm.	163
Figure 3.10: Illustration of novelty-suppressed feeding paradigm.....	164
Figure 3.11: Fmr1 KO show core symptoms of autism including impaired social interactions and stereotypies after social contact, which are reversed after acute, sub-chronic and chronic intranasal OXT treatment.....	176
Figure 3.12: OXTR binding.....	179
Figure 3.13: mGluR5 binding	182
Figure 3.14: NMDAR binding	185
Figure 3.15: Fmr1 KO and WT mice treated with OXT or saline display transcriptional alterations within several systems: the oxytocin and vasopressin neuropeptides, the endogenous opioid and glutamatergic system in the CPu.	188
Figure 3.16: Fmr1 KO and WT mice treated with OXT or saline display transcriptional alterations within several systems: the oxytocin and vasopressin neuropeptides, the endogenous opioid and glutamatergic system in the CeA.	191

Figure 3.17: Effect of chronic oxytocin on core symptoms of ASD assessed by 3-chamber sociability test and motor stereotypies assessment in cohort 2 (OXT-or saline treatment over 39 days).....	196
Figure 3.18: Effect of chronic oxytocin on comorbid symptoms of ASD in Fmr1 KO and WT mice.....	199
Figure 4.1: Representation of nAChRs subtypes distribution in the brain.....	222
Figure 4.2: Schematic of the chronic social defeat paradigm.....	232
Figure 4.3: Social interaction in mice subjected to chronic social defeat (SD) stress compared to non-social (NoSD) defeat.	237
Figure 4.4: Representative autoradiograms of $\alpha_4 \beta_2^*$ nAChRs binding in control non-social defeated (NoSD) and social defeated (SD) mice.....	239
Figure 4.5: Quantification of $\alpha_4 \beta_2^*$ nAChRs binding in control (NoSD) and SD mice.....	241
Figure 4.6: Representative autoradiograms of α_7^* nAChRs binding in control non-social defeated (NoSD) and social defeated (SD) mice.....	243
Figure 4.7: Quantitative autoradiographic analysis of α_7^* nAChRs binding in control (NoSD) and social defeated (SD) mice determined by [^{125} I] α -bungarotoxin binding.	244
Figure 4.8: Representative autoradiograms of D_2R binding in control non-social defeated (NoSD) and social defeated (SD) mice.	246
Figure 7.1: Opioid-induced locomotion and analgesia responses in Fmr1 $^{-/-}$ versus Fmr1 $^{+/+}$ mice.....	327
Figure 7.2: Opioid-induced responses in Fmr1 $^{-/-}$ versus Fmr1 $^{+/+}$ mice.	328

List of Tables

Table 1.1: A sample of preclinical models commonly used for the biomedical research of ASD. Adapted and updated from Belzung et. al. (2005).	48
Table 1.2: Clinical phenotypes of ASD analogous to rodent behavioural phenotype and the appropriate behavioural paradigms used for evaluation of preclinical animal models.	53
Table 2.1: Summary of the behavioural characteristics of the Fmr1 KO mouse displaying ASD symptoms conducted by a variety of behavioural tests from different studies.	97
Table 2.2: Primer sequences for qRT-PCR experiments.	106
Table 3.1: Summary of clinical trials of oxytocin treatment in autistic spectrum disorders (ASD) including Asperger's syndrome (AS), high functioning (HF) and low functioning (LF) individuals with ASD and, fragile X syndrome (FXS).	145
Table 3.2: Primer sequences for qRT-PCR experiments.	167
Table 3.3: Fold change differences of gene expression levels in the CPu of WT and Fmr1 KO mice treated with OXT or saline.	189
Table 3.4: Fold change differences of gene expression levels in the CeA of WT and Fmr1 KO mice treated with OXT or saline.	192

List of Abbreviations

AcbC	Accumbens Core
AcbSh	Accumbens Shell
ACC	Anterior Cingulate Cortex
ACTH	Adrenocorticotrophic Hormone
ADHD	Attention Deficit Hyperactivity Disorder
AMG	Amygdala
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOD	Anterior Olfactory nucleus-Dorsal
AOL	Anterior Olfactory nucleus-Lateral
AOM	Anterior Olfactory nucleus-Medial
AOV	Anterior Olfactory nucleus-Ventral
Arc	Activity regulated cytoskeletal-associated protein
Arpp21	cyclic AMP-regulated phosphoprotein, 21
AS	Asperger's syndrome
ASD	Autism Spectrum Disorder
AuCx	Auditory Cortex
AVP	Vasopressin
AVPR	Vasopressin Receptor
B3galnt1	β GlcNAc β 1,3-galactosaminyltransferase, polypeptide 1

BBB	Blood-Brain-Barrier
Bcl11b	B cell leukemia/lymphoma 11B
BDNF	Brain-derived Neurotrophic Factor
BG	Basal Ganglia
BLA	Basolateral amygdala
BMA	Basomedial Amygdala
BNST	Bed Nucleus of the Stria Terminalis
cAMP	<i>cyclic adenosine monophosphate</i>
CBT	Cognitive Behavioural Therapy
Cck	Cholecystokinin
Cckra	cholecystokinin A receptor
Cckrb	cholecystokinin B receptor
CeA	Central amygdala
c-fos	FBJ osteosarcoma oncogene
CgCx	Cingulate cortex
CL	Central Lateral
CI	Clastrum
CM	Central Media
Cnr1	cannabinoid receptor
CNS	Central Nervous System
CNV	Copy Number Variant

Col6a4	collagen, type VI, alpha 4
CPA	Condition Place Aversion
CPP	Condition Place Preference
Cpu	Caudate Putamen
CpuL	Caudate Putamen Lateral
CpuM	Caudate Putamen Medial
CRH	Corticotropin-releasing hormone
CSS	Chronic social defeat stress
CSF	Cerebrospinal Fluid
Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1
DA	Dopamine
DEn	Dorsal Endopiriform Nucleus
DG	Dentate gyrus
Dgkk	diacylglycerol kinase kappa
dHip	Dorsal hippocampus
DI	Deep layer
DLO	Dorsolateral Orbital Area
DOR	δ -opioid receptor
Drd	Dopamine receptor
D ₂ R	Dopamine receptor 2
DSM	Diagnostic and Statistical Manual of Mental Disorders

Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, Y-linked
EGF	Epidermal Growth Factor
EPI	External plexiform layer
Esrrb	estrogen related receptor, β
Fam40b	Striatin Interacting Protein 2
FDA	Food and Drug Administration
FMR1	Fragile X Mental Retardation
FMRP	Fragile X Mental Retardation Protein
Foxp1	Forkhead box P1
fr	fasciculus retroflexus
FrA	Frontal Association Cortex
FXS	Fragile X Syndrome
GABA	γ -amino-butyric acid
GAD	Generalized Anxiety Disorder
Galr	galanin receptor
GC	Glucocorticoids
GDP	Guanosine Diphosphate
GH	Growth Hormone
GI	Gastrointestinal
GPCRs	G-protein-coupled receptors
Gpr	G protein-coupled receptor

Grm	Glutamate receptor
GTP	Guanosine Triphosphate
Hip	Hippocampus
HPA	Hypothalamic–Pituitary–Adrenal
Hpca	hippocalcin
Htr6	5-hydroxytryptamine (serotonin) receptor 6
Hyp	Hypothalamus
IGI	Internal Granular layer
IMD	Intermediodorsal Thalamic Nucleus
InCx	Granular Insular cortex
InG	Intermediate gray layer of the superior colliculus
Insrr	insulin receptor-related receptor
lpn	Interpeduncular nucleus
IP3	<i>Inositol</i> trisphosphate
JNK	c-Jun N-terminal kinases
KO	knock-out
KOR	κ-opioid receptor
Lent	Lateral Entorhinal Cortex
LGP	Lateral Globus Pallidus
LO	Lateral Orbital area
LS	Lateral Septum

M1	primary motor cortex
M2	secondary motor cortex
mAChRs	metabotropic muscarinic Acetylcholine receptors
MAOI	Monoamine Oxidase Inhibitors
MDD	Major depression
Me	Medial amygdala
Medag	mesenteric estrogen dependent adipogenesis
MG	Medial Geniculate nucleus
mGluR	metabotropic Glutamate receptor
MHb	Medial Habenula
MO	Medial Orbital area
MOR	μ -opioid receptor
MPEP	Methyl-6-(phenylethynyl) pyridine
MS	Medial septum
MtCx	Motor cortex
Nac	Nucleus Accumbens
NA	Noradrenaline
nAChR	Nicotinic Acetylcholine receptor
Ndn	Necdin
Neurod6	neurogenic differentiation 6
NMDAR	N-methyl-d-aspartate receptors

NOR	Novel-Object Recognition
NoSD	Non-socially defeated
Nr4a1	Nuclear Receptor Subfamily 4 Group A Member 1
NSB	Non-specific binding
NSF	Novelty Suppressed Feeding
OCD	Obsessive Compulsive Disorder
OFC	Orbital Frontal Cortex
Oprd1	δ -opioid receptor
Oprk1	κ -opioid receptor
OPRM1	μ -opioid receptor
OXT	Oxytocin
OXTR	Oxytocin Receptor
PAG	Periaqueductal grey
PDD-NOS	Pervasive Developmental Disorder - not otherwise specified
Pde10a	Phosphodiesterase 10A
Pdyn	<i>Prodynorphin</i>
PENK	Proenkephalin
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
Pten	Phosphatase and tensin homolog
mPFC	Medial Prefrontal Cortex

Pir	Piriform cortex
PKC	Protein kinase C
PLC	Phospholipase C
PoA	medial Preoptic Area
PRhCx	Perirhinal Cortex
PrLCx	Prelimbic cortex
Prs	Presubiculum
PVN	Paraventricular nuclei
Re	Reuniens
REM	Rapid Eye Movement
RO	Reverse Osmosis
RSA	retrosplenial agranular cortex
RSCx	Retrosplenial Cortex
Rxfp1	relaxin/insulin-like family peptide receptor 1
S1	somatosensory cortex
S	subiculum
SAD	Social Anxiety Disorder
SD	Social defeat
SGA	Second-generation Antipsychotics
Shisa6	shisa family member 6
SI	Superficial layers

slc17a7	solute carrier family 17, member 7
SN	Substantia Nigra pars compacta
SNCA	α synuclein
SNP	Single Nuclear Polymorphism
SNRIs	Serotonin and Noradrenaline Reuptake Inhibitors
SON	Supraoptic nuclei
SsCCx	Caudal Somatosensory Cortex
SsRCx	Rostral Somatosensory Cortex
SSRIs	Selective serotonin reuptake inhibitors
SUG	Superficial Gray layer of the superior colliculus
Syt17	synaptotagmin XVII
TCA	Tricyclic antidepressants
Th	Thalamus
TSC	Tuberous sclerosis complex
Tu	Olfactory Tubercle
USV	Ultrasonic Vocalisations
VDB	Vertical limb of the Diagonal Band
Vdr	vitamin D receptor
ViCx	Visual Cortex
Vip	Vasoactive intestinal peptide
Vipr	Vasoactive intestinal peptid receptor

VO	Ventral Orbital area
VP	Ventral Pallidum
VTA	Ventral Tegmental Area
WHO	World Health Organization
Wnk	WNK lysine deficient protein kinase 4
WT	Wild-type

1. Chapter 1: General Introduction

1.1. Evolutionary implications of sociability

Man is a social animal with a natural urge to live an associated life with others and, society is a necessity for his existence or survival. It is accepted that humans have been living in society since time immemorial without though knowing the exact origin time (as described in (Hossain and Ali, 2014)). In classical antiquity even, the great Greek philosopher, Aristotle expressed that “Man is a social animal by nature and, the one who can spend his life outside society, must be either beast or God”. This statement fully reflects the importance of society to man’s existence and welfare. The social process starts from birth and continues throughout life. Healthy social interactions and social play are important behaviours throughout life that develop social communication skills, and ultimately create a ‘social’ person. Social communication encompasses behaviours such as facial expressions, eye contact, body language, which are influenced by both personality and sociocultural factors (Curenton *et al.*, 2004; Inglebret *et al.*, 2008) and a wide range of acceptable norms within and across individuals, families, and cultures contribute to that.

Human intelligence is a major element that peaked thousands of years ago and, which involves adaptation to a present environment and shaping of the present environment to fit to one’s skills interests or values. The natural selection on our intellect has contributed to the development of great human achievements. Evolution of human intelligence is closely related to the evolution of the human brain (Klug *et al.*, 2014). Various theories are formulated to explain and understand the evolution of human intelligence and, the ‘social brain’ hypothesis is one of them. According to Dunbar (1997), the ‘social brain’ theory supports the notion that human intelligence evolves primarily as a means of surviving and reproducing in large and complex social groups. Jerison (1973) was the first to point out that primates have unusually large brains in relation to their body size compared to other vertebrates. According to the ‘social brain’ hypothesis, primates evolved larger brains to cope with complex social behaviours (Dunbar, 1998). There are two predominant versions of

the 'social brain' hypothesis. One of them supports the theory that social transmission resulted from the necessity of establishing foraging skills (Reader and Laland, 2002), while the other expresses the notion that it is stemmed from processes underpinning social cohesion, with predation being the principal survival matter (Dunbar and Shultz, 2007).

A quantitative relationship has been revealed between social group size and brain size measurement (Dunbar, 1992, 1998). In most cases, the focus of the analyses is the size of the neocortex, as it is the only region that has expanded out of proportion as compared to other brain networks in primates (Finlay and Darlington, 1995). It has been found that neocortex is positively correlated with complex behaviours in social groups including male mating strategies (Pawłowski *et al.*, 1998), grooming clique size (Kudo and Dunbar, 2001; Dunbar and Shultz, 2007) and rates of deception (Byrne and Corp, 2004).

Conversely, a qualitative analysis of the 'social brain' theory revealed that mammals, who live in pair-bonded social systems (i.e. monogamous), have the largest brains (Dunbar and Shultz, 2007). It is assumed that there was a transition phase during the early stage of evolution in primates, in which a pair-bond relationship converted into a more complex form of bonded relationship (i.e. formation of friendships). This adds another dimension to previously described versions of the 'social brain' hypothesis, which encompasses only 'food-finding' or 'predation' matters. This new addition suggests that cognitive demands of pair-bonding in mammals triggered the enlargement of brain size, and these demands are responsible for the relationship between social-group-size to brain-size. Interestingly, social cognition is believed to have evolved through these processes and that takes its ultimate form in the "Theory of mind" in humans (Dunbar, 2009). "Theory of mind" is described as the ability to infer to other's mental states including beliefs, information, emotions, intuitions, plans, intentions and desires, which may differ from one's own (Premack and Woodruff, 1978). This theory implicates all skills required for typical development of social communication and relationships in humans such as verbally-mediated mindreading skills

emerging from the preschool period to later on in life developing skills of social competency (Slaughter, 2015).

Impairment affecting the “Theory of mind” ability is frequently seen in disorders that implicate social deficits affecting social cognition, social interactions and communication. These deficits can occur within the context of conditions such as autistic spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), learning, intellectual and developmental disabilities, specific language impairment and traumatic brain injury (Baron-Cohen *et al.*, 1985). In addition, conditions that involve psychological or emotional disorders such as social anxiety and depression can be involved in the spectrum of conditions characterised by social deficits, an impairment in social behaviour and communication often accompany these disorders.

1.2. Autistic spectrum disorders

1.2.1. Autism: Historical approach

The word ‘autism’ originates from the Greek word ‘εαυτός’ that means self, in an attempt to describe a condition where an individual is eliminated from social interactions therefore, an isolated self. The psychologist Eugen Bleuler was the first to utilise the term ‘autism’ in 1910 referring to one group of symptoms for schizophrenia with core symptoms being the split of personality, loosening of associations, detachment from reality and predominance of fantasy (Bleuler, 1912; Stotz-Ingenlath, 2000). Later on, the psychiatrist Leo Kanner (1943) described autism as a unique, standalone term based in a case study of observing 11 children with severe communication problems and repetitive behaviours. Shortly after, the paediatrician Asperger (1944) revealed a milder form of autism with social communication difficulties, the Asperger’s syndrome based on observations derived from a similar case study with children. It was included in diagnostic and statistical manual of mental disorders

(DSM-3) as autism disorder in 1987 (Weintraub, 2011). In the last 50 years, autism has been considered as a spectrum of neurodevelopmental disorder characterised by deficits in the social domain. For more information see Lord and Bishop (2015).

1.2.2. Epidemiological data of ASD

The prevalence of ASD in the adult population within the U.K. was estimated at 1.1 % in 2011 as illustrated by the Adult Psychiatric Morbidity Survey (The National Autistic Society, 2017) and at 0.76% of the worldwide population in 2010 (Baxter *et al.*, 2015). Notably, ASD is more prevalent in males than in females with a male: female range of ratio from 2:1 to 3:1 (Mattila *et al.*, 2011), and researchers have suggested that females may be protected against autistic behaviour (Robinson *et al.*, 2013). The first studies conducted in the 1960s and 1970s in Europe and the U.S., reported prevalence of autism estimated at 2-4 cases per 10,000 children (Treffert, 1970). Diagnosis of autism has climbed steadily since, with average prevalence estimated at 20.6/10,000 collected by 18 studies from Europe and the U.S. published between 2000 and 2009 (Rutter, 2005; Fombonne, 2009). While the exact reasons that caused the radical increase in autism cases over the years remain unknown, several factors have been linked to it, including changes in diagnostic criteria, diagnostic changes in the use of intellectual disability, greater awareness of the disorder and increased parental age (as reviewed by Rice *et al.* (2012)). It is estimated that the costs for supporting an individual with ASD (i.e. special education) and with intellectual disability during their lifespan is £ 1.5 million in the U.K. However, the cost for supporting an individual with an ASD without intellectual disability was estimated to be around £ 0.92 million in the U.K. (Buescher *et al.*, 2014). Annual costs associated with societal care for a child with ASD are estimated between 85% and 550% higher than annual cost of care for a typically developing child (Newschaffer *et al.*, 2007).

1.2.3. Clinical symptoms of ASD

According to the Diagnostic and Statistical Manual of Mental Disorders, 5th ed. (DSM-5) diagnostic criteria of the ASD are characterised by two core symptoms; impairments in social communication and repetitive, stereotyped behaviour. These symptoms are necessary for the diagnosis to be made. These symptoms must be present in early childhood, but may not become fully manifested until they limit and impair every day functioning. In particular, manifestation of deficits in social-emotional reciprocity (reduced sharing of interests, emotions), nonverbal communicative behaviours (lack of facial expression or gestures) and developing and maintaining relationships (difficulties sharing imaginative play and making friends) must be present. In addition, restricted or repetitive patterns of behaviour manifested by at least 2 symptoms including stereotyped or repetitive speech, motor movements, excessive adherence to routines, restricted/fixated interests and hyper- or hypo-reactivity to sensory input must be also present (American Psychiatric Association, 2013). Autism is a spectrum disorder, with symptoms ranging from mild to severe. Typically, it is diagnosed in children under the age of 3-5 and currently there is no effective treatment available. The spectrum of this disorder includes a wide range of behavioural communication and social impairment disorders such as Asperger's disorder, pervasive developmental disorder - not otherwise specified (PDD-NOS), Rett syndrome and childhood disintegrative disorder (Chmielewski and Beste, 2015).

Common associated features of ASD include atypical language development, motor abnormalities and excellent attention to detail (Baron-Cohen *et al.*, 2009; Fournier *et al.*, 2010; Maski *et al.*, 2011; Boucher, 2012), but these are not included in the DSM-5 criteria (American Psychiatric Association, 2013). These behavioural traits might transpire due to various common co-occurring conditions including intellectual disability, language disorders, motor abnormalities, ADHD and Tic disorders (Baron-Cohen *et al.*, 1999; Ming *et al.*, 2007; Green *et al.*, 2009; Boucher, 2012; Mahajan *et al.*, 2012). Medical conditions that often co-

occur with ASD include epileptic seizures, gastrointestinal problems, immune dysregulation, sleep disorders and genetic syndromes (Tuchman and Rapin, 2002; Benvenuto *et al.*, 2009; Buie *et al.*, 2010; Maski *et al.*, 2011). In fact, approximately 70% of those diagnosed with autism also have intellectual disability, and 25% have a seizure disorder (Tuchman and Rapin, 2002). Psychiatric symptoms such as anxiety, depression and eating disorders are also frequently observed in autistic individuals (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegård *et al.*, 2011). A summary of overlapping symptoms frequently associated with ASD can be seen in Figure 1.1.

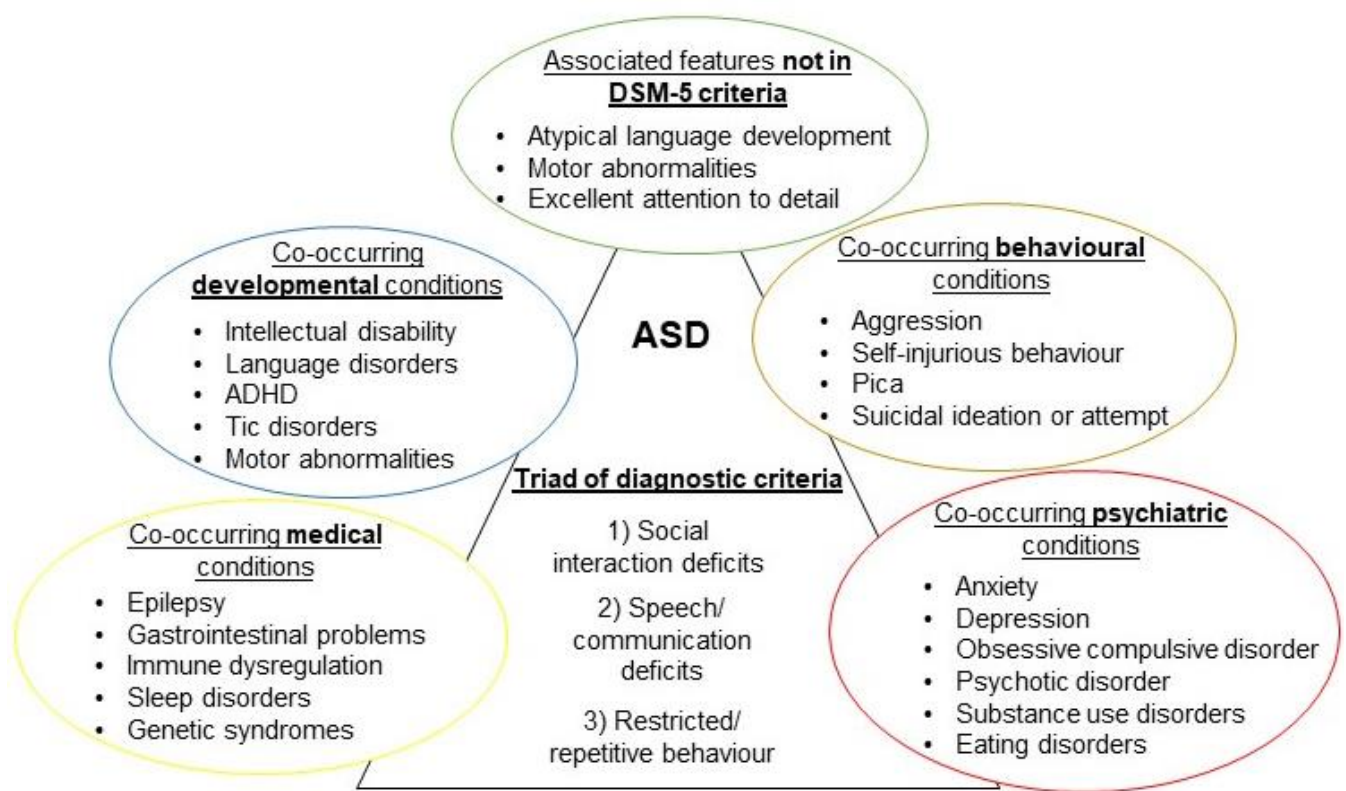


Figure 1.1: Core symptoms, frequent associated behavioural dysfunctions and co-occurring conditions in individuals with ASD. Modified from Lai *et al.* (2014).

1.2.4. Aetiology of ASD

ASD is widely considered to be a multi-factorial disorder resulting from genetic and environmental risk factors and their interaction. Approximately 10% of children with autism have been reported to be affected with ASD due to specific genetic, neurologic or metabolic disorders (Newschaffer *et al.*, 2007). The first family and twin studies that took place showed a strong genetic component with high concordance rates between the range of 82-92% in monozygotic twins in comparison to 1-10% in dizygotic twins, and heritability is estimated over 90% (Folstein and Rutter, 1977; Ritvo *et al.*, 1989). However, recent studies have illustrated that the concordance in dizygotic twins might be higher than 20% (Hallmayer *et al.*, 2011). Taken together, these findings strongly support a significant heritable component to the aetiology of autism. Nevertheless, the precise causes of pathology in ASD remain largely unknown. Genetic mutations with common mechanisms have been detected that can be treated with different classes of therapeutics, however no biomarkers are yet identified across each diagnosed case.

It is known that single rare medical or genetic conditions are associated with autism. For instance, syndromic ASD includes monogenic disorders such as Fragile X, Tuberous sclerosis complex (TSC), Rett syndromes (Pieretti *et al.*, 1991; Zoghbi *et al.*, 1999; Curatolo and Bombardieri, 2007) in which patients exhibit significant overlapping symptoms with ASD. Approximately 1% of cases account for diagnosis of ASD and comorbidity with other syndromic disorders, as many of them are quite rare, in comparison to the most common forms of idiopathic autism (Abrahams and Geschwind, 2008). Nevertheless, the discovery of the genetic causes of syndromic ASD was the first indication for the identification of a genetic component in the aetiology of ASD. The identification of single-gene disorders, which are associated with ASD allowed molecular genetic studies to investigate genetic underpinnings in ASD pathology by using mouse models that carry the same genetic

disruption. Yet it is important to note that ASD is commonly a polygenic disorder, and this factor has greatly complicated the identification of its genetic basis.

Genomic studies have revealed that *de novo* copy number variant (CNV) mutations affecting genes, which are expressed in the brain, are higher in individuals with ASD in comparison with the neurotypically developing population (Huguet *et al.*, 2013). Even though a great amount of research has been focusing on the identification of the genetic mechanisms underlying ASD, the identified genes associated with ASD are estimated to exceed 500-1,000 genes and this account for less than 1% of the individuals affected by the disorder (Iossifov *et al.*, 2012; Sanders *et al.*, 2012). Interestingly, two thirds of *de novo* mutations reported are associated with increase in the father's age at the time a child is conceived, and a year increase in the father's age increases by about two times the number of mutations (Kong *et al.*, 2012). Genome-wide linkage studies suggested potential autism susceptibility genes on chromosomes 2q, 7q, 15q, and 16p, however this has not been fully explained (Dykens *et al.*, 2004; Wassink *et al.*, 2004; Weiss *et al.*, 2008).

Chromosomal abnormalities contribute significantly to the genetic deficits of syndromic and idiopathic ASD. The transcription factor *ENGRAILED 2* gene, which is found on the autism-linked chromosomal region 7q36 (Alarcón *et al.*, 2002), has been associated with ASD displaying cerebellar morphological abnormalities (Gharani *et al.*, 2004). Chromosomal abnormalities on 15q have been associated with development of ASD and γ -amino-butyric acid (GABA) receptor subtype A (GABA_A) deficiencies. Genes encoding GABA_A receptors have been also associated with ASD with high confidence (Xu *et al.*, 2012). In addition, deletions or duplications in 16p chromosome including 16p11.2, 16p12.1 and 16p13.1 are linked to autism (Ballif *et al.*, 2007; Ullmann *et al.*, 2007; Eichler and Zimmerman, 2008).

Synapse dysfunctions are at the convergence between ASD and other neuropsychiatric disorders such as schizophrenia and intellectual disabilities, however their aetiology remains unknown. Mutations identified in both syndromic and idiopathic ASD involve synaptic genes

such as the *SHANK1*, *SHANK2* and *SHANK3* (Durand *et al.*, 2007; Leblond *et al.*, 2012; Sato *et al.*, 2012), *NLGN4X*, *NLGN3* (Jamain *et al.*, 2003; Laumonnier *et al.*, 2004) and *CNTNAP2* (Arking *et al.*, 2008). In addition, the *FMR1* mutated gene in the fragile X syndrome (FXS), the most commonly known single-gene disorder accounts for approximately 2-3 % of ASD cases (Reddy, 2005).

Other genes which are associated with ASD, include genes of the opioid system (opioid receptors and their endogenous ligands). In fact, an ASD patient has been detected with a genetic deletion including the *PENK* gene coding for proenkephalin, and another 10 ASD patients with CNVs that affect the μ -opioid receptor (*OPRM1*) gene (Kaminsky *et al.*, 2011; Sanders *et al.*, 2011; Halgren *et al.*, 2012; Battaglia *et al.*, 2013; Pellissier *et al.*, 2017). It is supported that social and affiliative behaviours are associated with the endogenous opioid system (Machin and Dunbar, 2011), and thus the involvement of the endogenous opioid system in the development of ASD needs to be further investigated. Furthermore, genes encoding for neuropeptide receptors that are implicated in social processes, including the oxytocin receptor (*OXTR*) and vasopressin receptors, *AVPR1A* and *AVPR1B* have been also studied as risk factors for ASD. Meta-analyses findings revealed a single nuclear polymorphism (SNP) on the *OXTR* gene associated with ASD, while genotype studies found evidence for associations of *AVPR1A* microsatellites and *AVPR1B* SNPs with ASD, suggesting the social neuropeptide system as potential target for establishing the neurobiologic and genetic basis of treatment for ASD (Yang *et al.*, 2010; LoParo and Waldman, 2015; Francis *et al.*, 2016). Taken these together highlight the profound importance to understand the role of opioids and OXT in ASD. The involvement of the endogenous opioid system as well as the OXT and AVP neuropeptides system in ASD are key objectives of this thesis and hence the current evidence linking these two neurotransmitter systems with disruption in the social context will be extensively discussed in

the introduction section of the result chapters of the present thesis; chapter 2 and chapter 3 respectively.

Metabolic defects such as purine metabolic disorders, creatine deficiency syndromes, phenylketonuria and mitochondrial dysfunctions are associated with less than 5% of individuals with ASD (Manzi *et al.*, 2008). High prevalence in ASD cases characterised with mitochondrial dysfunction was noted, suggesting that approximately 30% of children with ASD may experience metabolic abnormalities with one-third of autistic children having reported elevations in plasma lactate and/or the lactate-to-pyruvate ratio. Additionally, many other mitochondrial biomarkers including lactate, pyruvate, carnitine and ubiquinone had significantly different levels between ASD and controls (Rossignol and Frye, 2012), implicating dysregulation of major metabolic processes, and further support the mitochondrial hypothesis in the aetiology of autism.

In addition to genetic and metabolic causes, environmental factors such as prescription of medications, drugs of abuse and several heavy metals have been associated with ASD. Valproic acid, an antiepileptic drug that has been used in the treatment of schizophrenia and bipolar disorder has been linked to the development of ASD in humans (Moore *et al.*, 2000; Rasalam *et al.*, 2005). Estimated levels of airborne heavy metals such as mercury, cadmium and nickel at birth have been also reported to be moderately associated with ASD (Windham *et al.*, 2006). Moreover alcohol, smoking and illicit drug use during gestation have been also reported to be associated with ASD (Davis *et al.*, 1992; Aronson, Hagberg and Gillberg, 1997; Larsson *et al.*, 2005). In addition, prenatal cocaine exposure is suggested to lead to increased serotonin (5-HT) levels in serum, namely hyperserotonemia in fetuses, which is thought to be involved in the aetiological mechanisms implicated in autism (Whitaker-Azmitia, 2005). Perinatal factors such as low birth weight, birth asphyxia and abnormally short gestation length (Kolevzon *et al.*, 2007) as well as postnatal factors including viral infection, hypoxia and autoimmune disease (Ashwood and Van de Water, 2004; Kern and

Jones, 2006) are shown to be contributing-risk factors to the ASD. Inadequate nutrition is widely more common among children with ASD than the general population. Micronutrients including magnesium, zinc, selenium, vitamin A, vitamin B complex, vitamin D, vitamin E and carnitine have been found in lower levels in blood, hair or other tissue among children with ASD (Filipek *et al.*, 2004; Adams and Vogelaar, 2005; Strambi *et al.*, 2006; Pineles *et al.*, 2010; Gong *et al.*, 2013). For more information on chemicals and nutrition factors contributing to an ASD phenotype, see review by Fujiwara *et al.* (2016).

Several studies have reported evidence supporting the notion that various causality factors, from the ones previously mentioned, can affect boys and girls with ASD differently. In regard to micronutrients, the trend for ASD males having lower levels of vitamin D and 25(OH)D3 was noted in a study conducted in the Faroe Islands (Kočovská *et al.*, 2014). Similarly, another study showed that the gender difference of ASD on vitamin D metabolism might be explained on the basis of differential regulation of oestrogen and testosterone (Cannell, 2008). With regards to the genetics relationship and the development of ASD, as ASD develops in early childhood, girls seem to have a greater tolerance comparing to boys by aggregating genetic changes without developing ASD (Huguet *et al.*, 2013). Interestingly, many recent studies point to difficulties in females receiving clinical diagnosis even if they meet criteria for ASD (Knickmeyer *et al.*, 2008). These difficulties for their clinical diagnosis arise from the ability of females with ASD to demonstrate better emotionality and sociability as compared to males that might coverup some of the diagnostic features (Head *et al.*, 2014; Loomes *et al.*, 2017). These highly suggest that high-functioning females within the spectrum have been underdiagnosed, compared to males. Taken together, it is supported that gender differences in the aetiology of ASD contribute to the marked heterogeneity of clinical presentation among diagnosed individuals.

1.2.5. Neurobiology of ASD

1.2.5.1. Neuroanatomy/neurocircuitry

Enlarged total brain regions and volume have consistently been reported in individuals with ASD, after adjusting for height, IQ, and intra-cranial volume. This was observed since Kanner's first description in 1943, when he characterised some of the children diagnosed with autism with "large head". Children with ASD between the age of 18 months to 4 years have a 5-10% abnormal enlargement in total brain volume as indicated by imaging studies (Piven *et al.*, 1996; Sparks *et al.*, 2002; Hazlett *et al.*, 2005). It is common to observe macrocephaly in children and adults with ASD, which is defined as head circumference greater than the 97th percentile. Macrocephaly appears as abnormal brain overgrowth in white and gray matter, which develops during the first 2 years of life in children with autism, and these infants fall into the severe clinical spectrum of the disorder (Courchesne *et al.*, 2001; Courchesne *et al.*, 2004).

Also, morphological abnormalities in brain structures have been linked with ASD with respect to increased or decreased size. About subcortical structures, a volumetric meta-analysis showed that enlarged size of the caudate nucleus and a component of basal ganglia (BG) has been observed in ASD individuals (Duerden *et al.*, 2012) and, increased volume of the caudate nuclei observed after a magnetic resonance imaging (MRI) study in subjects with ASD, were significantly correlated with ritualistic-repetitive behaviours (Sears *et al.*, 1999). This suggests that caudate nucleus may be part of an abnormal distributed neural network sustaining stereotyped behaviours. Reduction in the size of thalamus in autistic patients has been also associated with stereotypical and repetitive behaviours (Tamura *et al.*, 2010; Estes *et al.*, 2011). Furthermore, increased cerebellar volume and reduced corpus callosum volume have been detected in ASD patients (Stanfield *et al.*, 2008; Frazier and Hardan, 2009). Enlarged volumes in amygdala have been detected mostly in high-functioning ASD

patients, however this was not consistent in all studies. In particular, meta-analyses studies showed decreased volume in amygdala (Nickl-Jockschat *et al.*, 2012), while increased volume was observed in younger patient groups (Bellani *et al.*, 2013). Outcomes from amygdala lesion studies, suggest that this region is implicated in fear processing, eye gaze when facing human and regulation of memory with emotional content (Adolphs *et al.*, 2005; Adolphs *et al.*, 2005; Spezio *et al.*, 2007). These suggest that amygdala abnormalities might be responsible for impaired emotional perception and regulation in ASD.

In addition, post-mortem studies on adults and adolescents with ASD, have shown reduced neuronal number in the amygdala, the fusiform gyrus, and the cerebellum. However, a study on young children showed significant increases in neuron number in the prefrontal cortex (PFC) (Courchesne *et al.*, 2011), a brain region associated with executive function. From an anatomical perspective, the PFC is a key region with important neuroanatomical characteristics, as it has projections to cortical, subcortical and brain stem sites (Alvarez and Emory, 2006). It has been shown that the medial PFC (mPFC), a subdivision of the PFC, which has reciprocal connections with the amygdala, hippocampus and sensory regions within temporal cortex, is involved in social cognition and social interaction (Wood and Grafman, 2003). This suggests that mPFC facilitates the neural basis of mentalizing (inferring to other's mental states/interests) and abnormalities detected in this brain region underline this dysfunction observed in ASD individuals (Frith and Frith, 2006). Disruption in communication within amygdala-mPFC circuitry induced memory-processing deficits, suggesting a key role of the mPFC in the development of ASD (Fuchs *et al.*, 2007; Mashhoon *et al.*, 2010).

Furthermore, thinning of several areas in the temporo-parietal region, a region central to the integration of social information, empathy and selective attention to salient stimuli, has been detected in individuals with ASD (McAlonan *et al.*, 2004; Scheel *et al.*, 2011; Greimel *et al.*, 2013). Increased cortical folding and cortical thickness in parietal lobes have been also

detected in brains of children with ASD (Kates *et al.*, 2009; Ecker *et al.*, 2013). On the contrary reduced cortical thickness, volume and surface area have been observed in the orbital frontal cortex (OFC) in individuals with ASD, which were associated with symptoms severity. From a functional perspective, the OFC is involved in sensory processing, goal directed behaviour, adaptive learning, and attachment formation and morphological alterations of this region may explain some of the aforementioned behaviours associated with ASD. The inferior and middle frontal gyri and the prefrontal cortex, found also in the frontal lobe, demonstrated also reduced cortical thickness in individuals with ASD (Hadjikhani *et al.*, 2005; Jiao *et al.*, 2010).

Additional studies have shown both increases and decreases in volume and thickness of the anterior cingulate cortex (ACC) in ASD, a highly interconnected brain region with the social brain network (Doyle-Thomas *et al.*, 2013). The ACC is situated in the frontal cortex, and has a role in self-perception, social processing, error monitoring, and reward based learning (Doyle-Thomas *et al.*, 2013; Ecker *et al.*, 2013), suggesting that abnormalities in this region is involved in social and reward deficits observed in ASD. Collectively, neuroanatomical abnormalities have been detected in several brain regions including the amygdala (AMG), substantia nigra (SN) basal ganglia (BG), and they are associated with manifestation of the core diagnostic symptoms, as illustrated in Figure 1.2. For more information on the neuropathology of autism see review from Amaral *et al.* (2008).

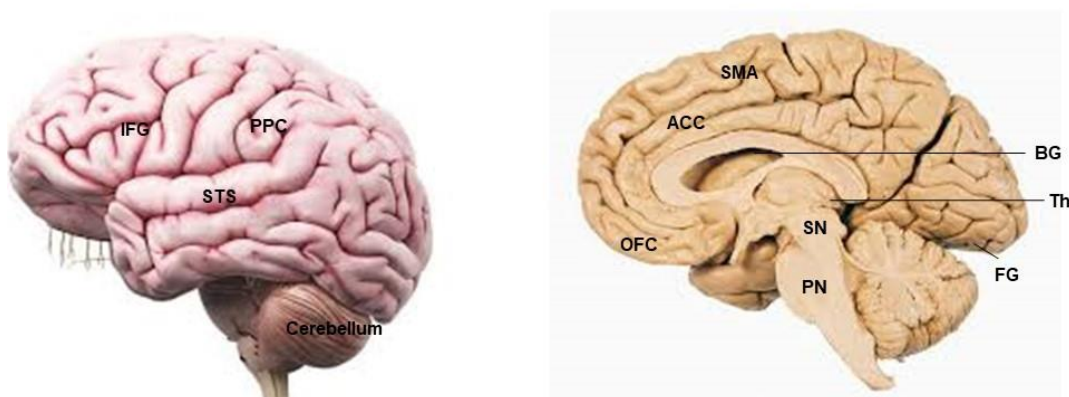
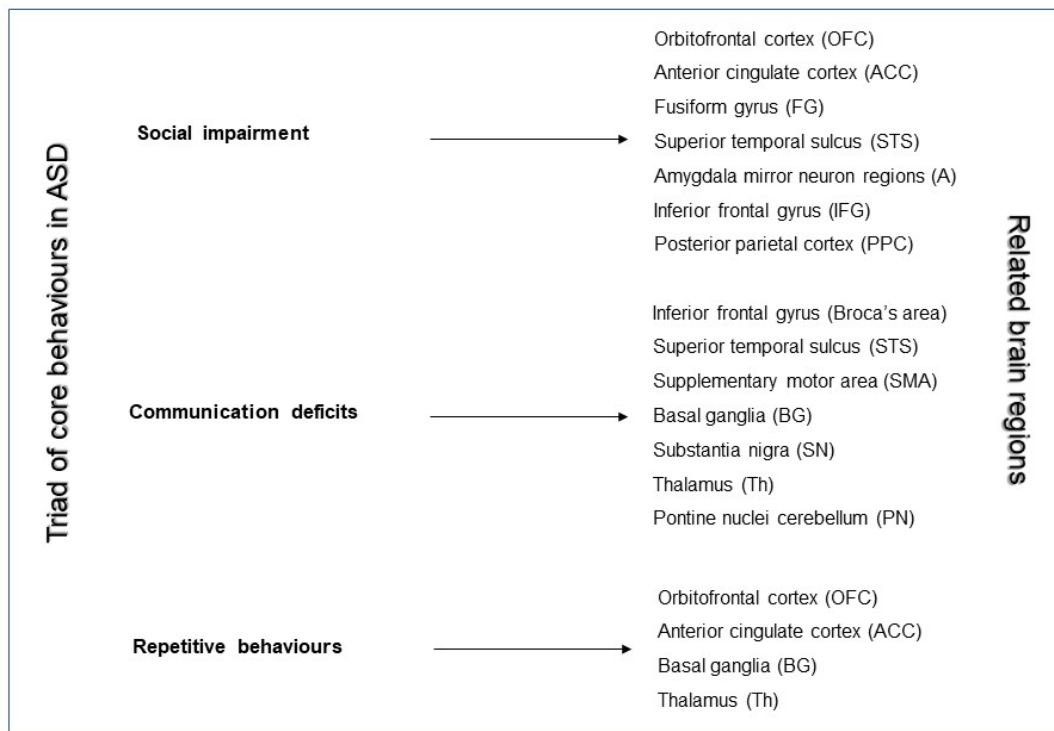


Figure 1.2: Major brain regions associated with core diagnostic symptoms of ASD. These form putative neural systems and are involved in the functions most impacted in ASD. Modified from Amaral *et al.* (2008).

1.2.5.2. Neurotransmitters and neuropeptides

Alterations in several neurotransmitter systems of the CNS have been reported in ASD patients. Emerging evidence suggest that ASD may be associated with abnormalities in excitatory glutamate and inhibitory GABA neurotransmission (Pizzarelli and Cherubini,

2011). The balanced interaction between glutamate and GABA transmission is essential for regulating cognition, memory, learning and emotional behaviours, whereas an imbalance between glutamate excitation and GABA inhibition, leading to hyperexcitation, has been linked to ASD (Gogolla *et al.*, 2009; Chao *et al.*, 2010; Coghlan *et al.*, 2012). In fact, Blatt and Fatemi (2011) detected significantly decreased levels of GABA_A and GABA_B receptor expression in the cerebellum, and lower levels of GABA_B have been found in the frontal and parietal cortex of individuals with autism. This is likely to have an effect on the excitatory/inhibitory balance in brain, and might further lead to seizures, a common comorbidity in ASD (Fatemi *et al.*, 2009).

Evidence of glutamatergic dysfunction in autism have been also reported. These support either a hyper-glutamate theory in the development of ASD (Fatemi, 2008) or a hypo-glutamate notion that has emerged from pharmacological effects of glutamatergic agonists and antagonists (Carlsson, 1998). In accordance to the hyper-glutamate theory, increased serum glutamate concentration has been detected in adults with ASD in comparison to healthy individuals (Shinohe *et al.*, 2006) and, also in a small sample of children with ASD (Aldred *et al.*, 2003), which seem to be related to the severity levels of social impairment (Shinohe *et al.*, 2006). In contrast, magnetic resonance spectroscopy studies have detected decreased concentration of glutamate/glutamine in the anterior cingulate cortex (ACC) in ASD patients (Bernardi *et al.*, 2011). Altogether, abnormalities in the central glutamatergic neurotransmission seem to be implicated in the pathogenesis of core and secondary symptoms of ASD.

Evidence for dopamine (DA) neurotransmitter abnormalities in ASD have been also reported. Positron emission tomography (PET) studies showed that children affected with ASD have low dopaminergic activity in the medial prefrontal cortex (Ernst *et al.*, 1997) than healthy controls, which is suggested to be linked to the disability of individuals with ASD to mentalize with others. Also, elevated DA transporter binding is detected in the orbitofrontal

cortex of high functioning ASD adults (Nakamura *et al.*, 2010). It has been also shown that disruptions in DA signalling originating in the VTA and projecting to limbic structures, including the nucleus accumbens (Björklund *et al.*, 1984) negatively impacts reward processing, a function important for developing social interactions (Curtis and Wang, 2005). Collectively, these findings highlight important ASD-related alterations in major neurotransmitter systems in the brain (mainly the glutamate, GABA and monoamines), which are likely to underlie some symptoms associated with ASD.

Dysregulation of 5-HT has been also associated with the neurobiology of ASD. In fact, hyperserotonemia has been reported in serum of children with ASD (Mulder *et al.*, 2004). In addition, significant higher expression of 5-HT_{2A} receptor subtype mRNA levels were found in platelets and the duodenum of autistic children with gastrointestinal (GI) problems (Kazek *et al.*, 2010). GI problems in ASD individuals appear to be responsible for maladaptive behaviours in ASD such as irritability, social withdrawal, hyperactivity and stereotypy and, those behaviour maladaptations may create or exacerbate GI problems (Chaidez *et al.*, 2014). In addition, while mice deficient for tryptophan hydroxylase 2, the rate-limiting enzyme of 5-HT synthesis, displayed reduced number of physical contacts (Beis *et al.*, 2015), administration of selective 5-HT reuptake inhibitors (SSRIs) prescribed to individuals with a form of ASD, improved core symptomatology of ASD (Kumar *et al.*, 2012) providing further evidence for the role of 5-HT in sociability behaviour.

Dysfunction of N-methyl-d-aspartate receptors (NMDAR) at excitatory synapses is also associated with ASD, as shown by human ASD-associated genetic variations found in genes encoding NMDAR subunits (Tarabeux *et al.*, 2011; Kenny *et al.*, 2014). It has been demonstrated that pharmacological modulation of NMDAR function ameliorates symptoms of social withdrawal and repetitive behaviour in ASD individuals (Posey *et al.*, 2004; Urbano *et al.*, 2014), suggesting that reduced NMDAR function may contribute to the development of ASD in humans.

As previously discussed, neuropeptide systems such as the OXT and AVP have been implicated in the neurobiology of ASD. In fact, low plasma OXT and AVP levels (Modahl *et al.*, 1998; Al-Ayadhi, 2005) and abnormal levels of peripheral OXT followed by OXT administration suggest that OXT signalling is disrupted in ASD (Andari *et al.*, 2010; Guastella *et al.*, 2010). Clinical findings using intravenous or intranasal administration of OXT in individuals with autism have demonstrated major improvements in some of the core symptoms of ASD (Hollander *et al.*, 2003; Andari *et al.*, 2010; Guastella *et al.*, 2010; Anagnostou *et al.*, 2012; Anagnostou *et al.*, 2014). However, prolonged OXT treatment seems to have detrimental effects on brain maturation providing not satisfactory evidence to allow OXT to be yet prescribed to patients. This thesis will focus on the understanding of the role of this social neuropeptide, as key objective, and hence extensive details covering the evidence linking this neurotransmitter system with ASD will be discussed in the introduction section of the result chapter 3.

Studies of response to drugs attempt to furthering our understanding of the underlying neurobiological networks that appear to be implicated in the neurobiology of ASD. The aforementioned studies that we have drawn our attention to, highlight the use of SSRIs, NMDAR agonists and OXT peptide in the management of ASD symptoms. It is, therefore, suggested that a unified approach incorporating modulation of these receptor systems (5-HT_{1A}, NMDAR, OXTR) might show greater treatment efficacy than a unilateral approach for the management of core symptomatology in the ASD pathology.

Neurobiological changes have also been noted in opioid receptors with clinical findings demonstrating increased opioid concentration in plasma (Leboyer *et al.*, 1994, Leboyer *et al.*, 1999, Tordjman *et al.*, 2009, Cazzullo *et al.*, 1999, Bouvard *et al.*, 1995), cerebrospinal fluid (Ross *et al.*, 1987, Gillberg *et al.*, 1985) and urine samples (Sokolov *et al.*, 2014, Reichelt *et al.*, 2012) from autistic patients. Recent evidence from clinical and preclinical studies have revealed a key role for endogenous opioids in ASD. Hence, this thesis will

focus on furthering the understanding of the role of the endogenous opioid system in order to shed light in their neurobiological basis in the development of ASD. For extensive evidence supporting the involvement of the opioids in ASD, see chapter 2.

Furthermore, neurotrophic factors such as the brain-derived neurotrophic factor (BDNF) and the epidermal growth factor (EGF) detected in blood have been involved in the pathophysiology of ASD (Qin *et al.*, 2016). In humans, increased BDNF levels were detected in serum of individuals with the disorder, while high expression of BDNF levels were detected in the valproic-acid rat model of autism (Schuch *et al.*, 2015). In addition, a significant reduction in serum levels of EGF has been shown in adult patients with high-functioning ASD as compared with controls (Suzuki *et al.*, 2007), while similar findings are observed in children with ASD (Russo, 2013). For more information on neurotransmitters associated with autism see review from Bethea and Sikich (2007).

1.2.6. Preclinical models of ASD

The high homology between mouse and human genomes and the genetic modifications that can be achieved in mice have raised interest over the years in generating knock-out (KO) and transgenic mouse models of human conditions such as ASD (Belzung *et al.*, 2005; Becker *et al.*, 2014). Defining mouse behavioural paradigms with high relevance to the diagnostic symptoms of autism has been a fundamental conundrum, as ASD is a human disorder (Crawley, 2004) with heterogeneous clinical phenotype incorporating a range of behaviours. In humans, ASD is assessed mainly by interviews from the individual with ASD and their environment (parents/carers/educators) and, speech is certainly a unique feature of communication. Specific human features such as eye contact, sharing of interests or facial expressions cannot be reflected in animals either (Bernardet and Crusio, 2006). Nevertheless, rodent models offer valuable preclinical tools in producing diagnostic and

ASD-associated symptoms by having evaluated animal related behaviour with validated experimental tests.

Three different criteria are evaluated for rodents before they are considered as good animal models for ASD: (i) construct validity, meaning that these models should mimic aspects of the aetiology, as autism is a multifactorial disorder (e.g., known mutations, neuroanatomical anomalies or environmental factors ASD-associated), (ii) face validity, which refers to similarity to clinical symptoms such as social deficits and repetitive behaviours that define ASD, and (iii) predictive validity referring to similarity of therapeutics between patients and animal models, such as similar drugs to improve social deficits or reducing stereotypies. However, as there is no approved drug treatment which can effectively treat the diagnostic symptoms of autism, predictive validity is not yet a determined criterion applied in animal models of ASD (Crawley, 2004; Belzung *et al.*, 2005). These ASD mouse models have been generally classified into four categories (Belzung *et al.*, 2005); (i) mutant mice with abnormalities in several neuropeptide or neurotransmitter systems involved in social behaviour and attachment, such as mice lacking OXT, AVP, opioid and GABA receptors; (ii) models that mimic epigenetic factors such as exposure of the foetus to anticonvulsants that increase the risk of autism in humans; (iii) animal models of neonatal lesions of brain areas characterised by autistic-related symptoms, including the cerebellum, amygdala and prefrontal cortex; (iv) models of human genetic diseases associated with autism such as the *Fmr1* KO mouse as a model of Fragile X syndrome, and *Shank3* KO as a model of microdeletion, the Phelan-McDermid syndrome, and (v) the Borna virus-induced model of autism, representing the neonatal infection-induced category (Belzung *et al.*, 2005; Bernardet and Crusio, 2006). Some of the rodent models of ASD, which are available for use in biomedical research along with their behavioural traits, are represented below (Table 1.1).

Table 1.1: A sample of preclinical models commonly used for the biomedical research of ASD. Adapted and updated from Belzung *et al.* (2005).

Model category	Animals	Behavioural traits	References
Neuropeptide deficits			
Vasopressin	Brattleboro rats (Mutant in <i>Avp</i> gene)	Impaired social recognition, no cardiovascular response to social isolation	Gardiner and Bennett, 1983; Engelmann and Landgraf, 1994
	<i>V1aR</i> KO mice	↓ social recognition	Bielsky <i>et al.</i> , 2004
	<i>V1bR</i> KO mice	↓ social recognition, ↓ aggression, modified social preference	Wersinger <i>et al.</i> , 2002, 2004
Oxytocin	<i>Oxt</i> KO mice	↓ social recognition, ↑↓ aggression, ↓ultrasonic vocalisation in new-borns, ↓discrimination and aversion to parasited male odour	(DeVries <i>et al.</i> , 1997; Winslow <i>et al.</i> , 2000; Ferguson <i>et al.</i> , 2001; Choleris <i>et al.</i> , 2003; Kavaliers <i>et al.</i> , 2003)
Opioid	<i>Oprm1</i> <i>-/-</i> mice (Mu-opioid receptor)	↓ultrasonic vocalisation in new-borns, no preference toward mother cue, stereotypic and perseverance, aggressiveness, ↑ anxiety, motor clumsiness, ↑ seizures susceptibility, spatial learning impairment, ↓ nociceptive thresholds, ↓ gastrointestinal mobility	Roy <i>et al.</i> , 1998; Gavériaux-Ruff and Kieffer, 2002; Jamot <i>et al.</i> , 2003; Moles <i>et al.</i> , 2004; Becker <i>et al.</i> , 2014
Prenatal toxicity			
5-Methoxytryptamin	Rat pups (E12 to P20)	↓ultrasonic vocalisation in new-borns, ↓ spontaneous alteration	Kahne <i>et al.</i> , 2002
Valproic acid	Rat pups (E9 or E12.5)	↓social behaviours, stereotypic-like hyperactivity	Schneider and Przewlocki, 2005
Neonatal infection			
Borna virus	Rats	↓social play behaviour, longer duration of social exploration, ↑ stereotypy scale	Hornig <i>et al.</i> , 1999; Pletnikov <i>et al.</i> , 2002
Early lesion			

Cerebellum	Rats (P10)	↓ preference for a compartment with their own soiled litter when compared with time spent in the compartment with conspecific odours, identical exploratory behaviour in a session with noise and a session without noise (persevering behaviour)	Bobée <i>et al.</i> , 2000
Amygdala	Rats (P7)	↓ in social play behaviour, ↓ in some social exploration, ↓ spontaneous alternation, stereotyped-like walking pattern	Volterink <i>et al.</i> , 2001; Diergaarde <i>et al.</i> , 2005
Medial prefrontal cortex	Rats (postnatal)	↓ social play (pinning behaviour) in juveniles, no deficit in amount of social interactions in adults but decreased conditioned place preference for places associated with social contacts, ↓ social grooming, spatial alternation deficit	Vicedomini <i>et al.</i> , 1982; Schneider and Koch, 2005
Models of genetic diseases associated with autism			
Fragile X syndrome	<i>Fmr1</i> tm1Cgr, KO mice	see Table 2.1 for behavioural traits	see Table 2.1 for references
<i>Dishevelled</i> gene	<i>Dvl-1</i> deficient mice	↓ social interaction, deficits in nest-building, ↓ bundling during home cage sleeping, subordinate responses in a social dominance test	Lijam <i>et al.</i> , 1997
<i>Reelin</i> gene	<i>Reeler</i> mutant mice	Normal social aggression, no difference in social exploration	Salinger <i>et al.</i> , 2003; Podhorna and Didriksen, 2004
Cerebellar and cortex abnormalities	GS Guinea pig	↓ social interaction, stereotyped behaviour in a motor learning task	Caston <i>et al.</i> , 1998
<i>Shank3</i> gene	<i>Shank3B</i> mutant mice	self-injurious repetitive grooming, anxiety -like behaviour in an elevated plus maze test, social interaction deficits in a social novelty test	Peça <i>et al.</i> , 2011
<i>Engrailed 2</i> gene	<i>Engrailed2</i> mutant mice	defective spatial learning, ↑ seizure susceptibility, ↓ social interaction, ↑ immobility in the forced swim test	Brielmaier <i>et al.</i> , 2012; Provenzano <i>et al.</i> , 2014

The development of behavioural assays has paved the way to allow assessment of several aspects of sociability in rodents. Mice and rats are social species, like humans, exhibiting a wide range of social behaviours that includes approach to familiar and new conspecifics after olfactory pheromones emitted by other mice. They engage in reciprocal social interactions, communication via ultrasonic vocalizations, communal nesting, parenting and sexual behaviours, territorial scent marking and aggressive behaviours (Carter *et al.*, 1992; Miczek *et al.*, 2001; Terranova and Laviola, 2005; Arakawa *et al.*, 2008; Silverman *et al.*, 2010). Standardized methods for scoring behavioural phenotyping have been utilized to address whether preclinical animal models display social deficits analogous to those seen in ASD in humans.

Rodents engage in reciprocal social interactions displayed by sniffing (nose to nose or body contact), paw contact, following behaviours assessed during e.g., direct social interaction paradigm (Peça *et al.*, 2011; Pobbe *et al.*, 2012). Also, three-chambered social approach task quantifies direct social approach behaviours when a subject mouse is assessed for its preference to spend time with either an unfamiliar mouse or an object (Nadler *et al.*, 2004). Rodents communicate primarily through olfactory pheromones and emit ultrasonic vocalizations during social interactions (Chabout *et al.*, 2012). Components of rodent ultrasonic vocalisations (USVs) that are analogous to human language communication, have been recorded and identified. Motor stereotypies and repetitive behaviours, as the second ASD diagnostic symptoms, are presented in mice models of ASD as high frequencies of behaviours such as circling, jumping, repetitive self-grooming, head shakes, rearing (Silverman *et al.*, 2010; Mattila *et al.*, 2011; Becker *et al.*, 2014).

In addition to the core symptoms of ASD diagnosis, there are associated symptoms that commonly occur as comorbid conditions, such as anxiety and depression. Common rodent behavioural tasks for the assessment of anxiety-like behaviours rely on the conflict between the tendency of mice' spontaneous exploratory behaviour versus innate aversion to brightly

illuminated open areas such as light/dark exploration test. The elevated plus-maze, in which mice are assessed for the time they spent in the two open arms of an elevated plus-maze as compared to the two enclosed arms of the maze, is another well-validated assessment of anxiety behaviour. In fact, anxiolytic drugs administered to mice increase the number of entries as well as time spent in the open arm of the maze in this paradigm. Furthermore, they also increase time spent in the light compartment as well as number of transitions between compartments in the light/dark apparatus, confirming predictive validity criterion (Crawley, 1985; Cryan and Sweeney, 2011). Several animal models have been also used for studying neurobiological events that lead to affective deficits and depressive disorders, which can potentially mimic human depression. The stress-induced social defeat is one of them, in which mice have undergone chronic stress by experiencing persistent loss in aggressive social encounters (Hollis and Kabbaj, 2014). The social defeat model is further discussed in section 4.1.

Exploration of all facets of ASD is essential before claiming that a model is relevant to ASD, yet most models have not been checked for the whole repertoire of ASD signs. Interestingly, several knock-out animals involving genes already associated with ASD such as *Engrailed* 2 gene null mice and the *Fmr1* KO mice have been developed (Bernardet and Crusio, 2006; Cheh *et al.*, 2006; Brielmaier *et al.*, 2012). Each of these candidate genes may be representative of a valuable model to be studied, despite that the classical ASD model is polygenic. The *Fmr1* KO mouse model displays strong construct validity as it recapitulates many of the physical, physiological and behavioural clinical features that are shared with the human FXS and autistic-like phenotype. This model was used in experiments presented in this thesis and extensive description and discussion of this model can be found in chapter 2, section 2.1.4.4. Also, rare ASD models satisfy some aspects of the face validity criteria. These include the prenatal 5-methoxytryptamine and the valproic acid model, three of early postnatal lesion models, the GS Guinea pig and the Borna virus-induced infection in

neonatal rats. Table. 1.2 below describes the clinical phenotypes, which are relevant to rodent behavioural phenotype and the appropriate behavioural assessments used to evaluate preclinical ASD models. For a comprehensive review on translational models of ASD see review by Kazdoba *et al.* (2016).

It is worth, however, to note that the aforementioned paradigms (e.g. elevated-plus maze, light/dark box) as well as others (e.g. the forced-swim test, open field test) have been developed and evaluated only for preclinical screening of psychotropic drugs. Therefore, they can be efficiently used for screening of compounds for their anti-depressant or anxiolytic action. Though, they have been widely used to assess behaviours in preclinical models on drug-free state that are further characterised as disorder-like, including anxiety- and depression-like disorders. Stanford, 2017, who has been very critical on this matter, strongly supports that data generated based on this misinterpretation should be considered as dubious, as the criteria for the validation of the tests as 'models of the disorder' are ignored. As it is further discussed by Stanford, 2017, the depression-like and anxiety-like symptoms, which are assessed in rodents do not reflect the symptoms which are presented in humans. In particular, in the elevated plus maze there is no justification why a mouse (in the drug free-state) that demonstrate increased activity in the open arms, is rather characterised as 'anxious' than a 'cautious' mouse (Stanford, 2017). These are valid and critical points to take into consideration when interpreting the data in our study.

Table 1.2: Clinical phenotypes of ASD analogous to rodent behavioural phenotype and the appropriate behavioural paradigms used for evaluation of preclinical animal models.

Clinical phenotype	Rodent behavioural symptoms	Assessment criteria for behavioural paradigms
I. Social interactions Communication deficits II. Stereotypy a) Motor stereotypies (hand flapping, toe walking, rocking) b) Repetitive behaviours/ interests	social sniffing, following, allogrooming olfactory pheromones, ultrasonic vocalizations circling, head shakes, jumping, repetitive self-grooming, rearing, Y-maze	three-chamber sociability, social interaction paradigm (Nadler <i>et al.</i> , 2004; Peça <i>et al.</i> , 2011; Pobbe <i>et al.</i> , 2012) recording ultrasonic vocalizations (Chabout <i>et al.</i> , 2012) Direct observation of stereotypies in mouse behaviour in a non-social context (Silverman <i>et al.</i> , 2010)
Comorbidity Anxiety Depression	Anxiety-like behaviour Depressive-like behaviour	elevated plus maze, light-dark exploration, novelty suppressed feeding (Crawley, 1985; Zhou <i>et al.</i> , 2010; Cryan and Sweeney, 2011) forced swim test, tail suspension test, social defeat model (Can <i>et al.</i> , 2012; Can <i>et al.</i> , 2012; Hollis and Kabbaj, 2014)

1.2.7. Treatment of ASD

The core and secondary symptoms of ASD can set limitations to the quality of life of individuals with the disorder, and concomitantly the lives of their families and caregivers. Therefore, early diagnosis and cognitive behavioural therapy (CBT) can improve the lives of these individuals (Howlin *et al.*, 2009). There is no current cure of the disease, but symptomatic management is available using pharmacologic agents for relieving some of the symptoms. Symptoms such as repetitive, restrictive and stereotyped behaviour, which overlap between patients with ASD and patients with OCD is known to be at least partly mediated by 5-HT dysregulation (Jacob *et al.*, 2009). This combined with evidence of 5-HT system dysregulation in individuals with ASD, prompted the use of SSRIs in adults and children with ASD (Todd, 1991; Reiersen and Todd, 2008). SSRIs and tricyclic antidepressants (TCAs) have been commonly used for symptomatic treatment of ASD including repetitive behaviours (Lewis and Kim, 2009). Interestingly, while treatment with fluoxetine and fluvoxamine demonstrated improvement on repetitive thoughts, actions and obsessive–compulsive-associated scale scores in adults (McDougle *et al.*, 1996; Hollander *et al.*, 2012), fluoxetine or citalopram did not show any improvement on these symptoms in children with ASD (Hollander *et al.*, 2005; King *et al.*, 2009) suggesting that improvement effects on repetitive behaviours after SSRIs treatment is age dependent.

The most common medications, which are FDA approved, are second generations antipsychotics (SGA) such as risperidone and aripiprazole (Siegel and Beaulieu, 2012; Spencer *et al.*, 2013) that target frequently co-occurring behaviours in individuals with ASD such as irritability, aggression, self-injurious behaviour and severe tantrums (see diagram for co-occurring behaviours of ASD in Figure 1.1). Risperidone, which acts on and blocks DA and 5-HT systems, has been shown to ameliorate stereotypical behaviour in children with ASD (McCracken *et al.*, 2002; McDougle *et al.*, 2005; Chavez *et al.*, 2006), yet this is not in

line with other studies (Tehrani *et al.*, 2014). The literature has varied findings on risperidone studies and only some of them suggest beneficial effects on core symptoms of ASD including improved social interactions and verbal communication skills, based on significant results (Scahill *et al.*, 2013; Tehrani *et al.*, 2014), whereas others showed improvements in repetitive, and stereotyped patterns of behaviour, interests, and activities of autistic children but not in social deficits (McDougle *et al.*, 2005). Meta-analytical evidence reveals that SGAs (i.e. risperidone) provide improvement in behavioural associated symptoms of Asperger's and high-function ASD (i.e. irritability, aggression) rather than core symptomatology, with most cases reporting weight gain as a potentially concerning adverse effect. This discrepancy in respect to the symptomatic treatment efficacy of SGAs among studies is potentially due to differences in experimental design used and, heterogeneity within ASD patients' samples assessed. While nosological changes are re-adjusting the diagnostic criteria of ASD, it is inevitable that study populations are categorized according to intelligence level, adaptive, functioning and language ability to help make more relevant and specific determinations of efficacy (Sochocky and Milin, 2013).

Development of pharmacological approaches to treat impairment of social functioning in ASD including limited social interactions, non-verbal communication difficulties (e.g., eye contact, gestures, body posture) and impaired pragmatic language did not progress as fast as in other areas. As a consequence, no medications are available for treatment of social impairment associated with ASD (Wink *et al.*, 2010). Nonetheless, therapies which include psychosocial support have been used to treat ASD individuals including social skills training and speech-language therapy, occupational therapy and CBT with varied efficacy. Several studies have examined the effects of social stories, verbal, and nonverbal social skills training, interactions with typical peers, CBT and computer face processing games as treatments for children with ASD (Barnhill *et al.*, 2002; Bauminger, 2002; Smith *et al.*, 2002; Carter *et al.*, 2004; Sofronoff *et al.*, 2004; Solomon *et al.*, 2004; Pakenham *et al.*, 2005).

However, few of these studies showed a positive impact on emotion recognition, life-problem solving and symptoms of depression in high-functioning ASD children after a laboratory/clinic based intervention (Solomon *et al.*, 2004). In addition, an intervention focused on parent management resulted in improved parent reports reporting fewer and lower behaviour problems in children with high functioning ASD and, improvement in the social interactions at home (Sofronoff *et al.*, 2004). CBT has shown remission of anxiety in children with ASD by cognitive-induced changes (Wood *et al.*, 2009). Altogether these suggest that there is discrepancy in efficacy in these interventions and this might be due to the different approaches used as well as the heterogeneity within patients' samples assessed. It is important to note that these and all other intervention research with children with high-functioning ASD are used in combination with psychotropic medications, which target symptoms of anxiety and depression (Martin *et al.*, 1999). These highlight the importance of applying combined pharmacotherapy and psychosocial interventions in the treatment of ASD symptomatology. Collectively, psychosocial and CBT therapies show some promising results in the treatment of core and secondary symptoms in ASD, which undoubtedly warrant further investigation.

Clinical trials have evaluated the efficacy of classes of compounds in treating ASD core symptoms that have been challenging due to the heterogeneity of the disorder. Pilot studies assessing effects of intranasal administration of the social neuropeptide OXT in individuals with ASD showed an improvement in emotional recognition and social awareness in ASD individuals as well as in the neurotypically developed (Hollander *et al.*, 2007; Bartz and Hollander, 2008; Rimmele *et al.*, 2009; Bartz *et al.*, 2010; Guastella *et al.*, 2010). Functional neuroimaging studies demonstrated that brain structures associated with sociability including the NAc, posterior cingulate, and premotor cortex showed enhanced activity in children with ASD following intranasal OXT administration, which suggests that this neuropeptide enhanced social behaviour responses and it is potential target for ASD patients (Gordon *et*

al., 2013). However, despite the beneficial effects of intranasal OXT administration in ASD subjects, there are gaps in terms of knowledge concerning prolonged OXT treatment. The present thesis will attempt to address this in chapter 3, in which extensive details covering the evidence linking OXT with ASD are discussed.

Arbaclofen, a selective GABA_B agonist, which stimulates neurotransmitter inhibition, was also studied as potential treatment in ASD individuals demonstrating improvement on social responsiveness and withdrawal (Erickson *et al.*, 2014). In addition, D-cycloserine, a partial agonist of the ionotropic glutamatergic NMDA receptor has been evaluated in studies, where children with ASD were assessed and showed significant improvement in social withdrawal (Posey *et al.*, 2004). Memantine, an NMDA receptor antagonist in open label retrospective studies demonstrated significant improvements in language function and social behaviour (Chez *et al.*, 2007; Erickson *et al.*, 2007), however the outcomes were not consistent (Owley *et al.*, 2006; Niederhofer, 2007). Clinical studies using cholinesterase inhibitors seem to be beneficial for the treatment of interfering behaviours in children with ASD, particularly aggression, behaviour dyscontrol and expressive language (Niederhofer *et al.*, 2002; Chez *et al.*, 2004; Nicolson *et al.*, 2006). For detailed information on potential treatments for ASD see review by Kazdoba *et al.* (2016).

Collectively, although risperidone and aripiprazole are two FDA-approved drugs for the treatment of associated symptoms of ASD such as irritability and aggression, yet medicine for treating autism's core characteristics need to be approved. However, medicines that can relieve irritability can concomitantly ease core symptoms of ASD by improving sociability and reducing aggressive outbursts, tantrums and self-injurious behaviours. Ultimately, aetiology-based treatments of ASD are warranted.

1.2.8. ASD comorbidity with anxiety and depression

ASD has been closely associated with mood disorders. Kanner (1943) demonstrated in his series of experiments that several children display symptoms of anxiety and fear of objects and events, while at least one showed a tendency to depression. Clinical studies show that depression is probably the most common comorbidity in individuals with ASD, even though its exact prevalence is not known (Ghaziuddin *et al.* 2002). Wing *et al.* (1992) was the first to suggest a link between self-awareness of social difficulties and depression in young people with ASD as they are more susceptible to emotional pain following social failures.

High prevalence of secondary psychopathologies is reported in about 72% of children with ASD (Leyfer *et al.*, 2006) with depression and anxiety being among the most common ASD comorbidities (Simonoff *et al.*, 2008). Interestingly, it has been reported that emotional comorbidities such as symptoms of depressive disorder might worsen core symptoms of ASD (Perry *et al.*, 2001; Matson and Nebel-Schwalm, 2007) and poor quality of social relationships often reported in this population might be related to increased levels of depressive and anxiety symptoms (White *et al.*, 2009; Whitehouse *et al.*, 2009). Taken these together, it is evident that social deficit, the core symptom in ASD can either act as a cause or the consequence to the development of depression and anxiety disorders.

A meta-analysis study has revealed that approximately 40% of ASD individuals suffered from a type of an anxiety disorder (White *et al.*, 2009b; van Steensel *et al.*, 2011). Specific phobia was the most common type affecting about 30% of autistic individuals, while OCD and SAD or agoraphobia occurring in 17% of the autistic population (van Steensel *et al.*, 2011). Remarkably, the same study suggests that children with a particular ASD subtype might be more vulnerable to develop a certain type of anxiety disorder over another. Studies, in which anxiety levels across ASD subtypes were compared, support the notion that anxiety varies in relation to ASD clinical severity. Indeed children with less severe ASD symptoms

would display more anxiety (Macneil *et al.*, 2009). Such examples are children and adolescents with Asperger's - syndrome with less severe manifestation of ASD- which display the highest anxiety levels, followed by children with PDD-NOS (Weisbrot *et al.*, 2005). In contrast, other studies failed to find differences in anxiety levels between ASD subtypes (Kim *et al.*, 2000; Gadow *et al.*, 2005; Pearson *et al.*, 2006; Sukhodolsky *et al.*, 2008).

To conclude, these are indicative of the complexity that these secondary psychopathologies add to the primary characteristics of the disorder. Hence, understanding the neurobiological basis of this comorbidity and taking into consideration differential diagnosis and classification of comorbid symptoms, will open new avenues of research implicating treatment innovations. As the results of this thesis focus on the neurobiology of social defeat which is associated with the manifestation of depression- and anxiety- like symptoms, we will focus this general introduction on these two disorders.

1.3. Other psychopathologies characterised by social deficits: Anxiety and depression

The 'Theory of mind' as discussed earlier in section 1.1, represents an evolved psychological capacity highly developed in humans and it is comprised by social cognition. This enables mammals to infer to others' mental states, emotions, social cues and attributional style (Premack and Woodruff, 1978; Allen *et al.*, 2008). Social neuroscience research targets treatment interventions that will improve patients' social cognitive skills. Although social interactions require a set of sophisticated skills, most social signals for majority of people are processed automatically, without awareness. The necessity of processing such crucial skills for successful social functioning, becomes obvious only when they are altered or insufficient. Various psychiatric disorders are characterised by impaired social cognition and social functioning like schizophrenia and addiction. There is growing

evidence that social cognitive deficits underlie poor functional outcomes in patients with psychiatric disorders and play an important role in the initiation and maintenance of the disorder (Dodge, 1993; Fett *et al.*, 2011). Social deficit is central for many psychiatric disorders and extensive research has been conducted in several of these including ASD, OCD, ADHD, addiction, post-traumatic stress disorder (PTSD), schizophrenia and mood disorders (i.e. depression). As the research chapters in this thesis will focus on the neurobiology underlining ASD and social defeat, we will concentrate the general introduction on the three disorders: ASD, depression and anxiety characterised by social deficits. Mood disorders including depression appear to be more common in those with ASD than in the general population (Ghaziuddin *et al.*, 2002). However, relatively little is known about its pattern of psychiatric comorbidity and diagnosing depression in ASD individuals remains a challenge given this population's difficulties coping with adversity, interacting socially and managing emotions. Therefore, the necessity for evaluating antidepressant treatment in the ASD population warrants more research.

1.3.1. Symptomatology and prevalence

1.3.1.1. Anxiety

It is widely believed that anxiety is a characteristic feature of our modern times and that the prevalence incidents have increased due to socio-politico-economical or environmental changes that have been implemented over the years (Bandelow and Michaelis, 2015). According to the DSM-5, anxiety disorders include disorders that share features of excessive fear and anxiety and related behavioural disturbances, which differ from normal feelings of nervousness or anxiousness. It is diagnosed as a psychopathology when the fear or anxiety exceeds the normal levels anticipated to a situation or it is age inappropriate and ultimately, hinders one's ability to function normally (American Psychiatric Association, 2013).

Around 8.2 million cases of anxiety were reported in the U.K. in 2013 (Fineberg *et al.*, 2013) with women being twice as likely compared to men of being diagnosed with anxiety disorders (Martin-Merino *et al.*, 2010). Genetic and neurobiological factors as well as psychosocial parameters such as chronic stressors or childhood sexual abuse have been discussed as possible causes for the increasing prevalence in women (Bandelow and Domschke, 2015). Anxiety disorders, including panic disorder with or without agoraphobia, generalized anxiety disorder (GAD), social anxiety disorder (SAD), phobias and anxiety from separation are the most prevalent amongst all mental health diseases, as reported by the World Health Organization (WHO) Mental Health Survey of over 60,000 adults from 14 countries (Kessler *et al.*, 1994; Demyttenaere *et al.*, 2004).

SAD is one of the most common of all anxiety disorders, with approximately 12% high rates of lifetime prevalence as compared to other anxiety disorders (Kessler *et al.*, 2005). According to DSM-5, SAD is defined as persistent fear, anxiety or avoidance of social or performance situations typically lasting 6 or more months in which the person is exposed to unfamiliar people (American Psychiatric Association, 2013). The National Comorbidity Survey revealed data showing that SAD is the third most common psychiatric condition following major depression and alcohol dependence (Kessler *et al.*, 2005).

1.3.1.2. Depression

According to DSM-5 criteria, depression is diagnosed when five or more symptoms, which are characteristic of 1) depressed mood such as feelings of sadness, hopelessness or 2) loss of interest or pleasure including significant weight loss/gain, decrease/increase in appetite are present longer than a 2-week period. These symptoms cause significant distress or social and professional life dysfunction and, are not caused by substance use or another medical condition (American Psychiatric Association, 2013).

Major depression is a debilitating, frequently occurring disorder linked to diminished quality of life, medical comorbidity and mortality (Spijker *et al.*, 2004; Ustün *et al.*, 2004). Information about the prevalence of depression have not been gathered for most countries, however lifetime prevalence estimates of major depression ranged from 1.0% in Czech Republic to 16.9% in the US (Moussavi *et al.*, 2007). Depression has been ranked as the 4th leading cause of disability worldwide, according to the WHO (Murray and Lopez, 1996), and it is estimated that it will be the second highest mortality cause by 2020 (Murray, 1997).

The increasing number of incidents of depression and anxiety disorders caused by the pressures of modern life is a financial burden to society: mood disorders including depression and anxiety disorders estimated annual costs in the UK rise up to £16 billion and £9.8 billion, respectively (Fineberg *et al.*, 2013).

1.3.2. Aetiology of other psychopathologies characterised by social deficit

Anxiety and depression are multifactorial conditions with genetic vulnerability and environmental factors, including psychosocial or physical stress being inextricably linked in their aetiology. Anxiety and depression are often comorbid conditions, but it is uncertain how this comorbidity develops. Anhedonia seem to be a key linking factor between anxiety and depression; being the intermediate stage of devolution from anxiety into depression. According to a cross-sectional study by Winer *et al.*, (2017), individuals suffering from anxiety start losing pleasure in avoiding activities which are anxiety-driven, and this leads to development of other depressive symptoms.

1.3.2.1. Depression

Family, twin and adoption studies have indicated an important role of genetic factors in the development of major depressive syndrome (MDD) and anxiety disorders. Heritability degree of 40% to 50% was suggested by twin studies and, 2-3-fold increase in the risk of developing

MDD among first-degree relatives by family studies (Lohoff, 2010). Interestingly, genetic predisposition may be shared among mood and anxiety disorders, with the individual clinical manifestation resulting from both genetic and environmental influences (Martin *et al.*, 2009). Comprehensive researching on the genetic origins of MDD used linkage studies, which are successful in detecting genetic risk factors for rare hereditary disorders with high penetrance. Genes involved in the serotonergic system including the 5-HT transporter gene (*SLC6A4*) are candidate genes for susceptibility to depression and anxiety disorders (Southwick *et al.*, 1994; Lesch *et al.*, 1996; Lesch and Mössner, 1998; Davis *et al.*, 1999; Brady *et al.*, 2000; Furmark *et al.*, 2004; Samochowiec *et al.*, 2004; You *et al.*, 2005) and contribute to an individual's response to a traumatic event (Caspi *et al.*, 2003; Lee *et al.*, 2005), given the fact that many antidepressant medications act on these systems (Fichtner *et al.*, 1994; Caspi *et al.*, 2010; Goldman *et al.*, 2010; Uher and McGuffin, 2010). Anxiety disorders hold a genetic link for genetic variants of DA beta-hydroxylase gene (*DBH*), as this enzyme plays a role in converting DA to noradrenaline (NA) as part of catecholamine synthesis (Bloom, 1982). Additionally, the discovery of the tryptophan hydroxylase isoform, the *THP2* gene, a rate-limiting enzyme in brain 5-HT synthesis links serotonergic systems with depression (Walther and Bader, 2003; Zill *et al.*, 2004). A meta-analysis study in depression collected from 183 articles found the presence of 393 polymorphisms in 102 genes showing a potential role for variants in the genes apolipoprotein E (*APOE*), guanine nucleotide-binding protein β -3 (*GNB3*), methylene tetrahydrofolate reductase (*MTHFR*), and 5-HT transporter (*SLC6A4*) (López-León *et al.*, 2008). Genome-wide studies were not successful to find significant correlations, although 11 SNPs were associated with MDD and were localised in a region within an overlapping gene, named *piccolo* gene, a presynaptic protein important in monoaminergic neurotransmission in the brain (Psychiatric GWAS Consortium Coordinating Committee, 2009).

Genetic and environmental components seem to be crucial for the development of MDD. For instance provided the robust correlation between stressful life events and risk of developing depressive symptoms, a specific gene variant was observed in MDD patients (Kendler *et al.*, 1995; Caspi *et al.*, 2010). In particular, individuals with one or more copies of the short allele of the *5HTT* promoter variants were at high risk of developing depression depending on the occurrence of adverse life events (Caspi *et al.*, 2003). Early life experiences are known to be linked to increased risk for depression including childhood abuse (Kendler *et al.*, 2002, 2006; Widom *et al.*, 2007) or poor emotional contact with partners (Roy, 1981; Slavich *et al.*, 2011). These early life experiences increase the risk of psychiatric disorders like depression and anxiety by implicating mechanisms that include biological processes, psychological and psychosocial constructs that convert temporary traumatic experiences into chronic vulnerabilities. For example poor parental care or parental loss results in low self-esteem and emotional instability (Kendler *et al.*, 2003; Avagianou and Zafiropoulou, 2008; Parsons *et al.*, 2010). These effects might limit the ability of the individual to form close relationships and therefore, weaken the quality of social support available in later life (Schoenfelder *et al.*, 2011). Evidence-based studies indicate that distinct but overlapping components of cortico-striato-thalamic neural circuits affect regulation of depression and insecure attachment (Galynker *et al.*, 2012). The trigger for a first episode of depression is potentially a physical event such as traumatic head injury or an internal event like hormonal challenges. However, it is more common the onset of depressive episodes following external events such as bereavement (Farmer and McGuffin, 2003; Slavich *et al.*, 2010, 2011) or chronic stresses from unemployment, poverty and poor family life (Monroe and Harkness, 2005; Harkness and Monroe, 2006).

Numerous studies have found that various environmental factors contribute to depression vulnerability. These factors include *in utero* exposure to infection, cannabis use, lack of nutrients, maternal stress, perinatal complications, social disadvantage, urban upbringing,

ethnic minority status, childhood maltreatment, bullying, traumatic events, and exposure to stress (Gámez-Guadix *et al.*, 2013; Cummings *et al.*, 2014; Gullander *et al.*, 2014; Lin and Wang, 2014; Feingold *et al.*, 2015). For an inclusive review on gene-environment interactions in depression see review by Lopizzo *et al.* (2015).

Several studies have shown that early adverse environment result in epigenetic marks via DNA methylation and histone modifications changes which may play a key role in the development of depression in subjects suffering from depression (Mill and Petronis, 2007; Tsankova *et al.*, 2007). Interestingly, these marks can be observed in DNA from samples taken from peripheral tissue either blood or epithelial cells, as well as from brain tissue or neuronal cells (Klengel *et al.*, 2012; Provencal *et al.*, 2012; Suderman *et al.*, 2012). The glucocorticoid receptor gene (GR), (*NR3C1*) has been associated with early life trauma-induced changes in DNA methylation profile of the promoter of the gene that is observed in brain tissue and blood cells (McGowan *et al.*, 2009; Labonté *et al.*, 2012; Suderman *et al.*, 2012; Melas *et al.*, 2013). The monoaminergic transporter genes were also the focus of other studies and, mainly the 5-HT transporter gene *SLC6A4*. Changes in methylation in the promoter of this gene have been reported after being exposed to environmental factors and in association with depression in peripheral tissue (Alasaari *et al.*, 2012; Zhao *et al.*, 2013). This provides a mechanistic explanation underlining the 'environment x genetic' interactions in depression. In addition, the gene encoding brain-derived neurotrophic factor (*BDNF*) has been also repeatedly investigated for epigenetic changes in depression. BDNF is a member of the neurotrophin family of growth factors that regulate development, maintenance and function of vertebrate nervous systems (Huang and Reichardt, 2001). BDNF has been associated with depression and treatment response to antidepressants (Murphy *et al.*, 2013). Reduction of BDNF transcripts in peripheral blood was observed in several studies of acutely depressed patients, which was reversed after antidepressant treatment (Menke, 2013). Furthermore, a higher *BDNF* promoter methylation profile was observed from a large

sample-size cohort of 108 depressed patients with history of suicidal attempts (Kang *et al.*, 2013). Yet another study demonstrated reduction in *BDNF* promoter methylation profiles found in the peripheral blood of depressed patients (Fuchikami *et al.*, 2011). For an extensive review on epigenetics and depression see review by Menke and Binder (2014).

1.3.2.2. Anxiety

Genes found to be associated with GAD have also been associated with MDD (Roy *et al.*, 1995). There is little evidence that early-life trauma influences the risk for developing SAD in adulthood, unlike in MDD (Hettema *et al.*, 2001). The focus of this section will be on the aetiology of SAD and bullying, as the most relevant subtypes of anxiety disorders and social defeat consequences, respectively that they are discussed in the results sections.

SAD has a high degree of familial aggregation with rates of estimated heritability being between 25 to 50%, indicating that environmental factors also play an important role in its development (Kendler *et al.*, 1999). The outcomes from genome-wide linkage analysis associated the region found near the NA transporter gene, on chromosome 16 with SAD. In addition, findings have included association of low extraversion, a characteristic trait of SAD, with a functional gene variant of β_1 -adrenergic receptor (*ADRB1*) and two SNPs and a three SNP haplotype in the Catechol-O-methyltransferase (*COMT*) gene -encoding enzyme that degrade catecholamines- in female patients with SAD (reviewed in (Stein *et al.*, 2008)). SAD is associated with shyness in childhood, low extroversion and high neuroticism. Genes associated with high behavioural inhibition include corticosterone-releasing hormone (CRH) and 5HTT (reviewed in (Stein *et al.*, 2008)). Additionally, a variation in the glutamic acid decarboxylase 1 (*GAD1*) gene, which is involved in synthesis of GABA from glutamate was found to be significantly associated with internalising-neuroticism behaviour (Hettema *et al.*, 2006).

1.3.3. Neurobiology of other neuropathologies characterised by social deficit: anxiety and depression

Mood and anxiety disorders are the result of various neuroendocrine, neurotransmitter and neuroanatomical disruptions. Shared neurobiological mechanisms might shed light in the overlapping symptomatology of depression and anxiety. Anxiolytic and antidepressant drugs are well-documented to act primarily on monoaminergic systems; hence they have implicated 5-HT, NA and DA in the pathogenesis of mood and anxiety disorders. Genetic studies have widely employed products, which regulate monoaminergic signalling in the pathophysiology of mood and anxiety disorders, and they play critical role for the mechanism of action of antidepressants.

1.3.3.1. Monoamine hypothesis

The monoamine hypothesis in depression initially postulated that low levels of monoamines are likely to be observed in depressed individuals, based on findings reporting that antidepressant drugs acutely increase their levels (Hirschfeld, 2000). Monoaminergic antidepressants are however widely used for first-line medication, though they do not exert their clinical benefit immediately, and benefit only some people and not others. This hypothesis emerged after the accidental discovery of the antidepressant effects of tricyclics and monoamine oxidase inhibitors (MAOIs). The first evidence, involves two structurally unrelated compounds (iproniazid and imipramine) developed for non-psychiatric conditions that demonstrated potent antidepressant effects in humans and enhanced central 5-HT and NA transmission (White and Roos, 1959). The second important factor adding evidence to the development of this hypothesis, however a controversial one (Baumeister *et al.*, 2003) was reserpine, an antihypertensive drug acting by depleting monoamines, which caused depressive symptoms in a subset of patients (Davies and Shepherd, 1955).

In experimental studies, in which they have ingested a mixture of tryptophan-deficient amino acid that causes the transient reduction of 5-HT levels in the brain, to patients in remission and patients who are under medications at the time of study have shown that they are both likely to experience a brief relapse of symptoms during tryptophan depletion. This suggests that a lowering of serotonin levels may result in depression (Ruhé *et al.*, 2007; Rot *et al.*, 2009). However, lowering serotonin levels by tryptophan depletion does not induce depression in all individuals. It has been reported that tryptophan depletion studies with individuals with an absence of personal or family history of depression did not show any mood changes (Ruhé *et al.*, 2007). Several factors could contribute to an individual's vulnerability to the effects of modified levels of 5-HT on mood (Jans *et al.*, 2007). These suggest that the relationship between monoamines and depression is not clear, and due to the complexity of the pathophysiology of depression, this hypothesis is now regarded as too simplified to explain the pathology. However, it has led to the development of antidepressants such as selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepinephrine reuptake inhibitors (SNRIs), which are widely used.

Today's antidepressant agents are designed to increase monoamine transmission acutely (Berton and Nestler, 2006), either via inhibition of neuronal reuptake such as SSRIs or by preventing degradation like MAOIs (Trivedi *et al.*, 2006). While MAOI and SSRIs increase immediately monoamine transmission, it requires weeks of treatment to induce their mood-enhancing properties. Serotonin-Noradrenaline Reuptake Inhibitors (SNRIs) have a similar mechanism of action to SSRIs but they block selectively the reuptake of 5-HT and NA. However, they have similar efficacy and side effects as SSRIs (Lambert and Bourin, 2002). On the other hand, the primarily used antidepressants; TCAs act by blocking reuptake of NA and to a lesser extent 5-HT. TCAs are more selective than the MAOIs, but not as selective as SSRIs/SNRIs (Tatsumi *et al.*, 1997; Gillman, 2007).

Monoamines appear to be implicated in the neurobiology of SAD, however there are not many clinical-based studies to support the preclinical findings. Medications including benzodiazepines, SSRIs, SNRIs and MAOI have some efficacy in the treatment of SAD. SSRI treatment is successful in treating SAD symptoms and reversing brain abnormalities such as elevated amygdala activity (Schwarz *et al.*, 1999; Mathew and Ho, 2006) and altered dopaminergic transmission evident in the mesolimbic reward pathway (Schneier *et al.*, 2000) that suggests a monoaminergic role in the aetiology of SAD (Stein *et al.*, 2008). In addition, studies supporting disrupted monoaminergic signalling in patients with SAD have demonstrated reduced 5HT_{1A} receptor binding in several brain regions such as the amygdala, nucleus accumbens core, insula and dorsal raphe nucleus, which suggests a 5-HT_{1A} disruption in the aetiology of SAD. It has been also reported that DA plays a role in the aetiology of SAD. In fact, neuroimaging analyses have revealed reduction in both the density of the DA transporter and binding capacity for the D₂R (as reviewed in (Engel *et al.*, 2009)). Interestingly, patients with Parkinson's disease have high rates of comorbid SAD, reinforcing a role of DA in SAD (reviewed in (Stein *et al.*, 2008)), however this co-morbidity could be caused by psychological factors such as insecurity due to display of the physical symptoms instead of a common aetiology of DA dysregulation.

There are several proteins that regulate monoaminergic signalling and seem to be involved in the pathophysiology of mood and anxiety disorders. These are monoaminergic regulators including transmitter receptors; vesicular monoamine transporter (vMAT), which packages the AVP and OXT neurotransmitters into vesicles (Egashira *et al.*, 2007). These neurotransmitters are then released in the CNS to regulate several physiologies such as stress and affiliative behaviours. Additionally, there are transmitter-specific reuptake transporters such as the 5-HT transporter (SERT) and DA transporter, the enzyme monoamine oxidase and the COMT enzyme (reviewed in (Martin *et al.*, 2009)).

1.3.3.2. The neuroendocrine hypothesis of depression

The HPA axis is importantly involved in the pathophysiology of depression. The HPA axis activity is regulated by the secretion of CRH and AVP from the hypothalamus, which then activates the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary and finally stimulates the secretion of the glucocorticoids (GC) (cortisol in humans and corticosterone in rodents) from the adrenal cortex. Activation of the HPA axis is self-limiting due to the inhibitory effects of GC on the stress response. The activated HPA axis regulates CNS as well as body peripheral functions (Fernández-Guasti *et al.*, 2012). Sustained stimulation of the HPA axis due to chronic stress or through dysregulation of GC-mediated negative feedback has been associated with mood disorders such as depression (McEwen, 2005). It is worth mentioning that Hans Selye was the first to classify the steroid hormones, including GC by performing extensive structure-activity studies in the 1930s-1940s and his preclinical work led to recognition of the "general adaptation syndrome", which was later called "stress response" characterised by physiological changes (Selye, 1936, 1941).

It has been reported that elevated levels of cortisol in saliva, plasma and urine were found in depressed patients as well as increased size and overactivity of the pituitary and adrenal glands (as reviewed in (Pariante and Lightman, 2008)). This increased activity of the HPA axis reported in depression is thought to be partially due to reduced feedback inhibition by endogenous GC. Through binding to the GC receptor (GR) and the mineralocorticoid receptor located in the HPA axis, the endogenous GCs serve as potent negative regulators of HPA axis activity (Pariante, 2006).

Several triggers such as early stressful life events may cause changes in the stress responses and thus of the HPA axis, which can continue during adulthood, predisposing individuals to develop psychopathology of anxiety and depressive disorders. In particular, increased plasmatic ACTH and cortisol levels have been observed during behavioural

despair in neonate non-human primates when separated from the mother (Levine *et al.*, 1985; Hennessy and Moorman, 1989). Furthermore, direct injection to the cerebral nervous system of CRH in laboratory animals produced effects of reminiscent of stress, depression, fear and anxiety via activation of specific brain regions (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Weiss *et al.*, 1994; Heim and Nemeroff, 2001).

Clinical observations from 90 subjects exposed to early parental loss in childhood reported higher cortisol and ACTH levels in plasma from subjects who were diagnosed with a psychiatric condition as compared to those who did not receive diagnosis (Breier *et al.*, 1988). In addition, observations from children who experienced permanent or long-term separations from parents, or parental death demonstrated hyperactivity of the HPA axis accompanied with increased basal salivary cortisol concentrations (Flinn *et al.*, 1996; Pfeffer *et al.*, 2007), cortisol non-suppression after the dexamethasone suppression test (Weller *et al.*, 1990), and from the combined Dex/CRH test (Tyrka *et al.*, 2008) further indicating that early life stress contributes to dysregulation of the HPA axis.

In addition, hyper-responsiveness of the HPA axis has been reported in patients with SAD, mainly during a psychosocial stressor which increased plasma cortisol (Furlan *et al.*, 2001; Condren *et al.*, 2002; Van West *et al.*, 2008; Roelofs *et al.*, 2009), while findings at baseline are similar to those of controls (Condren *et al.*, 2002; Roelofs *et al.*, 2009). Collectively these data support the notion that increase hypersensitivity of the HPA axis predisposes to depression and anxiety psychopathologies. For more information on the pathophysiology of depression see review by Hasler (2010), and by Krishnan and Nestler (2008). Also for the neurobiology of anxiety disorders see reviews by Martin *et al.* (2009) and Faravelli *et al.* (2012).

1.3.4. Anxiety and depression association with social defeat consequences

Psychiatric disorders like depression (Fava, 1998; Mammen *et al.*, 2002) and anxiety disorders (Eronen *et al.*, 1998) are associated with severe emotional disturbances as well as with impaired social functioning including impairment on social cognition and withdrawal as well as excessive aggression (reviewed in Kohn and Asnis, (2003; Swann, (2003; Neumann *et al.*, (2010)). This is biologically explained as there is high interconnection between neural circuits regulating social behaviours and emotions. In fact, social deficits causing excessive aggression might arise as a consequence of faulty emotional regulation (Davidson *et al.*, 2000). As anxiety and depressive behaviour were investigated as part of this thesis in relation to ASD and the neurobiology underlining social defeat, we will focus this section on understanding the association between emotional impairment and social deficit.

1.3.4.1. Definition and symptomology

Social defeat refers to stressor generated by continuous loss of social encounters among conspecific animals, or hostility behaviour among humans, potentially causing significant psychological and functional consequences in respect to control over resources, and hierarchical positions (Björkqvist, 2001). Hierarchical and territorial behaviour is prevalent in animals and humans, however these behaviours are not included in DSM1-5 (Rohde *et al.*, 2001). Living in a group, has both advantages and disadvantages, and while resources are not infinite in any ecosystem, distribution of access to such resources, and to peers is inevitably uneven among individuals belonging to a social group (Alexander, 1974; De Waal, 2000).

In humans, social defeat is most often associated with bullying in schools and in workplaces. The psychological distress resulting from bullying, both in children and adults has been associated with various negative health effects. Studies conducted on this topic as a branch of social psychology, focus on the concomitant psychosocial effects of social defeat

including depression, anxiety, loss of self-esteem, social phobia, sleeping problems, irritability and lack of concentration (Björkqvist, 2001; Arseneault *et al.*, 2010). Also, several studies have reported the association between bullying and symptoms of somatic effects like muscle-skeletal pain fatigue and GI symptoms (Bowling and Beehr, 2006; Nielsen *et al.*, 2012).

Experimental rodents like the social defeat mouse model are utilised and extended to include observations of human behaviour such as aggression and bullying in the social context (Björkqvist, 2001; Rohde *et al.*, 2001). Bullying is a subcategory of aggressive behaviour. It refers to a long-lasting and systematic form of interpersonal aggression and even violent behaviour by superiors, co-workers or other students directed against an individual over some period of time, and the target finds it difficult to defend themselves (Olweus, 1993; Einarsen and Skogstad, 1996). The literature identifies several forms of it such as physical, verbal, relational bullying and cyberbullying (Elinoff *et al.*, 2004; Stassen Berger, 2007). Bullying behaviour involves repeated exposure to aggression in forms of verbal hostility or threats, teasing, physical assaults and social exclusion (Einarsen, 2000; Solberg and Olweus, 2003; Juvonen and Graham, 2014). Bullying is a significant social stressor for many adults and children with estimated prevalence rate of 32% in schools (Nielsen *et al.*, 2015) and 15% in workplaces (Nielsen *et al.*, 2010). The high prevalence of bullying in the society is an indicator for representing the extent of the problem that further underlines major socioeconomic implications.

1.3.4.2. Neurobiology of social defeat consequences

Social defeat is a source of chronic stress in both animals and humans as addressed before that can cause behavioural, physiological and endocrine changes, and these changes can affect normal brain functioning and, overall the health of individuals (Björkqvist, 2001; Rohde, 2001; Allen and Badcock, 2003). As previously discussed in section 1.3.3,

monoamine theory depletion and HPA axis dysregulation have been majorly implicated in the pathogenesis of MDD and, subsequently in social defeat in humans. There is emerging evidence linking nicotinic acetylcholine receptors (nAChRs) with depression and, exposure to nicotine by tobacco smoking with depression. Certain nAChRs subtypes have been proposed for the treatment of depression. Yet, it remains unknown the neurobiological basis that connects nicotine with depression. In the present thesis, we will focus on investigating the role of DA and nAChR subtypes in the context of social defeat. For more information about the relationship between depression and nicotine, see the introduction section of chapter 4.

1.3.4.3. Social defeat as an animal model of depression

There are several animal models of anxiety, depression with different face, construct and predictive validity (for more information see review by Miczek *et al.* (2008)). As chapter 4 of this thesis will focus on the social defeat paradigm as a reliable model of depression induced by chronic psychosocial stress exposure, we will concentrate the general introduction on this paradigm alone. Social defeat model provides ethologic and ecologic validity, as it uses social conflict via repeated exposure to failed social encounters without prior habituation, in order to generate emotional and psychological stress (Tidey and Miczek, 1997). Social defeat induces a number of physiologic changes including increased heart rate, elevated blood pressure, and increased ACTH and corticosterone levels (Tornatzky and Miczek, 1993). Consistent with known biological consequences observed in depression (section 1.3.1), rodents that have undergone repeated exposure to social defeat display severe neurobiological modifications. These include morphological changes such as reduction in volume and cell proliferation in the hippocampus and the medial prefrontal cortex (Czeh *et al.*, 2001, 2007; Becker *et al.*, 2008; Van Bokhoven *et al.*, 2011) as well as HPA axis dysregulation evident by elevated corticosterone levels (Razzoli *et al.*, 2006; Buwalda *et al.*,

2011). Thus, face validity is provided in this model, as it mimics the symptomatology of stress-related disorders including depression in humans (Martinez *et al.*, 1998; Avgustinovich *et al.*, 2005). The development of social defeat in mice as well as employing of the social interaction paradigm has demonstrated that socially defeated mice display a long-lasting social avoidance, which is reversible after chronic, but not acute treatment with antidepressants (Berton and Nestler, 2006; Tsankova *et al.*, 2006), providing also predictive validity in this model of depression. Several therapeutic targets have been identified in social avoidance behaviour, and nAChRs seem to be among the most promising. For detailed information on the relationship between nAChRs and depression by utilising a social defeat paradigm, see chapter 4.

1.4. Focus of the thesis

This thesis focuses on understanding the mechanisms underlining the neurodevelopmental condition of ASD and also, on assessing the efficacy and neurobiological consequences of chronic OXT administration, as potential pharmacotherapy for the treatment of ASD. In addition, we have investigated the involvement of specific CNS targets in the neurobiology of social defeat which is a common psychopathology characterised by impairment in the social domain. Several neuronal systems have been implicated in the neuropathology of ASD, however, the underlining neurobiology remains unclear. As a result, more in depth understanding of the neurobiology underlining ASD is needed which will aid the development of pharmacological targets for its effective treatment. To this end we assessed the endogenous opioid system in a well-established model of ASD, the *Fmr1* KO as there is emerging clinical and preclinical evidence pointing towards a dysregulation of this system in ASD (evidence discussed extensively in chapter 2).

Pharmacotherapy is currently not available for the treatment of ASD but symptomatic management is available using pharmacologic agents for relieving some of the symptoms,

as discussed above. Emerging preclinical and clinical evidence have been supporting the role of intranasal OXT, as a novel therapeutic target in ASD (for extensive review of the evidence see chapter 3). Nonetheless, most studies focus on the acute effect of the drug and, the long-term effect of OXT exposure on related ASD-like symptomatology and brain neurochemistry is currently unknown. Long-term exposure of drugs often triggers compensatory alterations to CNS systems which may alter behaviour. Hence, we further investigated the behavioural and neurobiological effects of acute and chronic intranasal OXT administration in the *Fmr1* KO mouse and in WT animals, to assess the efficacy of OXT in mice with autistic-like phenotype as well as the effect of OXT in WT healthy mice. We carried out extensive behavioural, gene transcriptional and receptor autoradiographic binding studies in order to assess long term effects of OXT on this ASD model and WT animals. This is the first study with chronic OXT administration undertaken in the *Fmr1* KO mice, providing deeper understanding of long-lasting effects of OXT in the treatment of ASD.

Social defeat is a common psychopathology characterised by impairment in the social domain and is emerging as a more reliable and translationally relevant model of depression and social anxiety. Both social anxiety and depression often co-occur with the core symptoms of ASD. Although a lot is known about the neurobiology underpinnings of depression, pharmacotherapy available to treat this condition is often ineffective and problematic. Therefore, better understanding of the neurobiology underlining social defeat is needed in order to develop more effective targeted pharmacotherapy for the successful treatment of the condition. Emerging evidence support a role of nAChRs and DA receptors in social anxiety and depression (for extensive review on the evidence linking those neurotransmitter systems with depression, see introduction section of chapter 4). To this end, we investigated $\alpha_4\beta_2^*$, α_7^* nAChRs and the D₂R receptor binding levels in the brains of mice exposed to chronic defeat stress by autoradiographic binding. This study will contribute to shed light in the potential involvement of nAChRs and D₂Rs in the neurobiological

mechanisms underlining social defeat which point towards therapeutic interventions for this condition.

1.5. Aims and Hypotheses

The present thesis aims to shed light in neurobiological alterations that might occur at transcripts and receptor binding levels in animal models characterised by impairment in the social domain including ASD and depressive-like behaviour. In particular, the studies presented in this thesis assess specifically key central systems such as the oxytocinergic, the opioid, dopaminergic and the nACHR system in the neurobiological underpinnings of these pathologies as assessed in an ASD mouse model (*Fmr1* KO) and a social defeat mouse model, respectively. In addition, efficacy of intranasal OXT pharmacotherapy as well as the consequences on brain neurochemistry at mRNA and receptor binding level following the treatment are assessed in an ASD animal model. The specific objectives for each chapter are as follows:

Chapter 2: The involvement of the endogenous opioid system in the neurobiology of autism

- a) To assess levels of μ -opioid receptor (MOR), δ -opioid receptor (DOR) and κ -opioid receptor (KOR) receptors binding in the brains of well validated mouse model of autism, lacking the *Fmr1* gene by using quantitative autoradiography in the brains of WT and *Fmr1* KO mice. A global decrease of MOR, DOR and KOR binding levels, and most predominantly the MOR, is hypothesized to be observed in the brains of the *Fmr1* KO mice.
- b) To examine mRNA transcript levels of the genes of the endogenous opioid system (receptors and peptides) by performing quantitative Real Time PCR (qRT-PCR) in *Fmr1* KO mice in brain regions that play major role in reward and social behaviour such as the nucleus accumbens (NAc), caudate putamen (CPu), ventral tegmental

area (VTA), emotion processing such as the central amygdala (CeA) and cognition, the prefrontal cortex (PFC). It is hypothesized that the mRNA levels of the endogenous opioid ligands (prodynorphin and proenkephalin) and receptors (DOR, KOR, MOR) will be found down-regulated in the brains of the *Fmr1* KO mice.

- c) To assess levels of mRNA transcripts of genes involved in the modulation of glutamate and dopamine neurotransmission as well as the neuropeptides, oxytocin and vasopressin, which are all known to be involved in the ASD pathology, in *Fmr1* KO mice by utilising additional qRT-PCR experiments across the five aforementioned brain regions. It is hypothesized that transcripts of these genes will be also altered in the brains of the *Fmr1* KO mice as compared with WT controls.

Chapter 3: Effects of chronic intranasal oxytocin treatment on social behaviour, gene expression and receptor binding in an animal model of autism

- a) To assess the therapeutic potential of chronic intranasal administration of OXT in ASD by examining the effect of acute and chronic intranasal administration of OXT on the autistic-like behavioural phenotype in *Fmr1* KO mice including sociability, repetitive behaviour, anxiety and memory. A battery of behavioural paradigms including social interaction test, three-chamber sociability, motor stereotypies, novelty suppressed feeding, marble burying and novel object recognition were used to assess this objective. Intranasal OXT administration is hypothesised to improve behaviours, which are impaired in the *Fmr1* KO mice, acutely and chronically including social interactions, stereotypical behaviour, cognition and eliminate anxiety.

- b) To investigate the pharmacological effect of chronic intranasal administration of OXT, on OXTR, NMDAR and mGluR5, which are all known to be involved in ASD neurobiology, in the *Fmr1* model of ASD. Quantitative autoradiography was carried out to tackle this objective. Our hypothesis is that intranasal OXT administration in the *Fmr1* KO mice will induce increase in OXTR, and decrease in mGlu5R and NMDAR binding levels in the brains of the mutants.
- c) To investigate the expression of mRNA transcripts of several systems involved in the neurobiology of ASD including the endogenous opioid system, glutamatergic, OXTergic in *Fmr1* and WT mice chronically treated with OXT. qRT-PCR across two brain regions crucial in reward/motion and emotion processes; the CPu and the CeA was performed. It is hypothesized that intranasal OXT administration in the *Fmr1* KO mice will alter transcript levels of the endogenous opioid system, as well as the OXTergic, glutamate and dopaminergic systems, which are implicated in the etiopathology of ASD.

Chapter 4: Effect of chronic social defeat stress on nAChRs and D2 receptors: An autoradiography study in mice

To determine the effects of chronic social defeat exposure on 'healthy' mouse brain nicotinic acetylcholine receptors (AChRs) and dopamine receptor (D₂R subtype) systems with quantitative autoradiography in brains of socially defeated mice. It is hypothesized that chronic social defeat exposure will induce decrease in the nAChRs and D₂R binding levels in the brains of WT naïve mice.

2. Chapter 2: The involvement of the endogenous opioid system in the neurobiology of autism

2.1. Introduction

2.1.1. Pharmacology of the opioids

Exogenous opioids are a class of drugs derived from the opium poppy, where the biologically active alkaloid, morphine, is abundantly found. Opiates, namely the synthetic opioid drugs such as morphine, codeine or the semi-synthetic opiate heroin, act on three opioid receptor subtypes; the μ -opioid receptor (MOR), δ -opioid receptor (DOR) and κ -opioid receptor (KOR) (Martin *et al.*, 1976; Lord *et al.*, 1977). MOR, DOR and KOR are encoded by the *Oprm1*, *Oprd1*, and *Oprk1* genes respectively. The opioids neuropeptides are processed from large precursor proteins, encoded by *proenkephalin* (*PENK*), *prodynorphin* (*PDYN*) and *pro-opiomelanocortin* (*POMC*) genes. Opioid receptors are activated by endogenous opioid peptides, including β -endorphins which are selective for MOR, enkephalins which act on both MOR and DOR, and dynorphins acting on KOR.

The opioid receptors are G-protein-coupled receptors (GPCRs). The opioid peptides share a common NH₂-terminal sequence (Tyr-Gly-Gly-Phe) that allows them to bind to opioid receptors (Bradbury *et al.*, 1976). Opioid receptors are coupled to inhibitory G protein (G_i) and receptor activation thereby decreasing the intracellular cyclic adenosine monophosphate (cAMP) by the inhibition of the adenylate cyclase. Opioid receptors canonically couple to Kir3 and Ca²⁺ channels via G_{βγ} pathways. Activation of opioid receptor leads to an increase of intracellular potassium by activation of the Kir3 channel and blockade of the Ca²⁺ current sensitive P/Q-type, N-type and L-type channels (Diaz *et al.*, 1995; Torrecilla *et al.*, 2002) leading to inhibition of neuronal activity and/or suppression of neurotransmitter release.

Opioid receptors and endogenous opioid peptides are broadly expressed throughout the CNS and the periphery (Le Merrer *et al.*, 2009). The distribution pattern of opioid receptor expression in the brain is relatively similar for the MOR and KOR, yet more distinct for DOR. MOR in mice, is mostly expressed in the amygdala (AMG), excluding the central nucleus of

amygdala, thalamus (Th), mesencephalon, caudate putamen (CPu), nucleus accumbens (NAc) and some brain stem nuclei (Le Merrer *et al.* 2009). DOR is highly expressed in the olfactory tract, the cortex including neocortex, amygdala and the striatum (Pellissier *et al.* 2016). KOR is widely distributed in the basal anterior forebrain including endopiriform cortex (Den) and claustrum, striatum (CPu and NAc), olfactory tubercle, hypothalamus (Hyp), preoptic area and pituitary (Kitchen *et al.*, 1997). For extensive reviews on opioid pharmacology see reviews from Le Merrer *et al.* (2009) and Al-Hasani and Bruchas (2011).

Opioids have been traditionally used in the clinic for their analgesic effects. However, repeated use and abuse can result in the development of tolerance to certain effects of opioids, thus discontinuation of the use of opioids will be necessary. Another phenomenon that can be developed during repeated treatment with opioids is physical dependence and withdrawal symptoms (Al-Hasani and Bruchas, 2011). Therefore, opioids are prime targets for pain management yet can induce serious adverse effects. Opioids are also used for a number of other therapeutic indications including modulation of self-administration of drugs of abuse, brain stimulation reward and social behaviour (Panksepp *et al.*, 1980; Kreek, 1996; Van Ree *et al.*, 2000). Evidence has been presented that endogenous opioids play a role in the development of neurodevelopmental diseases such as autism, and this will be the key objective for appraisal of this chapter.

2.1.2. The concept of the ‘opioid theory’ in autism

The opioid hypothesis in autism was first described by Panksepp in 1979 as an emotional disorder characterised by excessive endogenous opioid activity (Panksepp, 1979; Leboyer *et al.*, 1994, 1999). Ever since it has been associated with elevated levels of the endogenous opioid peptide, β -endorphin, which was detected in plasma of autistic infants (Leboyer *et al.*, 1994). Interestingly, Panksepp formulated the idea that Kalat first tentatively suggested (Kalat, 1978) that the symptoms observed upon opiate withdrawal in dependent individuals,

i.e. symptoms of emotional lability, stereotypy, reduced socialization, correspond closely to those observed in autistic children (Panksepp *et al.*, 1978; Panksepp, 1979). Based on this, he further suggested that therapy with opioid antagonists will be beneficial for the treatment of the autistic syndrome (Panksepp, 1979). In line with the opioid hyperactivity theory, clinical findings have demonstrated an increased concentration of the endogenous opioid neuropeptide, β -endorphin, in plasma (Leboyer *et al.*, 1994, 1999; Bouvard *et al.*, 1995; Cazzullo *et al.*, 1999; Tordjman *et al.*, 2009) cerebrospinal fluid (Gillberg *et al.*, 1985; Ross *et al.*, 1987) and urine samples (Sokolov *et al.*, 2014) from autistic patients. Contradictory findings, however reveal decreased levels of opioid peptides in plasma (Weizman *et al.*, 1984, 1988) and cerebrospinal fluid (Gillberg *et al.*, 1990) of individuals with ASD. The contradictory findings support a dual directional approach of the opioid hypothesis in autism, which further suggests that different types of ASD have different aetiology. Collectively, these data together with the heterogeneity that characterises the nature of the pathology in autism, limit possibilities for therapeutic approaches to succeed, and subsequently do not reinforce the opioid theory in autism.

Numerous studies have examined the effects of opioid antagonists on individuals with ASD. Naloxone acts as a nonspecific opioid antagonist and naltrexone as MOR-selective and KOR-selective antagonist, and to a lesser extent DOR-selective (Emmerson *et al.*, 1994; Weerts *et al.*, 2008). Several clinical studies using naltrexone showed a modest effect on core symptoms of autism including improved communication and sociability, and reduced stereotypies (Leboyer *et al.*, 1988; Campbell *et al.*, 1989; Walters *et al.*, 1990; Panksepp and Lensing, 1991; Leboyer *et al.*, 1992; Bouvard *et al.*, 1995). Beneficial effects on secondary symptoms of autism have also been reported such as reduction in self-injurious behaviour, hyperactivity and impulsive behaviour (Campbell *et al.*, 1993; Aman and Langworthy, 2000; Sandman and Kemp, 2011). Together these findings highlight naltrexone as a promising agent for treating symptoms of autism.

Pellissier *et al.*, 2017 attempted to provide an explanation of the contradictory findings, which support that ASD is similar to opiate withdrawal and yet opiate antagonists are promising drugs for the pathology. This research group has presented a model of MOR balance and social behaviour, which suggests that there is an optimal window for adaptive, normal social function in between social withdrawal and social indifference behaviour. These behaviours are suggested to be dependent on the MOR balance and social aversion sociability. Therefore, deficient and excessive MOR function negatively influences social behaviour through an inverted -U relationship. This relationship can be readjusted by blockade of MOR stimulation (antagonists) or MOR stimulation (agonists) in the presence of low opioid tone in order to restore normal social behaviour (Pellissier *et al.*, 2017).

In contrast with the opioid excess theory, preclinical studies on the *Oprm1* gene reveal a strong association between the expression of this gene and autism. In particular, MOR gene knock-out mouse (*Oprm1*^{-/-}) is a well characterised monogenic model of autism (Oddi *et al.*, 2013; Becker *et al.*, 2014) displaying core symptoms of ASD including social interaction deficits, motor stereotypies. This model also displays comorbid symptoms such as aggressiveness, increased anxiety and susceptibility to seizures (Becker *et al.*, 2014), which altogether suggest an important role for MOR in the ASD pathology. In support of this, clinical studies also demonstrated a genetic link between opioids and autism such as mutations detected on the MOR (*OPRM1*) gene. Maternal origin or *de novo* mutations of deleterious or translocation events on the *OPRM1* gene (6q25-q27 loci) have been detected in low functioning ASD subjects (Meng *et al.*, 1992; Halgren *et al.*, 2012; Battaglia *et al.*, 2013).

2.1.3. Role of the endogenous opioid system in social reward

Social rewards are associated with positive feelings of self-worth, self-importance and they are reinforced via the establishment of social interactions (Hill, 1987). Preclinical evidence

from Panksepp *et al.* (1978) and clinical work from Liebowitz (1983) displayed similarities between drug addiction and social attachment by proposing for the first time that social and affiliative behaviours are associated with the endogenous opioid system (Machin and Dunbar, 2011). It is supported that during positive periods of social interactions, reward mediated effects might be regulated by the release of endogenous opioids (Herman and Panksepp, 1978). During negative stimulus such as social isolation, motivation for social contact is increased as a result of decrease in endogenous opioid release (Panksepp *et al.*, 1978; Knowles *et al.*, 1989; Kalin *et al.*, 1995). It is proposed that brain regions evolved in certain ways to modulate pain, either physical or emotional, as well as reward pathways (Panksepp *et al.*, 1997; Machin and Dunbar, 2011), which underscores the existence of common brain structures involved in these processes.

Social motivation deficits are present in most individuals with autism. Chevallier and colleagues (2012) have proposed the theory of social motivation in the pathology of autism. This theory posits that lack of understanding of the social cues in the early life of non-typically developed children diminishes the need for social interaction-seeking. As a consequence, deficit in perceiving the reward value develops, which further leads to deprivation of social inputs and learning opportunities for those children, and ultimately leads to cognitive dysfunction (Chevallier *et al.*, 2012). Social interactions activate brain regions within the cortico-basal ganglia circuit that induce feelings of pleasure and euphoria (Haber and Knutson, 2010; Kawamichi *et al.*, 2016). In particular, social interaction and social interaction-seeking activate cortical regions such as the orbitofrontal cortex (OFC), the medial prefrontal cortex (mPFC) and striatal regions like the nucleus accumbens (NAc) and the caudate putamen (CPu) in humans (Haber *et al.*, 2006; Haber and Knutson, 2010; Kawamichi *et al.*, 2016). Interestingly, DA neurons within the ventral tegmental area (VTA) and the dorsal raphe nucleus drive motivation for social interactions in rodents (Tsai *et al.*, 2009; Witten *et al.*, 2011; Gunaydin *et al.*, 2014; Matthews *et al.*, 2016). Social play, which has high rewarding properties, is a highly conserved behaviour among young mammals

(Vanderschuren *et al.*, 2016), thus studies exploring this behaviour will also be addressed in this section.

KOR anatomical distribution in the brain suggests a critical role for the endogenous KOR agonists in reward processes. KOR transcripts are abundantly detected in the VTA, substantia nigra (SN), NAc, CPu, Den, Hyp and the AMG of the rat (Meng *et al.*, 1993), as well as in the human brain (Simonin *et al.*, 1995; Zhu *et al.*, 1995). KOR and their dynorphin-like peptides play a major role in regulating the brain reward system by modulating DA release in the striatum (Di Chiara and Imperato, 1988; Maisonneuve *et al.*, 1994). In particular, dynorphin-like peptides inhibit DA release in the striatum and may thus, contribute to a negative feedback regulation of DA release (Bruijnzeel, 2009). Additionally, KOR antagonists stimulate DA release in the striatum and have anti-rewarding effects (You *et al.*, 1999; Pliakas *et al.*, 2001; Beardsley *et al.*, 2005). Immunohistochemistry studies suggested that KOR are produced in the VTA and the SN and, are further transported to the NAc and CPu, where they control presynaptically the release of DA (Mansour *et al.*, 1995; Britt and McGehee, 2008). Also, intracranial self-stimulation studies have shown that KOR agonists elevate reward thresholds in the rat brain (Todtenkopf *et al.*, 2004; Carlezon *et al.*, 2006; Tomasiewicz *et al.*, 2008), which further indicates that KOR agonists decrease sensitivity to rewarding electrical stimuli, thus inducing a negative mood state. Collectively the above strongly suggest a role of the KOR in reward function with KOR being considered as major anti-reward system (Wee and Koob, 2010; Lutz and Kieffer, 2013).

DORs are mostly known for their association with anti-anxiety and anti-depressive like properties, and they are less known for their rewarding properties (Lutz and Kieffer, 2013). However, DORs are abundantly found in brain regions modulating motivation and reward (mPFC, NAc, ventral pallidum (VP), VTA), and high levels of *Oprd1* transcripts are found in striatal regions, which suggests that DORs play a role in these processes. In particular, DOR blockade increases food reward in rodents, which points towards an inhibitory role of DOR on food reward (Pellissier *et al.*, 2016). Interestingly, pharmacological data have suggested

that reward processes are mediated by overlapping roles of MORs and DORs (Hutcheson *et al.*, 2001; Le Merrer *et al.*, 2009). Major evidence from use of DOR agonists shows increase in condition place preference (CPP) (conditioned measurement of reward) and in consumption of palatable substances, whilst their respective antagonists induce reduction in CPP in response to several drugs of abuse such as morphine. In fact, DOR ligands might produce some of their effects via activation of MORs (see review from Pellissier *et al.*, 2016). Interestingly, DOR agonists administered either directly into the NAc or systemically had no effects on social play (Vanderschuren *et al.*, 1995a; Trezza *et al.*, 2011) suggesting that rewarding effects via social play are not DOR-mediated. Taken together, the data suggest that DOR play a role in modifying reward processes.

MOR has been shown to play a key role in social play behaviour. Several studies have shown that systemic administration of MOR agonists under negative social conditions, such as separation from mother, social isolation and defeat, is sufficient to increase motivation for social play in juvenile rats (Loseth *et al.*, 2014; Pellissier *et al.*, 2017), further supporting that exposure to negative stimulation within the context of social behaviour increases motivation for social contact. Interestingly, MOR agonists directly administered into the NAc were able to enhance social play, whereas both MOR antagonists and KOR agonists decreased social play (Vanderschuren *et al.*, 1995a, 1995b; Trezza *et al.*, 2011). MOR and KOR seem to have opposite effects in regulating motivational processes, possibly by modulating common neuronal pathways; presumably via the mesolimbic pathway. In particular, systemic administration of the MOR agonist, morphine, induced CPP reflecting its reinforcing properties. In contrast, KOR agonist, U69593, induced condition place aversion (CPA) in a Pavlovian conditioning paradigm, supporting the anti-reward mechanism of this receptor (Shippenberg and Herz, 1986). KOR modulates social behaviour and comorbid depressive-like behaviours (Lutz and Kieffer, 2013). In fact, KOR signalling in the NAc mediates social rejection, while maintaining pair bonding in the monogamous prairie voles (Resendez *et al.*, 2012) and KOR antagonist seems to prevent social defeat in rats (McLaughlin *et al.*, 2006).

Pharmacological manipulations of MOR neurotransmission with naltrexone treatment in mouse pups demonstrated no preference for socially rewarding environment in these animals (Cinque *et al.*, 2012) supporting impairment in the social reward naturally obtained from social contact and affiliations. Genetic permanent disruption of MOR neurotransmission by employing *Oprm1*^{-/-} mice have demonstrated social deficits in this model, including reduced maternal attachment in mouse pups, reduced response to female vocalisation in adult males and modified social reward in juvenile mice (Moles *et al.*, 2004; Wohr *et al.*, 2011; Cinque *et al.*, 2012). In particular, *Oprm1*^{-/-} mice showed reduced interests in peers, which suggested impairment in social reward from social contact and affiliations (Cinque *et al.*, 2012). These strongly suggest that lack of MOR inhibits the establishment of experiencing reward via affiliative relationships. In addition, Becker *et al.* (2014), concluded that *Oprm1*^{-/-} mice recapitulate autistic-like syndrome symptomatology. In humans, adult carriers of a gain in function mutation (Sia *et al.*, 2008) on the A118G SNP of the *OPRM1* gene demonstrate increased capacity for experiencing social reward and neural sensitivity to social rejection (Way *et al.*, 2009; Troisi *et al.*, 2011), which further suggests that MOR can be modulating social reward in humans. In line with this, mice with the equivalent SNP, the A118G *OPRM1* knock-in (humanized), display motivation for social interaction and resilience to social defeat (Pellissier *et al.*, 2017). For an informative review on the involvement of MOR in social behaviour and reward, see updated review by Pellissier *et al.* (2017).

Taken altogether, a strong association between the endogenous opioid system in social reward processes is supported, yet more research in the field is warranted. The key objective of this chapter is furthering our understanding on the endogenous opioid system in ASD. For this purpose, we were challenged to select a translationally reliable animal model of ASD, and we anticipated that the *Fmr1* KO mouse was suitable.

2.1.4. *Fmr1* null mice as a model of autism spectrum disorders

2.1.4.1. Fragile X mental retardation disorder

FXS is the most common monogenic cause of autism and one of the most prevalent inherited forms of intellectual disability affecting approximately 1 in 4,000 men and 1 in 8,000 women (Peng *et al.*, 2004). It is an X-linked dominant disorder that was first identified in two brothers in 1969 and it was associated with the fragile X site 27.3 position of the chromosome (Xq27.3) (Lubs, 1969). FXS is caused by an insertion mutation of more than 200 cytosine-guanine-guanine (CGG) triplet repeats in the 5' untranslated region of the Fragile X mental retardation (*FMR1*) gene combined with extensive methylation of the *FMR1* promoter that contains the repeat sequence and upstream the CpG islands, leading to transcriptional silencing of *FMR1* and a partial (premutation) or full mutation of the Fragile X mental retardation (FMRP) protein (Sutcliffe *et al.*, 1992). Premutation has a length of 50-200 repeats and it is associated with excess production of *FMR1* mRNA levels but below normal FMRP protein synthesis (Tassone *et al.*, 2000a; Tassone *et al.*, 2000b) (Figure 2.1). There is no cure available for the FXS condition, current treatment is symptomatic. Two approaches have been investigated for FXS treatment; i) reactivating the *FMR1* gene and ii) compensating for the FMRP protein lack, with the latter approach in combination with mGluR5 antagonists (Pop *et al.*, 2014) having failed in clinical trial.

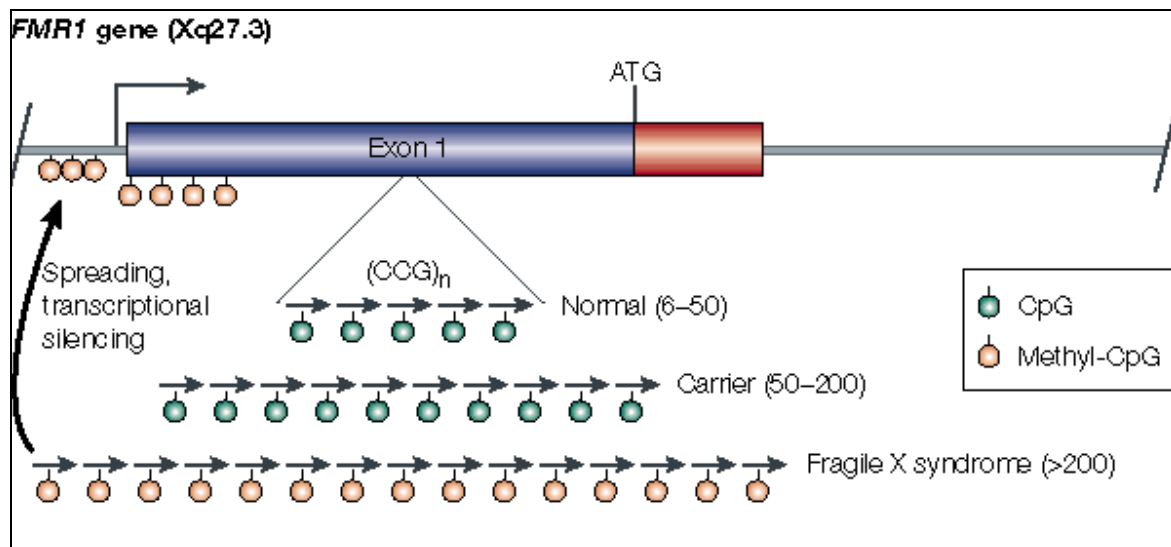


Figure 2.1: Schematic representation of the *FMR1* gene illustrating the location of the CCG triplet repeats within the 5'-untranslated region of exon 1 of chromosome 27. Adapted from Robertson and Wolfe (2000).

FXS is typically associated with symptoms of developmental delay in men (Jacquemont *et al.*, 2014). Early life hypotonia is often present and progresses to coordination difficulties during adulthood. Physical characteristics of the FXS involve macroorchidism in the majority of affected men and unusual craniofacial features such as long face, prominent jaw, forehead and ears, high arched palate and midfacial hypoplasia (Butler *et al.*, 1988; Lachiewicz and Dawson, 1994; Hagerman and Hagerman, 2002). Presence of hyperextensive joints, soft redundant skin on the palms, flat feet as results of the presence of loose connective tissue have been also identified (Davids *et al.*, 1990). Behavioural abnormalities associated with this disorder include attention deficits, irritability, a range of anxiety symptoms, increased susceptibility to seizures, hyperarousal and autistic-like traits such as social deficits, stereotypical behaviour and communication abnormalities (Garber *et al.*, 2008; Jacquemont *et al.*, 2014). Levels of severity of the symptoms in men carrying a full or premutation has been positively correlated with the percent of unmethylated *FMR1* alleles and FMRP levels (Gross *et al.*, 2015), while severity of cognitive impairment in females carrying a full mutation has been inversely related to the activation ratio of the normal *FMR1*

allele and the FMRP expression levels (De Vries *et al.*, 1996; Loesch *et al.*, 2004). Females with a full mutation are usually more mildly affected than males and this is due to the presence of FMRP in cells that express the normal X chromosome with the non-mutated *FMR1* allele (Jacquemont *et al.*, 2014). These suggest that severity of the manifestation of symptoms differs between males and females and, that it is closely related to expression levels of FMRP.

2.1.4.2. Comorbidity of FXS with other mental disorders

Numerous medical conditions have been associated with FXS. Behavioural and cognitive features of FXS often include intellectual weaknesses, impairment in visuospatial skills, working memory, attention, sequential information processing, while, on the other hand, simultaneous processing and long-term memory are improved in FXS (Kidd *et al.*, 2014). Attention disorders including impulsivity, inattention and hyperactivity are common among FXS patients and often lead to the development of a comorbid ADHD. Anxiety disorders are also very frequent with a prevalence percentage of 86.2% in males and 76.9% in females with FXS. Reported numbers of people with FXS that also have ASD have estimated prevalence between the range 5%–60% as results vary widely across studies (Devitt *et al.*, 2015), with additional reports showing that 90% of patients with FXS exhibit some type of autistic characteristic (Bailey *et al.*, 2000). Therefore, shared neurobehavioral symptoms of FXS with ASD as well as the prevalence of autism-related symptoms in FXS patients render FXS as a promising model for studying autism (Jacquemont *et al.*, 2014).

2.1.4.3. *FMR1* gene and FMRP functions

The *FMR1* gene has been highly conserved throughout evolution (Noto *et al.*, 2016). It is located on the X chromosome at the 27.3 position (Figure 2.1) and it codes for the FMRP.

This protein is ubiquitously expressed in the body and more predominantly in the brain and testes (Devys *et al.*, 1993; Tamanini *et al.*, 1997; Bakker *et al.*, 2000). It is localised in the cytoplasm or in the nucleus of the cell depending on alternative splicing (Verheij *et al.*, 1993) but it is also able to shuttle between these two compartments (Eberhart *et al.*, 1996), suggesting that it may be involved in transporting specific mRNAs out of the nucleus and further regulate their subsequent translation (Paradee *et al.*, 1999). In fact, FMRP interacts with mRNA (Brown *et al.*, 2001; Darnell *et al.*, 2011; Ascano Jr. *et al.*, 2012) and micro-RNA (Jin *et al.*, 2004; Edbauer *et al.*, 2010) at the synapses and suppresses protein synthesis (Santoro *et al.*, 2012). In the absence of FMRP, changes occur in neurotransmitter receptor function and intracellular signalling. Based on these known FMRP functions, therapeutic approaches have been proposed to inhibit the translation of certain FMRP targets (Lu *et al.*, 2004; Westmark and Malter, 2007; Gross *et al.*, 2010) or signalling molecules (Huber *et al.*, 2002; Osterweil *et al.*, 2010; Sharma *et al.*, 2010; Bhattacharya *et al.*, 2012).

Evidence suggests that FMRP deficiency displays defects in synaptic plasticity (Huber *et al.*, 2002; Zhao *et al.*, 2005) and the development of abnormally thin, long dendritic spines (Comery *et al.*, 1997; Nimchinsky *et al.*, 2001). During synaptogenesis, ribosomes aggregate on dendritic spines, where FMRP is mainly found (Weiler *et al.*, 1997). FMRP has been hypothesized to inhibit translation of mRNAs that encode for metabotropic glutamate (mGluR) receptor (Bear *et al.*, 2004). In particular, in normal dendrites FMRP inhibits translation of mRNAs that encode mGluR proteins, which are normally involved in the modulation of synaptic plasticity (Bear *et al.*, 2004; Gubellini *et al.*, 2004). This inhibition occurs by normal ionotropic glutamatergic receptor; α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) endocytosis. However, in FXS dendrites mGluR stimulation by glutamate causes excess local protein synthesis and altered synaptic plasticity with higher AMPAR endocytosis activity causing exaggeration of long term depression (LTD) of the synapses (Gladding *et al.*, 2009). These events cause the weakening of the synapses and the presence of increased numbers of immature dendrites.

This effect is reversed by the use of mGluR inhibitors or in FMRP KO/mGluR5 heterozygotes (Dolen *et al.*, 2007; Tabolacci *et al.*, 2016). Collectively, these observations have led to the formulation of the mGluR theory in the fragile X pathogenesis, in which functional loss of the FMRP protein in the brain results in excessive glutamatergic signalling in FXS (Fish *et al.*, 2013), which contributes to the increased number of long and thin immature dendritic spines. This is evident in both the *Fmr1* KO mice and the FXS patients (Portera-Cailliau, 2012), the abnormal dendritic spine morphology potentially reflects the intellectual disability phenotype in patients with FXS (Pop *et al.*, 2014).

Studies on the *Fmr1* KO mouse model have also implicated the AMPAR, N-methyl-D-aspartate receptor (NMDAR) and GABAR in FXS. FMRP loss of function has been associated with GABAergic inhibition in FXS with reduced levels of GABA_A β -subunit observed in the hippocampus and brainstem (El Idrissi *et al.*, 2005), which might explain the amygdala dysfunction reported in the *Fmr1* KO mice (Pop *et al.*, 2014). It has been reported that the *Fmr1* KO mice display differentially altered dopaminergic function in mesolimbic and nigrostriatal pathways, which can facilitate imbalance of reward and motor functions (Fish *et al.*, 2013). In particular, FMRP can modulate DA transmission in the forebrain and D1 receptor agonist has been shown to partially rescue hyperactivity and enhance the motor function of the *Fmr1* KO mice (Wang *et al.*, 2008). FMRP loss has been also linked to impairments in NMDAR-dependent synaptic plasticity in the dentate gyrus (DG) of the hippocampus and treatment with NMDAR co-agonists such as glycine or D-serine rescues these impairments in the *Fmr1* KO mouse (Bostrom *et al.*, 2015).

Interestingly, several studies have focused their attention on identifying mRNA targets of FMRP in an attempt to identify proteins that are misregulated in FXS (Darnell *et al.*, 2001) and have shown that FMRP can bind to RNAs that are untranslatable like BC1 (Zalfa *et al.*, 2003); an untranslated RNA abundant in dendrites which acts as a translation repressor (Wang *et al.*, 2002). Although it has been postulated that FMRP can modulate translation of several proteins that are linked to autism including NMDAR, mGluR5, tuberous sclerosis

complex 2, mammalian target of rapamycin (mTOR), neuroligin-3 and fragile-X related protein 2 (Darnell *et al.*, 2011; Ascano Jr. *et al.*, 2012; Iossifov *et al.*, 2012), the exact molecular mechanisms underlying the high prevalence of autism in the FXS-affected population are not yet fully characterised and require further investigation.

2.1.4.4. *Fmr1* KO mouse model

The first knock-out mouse model of FXS was constructed in 1994 by inducing a deleterious mutation in the *Fmr1* gene of the mouse, which has 98% homology with the respective human orthologue (The Dutch-Belgian Fragile X Consortium, 1994). It is the most widely used model for FXS, which totally lacks Fmrp expression after insertion of the neo cassette into exon 5 of the *Fmr1* gene. Furthermore, it largely recapitulates the human syndrome at protein level. For the purpose of our study however, a newer version and more versatile of the *Fmr1* KO model has been employed, which was generated with Cre-recombinase mediated deletion of exon 1 region in the *Fmr1* allele, as described by (Mientjes *et al.*, 2006), it also lacks Fmrp expression and detectable Fmr1 transcripts. However, neither mouse lines replicate the expansion of the CGG repeat, which is present in most human patients, and it might contribute in the pathology of the disorder in currently unknown ways (Ladd *et al.*, 2007; Khalil *et al.*, 2008). *Fmr1* KO mice display strong face validity as they recapitulate many of the clinical features of FXS including physical, physiological and behavioural that correspond to the human disease. Some of the common characteristics that they share with the human FXS are hyperactivity, macroorchidism and cognitive impairments as well as autistic-like phenotype including social interactions deficits, repetitive/compulsive behaviour and communication abnormalities (Bernardet and Crusio, 2006; Kazdoba *et al.*, 2016). Detailed account of phenotypic changes in the *Fmr1* KO mice with regards to the ASD domain are summarised in Table 2.1.

Predictive validity has been demonstrated in *Fmr1* KO mouse model by using pharmacological intervention studies. Treatment responses of several drugs i.e. minocycline and lithium in the *Fmr1* KO mice were evaluated and the findings were in parallel with those from human FXS open-label trials (Berry-Kravis *et al.*, 2008; Paribello *et al.*, 2010). The complete absence of the *Fmr1* gene and the FMRP product in the *Fmr1* KO mouse provides construct validity as it can incorporate aetiological similarities to the human disease (Leach *et al.*, 2016). Hence, this mouse model with an identified genetic mutation presenting ASD symptoms holds a promising strategy in the research of ASD pathology. To this end, we selected the *Fmr1* KO mouse model as a reliable model of ASD with high translational validity.

Table 2.1: Summary of the behavioural characteristics of the *Fmr1* KO mouse displaying ASD symptoms conducted by a variety of behavioural tests from different studies.

Behaviour	Phenotypic direction	Behavioural assays	References
Inappropriate social interactions	Passive social behavior↓ affiliative behaviour↓ Sniffing and receptive behavior↑	Direct social interaction	Spencer <i>et al.</i> 2005; Pietropaolo <i>et al.</i> 2011; Mineur <i>et al.</i> 2006; Spencer <i>et al.</i> 2008
	Time spent with unfamiliar WT the i) 1st day↓ ii) 3rd day ND, Time spent with familiar WT ND	Social dominance tube	Spencer <i>et al.</i> 2006; Spencer <i>et al.</i> 2008
	Time spent in familiar cage first 5 min↓ Time spent in familiar cage after 20min ↑	Influence of cage familiarity on response to unfamiliar social partners	Spencer <i>et al.</i> 2005
	No preference for novel mouse, sniffing duration with partner↓	Three chamber sociability	Dahlhaus <i>et al.</i> 2010; McNaughton <i>et al.</i> 2008
Perseverance	perseverance rate of learning↑	Water maze reversal learning-hidden platform, Plus-shaped water maze reversal learning	Paradee <i>et al.</i> 1999; Van Dam <i>et al.</i> 2000; The Dutch-Belgian Fragile X Consortium 1994; Kooy <i>et al.</i> 1996; Dhooge <i>et al.</i> 1997; Van Dam <i>et al.</i> 2000
	perseverance escape latencies↑	Water maze reversal learning-visible platform	The Dutch-Belgian Fragile X Consortium 1994; Dhooge <i>et al.</i> 1997
	perseverance↑	E-shaped water maze reversal learning, Five-choice serial reaction task	Kooy <i>et al.</i> 1996; Kramvis <i>et al.</i> 2013
	self-grooming↑	Social interaction	Pietropaolo <i>et al.</i> 2011; McNaughton <i>et al.</i> 2008
	number of buried marbles↑	Marble burying	Spencer <i>et al.</i> 2011; Gholizadeh <i>et al.</i> 2014; Veeraragavan <i>et al.</i> 2011
Anxiety	Time spent on open arms↓ ↑ ND	Elevated plus maze	Zhao <i>et al.</i> 2005; Mineur <i>et al.</i> 2002; Nielsen <i>et al.</i> 2002; Bilousova <i>et al.</i> 2009; Yan <i>et al.</i> 2004
	anxiety-like responses	Thigmotaxis in open-field, mirrored chamber open field	Yan <i>et al.</i> 2004; Spencer <i>et al.</i> 2005; Peier <i>et al.</i> 2000; Restivo <i>et al.</i> 2005
	Males: after 60min cort↑ Females: after 15min↓	Corticosterone response to acute stress	Lauterborn <i>et al.</i> 2004; Markham <i>et al.</i> 2006
	ND	Conditioned emotional response	Van Dam <i>et al.</i> 2000

Learning & memory	Performance↓	Passive avoidance: working memory	Ding <i>et al.</i> 2014; Veeraragavan <i>et al.</i> 2011; Yuskaitis <i>et al.</i> 2010
	Escape latencies ND	Cross-shaped water maze	Van Dam <i>et al.</i> 2000
	ND	Changing position of platform in water maze, E-shaped water maze, passive avoidance (latency to enter dark compartment), olfactory learning and memory tasks, novel object task	Paradee <i>et al.</i> 1999; Van Dam <i>et al.</i> 2000; Kooy <i>et al.</i> 1996; The Dutch-Belgian Fragile X Consortium 1994; Yan <i>et al.</i> 2004
	Escape latencies↑ Rate of learning ND	Morris water maze -hidden platform	The Dutch-Belgian Fragile X Consortium 1994; Yan <i>et al.</i> 2004; Kooy <i>et al.</i> 1996
	Escape latencies ND	Morris water maze -visible platform	The Dutch-Belgian Fragile X Consortium 1994; D'Hooge <i>et al.</i> 1997
	ND	Radial maze, Barnes maze, fear conditioning: context and conditioned cue	Yan <i>et al.</i> 2004; Mineur <i>et al.</i> 2002; Van Dam <i>et al.</i> 2000; Peier <i>et al.</i> 2000; Dobkin <i>et al.</i> 2000
	response↓	Trace fear conditioning, conditioned eyelid blink reflex	Zhao <i>et al.</i> 2005; Koekkoek <i>et al.</i> 2005; Ding <i>et al.</i> 2014
	object sniffing↓	Open field, novel object recognition	Mineur <i>et al.</i> 2002; Ventura <i>et al.</i> 2004
	entries↓	eight-arm radial maze: spatial memory	
	Conditioning learning ND, devaluation of reward and omission of lever press↑	Instrumental conditioning	Frankland <i>et al.</i> 2004
Communication	Vocalizations↓	Ultrasonic vocalization	Gholizadeh <i>et al.</i> 2014; Rotschafer <i>et al.</i> 2012; Roy <i>et al.</i> 2012
	Vocalizations↑		Spencer <i>et al.</i> 2011
Aggression	ND	Neutral cage aggression test	Mineur <i>et al.</i> 2002
Hyperactivity	↑	Open field activity, activity cage, motor activity test	Mineur <i>et al.</i> 2002; Spencer <i>et al.</i> 2005; Peier <i>et al.</i> 2000; The Dutch-Belgian Fragile X Consortium 1994; Ventura <i>et al.</i> 2004; Pietropaolo <i>et al.</i> 2011; Ding <i>et al.</i> 2014
	ND	Open field activity	Mineur <i>et al.</i> 2002; Spencer <i>et al.</i> 2005; Peier <i>et al.</i> 2000
Idiosyncratic responses to sensory stimuli	Variable outcomes ↑↓=	Auditory startle response	Frankland <i>et al.</i> 2004; Yan <i>et al.</i> 2004; Peier <i>et al.</i> 2000; Nielsen <i>et al.</i> 2002; Qin <i>et al.</i> 2005; Chen <i>et al.</i> 2001
	↑response	Prepulse inhibition	Frankland <i>et al.</i> 2004; Nielsen <i>et al.</i> 2002; Chen <i>et al.</i> 2001; Spencer <i>et al.</i> 2011; Ding <i>et al.</i> 2015; Pietropaolo <i>et al.</i> 2011
	↑response	Audiogenic seizures	Chen <i>et al.</i> 2001; Qin <i>et al.</i> 2005; Musumeci <i>et al.</i> 2000; Yan <i>et al.</i> 2004; Ding <i>et al.</i> 2014
	ND	Hot plate and tail-flick test	Zhao <i>et al.</i> 2005

The behavioural outcomes of the *Fmr1* KO mice presented are expressed in comparison to wild-type (WT) mice. Abbreviation ND used for no difference.

2.1.5. The opioid system in the *Fmr1* KO mouse

Given the clinical and preclinical evidence showing a role for the opioid system in the neurobiology of ASD, preclinical work conducted at the laboratory of Dr. Le Merrer and Dr. Becker investigated the effects of opioid drugs such as morphine in the *Fmr1* KO, which displays core symptoms of autism including social deficits, stereotypical behaviour and comorbid symptoms such as anxiety, hyperactivity, cognitive deficits and aggressiveness. They demonstrated that morphine-induced (5 mg/kg) hyper-locomotion was significantly reduced compared to WT controls, suggesting an interaction between the *Fmr1* gene and the endogenous opioid system (see data in appendix A). In support of this, the same group further demonstrated that while treatment-naïve *Fmr1* KO mice show significantly higher sensitivity to nociception compared with WT, using the tail immersion paradigm, morphine (5 mg/kg) induced higher analgesia in *Fmr1* KO mice vs. WT groups (see data in appendix B), suggesting an altered MOR system in the *Fmr1* KO mice. With respect to social behaviour, as expected *Fmr1* exhibited reduced social interaction behaviour compared to WT in the social interaction pair paradigm, and this was completely reversed following acute administration of morphine (5 mg/kg) (see appendix C). Similarly, reduction in duration of nose contacts and stereotypic grooming observed in *Fmr1* KO mice were completely abolished following morphine (5 mg/kg) administration (Appendix D and E respectively). Therefore, these alterations of the behavioural responses upon morphine administration in the *Fmr1* KO mouse, strongly support the presence of an interaction between the *Fmr1* gene and the endogenous opioid system. Thus, we embarked to further investigate the molecular nature of this interaction by analysing the potential alterations in transcripts and receptors of the endogenous opioid system in the brain of the *Fmr1* KO mouse.

2.2. Hypotheses and Objectives

2.2.1. Hypotheses

Emerging evidence suggest an association between alteration in the *Fmr1* gene and the endogenous opioid system, as illustrated by changes in sociability and stereotypy behaviours that were induced upon morphine administration in the autistic *Fmr1* KO mice. Therefore, the overarching hypothesis for this chapter is that the *Fmr1* KO mice will also demonstrate neurobiological changes on the opioid receptor binding level and mRNA transcript level. Our primary hypothesis is that decreased opioid binding levels will be observed for all opioid receptors, but predominantly for the MOR. Secondly, we hypothesize that the mRNA levels of the endogenous opioid ligands will be also down-regulated. Lastly, we hypothesize that neurotransmitter systems including glutamate and DA as well as the expression of neuropeptides implicated in social behaviour, oxytocin and vasopressin, which are known to be involved in the ASD pathology as discussed in chapter 1, will be differentially regulated in the *Fmr1* KO mice.

2.2.2. Objectives

Primarily, the aim of this study was to identify if there were alterations in a) MOR, DOR and KOR receptors binding in the brain of mice modelling autism, the *Fmr1* KO model by using quantitative autoradiographic binding in the brains of WT and *Fmr1* KO mice, and b) in mRNA transcripts of the different components of the endogenous opioid system (neuropeptide gene and receptors) by performing quantitative Real Time PCR (qRT-PCR) across brain regions that play major role in reward and sociality: NAc, CPu, CeA, VTA and PFC. Secondly, we aimed to assess other neurotransmitter systems including glutamate and DA as well as the expression of oxytocin and vasopressin, by utilising additional qRT-PCR

experiments across the five aforementioned brain regions of WT and *Fmr1* KO mice. These neurotransmitter components were carefully chosen based on their involvement in the neuropathology of ASD and possibly on the *Fmr1* KO phenotype.

2.3. Materials and Methods

2.3.1. Methods

2.3.1.1. Autoradiographic receptor binding

Animals and preparation of brain sections

In this cohort, we utilised male mice for assessing endogenous opioid receptor binding levels in the *Fmr1* KO mice. We proceeded with an initial screening of effects in order to further decide whether to continue with both sexes for the following cohorts. Brains from naïve WT and *Fmr1* KO male C57BL6 mice ($n=9$ per genotype, 6-8 weeks old), dissected at the University of Tours, France and shipped to the UK, were sectioned in a Microtome cryostat HM305E with an internal temperature of -21°C . Twenty-micrometre coronal sections were cut at 300 μm intervals from rostral to caudal levels and thaw-mounted onto gelatin-coated ice-cold microscope slides and processed for autoradiography. Adjacent sections were cut for determination of total and non-specific (NSB) binding. Sections were stored at -20°C prior to radioligand binding.

Autoradiography of MOR, DOR and KOR

For binding studies, sections were thawed and processed as per established protocols (Georgiou *et al.*, 2015, Kitchen *et al.*, 1997). Multiple adjacent sections from both groups were processed together in a paired binding protocol.

MOR binding: Sections were prewashed for 30 min in 50mM Tris-HCl pre-incubation buffer containing 0.9% w/v NaCl, pH 7.4 at room temperature to remove endogenous ligands. Binding was carried out in 50 mM Tris-HCl pre-incubation buffer, pH 7.4 at room temperature, in the presence of 4 nM [^3H] [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO)

(specific activity: 51.5 Ci/mmol) for 60 min. NSB was determined in adjacent sections with 4 nM [^3H] DAMGO in the presence of 1 μM naloxone for 60 min. At the end of the binding, slides were rinsed three times in ice-cold (4°C) medium of 50 mM tris-HCl buffer, pH 7.4 for 5 min for the washing out of the unbound excess ligands.

DOR binding: Slides were pre-incubated for 30 min in 50 mM Tris-HCl, containing 0.9% w/v NaCl, pH 7.4 at room temperature. The slides were then incubated in the same buffer in the presence of 7 nM [^3H] deltorphin (specific activity 50 Ci/mmol) for 60 min at room temperature. NSB was determined with 7 nM [^3H] deltorphin in the presence of 10 μM naloxone. Incubation was terminated by three rinses 5 min each in ice cold 50mM Tris-HCl, pH 7.4.

KOR binding: Slides were washed twice for 15 min in 50 mM Tris-HCl pre-incubation buffer, pH 7.4 at 4°C. Then incubation of the slides was followed in pre-incubation buffer in the presence of 5 nM of [^3H] U69593 (specific activity 37.4 Ci/mmol) for 90 min at room temperature. Adjacent sections were used to determine NSB with [^3H] U69593 in the presence of 10 μM naloxone. Incubations were terminated after 90 min by three rinses 5 min each rinse in ice cold pre-incubation buffer, pH 7.4.

2.3.1.2. Film exposure and development

Following the final incubation, sections were immediately dried under cold air for 2 hours. They were then placed in boxes containing anhydrous calcium sulphate for a minimum of seven days until completely dry. Adjacent total and non-specific sections were apposed to Kodak Biomax MR-1 film alongside autoradiographic ^3H microscale standards of known radioactivity concentration to permit the conversion of the optical densities of silver grains in autoradiograms to specific activity of tissue-bound ligand to femtomoles per milligram (fmol/mg) of tissue. The duration of the apposition stage of [^3H] DAMGO and [^3H] Deltorphin bound sections to films was 10 weeks, while [^3H] U69593 bound sections was for a year.

Sections for all treatment groups were processed in parallel in a paired protocol and apposed to the same film at the same time.

The end of the apposition was followed by the development stage. The films were covered with an aqueous solution of 50 % v/v Kodak D19 developer for 3 min. The reaction was terminated by 30 sec rinse in reverse osmosis (RO) water containing few drops of glacial acetic acid. Films were then fixed by submersion in Kodak rapid fix solution for 3 min. Final rinse of films was followed in RO water and films were then let to dry overnight in a fume cupboard.

2.3.1.3.Quantitative Image analysis

Films were analysed by video-based densitometry using an MCID image analysis software. In brief, fmol/mg specific activity of tissue-bound ligand equivalent for receptor binding was derived from ^3H microscale standards with known concentrations. This allowed for the calculation of the relationship between tissue reactivity and optical density by using an MCID software, with appropriate adjustments for radioactive decay of the standards and the radioligands. Specific receptor binding was derived by subtraction of NSB from total binding for MORs, DORs and KORs. Measurements were quantified bilaterally for each brain region; therefore, receptor binding represents determination for each brain region in duplicate, and the n values listed refer to the number of animals analysed. The following structures were analysed by sampling 5-20 times with a box tool cortex (8x8 mm) and hippocampus (5x5 mm), while all other regions were analysed by free-hand drawing. Brain structures were identified by reference to the mouse atlas of Franklin and Paxinos (2001).

2.3.1.4. Animals and Real-time quantitative PCR analysis

WT and *Fmr1* KO mice (n=5) aged 6-8 weeks males and females used were not subjected to any human handling or experimental procedures prior to sacrificing. In this separate cohort, we proceeded with mouse tissue from both sexes, which was pooled together in an experimental tube in order to have more material. These preliminary data only assess striking changes observed at mRNA level in the *Fmr1* KO mice brains, and are unable to show any sex differences at mRNA expression levels. Mice were culled by cervical dislocation and brains collected from both groups were dissected and immediately frozen in liquid nitrogen and kept at -80°C. Prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (NAc), central amygdala (CeA), ventral tegmental area (VTA) were punched out from sections of 1 mm thickness. When assessing mRNA levels of a collection of genes of interest, bilateral punches were taken and they were collected together in a test tube from a male and a female for each brain structure and genotype. Total RNA was extracted and purified using the Direct-zol™ RNA Miniprep. cDNA was synthesized starting from 30 µg total RNA using the ProtoScriptR first-strand cDNA synthesis kit. qRT-PCR was performed in quadruplets on a CFX384 Real-Time PCR Detection System using iQ-SYBR Green supermix kit with 0.25 µl cDNA in a 12.5 µl final volume, as described previously by (Le Merrer *et al.*, 2013; Becker *et al.*, 2014). β -actin, an abundant house-keeping gene, was used as a baseline for calculating changes in the expression of target genes. Gene-specific primers were designed using Primer3 software. (For list with primers see Table 2.2). PCR conditions were: 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec. A melting curve was tested by gradually increasing the reaction temperature from 48°C to 95°C after the last cycle of amplification to determine the number of PCR products. SYBR green fluorescence was measured during amplification and the cycle threshold (C_T) was determined. The fold change in the target gene in the experimental group, normalized to β -actin was calculated for each sample using the $2^{-\Delta\Delta C_T}$ method.

According to established protocol (Le Merrer *et al.*, 2012), qRT-PCR data were transformed before statistical analysis to obtain a symmetrical distribution centred on 0 (corresponding to no change in gene expression) using the following formula: if $x < 1$, $y = 1 - 1/x$; if $x > 1$, $y = x - 1$ (x : qPCR data; y : transformed data).

Table 2.2: Primer sequences for qRT-PCR experiments.

RefSeq	Gene name	Gene title	Forward oligonucleotide	Reverse oligonucleotide
NM_013622	opioid receptor, delta	<i>Oprd1</i>	TTTGTGTTGGATGGGGCATG	CTGACTGGAGTTCAACCCCT
NM_001204371	opioid receptor, kappa 1	<i>Oprk1</i>	CCTGGCATCATCTGTTGGTA	GGAAACTGCAAGGAGCATTC
NM_011013	opioid receptor, mu	<i>Oprm1</i>	ATCCTCTCTGAAGCCAACCC	TTCTGTCTTGGGCCATCAT
NM_018863	prodynorphin	<i>Pdyn</i>	TTTGGCAACGGAAGAATC	TAGCGTTTGGCCTGTTTTCT
NM_001002927	preproenkephalin	<i>Penk</i>	ATGCAGATGAGGGAGACACC	GCTTCTGCAGCTCTTTTCT
NM_009732	Arginine vasopressine	<i>Avp</i>	ACACTACGCTCTCCGCTTGT	CACTGTCTCAGTCCATGTCA
NM_016847	Arginine vasopressine receptor 1A	<i>Avpr1a</i>	GGAGAAACGGGAGACAGACA	AAGCCCATGTACAGCCCAAG
NM_011924	Arginine vasopressine receptor 1B	<i>Avpr1b</i>	AGGAGGAAGAGGAGGAGGAA	ACTGAAGGCAGGAAGTGAAG
NM_011025	oxytocin	<i>Oxt</i>	CTGCTTGGCTTACTGGCTCT	GGGAGACACTTGCAGATATC
NM_001081147	oxytocin receptor	<i>Oxtr</i>	CTTAGGGCCAAAAGGTGTCA	GCAGGTTTCTATGCCCTCTG
NM_010076	dopamine receptor D1A	<i>Drd1a</i>	AGATCGGGCATTGAGAGAG	GGATGCTGCCTCTTCTTCTG
NM_010077	dopamine receptor D2	<i>Drd2</i>	TGCCATTGTTCTTGGTGTGT	GTGAAGGCGCTGTAGAGGAC
NM_007877	dopamine receptor D3	<i>Drd3</i>	GCATCCAACTTCGAAGGAG	GTGGATAACCTGCCATTGCT
NM_007878	dopamine receptor D4	<i>Drd4</i>	GTCTGCAGAGGTCAAGGAG	AACCCCAAGCCTTCATAAGT
NM_013503	dopamine receptor D5	<i>Drd5</i>	ACAAGGGCCCTTGAGGAAAT	GCTTGAGAATCCCGCATCTA
NM_016976	glutamate receptor, metabotropic 1	<i>Grm1</i>	TTCCACCAAGTTGAGAAACC	AAAGCCCTGACTGCACAAGT
NM_001160353	glutamate receptor, metabotropic 2	<i>Grm2</i>	CTTGTAAGCTATGCCCCGTGT	GACTGGAAGCACCTTTCAT
NM_181850	glutamate receptor, metabotropic 3	<i>Grm3</i>	GTGGTCTTGGGCTGTTTGTT	TGCTTGCAGAGGACTGAGAA
NM_001013385	glutamate receptor, metabotropic 4	<i>Grm4</i>	CTTCCTTAGCCAGGGTCTCC	CATCCCTTCGGACACAGTTT
NM_001143834	glutamate receptor, metabotropic 5	<i>Grm5</i>	CCACTGACGACTTGACAGGTT	CAGTAACGAAGAGGGTGGCTA
NM_177328	glutamate receptor, metabotropic 7	<i>Grm7</i>	CCAAAACTGTGCTGGGAAG	GCATGCATTCAAAGATCAA
NM_008174	glutamate receptor, metabotropic 8	<i>Grm8</i>	TCCTGTCCCACTGTTCAA	AAAATTGCATGCGTCAATCA
NM_011702	vasoactive intestinal polypeptide	<i>Vip</i>	AGGTACTCCATCTCTCACGGA	CCAGATTGGACGATGGGCTT
NM_011703	Vasoactive intestinal peptid receptor 1	<i>Vipr1</i>	GGGAGTGCTCCTAGAAATCCA	CATCTCTCAGCCTCATCGGA
NM_009511	Vasoactive intestinal peptid receptor 2	<i>Vipr2</i>	TCCAGGGCTACAAGGCTAGA	TAGCATCCACCTATCGCCAC

2.3.1.5. Data analysis and statistical analysis

All autoradiographic receptor binding data are presented as the mean $\pm$ standard error of the mean (SEM) and were analysed using GraphPad Prism 6. Two-way ANOVA for factors 'Genotype' (*Fmr1* KO vs. WT) and 'Brain Region' effect was used for comparison of specific binding. Sidak's multiple comparisons *post-hoc* test was carried out if ANOVA was

significant to investigate the presence of a genotype effect in any brain regions analysed. Significance defined as $p < 0.05$.

A one-sample t-test was performed to assess the statistical significance of the transformed qRT-PCR data. Calculated p values indicated probability for a regulation to differ from 0. Only regulations over +1 or below -1 (fold-change) were retained as significant.

2.3.2. Materials

Authoradiographic analysis by receptor binding

[³H] DAMGO, [³H] Deltorphin and [³H] U69593 radioligands were purchased from PerkinElmer Life sciences (Massachusetts, USA), naloxone from Tocris Bioscience (Bristol, UK), Tris-HCl and NaCl from Sigma-Aldrich (Poole, UK). Microtome cryostat HM305E used was purchased from Zeiss Hyrax C 25, Carl Zeiss Microimaging GmbH, (Germany) and the MCID image analyser from Image Research (Canada).

Quantitative Real Time PCR

Direct-zol™ RNA Miniprep was purchased from the Epigenetics company, Zymo research, ProtoScriptR first-strand cDNA synthesis kit from Lifetechnologies (France), iQ-SYBR Green supermix and Real-Time PCR Detection System from Bio- Rad (Marnes-la-Coquette, France).

2.4. Results

In an attempt to reveal possible relationships between the endogenous opioid system and the absence of the *Fmr1* gene, we looked for modifications in the receptors of the opioid system (MOR, DOR and KOR) both on receptor binding and transcripts level in specific brain regions of the *Fmr1* KO mice. We further aimed to investigate alterations on transcription level between genotypes on a collection of genes involved in the control of social behaviour and reward. Several genes have been associated with the aetiology of autism and we quantified a selection of these in the WT and *Fmr1* KO mice. We greatly focused on genes involved in the dopaminergic and glutamatergic system, as well as genes coding for neuropeptides implicated in social behaviour, oxytocin (OXT) and vasopressin (Avp).

2.4.1. Autoradiographic analysis by receptor binding

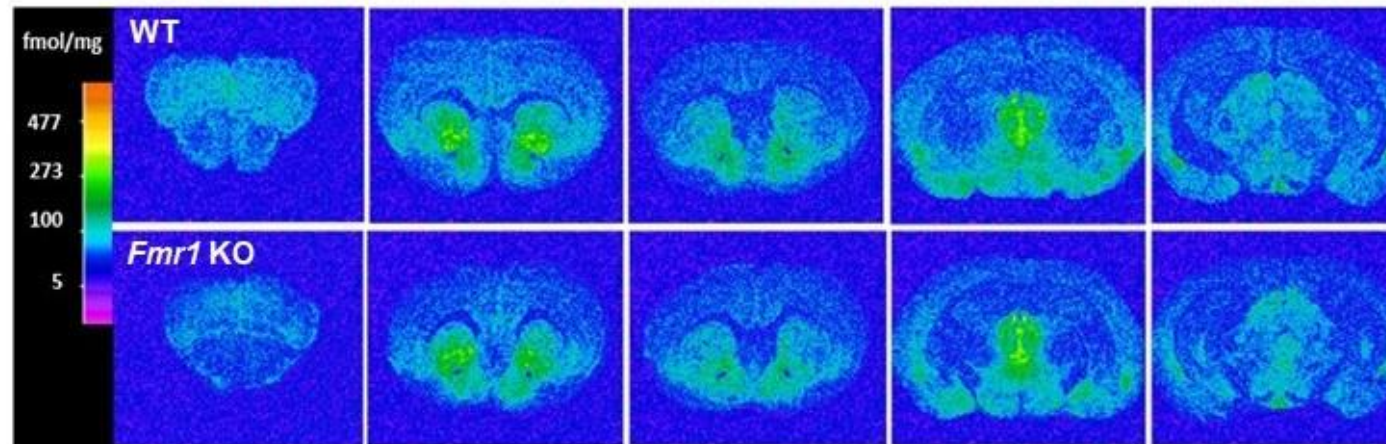
The quantification of the density levels of [³H] DAMGO, [³H] Deltorphin and [³H] U69593 in the brains analysed is expressed relative to the [³H] microscale standards used. NSB was at background levels with no difference between genotypes. Brain sections are shown relative to bregma points of the coronal sections, in accordance to the Paxinos and Franklin brain atlas (2001).

Our data demonstrate that the distribution of MOR in the brains of the *Fmr1* KO mice was not significantly different from their WT controls. High levels of MOR binding (99.5– 210 fmol/mg) were detected within the intermediodorsal thalamic nucleus (IMD) and the interpeduncular nucleus (Ipn), moderate levels (55.9 – 96.6 fmol/mg) in the lateral septum (LS) and the ventral pallidum (VP), and low levels (20.18 – 50.87 fmol/mg) in the superficial layer of the motor cortex (MtCxSI) and the hippocampus (Hip) in both groups. Although, two-way ANOVA revealed a significant effect on brain region ($F_{(50, 815)}=102.3$, $p<0.0001$), it did

not show a significant effect of 'genotype' ($F_{(1, 815)} = 0.836$, $p > 0.05$) or 'brain region' x 'genotype' interaction ($F_{(50, 815)} = 0.1978$), $p > 0.05$). Figure 2.2 illustrates MOR binding results. Similarly, DOR binding in the brains of the *Fmr1* KO mice showed no difference in receptor distribution compared to WT mice respectively, in any region analysed. High binding was observed in the internal granular layer (IGI) (137.2–258.4 fmol/mg), moderate levels (112.2–136.9 fmol/mg) within the caudate putamen (CPu) and the deep layer of the granular insular cortex (InCx), and low levels (19.6–104.8 fmol/mg) in the substantia nigra (SN) and the ventral tegmental area (VTA) in both groups. Two-way ANOVA did not show a significant difference on genotype effect ($F_{(1, 815)} = 0.022$, $p > 0.05$) or 'brain region' x 'genotype' interaction ($F_{(50, 815)} = 0.435$, $p > 0.05$), however, it revealed a significant brain region effect ($F_{(50, 815)} = 73.50$, $p < 0.0001$) (Figure 2.3).

Consistent with our data demonstrating no change in MOR and DOR receptor distribution in the *Fmr1* KO mice compared to WT, KOR binding also did not show any differences between genotypes. High binding of KOR was found in the claustrum (Cl) and the dorsal endopiriform cortex (DEn) (41.86–44.65 fmol/mg), moderate levels within regions such as the cingulate cortex (CgCx), the nucleus accumbens (NAc) and the medial septum (MS) (31.18–35.73 fmol/mg), while low levels were detected in the basolateral amygdala (BLA) and the hippocampus (Hip) (20.15–28.98 fmol/mg). Two-way ANOVA did not reveal a significant difference on genotype effect ($F_{(1, 320)} = 2.908$, $p > 0.05$) or 'brain region' x 'genotype' interaction ($F_{(19, 320)} = 0.394$, $p > 0.05$), although it showed a significant brain region effect ($F_{(19, 320)} = 7.454$, $p < 0.0001$) (Figure 2.4). Overall, there was no significant difference of the binding levels of the opioid receptors between genotypes throughout the brain.

A



B

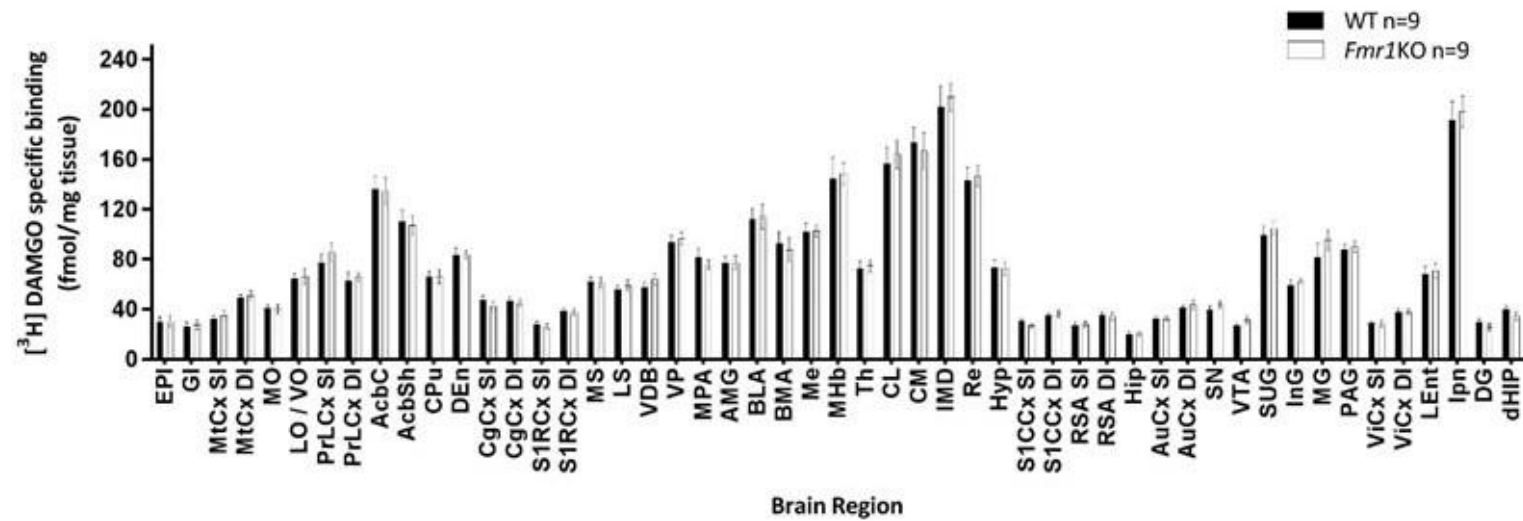
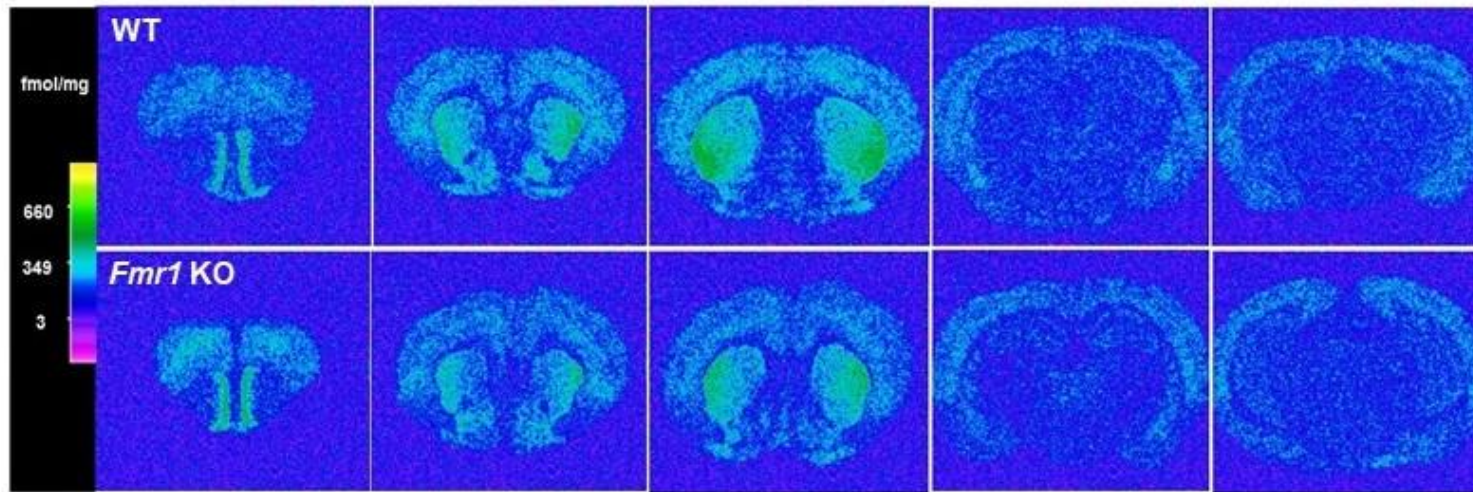


Figure 2.2: [³H] DAMGO MOR binding in WT and *Fmr1* KO mice.

A) Representative images of adjacent coronal brain section from complete mapping of the brain at the level of the olfactory nuclei (column 1) (Bregma 3.08), nucleus accumbens (column 2) (Bregma 1.10), medial septum (column 3) (Bregma 0.74), thalamus (Bregma -1.46) and the ventral tegmental area (column 4) (Bregma -3.40). NSB was undetectable (at background levels). The colour bar illustrates a pseudocolour interpretation of black and white image density calibrated in fmol/mg of tissue equivalent. Top row; from WT and bottom row; *Fmr1* KO mice sections. B) Quantitative MOR autoradiographic binding in brain regions of naïve WT and *Fmr1* KO mice. Two-way ANOVA ($p > 0.05$) indicated no significant difference between genotypes or 'genotype x brain region' interaction. Data are expressed as mean $\pm$ SEM (n=9) of [³H] DAMGO specific binding (fmol/mg tissue). For abbreviations see p.17-26.

A



B

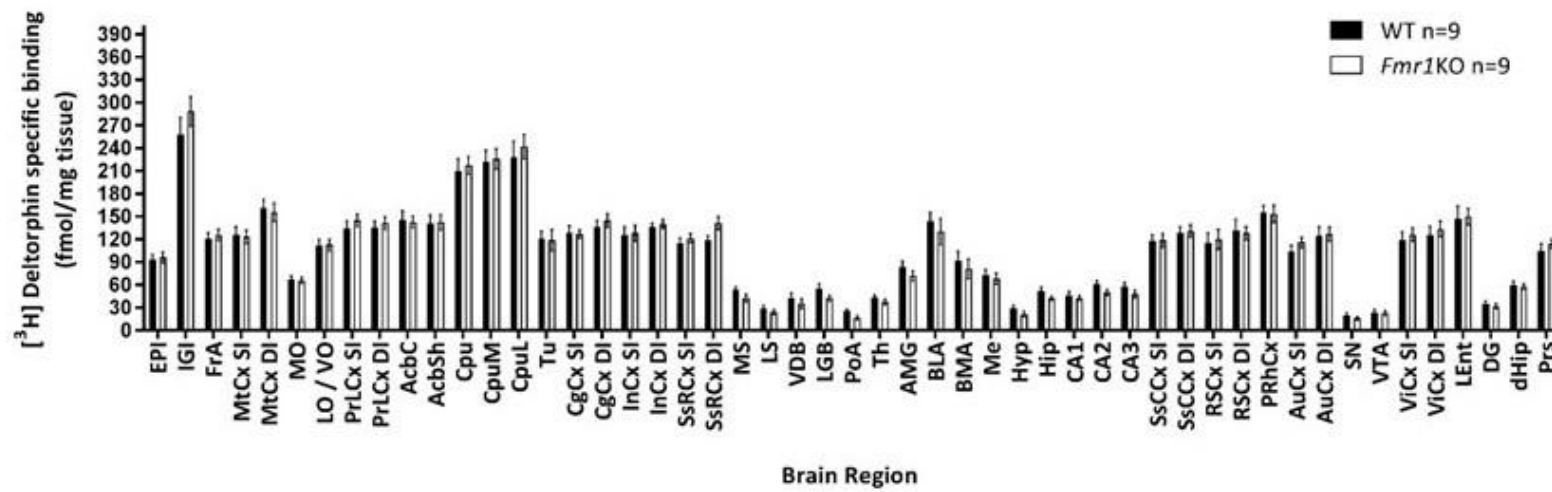


Figure 2.3: [³H] Deltorphan DOR binding in WT and *Fmr1* KO mice.

A) Representative images of adjacent coronal brain section from complete mapping of the brain at the level of the olfactory nuclei (Bregma 3.08), nucleus accumbens (column 2) (Bregma 1.10), medial septum (column 3) (Bregma 0.74), thalamus (Bregma -1.46) and the ventral tegmental area (column 4) (Bregma -3.40). Top row; from WT mice sections and bottom row; *Fmr1* KO mice sections. The colour bar illustrates a pseudocolour interpretation of black and white image density calibrated in fmol/mg of tissue equivalent. B) Quantitative DOR autoradiographic binding in brain regions of naïve WT (top row) and *Fmr1* KO (bottom row) mice. Two-way ANOVA ($p > 0.05$) indicated no significant difference between genotypes or “genotype x brain region” interaction. Data are expressed as mean $\pm$ SEM (n=9) of [³H] Deltorphan specific binding (fmol/mg tissue). For abbreviations see p.17-26.

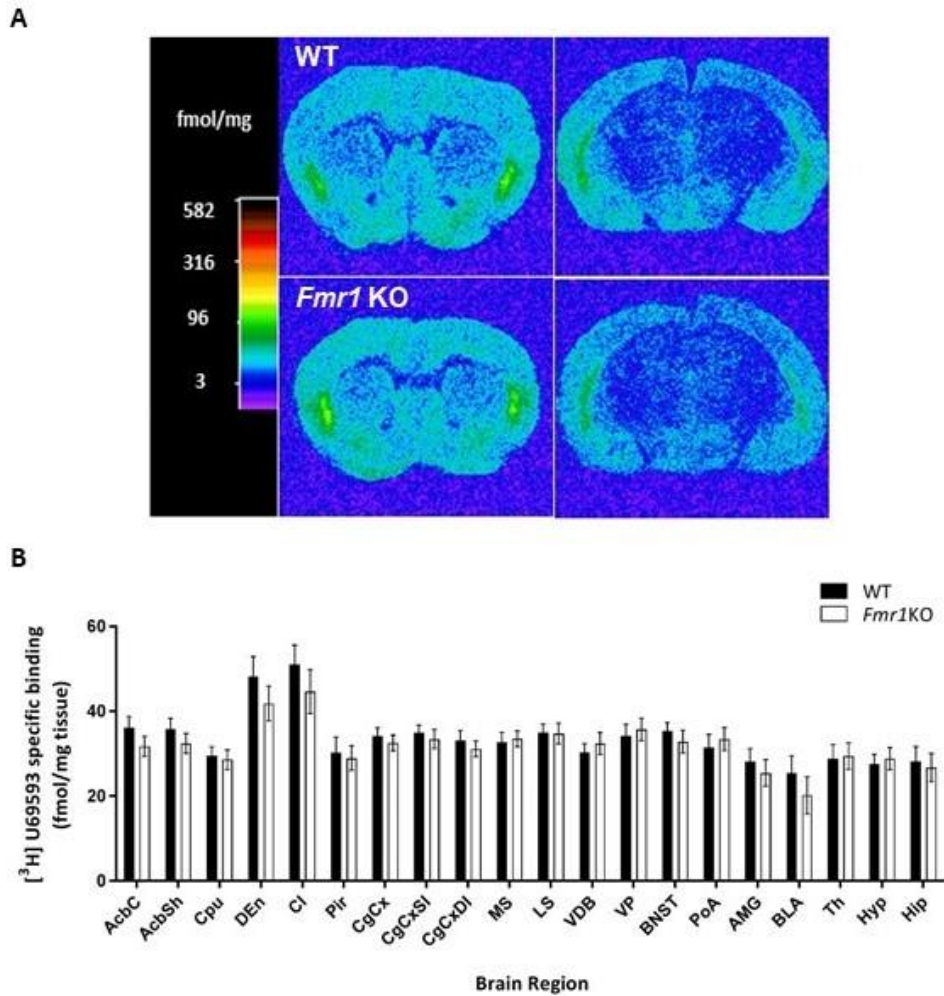


Figure 2.4: [^3H] U69593 KOR binding in WT and *Fmr1* KO mice

A) Representative images of adjacent coronal brain section from complete mapping of the brain at the level of the striatum (column 1) (Bregma 1.10) and the hypothalamus (column 2) (Bregma -1.46). Top row; from WT mice sections and bottom row; *Fmr1* KO mice sections. The colour bar illustrates a pseudocolour interpretation of black and white image density calibrated in fmol/mg of tissue equivalent. B) Quantitative KOR autoradiographic binding in brain regions of naïve WT and *Fmr1* KO mice. Two-way ANOVA ($p > 0.05$) indicated no significant difference between genotypes or “genotype x brain region” interaction. Data are expressed as mean $\pm$ SEM ($n=9$) of [^3H] U69593 specific binding (fmol/mg tissue). For abbreviations see p.17-26.

2.4.2. Real-time Quantitative PCR analysis

Several neurotransmitter systems might be responsible for the marked behavioural alterations detected within the opioid system in the *Fmr1* KO mice. As discussed before, modulatory circuitry of social motivation, which includes the endogenous opioids, DA, OXT, AVP (Nelson and Panksepp, 1998) and glutamate system is integrated into a network of neurobiological mechanisms whose alterations could cause the social deficits in autism (Neuhaus *et al.*, 2010). There is also evidence that the neuropeptides implicated in social behaviour, OXT and AVP, are involved in the aetiology of autism. Indeed, transcription of these candidate genes for ASD is altered in the *Fmr1* KO mice across five brain regions critical for motivation/reward, emotion and cognitive control: notably the NAc, CPu, VTA, CeA and PFC.

Interestingly, mRNA levels for DOR (*Oprd1*), KOR (*Oprk1*), prodynorphin (*Pdyn*) and proenkephalin (*Penk*) were significantly down-regulated in some of the brain regions of *Fmr1* KO vs. WT examined (Figure 2.5). Significant reduction of transcripts was observed in the VTA for *Oprd1* (~1.7 fold, $p < 0.001$), *Oprk1* (2 fold, $p < 0.05$) and *Pdyn* (~1.5 fold, $p < 0.05$), in the CeA for *Oprd1* (~1.2 fold, $p < 0.01$) and *Oprk1* (<2 fold, $p < 0.05$), and in the PFC for *Oprk1* (~1.5 fold, $p < 0.01$), *Pdyn* (~1.2 fold, $p < 0.05$) and *Penk* (~1.8 fold, $p < 0.01$). MOR (*Oprm1*) transcript levels were consistent with binding data (Figure 2.2) showing no significant changes across all brain regions. Notably, the *Penk* gene coding for the precursor protein which binds to MOR and DOR was down-regulated (~1.7 fold, $p < 0.01$) in the CPu of *Fmr1* KO mice.

Transcript levels were also modified for genes coding for vasopressin receptor (*Avpr1a*) in the CPu and the CeA, the vasopressin receptor (*Avpr1b*) in the PFC and the oxytocin peptide (*Oxt*) and its receptor (*Oxtr*) in the NAc, PFC, VTA and the CeA (Figure 2.6A). In particular, up-regulation of *Avpr1a* (3 fold, $p < 0.05$) mRNA in the CPu and down-regulation in

the CeA (~1.7 fold, $p<0.01$) were detected, as well as reduction of *Avpr1b* (~ 1.7 fold, $p<0.05$) of mRNA in the PFC. No alteration of *Avp* mRNA was detected across all brain regions examined. Interestingly, a significant 3-6 fold decrease in *Oxt* transcripts was found in the PFC ($p<0.01$) and in the NAc ($p<0.05$), and ~1.5 fold significant decrease was observed in *Oxtr* in these regions (PFC ($p<0.01$), NAc ($p<0.05$)). *Oxtr* mRNA was also altered in the CeA ($p<0.01$) and the VTA ($p<0.05$) with ~1.5 fold decrease in the *Fmr1* KO mice.

With regards to the dopaminergic system, significant decrease in *Drd1a* (1.5 fold, $p<0.001$) and *Drd5* (>1.5 fold, $p<0.01$) mRNA was noted in the CeA, and a significant reduction was also marked in *Drd2* (~1.5 fold, $p<0.05$) and *Drd5* (<1.5 fold, $p<0.01$) mRNA in the PFC. *Drd1a* transcripts were decreased by ~1.2 fold ($p<0.05$) in the CPu, and *Drd5* transcripts were increased by ~1.2 fold ($p<0.05$) in the Nac (Figure 2.6B). With respect to the metabotropic glutamatergic system, significant down-regulation of several mGluR subtypes was observed in several brain regions of *Fmr1* KO mice with only *Grm1* ($p<0.01$) and *Grm4* ($p<0.05$) being up-regulated by ~1.5 fold in the VTA and NAc of *Fmr1* KO mice, respectively (Figure 2.6C). In particular, significant reduction of transcripts was observed in the CPu for *Grm1* (~1.2 fold, $p<0.01$), *Grm5* (~1.2 fold, $p<0.05$) and *Grm8* (~2 fold, $p<0.05$), in the PFC for *Grm4* (~1.2 fold, $p<0.05$), *Grm5* (1.5 fold, $p<0.01$), *Grm7* (~1.2 fold, $p<0.05$) and *Grm8* (1.5 fold, $p<0.01$), in the NAc for *Grm2* (1.5 fold, $p<0.01$), *Grm5* (~1.2 fold, $p<0.05$) and *Grm7* (<1.2 fold, $p<0.01$), in the VTA for *Grm3* (~1.7 fold, $p<0.01$), *Grm5* (~1.3 fold, $p<0.05$), *Grm7* (~ 1.3 fold, $p<0.05$) and *Grm8* (1.5 fold, $p<0.05$), and in the CeA only for *Grm7* (~1.2 fold, $p<0.01$).

Significant transcriptional alterations were also noted in the vasoactive intestinal peptide (*Vip*) and its receptors (*Vipr1*, *Vipr2*) in the *Fmr1* KO mice. Significant increase was observed in the *Vip* mRNA by 1.2 fold in the CeA ($p<0.001$), while decrease by 1.5 fold was detected in the mRNA of *Vipr1* in the PFC ($p<0.05$), in the CPU ($p<0.05$) and in the CeA

($p < 0.001$). Also, *Vipr2* mRNA was down-regulated by 1.7 fold in the PFC ($p < 0.01$), 1.3 fold in the NAc ($p < 0.001$) and ~1.8 fold in the CPu ($p < 0.001$) and the VTA ($p < 0.05$) (Figure 2.6D).

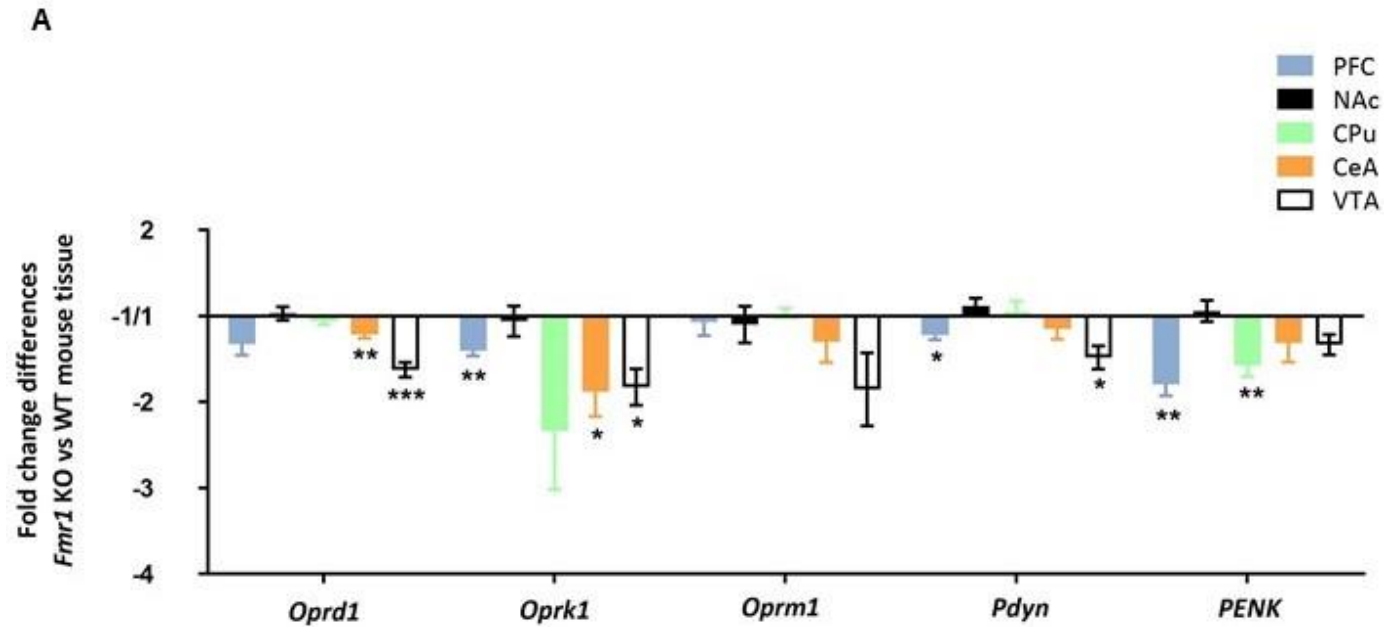
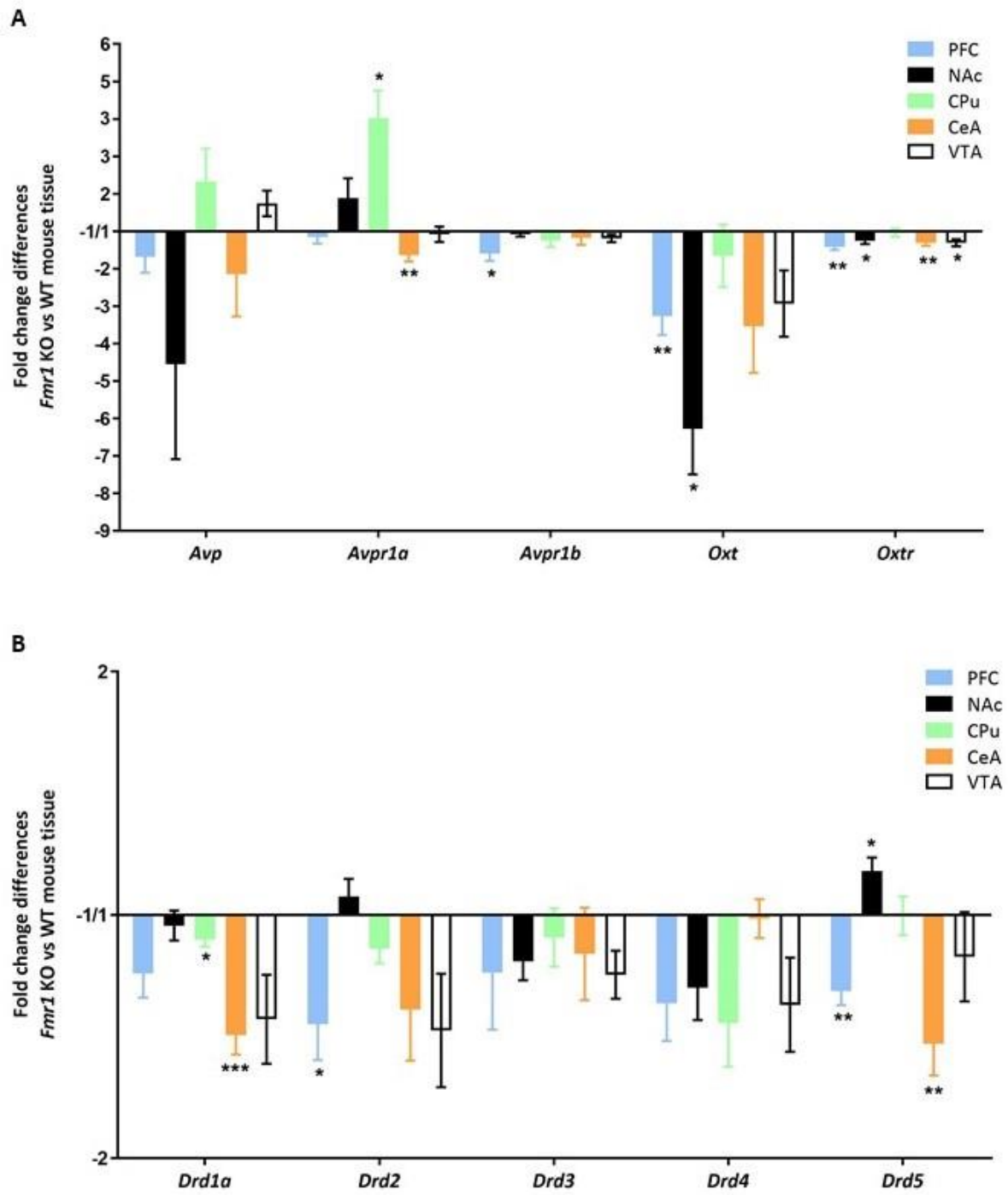


Figure 2.5: *Fmr1* KO mice display transcriptional alterations within the endogenous opioid system.

A) Brain-region specific mRNA extracted from tissues of WT and *Fmr1* KO mice (n=5) for five regions; the NAc, CPu, PFC, CeA and the VTA. mRNA was amplified for DOR (*oprd1*), KOR (*oprk1*) and MOR (*oprm1*) opioid receptors, prodynorphin (*Pdyn*) and preproenkephalin (*Penk*) primers displaying a significant down-regulation of the respective genes of the opioid system. Data analysis was performed using a comparative C_t method. Significance of qRT-PCR results was assessed after transformation using a one sample t-test ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).



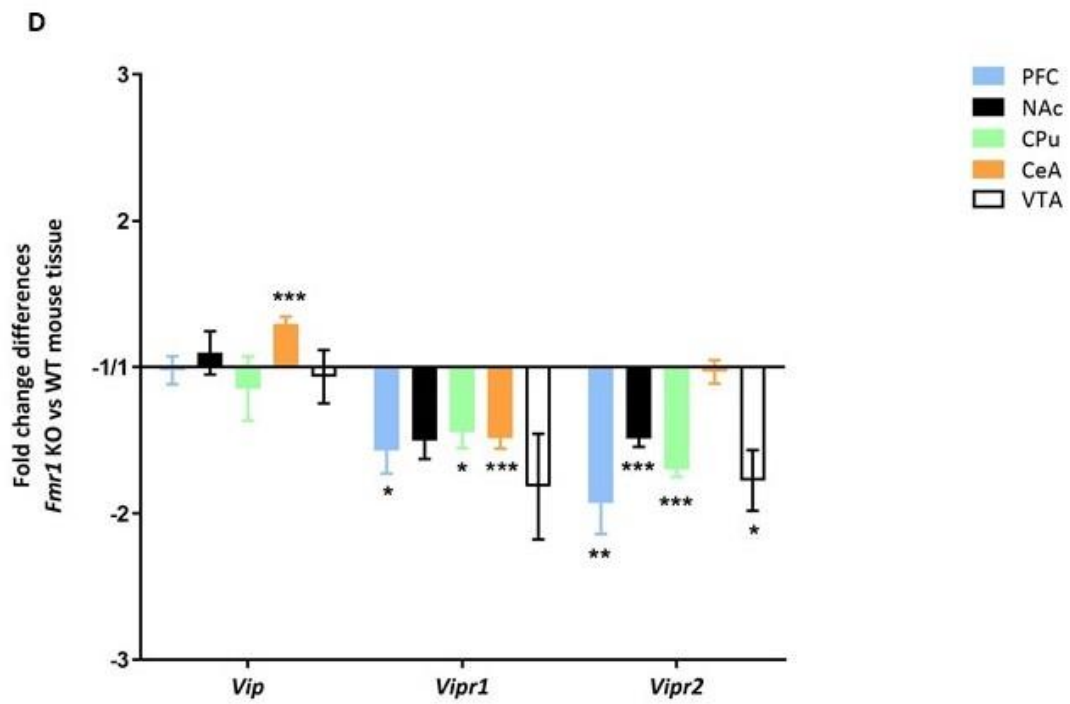
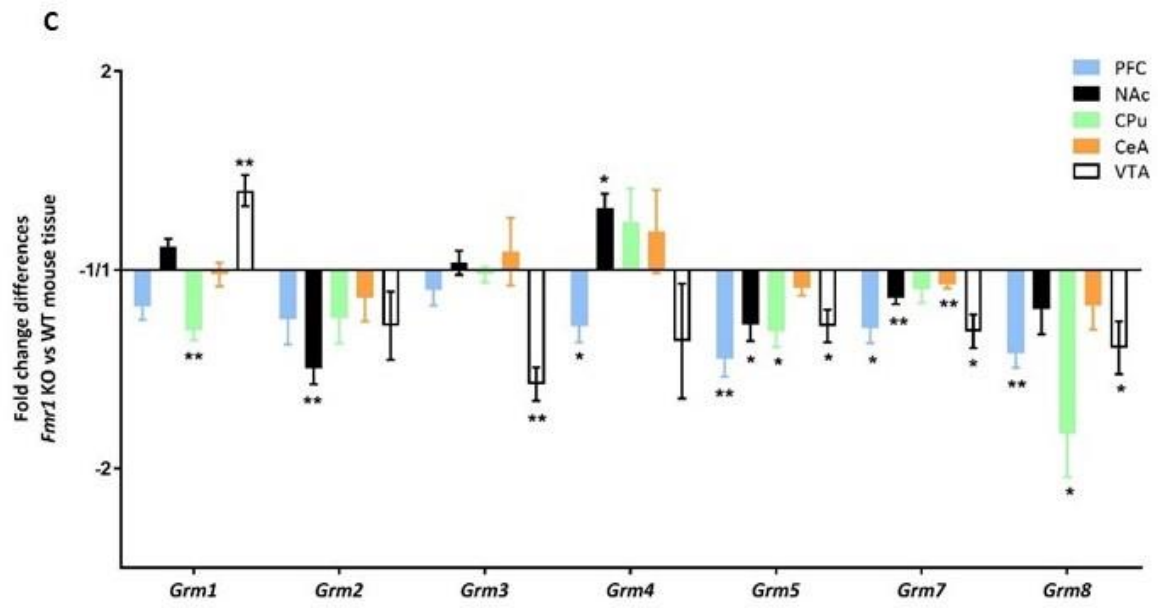


Figure 2.6: qRT-PCR data analysis of candidate genes across five regions; the nucleus accumbens (NAc), caudate putamen (CPu), prefrontal cortex (PFC), central amygdala (CeA) and the ventral tegmental area (VTA) in the *Fmr1* KO mice.

Brain-region specific mRNA extracted from tissues of WT and *Fmr1* KO mice (n=5). *Fmr1* KO mice display transcriptional alterations A) of OXT and AVP; mRNA was amplified for *Avp*, *Avpr1a* and *Avpr1b*, *Oxt* and *Oxtr* primers B) of the mGluRs; mRNA was amplified for mGluRs 1-5 and 7-8. (*Grm1-5* and *Grm7-8*) primers C) in the dopaminergic receptor system; mRNA was amplified for DA receptors (*Drd1a*, *Drd2-5*), D) for genes of vasoactive intestinal peptide (*Vip*) and their receptors (*Vipr1* and *Vipr1*) displaying an overall down-regulation of the respective genes of the dopaminergic system. Data analysis was performed using a comparative C_t method. Significance of qRT-PCR results was assessed after transformation using one sample t-test ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

2.5. Discussion

Overall our data demonstrate global down-regulation of mRNA levels in the majority of genes analysed in the *Fmr1* KO mouse including transcripts of genes of the opioidergic, dopaminergic, glutamatergic systems, the neuropeptides implicated in social behaviour and the vasoactive intestinal peptide. With respect to opioid receptors, these changes in mRNA levels did not translate to receptor binding changes, which were unaltered. This widespread down-regulation of the transcripts in the *Fmr1* KO mouse, is seemingly contradictory to the role of the Fmrp protein which suppresses translation of proteins, thus in the absence of which excess translation occurs (Jacquemont *et al.*, 2014). Nonetheless, it needs to be noted that the Fmrp has a suppressive role at the translation level (Li *et al.*, 2001), not the transcriptional level, and thus without additional experiments e.g. employing western blotting and double labelling immunohistochemistry to measure protein levels of these genes, it would be difficult to ascertain, if the down-regulation of gene expression actually translates to down-regulation of protein levels.

2.5.1. Regulation of the endogenous opioid system in the *Fmr1* KO

Our data demonstrate that inactivation of the *Fmr1* gene does not influence MOR, DOR and KOR expression at a receptor binding level (Figures 2.2, 2.3 and 2.4 respectively); however, it alters the transcript levels of their respective genes but not of the *Oprm1* gene (Figures 2.5; 2.6 and Table 2.2). Work conducted at the laboratory of Dr. Becker and Dr. Le Merrer suggests that the endogenous opioid system is differentially regulated in the *Fmr1* KO mouse model, as indicated by the alterations in the behavioural responses upon morphine administration. In particular, acute morphine (5 mg/kg) administration reversed the social deficits (Appendix C, D for data) and suppressed self-grooming behaviour (Appendix E for data), indicator of stereotypy behaviour, in *Fmr1* KO mice. These findings suggest an

impairment of the endogenous opioid system in this model of ASD, which may underline the observed autistic-like behaviour. Additionally, morphine-induced locomotion was significantly lower in the *Fmr1* KO mice in comparison to WT and morphine-induced analgesia was found to be significantly higher in the mutated mice compared with WT controls (Appendix B for data). These findings reinforce the suggested dysregulated opioid system in this mouse model of autism.

In line with this, our data demonstrate that several alterations on transcript levels have been detected for the DOR (*Oprd1*), KOR (*Oprk1*) *Penk* and *Pdyn* genes in some of the brain regions examined (Figure 2.5). Interestingly, a genetic polymorphism of gain in function on the *PENK* gene in human was found to be protective over social deficits (Troisi *et al.*, 2011) and CPu is a key region for behaviours relevant to ASD (Langen *et al.*, 2009) including sensorimotor gating and social interaction sensitivity (Wyers *et al.*, 1968, Swerdlow *et al.*, 2001, Peeke and Herz, 1971). Also, *Penk* and *Pdyn* transcripts that bind to MOR/DOR and KOR, respectively, are down-regulated in many regions of the *Fmr1* KO mice including the CPu and PFC. However, the receptor binding (protein) levels remained intact in the *Fmr1* KO mice. This suggests if there is any impairment in the endogenous opioid receptor system, it should be found in downstream of the signalling cascade and this might underline some of the behavioural symptoms observed in *Fmr1* KO mice. Furthermore, the marked decrease in the *Pdyn* transcripts may negatively affect striatal DA levels and social reward and motivation. It has been shown that dynorphin-KOR systemic activity play a critical role in regulating the state of the brain reward by modulating DA release in the striatum (Di Chiara and Imperato, 1988a). Notably, decrease in dopaminergic activity following KOR activation is associated with aversive behaviour in mice (Shippenberg *et al.*, 1992; Bals-Kubik *et al.*, 1993; Shippenberg and Elmer, 1998). Interestingly, no alterations were detected on the DOR and KOR protein level but down-regulation of their transcripts suggesting a lack of concordance between mRNA and protein level.

With regards to the implication of the MOR system in the *Fmr1* KO mouse brain, the MOR deficient mouse model, *Oprm1*^{-/-} has been shown to recapitulate an autistic-like phenotype (Becker *et al.*, 2014). This would thus suggest that changes in this receptor system are anticipated to be observed in the *Fmr1* KO mouse. Nevertheless, no alterations were found in the MOR protein and mRNA expression in any of the regions analysed indicating lack of disruption of MOR at protein and mRNA levels, which further suggests that *Fmr1* does not regulate MOR transcription or MOR receptor density. Hence impairment of the MOR system implicated by the behavioural data in the *Fmr1* KO is not due to disruption of MOR at protein or mRNA level. Subsequently, we speculate that alterations are found at intracellular signalling and second messenger level. MOR stimulating GTPγS binding assay in the *Fmr1* KO mouse brain would clarify whether there is disruption in the MOR activity in this model. In conclusion, our findings report modifications in mRNA levels of the opioid receptors, which are not translated to changes of their respective proteins in the *Fmr1* KO mouse, as it has been investigated in this study using receptor binding studies. This was not anticipated to be observed in the *Fmr1* KO mice while excess synthesis of other proteins has been detected in an FXS mouse model and cells from patients (Gross and Bassell, 2012). However, receptor expression measured by ligand binding provides us with information more relevant to function than mRNA expression, so we are unable to argue much importance of the mRNA changes observed. Therefore, whatever the aetiology behind the alterations in mRNA levels, due to the lack of change in the protein receptor level it would be safe to conclude that the behavioural alterations observed in the *Fmr1* KO mice are not due to any alterations of DOR, MOR or KOR levels.

2.5.2. Regulation of oxytocin and vasopressin in the *Fmr1* KO

Other neurotransmitter systems may be also implicated in the marked behavioural alterations in the *Fmr1* KO mice. The neuropeptides OXT and AVP have been involved in

the aetiology of autism (Insel *et al.*, 1999), therefore these are also potential targets for investigating the neurobiology of the *Fmr1* KO. Modahl *et al.* (1992) suggested that an interplay of deficit in the OXT systems and the modulation of opioids might underline the social deficits in autism. Moreover, the opioid antagonists, naloxone and naltrexone, potentially increase OXT levels in the brain whilst they do not affect AVP levels, indicating regulatory mechanisms underlining the relationship of opioids and OXT (Bicknell *et al.*, 1985; Vuong *et al.*, 2010). Interestingly, a study by Dr. Chang *et al.* 2017 is underway investigating the combinatorial benefit of administering OXT in conjunction with naloxone or naltrexone in a nonhuman primate model and whether this treatment will promote optimal prosocial effects including social attention and natural social interaction (<https://sfari.org/funding/grants/abstracts/optimizing-social-effects-of-oxytocin-with-opioid-blockers>). Whether dysregulation of the oxytocin system is driven by the dysregulation of opioid peptide levels cannot be determined from this study but further investigation is warranted on the interplay between opioid and OXT in the CNS.

OXT receptor is encoded by the *Oxtr* and there are three subtypes for AVP receptors which are encoded by the genes *Avpr1a*, *Avpr1b* and *Avpr2*. With respect to the involvement of neuropeptides implicated in social behaviour in the *Fmr1* KO, our data reveal profound decrease of *Oxt* transcripts but to a lesser extent on *Oxtr* in the PFC and NAc reward brain regions. Both human and animal data, point towards OXT deficit at least partly to be responsible for some ASD symptoms (see section 3.1.4). This evidence together is in line with our data, as we display a significant decrease of *Oxt* and *Oxtr* concomitant with social deficit in *Fmr1* KO (Figure 2.6A) that further support the OXT deficiency theory in autism. Nonetheless, decrease in *Oxt* mRNA does not necessarily mean decrease in the OXT peptide availability in these brain regions or even in neuronal OXT release. Further microdialysis experiments would reveal whether changes in mRNA translate to changes in OXT peptide release in the NAc and PFC of the *Fmr1* KO mice.

In addition, AVP receptors are also implicated in ASD. *AVPR1A* has been known for its role in modulating a range of behaviours including stress, social bonding and autism (Charles *et al.*, 2014). Autism-associated variants in the *AVPR1A* gene are linked to abnormal activity in the amygdala (Meyer-Lindenberg *et al.*, 2009). Ongoing preclinical work from Roche pharmaceuticals reveals that *Avpr1a* antagonist restores social interaction in a rat model of autism (Grundschober, Roche and Pharmaceutical, 2015). Altogether these findings which support our data, suggest that the *Avpr1a* is likely to contribute to the pathology of autism and must be further investigated. In our study, we found that although *Avp* mRNA is not altered in any of the regions analysed, *Avpr1a* transcription is up-regulated in the CPu and down-regulated in the CeA, and *Avpr1b* is down-regulated in the PFC (Figure 2.6A) suggesting a dysregulation of the central endogenous AVP system in the *Fmr1* KO mice. This is in line with the evidence associating CNS AVP dysregulation and autism (Leboyer *et al.*, 1992; Boso *et al.*, 2007). It is not possible to know from this data alone if dysregulation of the AVP receptor expression is involved in the ASD pathology, and future studies employing receptor binding and/or western blots experiments would be interesting to further investigate this relationship.

2.5.3. Regulation of the dopamine and metabotropic glutamate receptors

There is substantial evidence to suggest that *Fmr1* deletion affects the dopaminergic and glutamatergic neurotransmitter systems that may contribute to the impairment of social reward processes in the brain of these mice. Changes in DA activity is associated with numerous core deficits in autism and comorbid symptoms including seizures (Starr, 1996; Tuchman and Rapin, 2002), motor problems (Rinehart *et al.*, 2001, 2006) and repetitive behaviours (Canales and Graybiel, 2000; Ralph *et al.*, 2001; Ralph-Williams *et al.*, 2003). In particular, loss of *Fmr1* differentially alters dopaminergic function in the mesolimbic and nigrostriatal pathways, and decreases dopaminergic activity thus leading to dysregulation in

both reward and movement (Wang *et al.*, 2008; Fish *et al.*, 2013). Dysregulation of the DA system has been reported in children with autism and ADHD demonstrated either by low DA levels that cause impaired attention and focus or high DA levels which result in hyperactivity and increased sensory inputs, causing ultimately an overload on the brain processing ability (Lake *et al.*, 1977; Pasini and D'Agati, 2009). In line with the evidence showing dopaminergic dysregulation in ASD and ADHD, our data demonstrate an overall down-regulation of the mRNA levels encoded by the DA receptors (*Drd1*, *Drd2* and *Drd5*) genes in the *Fmr1* KO mice across the majority of the brain regions but the *Drd5* increased transcripts in the NAc (Figure 2.6B). Our results thus suggest a hypofunction of dopaminergic activity across all brain regions analysed in this animal model of ASD, which appears rather as a paradox, as antipsychotics are used for the management of symptoms in ASD. However, it has to be noted that ASD is manifested with varied symptoms in individuals affected by the pathology. It is reported that antipsychotics used such as risperidone are mostly effective for children with autism and comorbid irritability and aggressive behaviour (Sharma *et al.*, 2012). Thus it might be that these discrepancies lie in the wide spectrum of neurobiological systems that contribute to the development of ASD, and treatment with antipsychotics is not effective for all ASD individuals.

Interestingly, *Fmr1* KO mice exhibit increased DA turnover in the striatum and cortex regions (Gruss and Braun, 2004) further supporting the presence of a dopaminergic dysregulation in this ASD model. Moreover, *Drd5* was identified as risk factor gene for ADHD and significant down-regulated mRNA levels of the receptor were identified in whole blood counts in ADHD subjects in a population of Chinese Han children (Xu *et al.*, 2015). It would be intriguing to investigate whether the decrease in *Drd5* transcript in the CeA and PFC of *Fmr1* KO underline any ADHD related symptoms which are prominent in ASD. Whether down-regulation of DA receptors transcript translates to changes in DA receptor protein and activity is a matter which warrants further investigation.

Absence of FMRP protein has been shown to induce exaggerated local protein synthesis (Jacquemont *et al.*, 2014), which is contradictory to our results showing a down-regulation of gene transcripts in the majority of the genes analysed. Notably, glutamate transmission abnormalities derived from dysregulated glutamate receptor numbers and function have been extensively reported in models of ASD and thus, mGluRs antagonists are considered potential therapeutic targets for the treatment of ASD (Carlson, 2012). Our data illustrate that majority of the mGluR transcripts tested were decreased in most of the brain regions analysed, except for the *Grm1* and the *Grm4* (Figure 2.6C), which maybe in line with findings reporting increases in glutamate levels of ASD patients (Zheng *et al.*, 2016). However, *Gmr4* was shown to be down-regulated in the striatum of opioid deficient model of ASD, notably the *Oprm1*^{-/-} mice (Becker *et al.*, 2014), which is contradictory to our findings that support the presence of up-regulated *Grm4* in the striatum of *Fmr1* KO mice. Nonetheless, it needs to be noted that although both KO mice strains display an ASD phenotype, the deleted gene responsible for this phenotype is distinct. Therefore, it could be that the *Fmr1* gene and Fmrp protein may have completely the opposite effect on *Gmr4* regulation compared to *Oprm1*. Whether the alterations of *Gmr4* transcripts translate to changes in Gmr4 receptor protein levels is not known, but warrants further investigation.

We also assessed the expression levels of the vasoactive intestinal peptide (*Vip*) peptide and its receptors that are implicated in ASD (Figure 2.6D). Emerging preclinical and clinical evidence support that alterations in the *Vip* gene is associated with deficits in social behaviour and stereotypies observed in individuals with autism (White *et al.*, 2010). Our data reveal an increase on mRNA levels encoded by *Vip* and an overall down-regulation of its receptors gene transcripts across the five brain regions, further supporting in this way a relationship between these genes and autism. It might be that the up-regulated *Vip* triggers the down-regulation of the receptors *Vipr1* and *Vipr2* via a homeostatic compensatory mechanism. Future research should focus on assessing the potential link between the

aforementioned dysregulated genes and ASD with the vision of developing novel targets for its treatment.

2.6. Conclusion

Finally, our findings conclude that while there are no alterations of opioid receptor binding levels in the *Fmr1* KO mouse model, changes on transcripts within the endogenous opioid system in the brain are detected. In addition, transcription levels of other major neurotransmitter systems (OXT, DA and glutamate) discussed are also differentially regulated in the *Fmr1* KO, which points towards potential targets for the development of novel treatment approaches. Whether the observed changes in the social behaviour in the *Fmr1* KO mice are concomitant to these transcription alterations warrants further investigation.

3. Chapter 3: Effects of chronic intranasal oxytocin treatment on social behaviour, gene expression and receptor binding in an animal model of autism

3.1. Introduction

The natural neuropeptide OXT is an attractive therapeutic target for psychiatric disorders with impairment in the social domain, such as autism. While OXT has a long-established role in milk ejection, uterine contraction during labour as well as in regulating the function of peripheral target organs, relatively recent research suggests that OXT and its closely related peptide, AVP, play an important CNS role in the development of normal cognition and in complex social behaviour (Popik and van Ree, 1991; Carter *et al.*, 1992; Insel and Hulihan, 1995). OXT plays a crucial role in social exploration and recognition, attachment, anxiety and stress-related behaviours (Meyer-Lindenberg *et al.*, 2011). While the two peptides are closely regulated and often demonstrate compensatory functional roles, this chapter is focussed on the role of the OXT peptide and its involvement in autism, with AVP occasionally included in the discussion.

The heterogeneity that characterises ASD pathology calls for diagnosis-based pharmacotherapies. It is not yet clear whether a compromised OXT system contributes to ASD. Also, the consequences of exposure to chronic OXT treatment are not yet known and further studies are essential. Studies have shown that chronic intranasal administration reduces OXTR binding in various brain regions (i.e. NAc, AMG) and social behaviour in WT healthy mice (Huang *et al.*, 2014), as well as causes long-term impairments in formation of typical social bonding in WT healthy male prairie voles (Bales *et al.*, 2013). These together suggest that long-term changes in social behaviour induced by chronic OXT treatment seem to diminish rather than promote social behaviours under physiologic conditions and these effects might persist long after the treatments themselves. Further studies are essential to shed light on the potential detrimental effects of chronic OXT treatment on brain maturation. Therefore, the therapeutic value of intranasal OXT remains dubious, as evidence is not yet sufficient to allow OXT to be prescribed to patients. Nevertheless, animal studies are

essential tools to reinforce our knowledge via investigating mechanisms of actions, determining the right dose and age, optimizing current therapies/ administration methods and developing next-generation approaches.

3.1.1. Neurobiology of oxytocin: structure, pharmacology, anatomy and physiology

OXT and AVP were identified as neurohormones of the hypothalamic neurohypophyseal system (Bargmann and Scharrer, 1951). OXT was primarily known for its importance in stimulating uterine contraction during parturition and milk ejection during lactation (Burbach *et al.*, 2001), while AVP was mainly known as an antidiuretic hormone that regulates water homeostasis and as a vasoconstrictor (Kozniowska and Romaniuk, 2008). In the CNS, AVP affects memory by facilitating storage and retrieval of acquired information, whilst OXT has the opposite effect possessing amnesic properties (Bohus *et al.*, 1978). Later studies have revealed OXT's key roles in the control of more complex behaviours such as aggression, fear conditioning, anxiety, social and affiliative behaviours (Carter, 2003; Donaldson and Young, 2008; Carter *et al.*, 2009; Ebstein *et al.*, 2009; Insel, 2010).

OXT is synthesized in magnocellular neurons of the supraoptic- (SON) and the paraventricular (PVN) nuclei of the hypothalamus, which project through their axons to the posterior pituitary, where they are released into blood circulation to further act on distant organs of the periphery. In addition, dendritic release of OXT modulates local activity of the hypothalamic neurons but also reaches distant targets of the brain through diffusion. Parvocellular OXT-containing neurons in the PVN also have projections to other regions of the brain such as the AMG, Hip, suprachiasmatic nucleus, striatum, bed nucleus of stria terminalis (BNST) and brainstem and they modulate neurotransmission in these regions (Meyer-Lindenberg *et al.*, 2011) (Figure 3.1). OXT bound to OXT receptors initiates autoregulatory positive feedback of OXT release via increased influx of intracellular Ca^{2+} mechanisms in the Hyp and posterior pituitary (Dayanithi *et al.*, 2000; Ludwig *et al.*, 2002). It

has been demonstrated that OXT release from the SON increases in response to social defeat, whereas peripheral OXT release was not affected (Engelmann *et al.*, 1999) suggesting that central and peripheral oxytocin act either in synchrony or independently during neuroendocrine-mediated behaviours to exert their behavioural effects (Baskerville and Douglas, 2010).

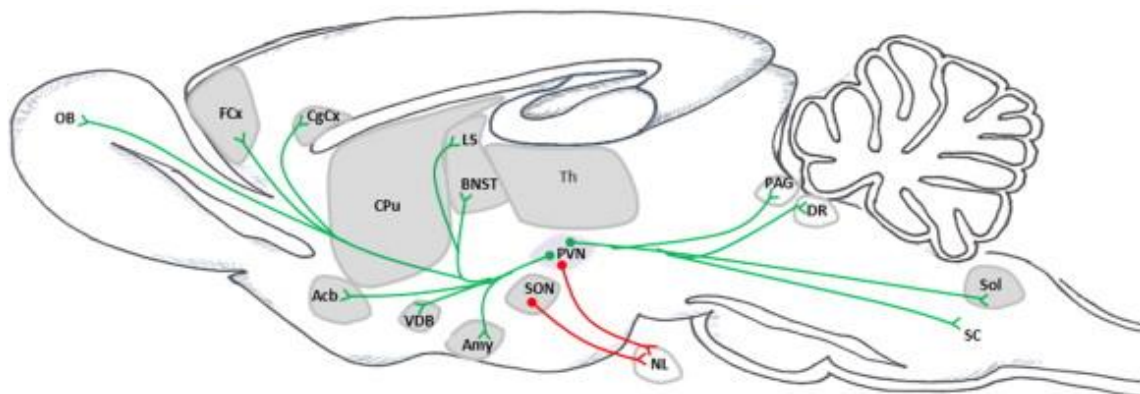


Figure 3.1: Representation of the oxytocinergic projections circuitry in the brain of rodents.

OXT is synthesized in the SON and the PVN nuclei of the hypothalamus, which project through their axons to the posterior pituitary, where they act on distant organs of the periphery. Parvocellular OXT-containing neurons in the PVN have projections to other regions of the brain such as the amygdala, hippocampus, suprachiasmatic nucleus, striatum, bed nucleus of stria terminalis and brainstem, where they modulate neurotransmission. Adapted from Zanos *et al.* (2014).

OXT is synthesized by proteolytic cleavage from its precursor protein, neurophysin-1, and it is a nine amino-acid peptide widely abundant in reproductive related-tissue and the brain. The *OXT* gene was sequenced in 1984 (Ivell and Richter, 1984) and OXT was the first peptide hormone to be synthesized chemically in its biologically active form (Du Vigneaud *et al.*, 1953) (Figure 3.2).

The *OXT* receptor (*OXTR*) gene has been detected in several species. It was primarily isolated, cloned and identified in human (Kimura *et al.*, 1992), and later in mouse (Kubota *et al.*, 1996) and rat (Rozen *et al.*, 1995). OXT binds predominantly to its only receptor, the *OXTR*, and it shows lower affinity towards AVP receptors (*AVPR*). After sequence comparison, the mouse *oxtr* gene shows 91% homology to the human and 97% to the rat homology (Kubota *et al.*, 1996; Kumsta *et al.*, 2013). In the mouse brain, *OXTR* is abundantly found in the AMG, olfactory nuclei, Hip and Hyp (Gould and Zingg, 2003). It is suggested that *OXTR* act at sensory modalities to modulate social approach and behaviour by utilising olfactory inputs in rodents (Grinevich *et al.*, 2016) and visual inputs in primates (Freeman *et al.*, 2014).

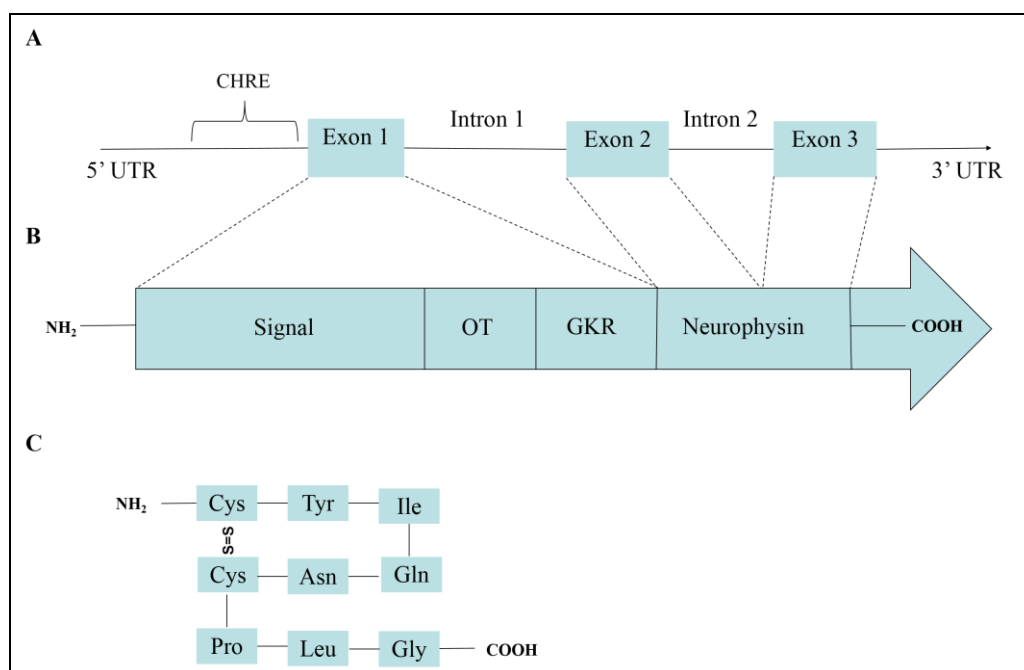


Figure 3.2: Representation of the A) OXT gene, B) mRNA and C) the neuropeptide itself.

Modified from Gimpl and Fahrenholz (2001).

The OXTR belongs to the family of seven transmembrane $G_{\alpha/q11}$ protein-coupled receptors (Kubota *et al.*, 1996). Upon activation of the OXTR, the α subunit of the G protein dissociates from $\beta\gamma$ subunits and activates phospholipase (PLC_{β}) isoform in the cytoplasm, which generates inositol phosphatase (IP_3) and 1,2 diacylglycerol (DAG_{1-2}) products. IP_3 triggers Ca^{2+} release from intracellular stores of the endoplasmic reticulum and mitochondria, while DAG activates protein kinase C (PKC) that further phosphorylates other proteins. Many cellular responses can be initiated directly or indirectly through this molecular pathway (Gimpl and Fahrenholz, 2001). For an extensive review on the OXTR pharmacology see Gimpl and Fahrenholz (2001) and Devost *et al.*, (2008).

3.1.2. Routes of oxytocin administration

OXT agonists and antagonists are commonly administered via the intravenous, intramuscular and intranasal route and they are routinely used in obstetrics and/or preclinical research. However, the highly significant homology between the OXTR, AVP peptides and their receptors, OXTRs and AVPRs (V1a, V1b, V2) respectively, makes it difficult to distinguish their specific action (Manning *et al.*, 2008). The experimental and therapeutic use of neuropeptides in CNS disorders and CNS translational research has been hampered by their inability passing through the blood-brain-barrier (BBB), and causing potent hormone-like side effects, when administered systemically (Illum, 2000). Oral OXT administration is not considered as a sustainable route due to the elimination of the peptide en route to the 'first pass metabolism' (Lee, 1988). Additionally, parenteral OXT injections are unlikely to bypass the BBB and reach the brain in significant concentrations due to the large size of the OXT peptide (1008Da) (Ermisch *et al.*, 1985). The BBB restricts free diffusion of lipophilic molecules from blood circulation to the brain, when molecules are larger than 500Da (Pardridge, 2002). Initially, it was mistakenly thought that there was free communication between CSF and the extracellular fluid in the CNS, allowing for the determination of peptides levels in the brain through the concentration of these hormones in the CSF. Preclinical work demonstrated that only 0.01% of the OXT peptide is able to cross the BBB after intravenous administration (Kendrick *et al.*, 1991) and peptides, which are administered peripherally have a short half-life (Mens *et al.*, 1983). Indeed, it has been shown that intravenous OXT administration in high doses is able to enter the brain in minimal concentrations via areas with tight capillary endothelial junctions and in significant concentrations via circumventricular organs with leaky capillary endothelial junctions (Landgraf *et al.*, 1979; Ermisch *et al.*, 1985). Additionally, intravenous OXT administration is an unfavourable administration route for treatment of CNS disorders in humans due to high risk of cardiovascular side-effects (Dyer *et al.*, 2011). Following these findings, the most

reliable animal studies investigating functional roles of OXT had used intracerebroventricular or intracerebral administration routes. However, this is an invasive method with no feasibility and high risk of potential complications in human research.

Intranasal administration of neuropeptides such as OXT and AVP seems a promising administration route for treatment of neurogenic diseases. Direct delivery of biologically effective quantities to the brain via the nasal passage offer major advantages over other administration routes including increased compliance (Guastella *et al.*, 2013), ease of use, faster drug onset without causing systemic adverse effects (Yamamoto *et al.*, 2001) and greater bioavailability (Miller *et al.*, 2008). It has been shown that the OXT's highly homologous peptide, AVP and other hormone-like peptides can cross the BBB and elicit elevated concentrations in the CSF and blood following 15-30 min of intranasal administration (Born *et al.*, 2002). Thereafter, break-through studies have also reported that intranasal administration of OXT increased OXT concentration in the CSF and blood in humans (Striepens *et al.*, 2013) and the CSF in macaques (Monte *et al.*, 2014). Preclinical studies have also demonstrated that intranasal administration elevated levels of OXT in the CSF (Born *et al.*, 2002), as well as in plasma and microdialysates within the dorsal hippocampus and the amygdala in rats and mice (Neumann *et al.*, 2013).

There is great controversy on how intranasal OXT administration mediates its functional effects within the CNS. Whether increased blood OXT levels are caused via direct nose to brain penetration or via indirect afferent feedback from the periphery that stimulates endogenous OXT release within the brain and in parallel, secretion into the periphery remains an open question. It has also been shown that increased OXT concentration in blood might arise from vasculature absorption in the nasal mucosa and ends in the peripheral circulation (MacDonald and Feifel, 2013). The anatomical and physiological characteristics of the olfactory region provide intracellular and extracellular pathways through the nasal cavity into the CNS, which circumvent the BBB. There are three suggested

passages that OXT is transmitted via the nasal transportation to the CNS; either directly via olfactory and transgeminal nerve fibres (intracellular and extracellular transport) or indirectly through systemic circulation. Intracellular transportation involves endocytosis mechanisms of the olfactory sensory neurons that deposit the substance particles on the olfactory epithelium (Thorne *et al.*, 1995). OXTR have been found in the olfactory nuclei of humans and rats and the olfactory bulb of the rat (Yu *et al.*, 1996). However, as OXT is a hydrophilic peptide with a large molecular weight, it is less likely to use this mode of transport. Millions of olfactory sensory neurons receptors have been detected in the olfactory epithelium (Doty and Bromley, 2007), which have large projections to the mucous layer of the olfactory epithelia located in the upper posterior nasal cavity and in this way access the external environment. Thereafter, the filia olfactoria is formed by convergent arrangement of the olfactory cell axons, which cross the threshold of the skull via a gap between the ethmoid bone to the olfactory bulbs (Kalogjera and Dzepina, 2012; Gerhard, 2013). Extracellular transport occurs via paracellular diffusion for the olfactory and respiratory epithelia and mainly favours hydrophilic substances with a large molecular weight, thus it might potentially favour the OXT peptide (Balin *et al.*, 1986). Extracellular deposition of molecules can directly take place from the route of the olfactory epithelium to the olfactory bulb (Li *et al.*, 2005) or be facilitated by regeneration of the olfactory sensory neurons (Balin *et al.*, 1986). The third potential passage suggests that delivered substances travel indirectly to the brain crossing the BBB via the microvasculature facilitating rapid uptake to the surrounding capillary networks and ending into systemic circulation (Chow *et al.*, 1999; Wolburg *et al.*, 2008), from where they cross into the CSF and across the BBB (Wallum *et al.*, 1987) (Figure 3.3)

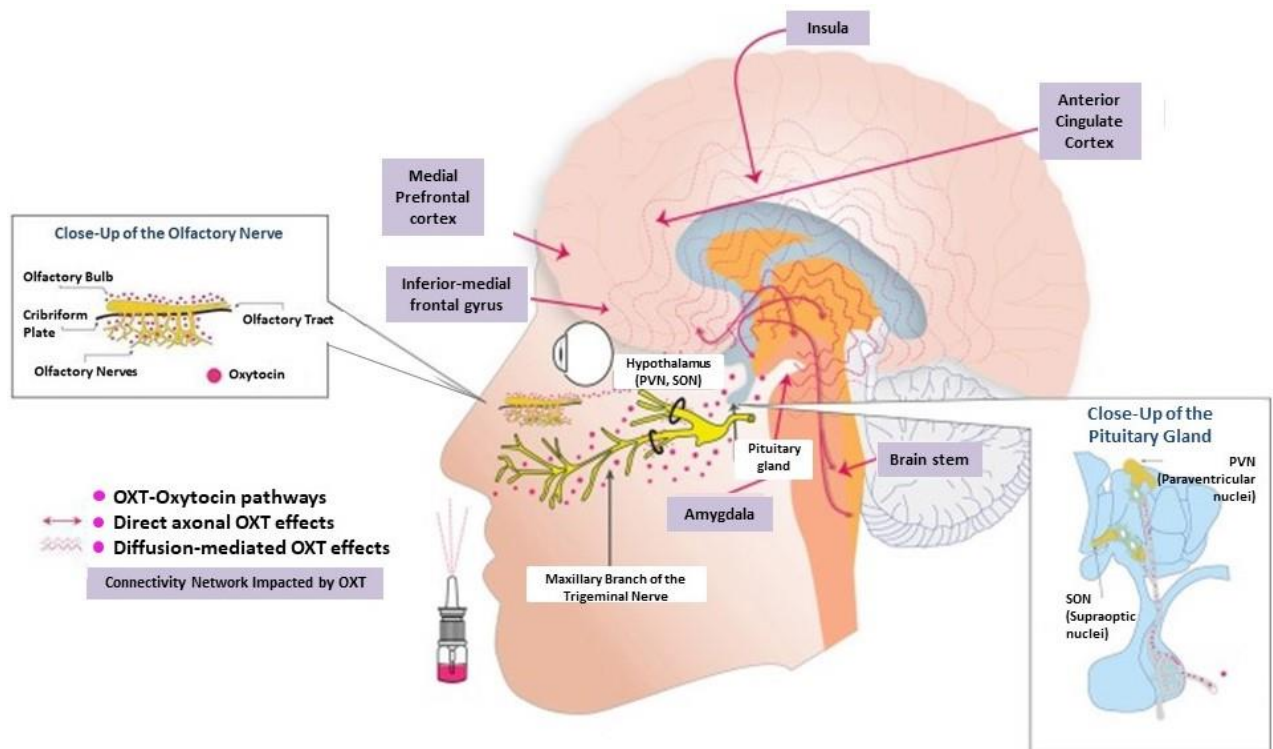


Figure 3.3: Intranasal oxytocin administration potential routes for therapeutic regulation of brain function in the ASD.

Potential aspects of how intranasal OXT treatment exerts its functional effects in autism are represented. One potential way is via extraneuronal/perineuronal routes along trigeminal or olfactory nerve pathways, which allow the crossing of the BBB directly and cause central effects. Other mechanisms of OXT's entry in the brain are discussed in detail in the text. Intranasal OXT may cause some of its central effects by stimulating the endogenous OXT system, which secretes OXT into the periphery and has direct-axonal, diffusion-mediated as well as OXT-OXT pathways mediated central effects (Landgraf and Neumann, 2004; Stoop, 2012). Intranasal OXT treatment impacts the function of important brain regions and, a few of these are represented here including the amygdala, insula, anterior cingulate, and the medial prefrontal cortex. Adapted from MacDonald and Feifel (2013).

OXT intranasal infusion is the preferred administration route for most studies on autism, as it has been shown that it can modulate a wide variety of complex social behaviours with only minimal side effects. Notably, chronic administration of intranasal OXT administered in high doses of 320IU within a period of 13 weeks caused only minor side effects (Epperson, McDougle and Price, 1996; Ohlsson *et al.*, 2005; Feifel *et al.*, 2010, 2012; Pedersen *et al.*, 2011). Translational research needs to overcome various limitations in relation to OXT based therapeutics in autism including limitations related to OXT dosage, chosen measurement techniques and biological medium analysed such as blood, CSF, urine, saliva (Carter *et al.*, 2007; Szeto *et al.*, 2011). Interestingly, a highly promising technique for detecting OXT in plasma in humans using a novel 2D liquid chromatography-tandem mass spectrometry assay has been reported (Zhang *et al.*, 2011).

3.1.3. Oxytocin interactions with motivational reward circuitry

The motivational theory in autism, as discussed earlier (chapter 1 and chapter 2), proposed by Chevallier *et al.* (2012), implicates the action of certain neuropeptides and neurotransmitters such as OXT, DA and opioids in the mesocorticolimbic circuit, which encompasses the VTA, NAc, AMG and PFC brain regions. In support of this, Gordon *et al.* (2016) proposed that diminished social motivation observed in ASD individuals mirrored decreased connectivity on this circuit. Therefore, it was proposed by his group that intranasal administration of OXT increased connectivity between subcortical (NAc, AMG) and cortical (PFC) regions within the circuitry by stimulating reward value from positive auditory (i.e. music) or visual (i.e. happy faces) stimuli (Gordon *et al.*, 2016). Studies in mammals suggest that OXT enhances reward via DA-dependent mesolimbic reward pathway (Donaldson and Young, 2008). In the mesolimbic pathway, DA is synthesised in the VTA and is released from projections to the NAc, AMG and the PFC. OXT which is synthesised in the PVN also projects to the VTA, NAc and the AMG regions and there is evidence to suggest that it can

potentially modulate DA activity along this circuit (Gordon *et al.*, 2016). The location of the OXTRs expression, which is abundant in the mesocorticolimbic DA system, places OXT in an ideal position to influence a wide range of motivated behaviours (Love, 2014). Interestingly, injection of OXT into the VTA of rats increased DA release in the NAc (Melis *et al.*, 2007; Shahrokh *et al.*, 2010), which suggests that OXTR stimulate DA transmission in the mesolimbic pathway (Peris *et al.*, 2017).

Preclinical research supports the notion that OXT affects social recognition (Choleris *et al.*, 2003; Ross and Young, 2009). While OXT administration into the hippocampus or medial preoptic area of the hypothalamus improves social recognition, intracerebroventricular administration of OXT antagonists results in social recognition deficits (Popik and van Ree, 1991; Van Wimersma and Maigret, 1996); OXT effects appear to be context-dependent. OXT administration in rodents increases positive social behaviours towards offspring and mates (i.e. in group) but also increases aggression towards strangers and conspecifics (i.e. out-group) (Love, 2014). Similarly, OXT administration has been associated with increased altruistic responses between members of the group they belong to and increased aggression towards members of other groups (De Dreu, 2012).

OXT acts on the mesocorticolimbic circuit and promotes the expression of human affiliative social behaviour between peers by increasing trust, and eliminating fear responses via reducing amygdala activity (Kirsch *et al.*, 2005; Lim and Young, 2006; Petrovic *et al.*, 2008), thereby greatly increasing benefits from social interaction (Kosfeld *et al.*, 2005). It has been demonstrated that OXT increases processing of facial stimuli in human faces via increased eye gazing (Guastella *et al.*, 2008) and, also increased ability of inferring others mental/emotional state from social cues of the eye region (Domes *et al.*, 2007), further illustrating its impact on affiliative social behaviour. Human research has also shown action of the OXT nasal spray on face processing networks like the amygdala that increased attention to positive social cues, such as exposure to happy over angry faces, and overall

improved emotion recognition (Domes *et al.*, 2007; Domes *et al.*, 2010, 2013). OXT might improve social cognition by enhancing gaze on cues that are important for social communication and the reward associated with engaging with the social cues (Guastella *et al.*, 2008). Interestingly, a single-dose study in healthy volunteers showed that OXTR gene polymorphisms alter the sensitivity to reward-relevant features and/or their aversive properties in infants (Marsh *et al.*, 2012). OXT administration has been also shown to enhance memory for social over other stimuli (Rimmele *et al.*, 2009). OXT has also established close interactions with the mesocorticolimbic DA system (Baskerville and Douglas, 2010). In particular, OXT enhances motivational salience towards social stimuli and carries signals related to motivational value. Thus, by modulating dopaminergic activity within this network, OXT may encourage changes in both motivational salience and valence (Love, 2014). In light of this evidence supporting a role of OXT in the motivation and reward circuitry, we hypothesize that by targeting this system by using pharmacological manipulations, there are potentials to develop novel approaches in the treatment of ASD pathology.

3.1.4. The relationship between oxytocin and autism

Both neuropeptides OXT and AVP have been found to be involved in the development of social attachments. OXT plays an important role in facilitating partner preference in the absence of mating in female prairie voles, while administration of OXT antagonist prevented partner preference without interfering with mating (Insel and Hulihan, 1995). Although several studies investigated OXT and social behaviour, we decided to focus on the relationship between OXT and ASD for this thesis.

OXT dysregulation has been reported in children with autism. Modahl *et al.* (1998) was the first to show that autistic children display significantly lower plasma OXT levels compared to typically-developed children. Autistic children failed to display increased OXT blood levels

during development; a physiological response associated with normal establishment of social interaction skills later in life (Modahl *et al.*, 1998). It has been suggested that autistic patients are distinguished by different social interaction styles; 'aloof', for individuals displaying avoidance towards physical proximity with others, 'passive' individuals who neither engage in social relations nor reject, and 'active-but-odd' individuals who display inappropriate behaviour (Wing and Gould, 1979). Taking into consideration this classification, Modahl *et al.* (1998) demonstrated that higher OXT levels are significantly associated with lesser development of social and communication skills in the subgroup of 'aloof' autistic children, but not in the 'active-but-odd' children. Together these suggest that a degree of heterogeneity in the responses to OXT treatment might be due to these different social interaction profiles. In a follow up study, higher levels of precursor proteins of the OXT peptide were found in plasma of a sample of autistic children, suggesting that autism is linked to alterations occurring during the OXT peptide processing (Green *et al.*, 2001). However, opposing studies have shown overall elevated OXT levels in plasma of autistic individuals (Jansen *et al.*, 2006), and marked behavioural differences between gender (Miller *et al.*, 2013). In particular, Miller *et al.* (2013) measured plasma OXT in both typically developing children and high-functioning autistic subjects, aged between 8 and 18 years reported increased OXT levels only in girls, and higher levels of AVP in all boys. The observed increase in OXT levels was linked to higher anxiety levels in all girls and to better social language skills in all individuals (Miller *et al.*, 2013), highlighting a dimorphism of the endogenous OXT system between the two genders. Notably, variability of OXT plasma levels has been reported in normative populations (Zhong *et al.*, 2012). Therefore, the heterogeneity of autism in clinical symptoms, accompanied by differences in the OXT level amongst individuals and between genders, are parameters to be considered in order to develop selective and personalised use of OXT treatment following essential genetic and pharmacological screenings.

Based on disrupted OXT function in autism, OXT administration was proposed as a treatment (Andari *et al.*, 2010; Guastella *et al.*, 2010). Clinical findings using intravenous or intranasal administration of OXT in individuals with autism have demonstrated reduction in restricted repetitive behaviour (Hollander *et al.*, 2003; Anagnostou *et al.*, 2012), improved social cognition (Anagnostou *et al.*, 2012) and recognition of emotions (Guastella *et al.*, 2010), increased social approach and social comprehension as well as cooperation (Andari *et al.*, 2010). In contrast to these findings, Dadds *et al.* (2014) failed to show improvement in social interaction, emotion recognition or repetitive restrictive behaviours in high-functioning autistic individuals, as improvements over time of OXT administration were noted in both OXT and control groups. Notably, an open label trial of administration of intranasal OXT in autistic children and adolescents demonstrated improvement in social learning, social cognition, face recognition and decrease in repetitive behaviour over 12 weeks of treatment that persisted three months after discontinuation of OXT administration (Anagnostou *et al.*, 2014). Administration of single dose of intranasal OXT in autistic individuals significantly increased the rate in inferring others' social emotions (Aoki *et al.*, 2014). Additionally, in line with studies on autistic patients, Hall *et al.* (2012) demonstrated that administration of intranasal OXT was sufficient to increase eye gaze frequency and decrease salivary cortisol levels in low functioning FXS patients suggesting that intranasal OXT administration may ameliorate social anxiety symptoms in FXS patients. Also see Table 3.1 for a detailed summary of the use of OXT in clinical trials for ASD and FXS treatments.

Table 3.1: Summary of clinical trials of oxytocin treatment in autistic spectrum disorders (ASD) including Asperger's syndrome (AS), high functioning (HF) and low functioning (LF) individuals with ASD and, fragile X syndrome (FXS).

Subjects	Administration	Dose	Clinical trial	Assessment	Behaviour	Reference
5♂ with ASD or AS (16-19 y.o)	Intranasal	24IU	randomized double-blind crossover	RMET	no difference	Guastella <i>et al.</i> 2010
11♂ with ASD or AS (12-15 y.o)		18 IU	randomized double-blind crossover	RMET	↑ emotion recognition	Guastella <i>et al.</i> 2010
15 with ASD or AS 14♂ 1♀ (mean age =19-56)	Intravenous	10U/ml	randomized double-blind, placebo controlled crossover design	YBOCS compulsion severity scale	↓ repetitive behaviour	Hollander <i>et al.</i> 2003
14♂ with ASD or AS (19-56 y.o)	Intravenous	10U/ml	randomized double-blind crossover	Affective speech score	retained social information	Hollander <i>et al.</i> 2007
11♂ 2♀ with HF-A or AS (mean age =26)	Intranasal	24IU	randomized double-blind, placebo controlled within subject design	Social ball tossing game	↑ eye gazing and trust promotes affiliation and cooperation	Andari <i>et al.</i> 2010
16♂ 3♀ with ASD	Intranasal	24IU	Randomized double-blind, placebo-controlled, parallel design trial over six-weeks	Clinical global impression, DANVA, RMET, Social Responsiveness scale	no significant decrease in restrictive repetitive behaviour, significant improvement in social cognition and quality of life	Anagnostou <i>et al.</i> 2012
38♂ with ASD (mean age =11.79) Placebo (mean age =10.74)	Intranasal	12IU if < 40kg 24IU if > 40kg	Randomized double-blind, control trial over five days	Social skills rating scale, Social reciprocity scale, UNSW Facial Emotion task, OSU, Childhood Ratings scale, DISCAP-ASD	No difference in social interaction, emotion recognition or general behavioural adjustment	Dadds <i>et al.</i> 2014
11♂ 4♀ with HF-ASD or AS (mean age =13.8)	Intranasal	0.2 < 0.4IU	Modified Maximum Tolerated Dose-open label trial over 12 weeks	DANVA-2, BFRT, VYFPB, Revised eyes test, Strange stories task, Real and Apparent Emotion task, Irony and Empathy Task, Social Responsiveness Scale, CGI-I-Social, BASC-2, Yale-Brown Obsessive-Compulsive Scale, Repetitive Behavior Scale-Revised, Child and Adolescent Symptom Inventory	↑ social learning, social cognition, face recognition, ↓ repetitive behaviour and anxiety	Anagnostou <i>et al.</i> 2014
20♂ with ASD	Intranasal	24IU	double-blind placebo-controlled within subject	Story fixation task	↑ inferring with others' social emotions	Aoki <i>et al.</i> 2014
8 LF♂ with FXS (13-18 y.o)	Intranasal	24IU 48IU	randomised double-blind placebo-controlled trial	eye gaze frequency, heart rate, respiratory sinus arrhythmia (RSA), heart rate variability (HRV) and salivary cortisol	↑ eye gazing, ↓ salivary cortisol, no difference for heart rate, heart rate variability, respiratory sinus arrhythmia	Hall <i>et al.</i> 2012

Despite the great interest and the deluge of reports investigating the social behaviour benefits following intranasal OXT administration, great scepticism has arisen in relation to this topic including, the methodological approaches used, statistical interpretation of outcomes, dosage regimen and sampling collection times. Leng and Ludwig (2016b) have extensively criticized the reliability of the outcomes from several of the aforementioned studies by questioning the amount of exogenous OXT administered ($> 24\text{IU}$), in all cases greater than the human physiological pituitary content that is estimated to be approximately 14IU ($28\mu\text{g}$) (Heller and Zaimis, 1949), which was associated with modest increased concentrations found in the CSF. Besides, Leng and Ludwig (2016b) further suggest that higher OXT concentrations are essential for the stimulation of CNS effects as compared to peripheral effects because agonist stimulation of OXT receptors leads to desensitization (Conti *et al.*, 2009), while receptors at peripheral sites are exposed to lower concentrations of OXT ($1\text{-}10\text{pg/ml}$) than receptors located in the brain (Leng and Ludwig, 2008); therefore, they are highly sensitive to the release of the peptide (Leng and Ludwig, 2016a). Conclusively, even Leng and Sabatier (2017), the greatest sceptics in the topic of 'how intranasal OXT reaches the brain', seem to agree with the notion that with enormous amounts administered, biologically significant peptide concentrations can enter the brain with a tiny penetration rate that can stimulate behavioural responses.

3.1.4.1. Genetics and epigenetic findings on the OXTR gene expression

Numerous studies have associated variants of the *OXTR* gene with autism. Gregory *et al.* (2009) was the first to report deleterious mutation of *OXTR* gene in an autistic proband and his mother. However, no such deletion was detected in the proband's affected sibling but instead found increased methylation of *OXTR* compared with the non-autistic sibling, suggesting that epigenetic silencing of this gene by DNA methylation might be involved in the causality of autism. DNA methylation analysis further detected the hypermethylated

pattern of the *OXTR* gene in the peripheral blood mononuclear cells and the temporal cortex of autistic individuals (Gregory *et al.*, 2009). Numerous cohorts have also reported positive associations between polymorphisms at the *OXTR* gene and autism (<https://gene.sfari.org/GeneDetail/OXTR>), including family based studies with Chinese Han, Caucasian, Japanese, German and Israeli populations (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.*, 2008; Liu *et al.*, 2010; Kranz *et al.*, 2016). CD38 is a transmembrane glycoprotein widely distributed throughout the hypothalamus and found in OXT neurons, which has been shown to regulate OXT secretion (Jin *et al.*, 2007). Ebstein *et al.* (2009) reported that CD38 has been associated with OXT detected in plasma from autistic patients. In particular a significant association was detected between SNPs of the *CD38* gene and the measures for assessing ASD categories (Lord *et al.*, 1994). Munesue *et al.* (2010) also reported the association of a CD38 SNP with high-functioning autistic individuals from cases mainly recruited to the Autism Genetic Resource Exchange. Yrigollen *et al.* (2008) reported a significant SNP in the OXT/AVP region linked to stereotypical behaviour. Finally, Campbell *et al.* (2011) detected 3 SNPs on the *OXTR* gene that are associated with both ASD diagnostic criteria as well as subphenotype measurements of social impairment in autism.

3.1.4.2. Preclinical findings

Genetic studies have been conducted in rodents for advancing our understanding of the OXT system with targeted genetic disruptions of their OXT system and evaluate social behaviours (Young *et al.*, 1997; Ferguson *et al.*, 2000; Jin *et al.*, 2007). Mice lacking the *oxtr* or *oxtr* gene and *CD38* gene -involved in OXT secretion- demonstrate similar and profound behavioural abnormalities, which together resemble those of human autism. As mentioned earlier in section 3.1.41, evidence from clinical studies have associated CD38 with ASD, thus the involvement of this gene in ASD is worth discussing.

Significant reduction in ultrasonic vocalisation following maternal separation was reported in oxytocin knockout mice (*Oxt* KO) pups suggesting that OXT deficiency fails to establish social affiliative behaviour (Young *et al.*, 1997). *Oxt* KO mice display social memory deficits, reduced aggression and, normal social approach as compared with controls (DeVries *et al.*, 1997; Ferguson *et al.*, 2001; Winslow and Insel, 2002; Ricceri *et al.*, 2007). Evidence accrued from studies in rats reported that OXT administered intracerebroventricularly induces stereotypical behaviour such as repetitive grooming and paw suckling (Drago *et al.*, 1986; Nelson and Alberts, 1997), suggesting a relationship between OXT and stereotypical behaviour.

Oxtr KO mice were first generated by Takayanagi *et al.* (2005) where his study revealed that fewer ultrasonic vocalisations were emitted in comparison to WT littermates following social isolation. Later on, Sala *et al.* (2011) recapitulated that *Oxtr* KO mice exhibit relevant symptoms of autism, including impaired social interactions, decreased cognitive flexibility, increased aggressive behaviour and increased susceptibility to seizures; a common comorbidity in autistic patients. However, these behavioural responses were rescued after intracerebral OXT infusion, which attenuated aggressive behaviour and reversed social recognition defects likely via V1A receptors, while seizure susceptibility was antagonized by peripheral OXT administration (Sala *et al.*, 2011). It might be that in the absence of OXTR, OXT acts as a partial agonist on V1A receptor to induce compensatory upregulation and/or downregulation of the receptor. Other studies, however did not demonstrate change in V1A expression in *Oxtr* KO mice (Ferguson *et al.*, 2000). Pobbe *et al.* (2012) observed widely reduced social interactions in the *Oxtr* KO mice such as reduced sniffing behaviour, increased time spent alone when compared to their WT littermates. In addition, overall reduction in communication displayed by a reduced number of urinary scent marks across social and non-social contexts. However, these mice failed to show patterns of repetitive stereotypical behaviour in a repetitive novel object contact task. These together, reinforce

the autistic-like phenotypic traits of the *Oxtr* KO mice as indicated by robust social deficits and reduced communication levels (Pobbe *et al.*, 2012).

CD38 KO mice display deficit in the social recognition responses to the cues provided. Significantly lower OXT levels were reported in plasma and CSF of the *CD38* KO mice as compared to WT littermates, while increased OXT concentration were found in the hypothalamus and neurohypophysis of these mice. These physiological responses were reversed following restoration of *CD38* in the knock-out mice after infusion of lentiviral vectors carrying human *CD38* gene (lenti-*CD38*) in the third ventricle (Jin *et al.*, 2007) suggesting that administration of OXT can restore dysregulation of the central oxytocinergic system in these mice. OXT has been reported to rescue social deficits in both *Oxt* KO and *CD38* KO mice (Jin *et al.*, 2007, Ferguson *et al.*, 2000) suggesting restoration of the endogenous OXT levels as a promising therapeutic strategy for conditions with impairment in the social domain, such as autism.

Studies on animal models characterised as good ASD models such as the BALB/cByJ and the C58/J (Brodkin, 2007; Ryan *et al.*, 2010) have shown that subchronic intraperitoneal administration of OXT increases social interactions in both genotypes, while acute single dose of OXT is sufficient to reduce repetitive behaviours in the C58/J mice (Teng *et al.*, 2013).

While OXT is emerging as a promising target for the treatment of some core symptoms of ASD, the behavioural and long-term consequences of chronic exposure to OXT are not well known and further studies are essential. In an attempt to answer these questions, this chapter focuses on the behavioural and neurobiological consequences of chronic intranasal OXT administration in a well-validated model of autism, the *Fmr1* KO model. We particularly focus on the OXT's neurobiological underpinnings of behavioural deficits in autism and the therapeutic potential of intranasal OXT administration. This is the first study to investigate

the biological and behavioural-induced effects of acute and chronic intranasal OXT administration in the *Fmr1* KO autistic mouse model.

3.2. Hypothesis and objectives

3.2.1. Hypotheses

OXT is a promising therapeutic target for the treatment of ASD as illustrated by preclinical and clinical based studies. It has been demonstrated that OXT is sufficient to relieve core symptoms of autism including social interaction deficits and stereotypy, as well as symptoms of anxiety and cognitive impairment. Receptor targets including metabotropic glutamate receptor 5 (mGluR5) and N-methyl-d-aspartate (NMDA) receptors seem to be implicated in models of fragile X with ASD phenotypes. Interestingly, mGluR5 and NMDA hyperactivity underlies some of the fragile-X and ASD-associated symptomatology in experimental rodent models. Therefore, we hypothesized that OXT treatment in the ASD *Fmr1* KO mouse model will induce:

- (a) beneficial effects against the autistic-like behaviours (mentioned above)
- (b) changes in OXT receptor, mGluR5 and NMDA receptor binding levels in the brain
- (c) transcript changes in the endogenous opioid system, the oxytocinergic, glutamate and other systems, which are involved in the etiopathology of ASD.

3.2.2. Objectives

This study investigated if there are beneficial changes of acute and chronic OXT in the autistic-like phenotype of the *Fmr1* KO mice including sociability and repetitive behaviour (core symptoms of ASD) and comorbid symptoms such as anxiety and cognition impairment by assessing them using a battery of behavioural paradigms including social interaction, three-chamber sociability and motor stereotypies tests, marble burring, novelty suppressed feeding and novel object recognition tests. Additionally, we assessed in the *Fmr1* KO mice, the effect of chronic OXT administration on receptor systems, which are involved in the

etiopathology of ASD including the OXT, NMDA and mGluR5 receptors. We utilised quantitative autoradiographic receptor binding to assess the presence or absence of any receptor binding changes following chronic OXT administration in mice. Lastly, we investigated the expression of mRNA transcripts in *Fmr1* KO mice following chronic OXT administration of several gene components of systems involved in the neurobiology of ASD including the endogenous opioid system, glutamatergic, oxytocinergic and others. qRT-PCR was performed across two crucial brain regions involved in reward/motor behaviour (CPu) and emotional processing (CeA).

3.3. Materials and Methods

3.3.1. Methods

3.3.1.1. Effect of chronic intranasal OXT administration on core and secondary symptoms of ASD in *Fmr1* KO and WT mice

Male and female WT and *Fmr1* KO mice aged 5–9 weeks generated at the lab of Julie Le Merrer and Jerome Becker (INRA-0085 CNRS-7247 INSERM, Université de Tours Rabelais, Nouzilly, France) were bred in-house on a homogeneous C57BL/ 6J background. For the purpose of the study, *Fmr1*^{+/+} and *Fmr1* KO pups were nurtured by *Fmr1*^{+/+} and *Fmr1* KO parents, respectively. Same gender animals were group housed (4-5 animals per group) and maintained in a climate-controlled animal facility (21°C±1°C) and maintained on a 12-hour light/dark cycle with food and water available *ad libitum*. All procedures were conducted in accordance with the C2EA-19- Ethics Committee for animal experiments of the Val de Loire. All behavioral, qRT-PCR and autoradiography experiments carried out were performed by myself at the lab of Dr. Le Merrer and Dr. Becker (behavioral, qRT-PCR) and at University of Surrey (autoradiography). As previously discussed (section 2.3.1.4), we utilized both sexes for the experiments carried out in this chapter, by following the same approach (not considering sex differences) in the qRT-PCR, autoradiography and the behavioural experiments carried out in the *Fmr1* KO mouse model that provided us with preliminary data.

3.3.1.2. Intranasal administration

Male and female mice were divided into four groups; *Fmr1*^{+/+} chronic saline, *Fmr1*^{+/+} chronic OXT, *Fmr1* KO chronic saline and *Fmr1* KO chronic OXT. All procedures were carried out according to previously established protocols with minor modifications (Bales *et al.*, 2013;

Huang *et al.*, 2014). OXT was dissolved in saline (0.9% NaCl) and administered intranasally in a volume of 5 µl to each mouse in doses of 0.3 IU/5 µl (OXT 0.3IU). The dose used is similar to the higher range of intranasal OXT doses given to adolescent prairie voles (Bales *et al.*, 2013). Also, it is similar to the total daily dosage being used currently in clinical trials (Bakermans-Kranenburg and van IJzendoorn, 2013; Anagnostou *et al.*, 2014; Dadds *et al.*, 2014) and other studies with clinical populations (Goldman *et al.*, 2011; MacDonald and Feifel, 2013). Control mice received the same volume of saline (vehicle). Saline and OXT administration was carried out once daily in the morning between 0700 and 1200 h throughout the duration of the study. For most experiments, group sizes were 4-5 mice arranged by gender, genotype and treatment. For the intranasal administration, a P10 Eppendorf pipette with 1-20 µl standard loading tips were used for administration. The animal was held still and drops of the 5 µl solution were gently placed equally on both nostrils of each mouse, which were taken in when they reflexively inhaled. Administration was rapid (less than 30 s) and handling was consistent across treatment groups. Following administration, the animal was returned to its home-cage with its familiar cage-mates. Behavioral interaction assessment was performed 5 min after the administration twice weekly, on days 1, 5, 9, 12, 16 and 20 of a 20-day period. The timeline for treatments on different cohorts is given in Figures 3.5; 3.6. For the intranasal treatments, mice were administered with OXT only once daily, 5 min before the start of the behavioural tests. The delay of only 5 min was based on OXT's very rapid pharmacokinetics with effects expected to appear already within a few minutes following intranasal administration, as previously indicated by Veening and Olivier (2013).

3.3.1.3. Behavioral Assessments

Social abilities were explored using direct social interaction and three-chamber sociability tests as described previously by established protocols (Peça *et al.*, 2011; Pobbe *et al.*, 2012;

Becker *et al.*, 2014). Stereotyped behavior was assessed by scoring motor stereotypies as described previously (Silverman *et al.*, 2010; Becker *et al.*, 2014). Anxiety-like behavior was evaluated in the marble burying (Amodeo *et al.*, 2012) and novelty suppressed feeding (NSF) (Zhou *et al.*, 2010) tests as described by previous protocols. Learning and memory behavior was assessed by the novel-object recognition (NOR) paradigm for short-term memory in accordance to previously established protocols (Le Merrer *et al.*, 2013). Two separate cohorts of mice underwent the same administration paradigm with differences on the duration of administration and the behavioral experiments utilized. Measurements of behavioral outcomes were unbiased (blinded to groups).

Direct social interaction: For cohort #1 (n= 6-8 per group), mice were assessed in a 10-min direct social interaction paradigm, where they were exposed to a “stimulus” mouse. ‘Stimulus’ mice used (8-10 weeks old) for first series of experiments were gender-matched, grouped-housed WT mice, socially naive to the experimental animals. In all cohorts, behavioral interaction assessment was performed 5 min after the administration of either saline or OXT twice weekly, on days 1, 5, 9, 12, 16 and 20 of a 20-day period. (For timeline see Figure 3.5). During this test, experimental animals met wild-type age- and gender-matched unfamiliar stimulus mice. In cohort 2, social interaction was further assessed on day 36 to verify maintenance of OXT effects (For timeline see Figure 3.6). Testing started when experimental and stimulus animals were introduced in an arena for 10 min session of direct social interaction (SI) under 15 lx. Also for an illustration of the paradigm please see Figure 3.4. Total time spent in close contact (nose and paw contacts), number and duration of nose and paw contacts (crawling over, mounting, stepping on, pushing) and self-grooming, as well as frequency of occurrence of the following, rearing and circling episodes for the 10 min duration were filmed and scored from visualizing the video recordings (Pobbe *et al.*, 2012). The timeline of social interaction assessments was performed according to the timeline illustrated on Figure 3.5. Forty-five min following the final OXT or saline

administration, mice were euthanized by cervical dislocation and decapitation and brains were collected and immediately frozen in liquid nitrogen (-80°C), which were processed for qRT-PCR experiments or quantitative autoradiography.

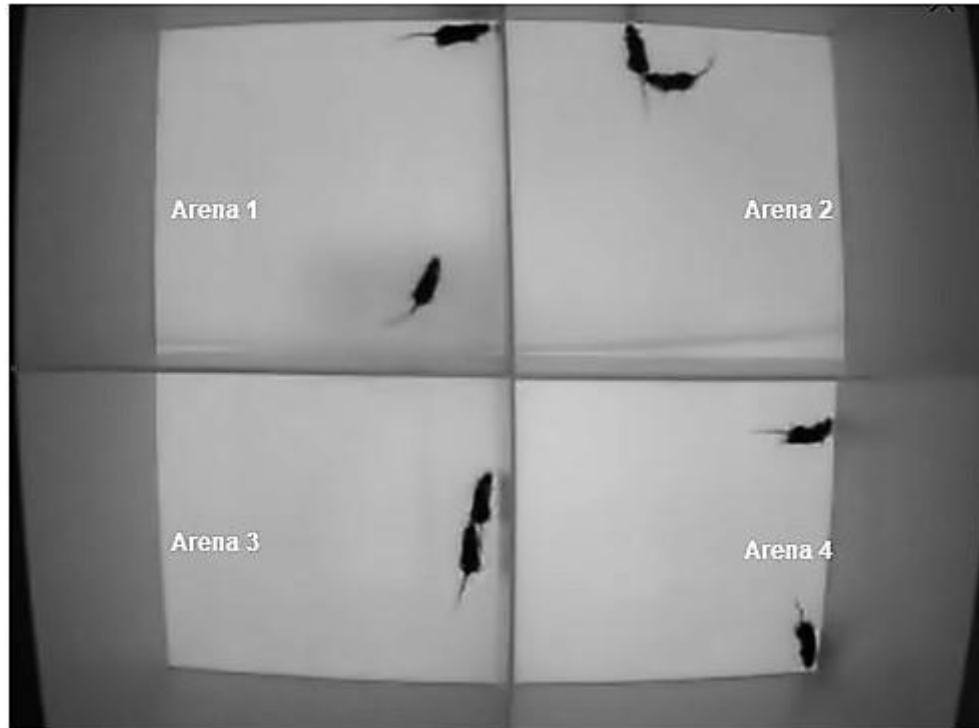


Figure 3.4: Platform consisting of four arenas, in which direct social interaction sessions were performed.

In order to distinguish between the two mice, the experimental mouse was placed in the arena first, following the start of the camera recording. Then, the 'stimulus' mouse was also placed in the arena. Social interactions of sessions including up to four experimental animals were run each time, each mouse pair (stimulus, experimental) placed on individual arenas, as indicated on the image. Image captured from the recorded videos, which were scored and analyzed. Measurement of outcomes were unbiased (blinded to groups).

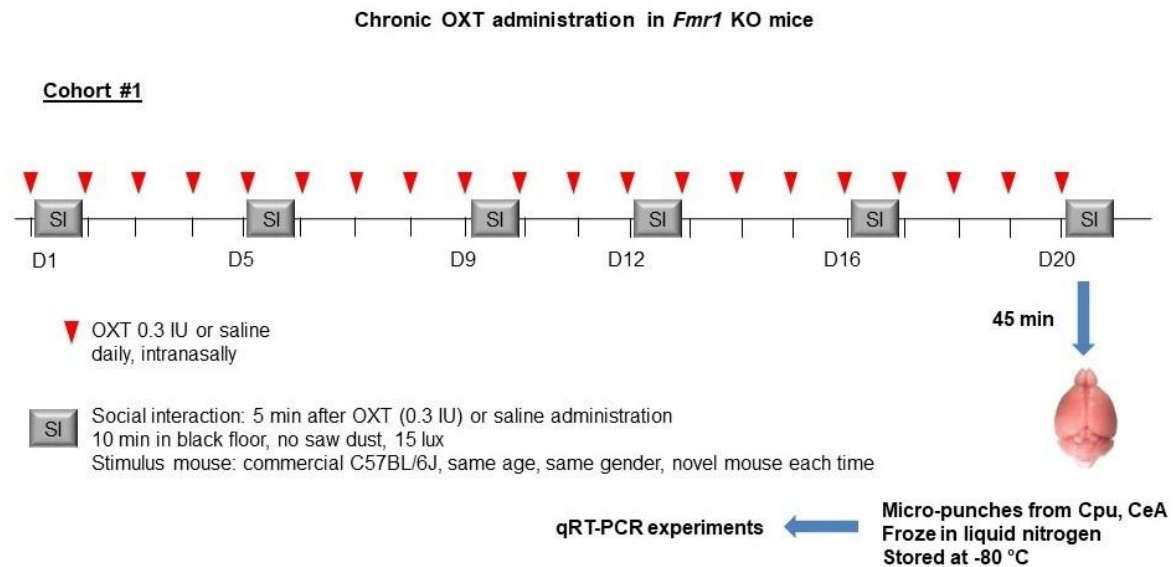


Figure 3.5: Cohort #1 timeline of chronic oxytocin administration or saline in *Fmr1* KO and wild-type mice for the social interactions (SI) assessments.

For cohort #2 (n=8-9 per group), mice followed the same administration protocol as previously described in section 3.3.1.2. However, in order to evaluate more behaviors on the WT and *Fmr1* KO mice treated with OXT or saline, OXT treatment lasted longer and the previous protocol was adjusted appropriately. Mice were administered OXT for a period of 39 days, during which additional behavioral paradigms including the three-chamber sociability (or Crawley's) test, scoring motor stereotypies, marble burying, novelty-suppressed feeding (NSF) and novel object recognition (NOR) tests assessed core and comorbid symptoms of autism. For a timeline diagram, please see Figure 3.6. Direct social interaction, novel object recognition and novelty suppressed feeding were performed in 4 equal square arenas (50 x 50 cm) separated by 35cm-high opaque grey Plexiglas walls over a white Plexiglas platform (View Point, Lyon, France). While several animals were tested concurrently and thus, olfactory stimuli from animals in adjacent sets of apparatus were present, it has to be noted that this is a factor that could not affect the outcomes, as it was a consistent parameter amongst all groups.

Chronic OXT administration in *Fmr1* KO mice

Cohort #2

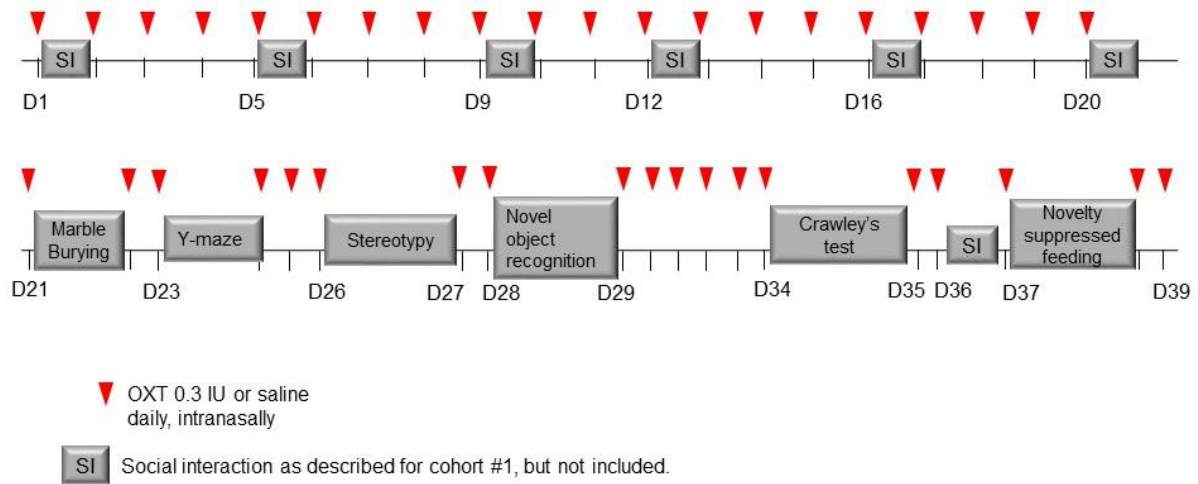


Figure 3.6: Cohort #2 timeline of chronic oxytocin administration or saline in *Fmr1* KO and wild-type mice and a battery of behavioral tests assessed.

Social interaction and Y-maze tests haven't been scored and analyzed yet, thus they are not included in the methods or results sections.

Three-chamber sociability test (or Crawley's test): Social approach was assessed in a box separated in three chambers with two sliding doors (8 x 5 cm) that allowed transitions between chambers. The experimental protocol was adapted from Peca *et al.* (2011). Two small identical cages (18 x 9 cm, 0.5 cm diameter-rods spaced 1 cm apart) comprised of 1 cm-spaced vertical bars, were placed in each end-chamber and secured with a weighted cup. Stimulus mice were habituated in these restricted cages for 2 days (20 min/day) prior to the experiment. The test started with a 10-min habituation phase, where the experimental mouse was placed into the middle chamber (40x20x22.5 cm) of a transparent acrylic box consisting of partitions that divided the box into three interconnected equal chambers, each of the lateral chambers containing an empty wire cage. During this phase, the experimental mouse was free to explore the entire apparatus (wire cages empty) for 10 min. In a second phase, an unfamiliar 'stimulus' gender- and age-matched mouse (stranger) was placed into one of the two wire cages ("social cage") while the other cage contained a soft mouse toy.

The experimental mouse was placed back into the centre chamber and was free explore the apparatus for 10 mins (sociability phase). Also see illustration of the paradigm on Figure 3.7. The number of entries in each compartment, the time spent in each chamber and in nose contact with the stimulus mouse or the wired cage, as well as duration of each nose contact episode was scored by analysing the video recordings after 10 min duration of the test. The analyses of the video recordings were performed manually on video recordings (Viewpoint, Lyon, France) and measurements were validated by an observer blind to the treatment groups. Social preference was determined by calculating the time that the “experimental” mouse spent around the cage containing the unfamiliar mouse (stranger 1) vs. the time spent around the cage containing the toy during the social interaction phase of the test. The test was carried out in dim light, at 15 lux. After the end of the test, the mouse was placed back in its home cage.

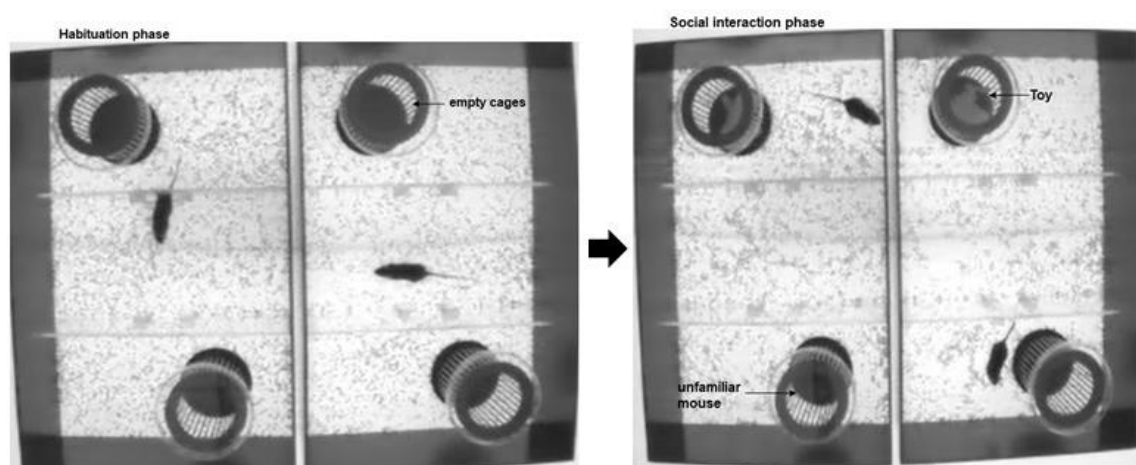


Figure 3.7: Illustration of habituation and social interaction phases of the three-chamber sociability test.

During the sociability phase, the experimental mouse is given a choice to interact either with an unfamiliar gender-matched adult mouse (‘stranger’), contained in one of the wired cages placed in the one-end chamber, or with a soft toy mouse contained in the other smaller cage located in the anti-diametrical chamber. Image captured from the recorded videos that were scored and analyzed. Measurement of outcomes were unbiased (blinded to groups).

Novel object recognition: This paradigm was used to assess learning and memory in cohort #2 (see timeline diagram Figure 3.6), as previously described by Le Merrer *et al.* (2013). The recognition test consisted of 3 sessions of 10 min separated by 2 inter-trial intervals of 5 min. During the familiarization phase (session 1), the experimental mouse was presented with two copies of an unfamiliar object. Stimuli objects used sized 1.5-3 x 2-3 cm and included dices and Lego® bricks. The mouse was then removed to an opaque holding container for 5 min, and one of the objects was displaced to a novel location for the next session (location-dependent phase). After 5 min exploring during session 2, the mouse was placed in the holding container and only the object from session 1 was replaced with a novel object - introduced for the first time (novelty-dependent). The identity of the objects and their spatial location were the same for all subjects throughout the three sessions. Also see illustration of the paradigm on Figure 3.8. The number of visits and the time spent to explore each object were scored on video recordings for sessions 2 and 3. During session 2, short-term memory associated with spatial awareness (hippocampus-dependent) was assessed illustrated by frequency of exploration of displaced object. During session 3, short-term memory associated with novelty was assessed as indicated by the frequency of exploration of the novel object. Sniffing duration ratio measurement was used for all sessions.

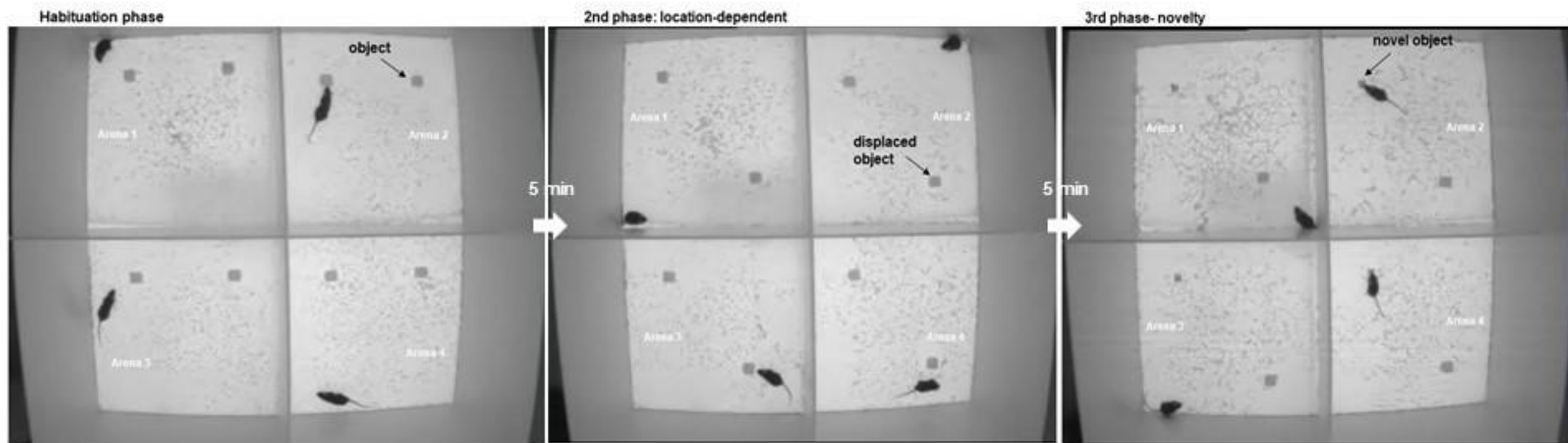


Figure 3.8: Representation of the three phases of the novel object recognition paradigm; habituation session, displaced object and novel object sessions.

Exploratory behaviour was defined as any behaviour directed at the object including sniffing (within ~ 5 cm), touching, chewing, pawing (directed touch), rearing or standing on the object. Measurement of outcomes were unbiased (blinded to groups).

Motor stereotypies: This paradigm was used to assess motor stereotypies in cohort #2 (see timeline diagram Figure 3.6), as previously described by Silverman *et al.* (2010) and Becker *et al.* (2014). In order to detect motor stereotypies in *Fmr1* KO and WT mice treated with OXT or saline, animals were individually placed in clear standard home cages (21×11×17 cm) filled with 3-cm deep fresh sawdust for 10 min. Light intensity was set at 30 lux. Numbers of head shakes, as well as rearing, grooming, circling episodes and total time spent digging were scored by direct live observation for 10 min duration of the test.

Marble-burying: The procedure for marble burying was adapted from Amodeo *et al.* (2012) and this paradigm was used to evaluate anxiety levels of the animals (Becker *et al.* 2014). Mice were introduced individually in transparent cages (21×11×17 cm) containing 20 glass marbles (diameter: 1.5 cm) evenly spaced on 4-cm deep fresh sawdust. Also see illustration of the test (Figure 3.9). In order to prevent escapes, each cage was covered with a filtering lid. Light intensity in the room was set at 40 lux. The animals were removed from the cages after 15 min, and the number of marbles buried under more than half of the sawdust was counted.

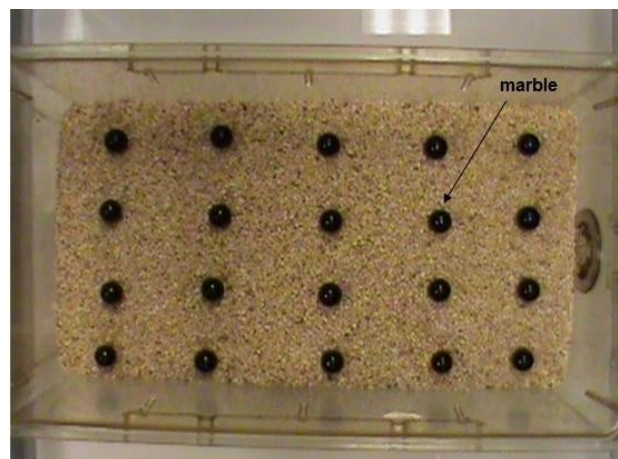


Figure 3.9: Illustration of marble-burying paradigm.

Mice were assessed for their marble-burying performance in transparent standard cages during a 10-min session, used as an index of anxiety behaviour. Measurement of outcomes were unbiased (blinded to groups).

Novelty-suppressed feeding: Experimental protocol was adapted from Zhou *et al.* (2010) and Becker *et al.* (2014). Mice were food deprived for 24 h prior to the test. Then they were placed individually in a standard housing cage for 30 min before individual testing. Three pellets of ordinary lab chow were placed on a white tissue in the center of each arena. Light intensity in the room was set at 60 lx. Each mouse was placed in a corner of an arena and was allowed to explore for a maximum time of 15 min. Latency to feed was measured as the time needed to bite a food pellet. Immediately after an eating event, the mouse was transferred back to their home cage (See illustration on Figure 3.10).

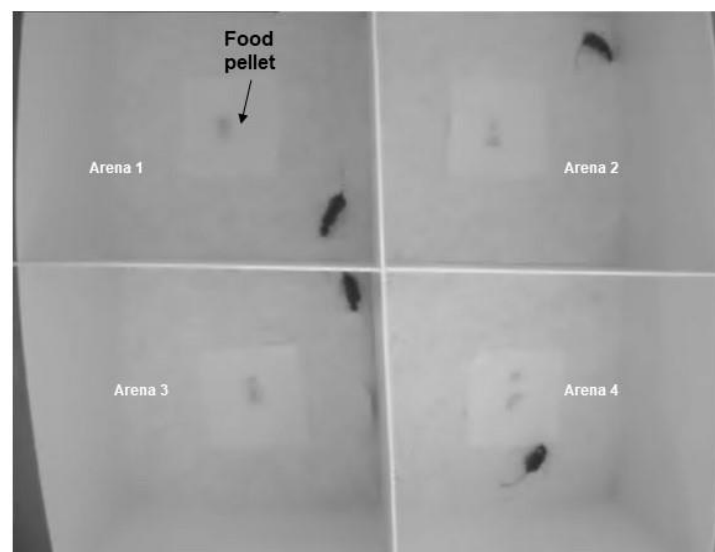


Figure 3.10: Illustration of novelty-suppressed feeding paradigm.

Food-deprived mice for 24 h were assessed for their latency to feed on the only 2-3 pellets placed in the middle of the chamber, while being in a stressful environment of light intensity at 60 lux. Image captured from the recorded videos that were scored and analyzed. Measurement of outcomes were unbiased (blinded to groups).

3.3.1.4. Biochemical assays applied in the brains of wild-type and *Fmr1* KO mice treated with chronic intranasal oxytocin or saline

3.3.1.4.1. Real-Time Quantitative PCR Analysis

After completion of OXT treatment in cohort #1 (see diagram on Figure 3.5), mice brains were collected and the PFC, CPu, CeA, MeA (medial amygdaloid nuclei) and VTA/SN

(substantia nigra) brain regions were extracted to be processed for qRT-PCR experiments. Here we focus on two brain regions, CPu and CeA, as they play a critical role in the pathophysiology of ASD (Langen *et al.*, 2009). In addition, for the qRT-PCR experiments we did not consider potential gender differences amongst the groups as we did not find significant behavioral differences between males and females amongst the groups in any of the tests. Therefore, when transcripts were assessed for a collection of genes of interest, tissue was pooled from one male and one female mouse of same genotype and treatment, separately for each brain structure.

3.3.1.4.2. Micro-punches

Procedures followed for the micro-punches were in accordance to previously established protocols (Le Merrer *et al.*, 2013). Mice brains were removed after cervical dislocation, placed into a brain matrix and, with the use of razor blades, coronal sections through the brain were sliced at intervals of 1mm. Then, sections containing the CPu and the CeA were located at -0.34 mm and -1.34 mm bregma points, respectively, by using the mouse brain atlas Paxinos and Franklin (2001). These brain section were then placed on a microscope slide in order to allow selected brain regions to be punched out. Biopsy punches of 2-mm diameter and 0.8-mm diameter were used for the extraction of the CPu and CeA brain regions, respectively. Tissue containing these brain regions were collected in experimental tubes and they were immediately frozen in liquid nitrogen followed by dry ice and, storage at -80°C.

3.3.1.4.3. qRT-PCR

Tissue was pooled from one male and one female mouse of the same genotype and treatment, separately for each brain structure (n=3-4/genotype/ treatment group). Procedures about RNA processing, cDNA synthesis and qRT-PCR reaction and conditions,

were performed same as previously described in section 2.3.1.2. Primer sequences are displayed in Table 3.2.

Table 3.2: Primer sequences for qRT-PCR experiments.

RefSeq	Gene name	Gene title	Forward oligonucleotide	Reverse oligonucleotide
NM_011013	opioid receptor, mu	<i>Oprm1</i>	ATCCTCTCTGAAGCCAACCC	TTCTGTCTTGGGCCATCAT
NM_018863	prodynorphin	<i>Pdyn</i>	TTTGCCAACGGAAAAGAATC	TAGCGTTTGGCCTGTTTTCT
NM_001002927	preproenkephalin	<i>Penk</i>	ATGCAGATGAGGGAGACACC	GCTTCTGCAGCTCTTTTGCT
NM_009732	Arginine vasopressine	<i>Avp</i>	ACACTACGCTCTCCGCTTGT	CACTGTCTCAGTCCATGTCA
NM_016847	Arginine vasopressine receptor 1A	<i>Avpr1a</i>	GGAGAAACGGGAGACAGACA	AAGCCCATTTGACAGCCCAAG
NM_011924	Arginine vasopressine receptor 1B	<i>Avpr1b</i>	AGGAGGAAGAGGAGGAGGAA	ACTGAAGGCAGGAACTGAAG
NM_011025	oxytocin	<i>Oxt</i>	CTGCTTGCTTACTGGCTCT	GGGAGACACTTGCATATC
NM_001081147	oxytocin receptor	<i>Oxtr</i>	CTTAGGGCCAAAAGGTGTCA	GCAGGTTTCTATGCCCTCTG
NM_001160353	glutamate receptor, metabotropic 2	<i>Grm2</i>	CTTGAGCTATGCCCCGTGT	GACTGGAAGCACCTTTGCAT
NM_001013385	glutamate receptor, metabotropic 4	<i>Grm4</i>	CTTCCTTAGCCAGGGTCTCC	CATCCCTTCGGACACAGTTT
NM_008174	glutamate receptor, metabotropic 8	<i>Grm8</i>	TCCTGTCCCACTGTTCAA	AAAATTGCATGCGTCAATCA
NM_011702	vasoactive intestinal polypeptide	<i>Vip</i>	AGGTAATCCATCTCTACGGA	CCAGATTGGACGATGGGCTT
NM_011703	Vasoactive intestinal peptid receptor 1	<i>Vipr1</i>	GGGAGTGGTCCTAGAATCCCA	CATCTCTCAGCCTCATCGGA
NM_009511	Vasoactive intestinal peptid receptor 2	<i>Vipr2</i>	TCCAGGGCTACAAGGCTAGA	TAGCATCCACCTATGCCAC
NM_018790	activity regulated cytoskeletal-associated protein	<i>Arc</i>	CCAGGAGAATGACACCAG	TTCAGGAGAAGAGAGGATG
NM_020026	UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 1	<i>B3galnt1</i>	AAGGCACAGAAGGCTGAGAC	AAAACACACACTGGGAGGGT
NM_007540	brain derived neurotrophic factor	<i>Bdnf</i>	GTGACTGAAAAAGTTCCACC	GACGTTTACTTCTTCATGGG
NM_001284508	cholecystokinin	<i>CCK</i>	ACAACCACACATACGACCCC	CTTCCGACCACACAGTAGG
NM_009827	cholecystokinin A receptor	<i>Cckar</i>	TGCAATGGATATTGGAACCAAC	AAGAGCTGTAACTTTAATTTTGC
NM_007627.5	cholecystokinin B receptor	<i>Cckbr</i>	CCAACAAATGTGGTCCGTGC	GGTAGAGTTCGCGGAGATG
NM_010234	FBJ osteosarcoma oncogene	<i>Fos</i>	GAAGGGAACGGAATAAGATG	CATCTTCAAGTTGATCTGTCTC
NM_026763	collagen, type VI, alpha 4	<i>Col6a4</i>	AGGCCAACCAGTCTGAAACC	AACGCCAGTCTCACTGTCTC
NM_175475	cytochrome P450, family 26, subfamily b, polypeptide 1	<i>Cyp26b1</i>	TGTGGCATGTGACTCTGGTC	TTCTTCACACTTCTCGGC
NM_012011	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	<i>Eif2s3y</i>	CAGTCAAGGCAGATTGGGT	GCCTCTTCTTATCTGGCCCC
NM_001159500	estrogen related receptor, beta	<i>Essrb</i>	CTAGTGATGCAAGCACCCCA	GGCTCTACCTCTGACTCCA
NM_008082	galanin receptor 1	<i>Galr1</i>	AACTCCATGGAAGTCTGCCG	CCTCTTTGGAGACGCAGCTA
NM_010254	galanin receptor 2	<i>Galr2</i>	ACTTCCCACTGCACAACCT	ATGACCCCCAGGTATCGTCT
NM_008117	growth hormone	<i>Gh</i>	GCTGCTCTCTGCTTCAAGA	GCACAGCTGCTTCCACAAA
NM_175493	G protein-coupled receptor 68	<i>Gpr68</i>	GGATGCCGGAGAGATGTAG	AGGAAGTGGCTAAGGACGC
NM_011832	insulin receptor-related receptor	<i>Insrr</i>	CTCCCCCTCTGTCCCACATA	AAGAAGGAGGAGCACCAAGC
NM_027519	mesenteric estrogen dependent adipogenesis	<i>Medag</i>	AACCTTGGGTACACAGCAAGA	TTTCTGGGGTGATTACTGACT
NM_010882	necdin	<i>Ndn</i>	GCAGGACTTAACAGCAACGC	AGTCCATTCCACATGGATGCT
NM_009717	neurogenic differentiation 6	<i>Neurod6</i>	GTCCCTTAAGTCTCCCCCA	GGGTGGCACTGCACAGTAAT
NM_212452	relaxin/insulin-like family peptide receptor 1	<i>Rxfp1</i>	TCCATCAACTCTGGCACAAC	GGCCACATTTCCACCCAGAT
NM_021358	5-hydroxytryptamine (serotonin) receptor 6	<i>Htr6</i>	GTCTGACCACCAAGCATAGCA	AGTCACATACGGCCTGAGC
NM_001034874	shisa family member 6	<i>Shisa6</i>	GCTGAAGCTCCACCTTGACT	ACATGAAGAGGGGCCGAAAA
NM_182993	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	<i>Slc17a7</i>	TCAGTGGCCATTGTGTGT	ACCCCCACCCAGATTTCTAC
NM_138649	synaptotagmin XVII	<i>Syt17</i>	TCAGTCTCCTCCCCAAAATG	CAGCTGGACTCATAGCACCT
NM_009504	vitamin D receptor	<i>Vdr</i>	CTGCGTGGGAGAAAGTAGGG	AGCCATGCTGACTTCCACC
NM_175638	WNK lysine deficient protein kinase 4	<i>Wnk4</i>	GGGGATATTGGCAGGATGTG	ACTCAGTGCTGGGGTGTCT

3.3.1.4.4. Autoradiographic receptor binding

Preliminary pilot studies performed in *Fmr1* KO mice and their wild-type controls at the lab of Dr. Le Merrer and Dr. Becker showed that chronic intranasal OXT treatments produced increase in social interactions (data not shown). The same experiment was repeated (as cohort #1 in this chapter), as illustrated in Figure 3.5). After completion of this study, mice (n=4-8 per group) were culled 45 mins after the end of the 20th day chronic intranasal OXT or saline administration with cervical dislocation and brains were removed and processed as previously described on chapter 2, section 2.3.1.1.1. As all previous procedures were carried out in the lab of Dr. Becker and Dr. Le Merrer, the brains were shipped to University of Surrey, where I processed them for autoradiographic binding experiments. Procedure of tissue preparation was carried out as detailed on section 2.3.1.1.1.

Oxytocin receptor binding

OXTR binding was carried out on sections from chronic saline- and OXT- treated WT and *Fmr1* KO mice, both male and female, in accordance with previously described protocol by Jarrett *et al.* (2006) with minor modifications. Sections were rinsed twice for 10 min in a buffer solution (50 mM Tris-HCl, pH 7.4) at room temperature to remove endogenous OXT. Total binding was determined by incubating sections with 50 pM [¹²⁵I] - ornithine vasotocin (OVTA), a highly selective OXTR antagonist, in an incubation buffer medium (50 mM Tris-HCl, 10 mM MgCl₂, 0.1 % w/v bovine serum albumin, 0.05 % w/v bacitracin, pH 7.4) at room temperature. Adjacent sections were incubated with [¹²⁵I] - OVTA (50 pM) in the presence of 50 μM unlabelled (Thr⁴,Gly⁷)-OXT, to determine non-specific binding (NSB). Following a radioligand binding period of 60 min, slides were rinsed for 3 x 5 min in ice-cold rinse buffer (50 mM Tris-HCl, 10 mM MgCl₂, PH 7.4) followed by a 30 min wash in the ice-cold rinse buffer. That was followed by a 2-sec wash in ice-cold distilled water. Slides were then dried

under a stream of cool air for 2 hours and stored in sealed containers with anhydrous calcium sulphate for 7 days.

Metabotropic glutamate receptor 5 binding

Metabotropic glutamate receptor 5 (mGluR5) binding by quantitative autoradiographic analysis was carried out in accordance to previously established protocols (Georgiou *et al.*, 2015; Wright *et al.*, 2016). Sections were rinsed for 20 min in a buffer solution 50 mM Tris-HCl containing 120 mM NaCl, 5mM KCl, 2mM CaCl₂ and 1mM MgCl₂, pH 7.4 at room temperature to remove endogenous ligand. Total binding was determined by incubating sections with 10 nM [³H]-2-Methyl-6-(phenylethynyl) pyridine (MPEP), a selective mGluR5 antagonist, in 50 mM Tris-HCl buffer solution, pH 7.4 at room temperature for 60 min on ice. Adjacent sections were incubated in the same buffer in the presence of 10 nM [³H]-MPEP and 10μM fenobam, an mGluR5 antagonist to determine NSB sites. Incubations were terminated by rapid rinses of 2 x 1 min in ice-cold 50 mM Tris- HCl buffer, pH at 7.4 followed by a 1 min wash in ice-cold distilled water. Slides were then dried under a stream of cool air for 2 hours and stored in sealed containers with anhydrous calcium sulphate for 7 days.

N-methyl-D-aspartate receptor binding

N-methyl-D-aspartate receptor (NMDAR) binding by quantitative autoradiographic analysis was carried out in accordance to established protocol by Reynolds (2001). Sections were rinsed in 50 mM Tris- HCl, pH 7.4 containing 50μM glutamate, 50 μM glycine, 50 μM spermidine (pre-incubation buffer) for 20 min at room temperature. Total binding was determined by incubating sections with 70 nM [³H] (+)MK801 in a pre-incubation buffer for 60 min on ice. Adjacent sections were incubated with 70nM [³H] (+)MK801 in the same buffer in the presence of 1mM (+)-MK801 to determine NSB for the same amount of time. Then, radioligand binding incubations were terminated by rapid rinses of 2 x 30 sec in ice-cold 50 mM Tris- HCl buffer, pH at 7.4, followed by three 3 x 5 min rinses in ice-cold buffer (50 mM

Tris-HCl, 10 mM MgCl₂, pH 7.4) and a sequential 30-min final wash in ice-cold distilled water. Slides were then dried under a stream of cool air for 2 hours and stored in sealed containers with anhydrous calcium sulphate for 7 days.

3.3.1.4.5. Film apposition and development

For the OXTR receptor binding, slides were apposed for 3 days to Kodak MR-1 films in Hypercassettes with autoradiographic [¹⁴C] microscales of known concentrations that were converted to the equivalent concentrations for [¹²⁵I] microscales, as described by Miller and Zahniser (1987). For the film apposition of mGluR5 and NMDAR experiments, slides were apposed with autoradiographic [³H] microscales of known concentration for 3 weeks. Sections for all treatment groups were processed in parallel in a paired protocol and apposed to the same film at the same time. Film development was carried out as previously described in section 2.3.1.2.

3.3.1.4.6. Image analysis

Image analysis was carried out as described previously in section 2.3.1.3. Specific binding was determined by subtracting the NSB from the total binding in the images of the brain sections for OXTR binding and mGluR5 binding.

3.3.1.5. Statistical Analyses

Data represents mean $\pm$ SEM for each treatment group. Statistical analyses were performed using the Graphpad Prism 7.0 software (StatSoft, UK). Statistical significance in behavioral assessments was assessed using two-way ANOVA for factors 'genotype' and 'treatment', followed by Holm Sidak multiple comparisons post hoc test or three-way ANOVA for factors 'genotype', 'treatment' and 'preference', followed by Holm Sidak *post hoc* analysis. Quantification autoradiographic binding data were analysed with two-way ANOVA followed by Holm Sidak multiple comparisons post hoc. Significance of qRT-PCR results was assessed after transformation with one sample t-test. For all comparisons, values of $p < 0.05$ were considered as significant. Data are presented as fold changes between WT OXT vs. WT saline, *Fmr1* KO saline vs. WT saline and *Fmr1* KO OXT vs. *Fmr1* KO saline-treated groups.

3.3.2. Materials

[¹²⁵I]-OVTA, [³H]- MPEP and [³H] (+)MK801 radioligands were purchased from PerkinElmer Life sciences (MA, USA). OXT was purchased from Sigma-Aldrich (France), unlabelled (Thr4,Gly7)-OXT from Bachem (Germany) and anhydrous calcium sulphate from Drierite-BDH Chemicals (Dorset, U.K.). MgCl₂, bovine serum albumin, bacitracin, fenobam, (+)-MK801, glutamate, glycine, spermidine from Sigma-Aldrich (Poole, UK). For the qRT-PCR experiments, brain matrix was purchased from ASI Instruments Warren (MI, USA), 2mm thickness biopsy punch, Direct-zol™ RNA Miniprep from the Epigenetics company, Zymo research (France), ProtoScript first-strand cDNA synthesis kit from the New England, Biolabs (France) and the DyNAmo SYBR Green qPCR Kit F-400S was purchased from M.J. Research Inc. (France). 96-well plates used were purchased from DNA Engine Opticon System, MJ Research Inc. (France). RNA concentration was measured on a spectrophotometer (Milton Roy Co., Spectronic 601). Behaviours were analysed with Viewpoint behaviour technology software (Montpellier, France).

3.4. Results

3.4.1. Effect of chronic intranasal OXT administration in WT and *Fmr1* KO on core symptoms of ASD

In order to evaluate the effect of acute, sub-chronic and chronic OXT (0.3 IU/5 μ l) in the *Fmr1* KO mouse model of autism, WT and *Fmr1* KO mice (cohort #1) were treated daily with saline or OXT for 20 consecutive days and were assessed in a social interaction paradigm 6 times during the 20-day treatment. Social and non-social behaviours including time spent in social interaction (paw and nose contact), following, grooming, rearing and grooming after social contact were analysed with two-way ANOVA for factors 'genotype' and 'treatment', followed by Holm-Sidak post hoc analysis on each of the social interaction assessment days.

In terms of time in social interaction, two-way ANOVA revealed a significant treatment effect for social interaction session on day 1, ($F_{(1,28)} = 62.33$, $p < 0.0001$) and day 20 ($F_{(1,21)} = 22.42$, $p < 0.001$), a significant genotype effect ($F_{(1,28)} = 49.31$, $p < 0.0001$) on day 1 and day 20 ($F_{(1,21)} = 19.47$, $p < 0.001$), and 'treatment x genotype' interaction effect on day 1 ($F_{(1,28)} = 53.26$, $p < 0.0001$), day 3 ($F_{(1,28)} = 176.9$, $p < 0.0001$), and day 20 ($F_{(1,21)} = 447.7$, $p < 0.0001$). Our data clearly demonstrates a profound impairment of social interaction behaviour in the saline treated *Fmr1* KO mice on day 1, 3 and 20, as illustrated by a reduced time spent in social contact (paw, nose contact) with an unfamiliar mouse vs. their WT counterpart (Figure 3.11A). This social deficit was reversed by acute (day 1, first OXT administration; mean diff. = -71.98, $p < 0.0001$ vs. *Fmr1* KO saline), sub-chronic (day 3, mean diff. = -52.72, $p < 0.0001$ vs. *Fmr1* KO saline) and chronic (day 20, mean diff. = -82.64, $p < 0.0001$ vs. *Fmr1* KO saline) OXT administration. Interestingly, while acute OXT administration did not affect sociability behaviour in WT mice (mean diff. = -2.898, ns vs. WT saline on day 1), subchronic and chronic administration of OXT significantly reduced social interaction in the WT mice (mean

diff.= 56.08, $p < 0.0001$ vs. WT saline on day 3; mean diff.= 52.42, $p < 0.0001$ vs. WT saline on day 20). In contrast, in *Fmr1* KO mice, OXT administered either acutely (day 1), sub-chronically (day 3) or chronically (day 20) had persistent prosocial effects. Interestingly, while WT mice treated with OXT did not reveal any difference on their social phenotype on Day 1 of OXT administration, a significant decrease in time spent during social interaction on Day 3 was evident, which persisted until Day 20 displaying an identical socially impaired phenotype with the *Fmr1* KO mice saline-treated mice (Figure 3.11A). Similarly, the number of followings was significantly increased in the *Fmr1* KO OXT-treated mice as compared with the *Fmr1* KO saline-treated on all days of social interaction assessments. Two-way ANOVA revealed a significant treatment effect for number of followings for session ($F_{(1,28)} = 4.932$, $p < 0.05$) on day 1, day 3 ($F_{(1,28)} = 5.298$, $p < 0.05$), and day 20 ($F_{(1,21)} = 44.07$, $p < 0.0001$), genotype effect only on day 3 ($F_{(1,28)} = 10.7$, $p < 0.01$), day 20 ($F_{(1,21)} = 21.42$, $p < 0.0001$), but not on day 1 ($F_{(1,28)} = 3.531$, ns) and 'treatment x genotype' interaction effect on day 3 ($F_{(1,28)} = 96.28$, $p < 0.0001$), day 20 ($F_{(1,21)} = 136.5$, $p < 0.0001$) but not on day 1 ($F_{(1,28)} = 3.859$, ns). Interestingly, consistent with the social interaction data, WT OXT-treated mice displayed reduced number of followings on day 3 (mean diff.=3.875, $p < 0.0001$) and day 20 (mean diff.=3.762, $p < 0.01$), but not on day 1 (mean diff.= -0.375, ns), as compared with WT saline-treated animals. The decrease in the number of followings is one more parameter that clearly demonstrates reduced social interactions in the *Fmr1* KO mice on days 1, 3 and 20, which was reversed by acute and chronic OXT treatment. However, in the WT mice, chronic OXT administration decreases social interaction and thus induces a social impaired phenotype, as illustrated on Figure 3.11B).

Also, social behaviour of self-grooming after social contact was significantly higher in mutants treated with saline on day 1 (mean diff.= -2.375, $p < 0.0001$); day 3 (mean diff.= -1.625, $p < 0.01$); and day 20 (mean diff.= -1.881, $p < 0.001$), as compared with WT saline-treated mice for the three time-points assessed, which shows that social contact was

aversive in these animals. Interestingly, OXT treatment in mutants abolishes this behaviour as illustrated on all days of assessment (day 1, mean diff.= 2.375, $p < 0.0001$; day 3, mean diff.= 1.625, $p < 0.01$; and day 20, mean diff.= 2.167, $p < 0.0001$; vs. *Fmr1* KO saline), which indicates a prosocial effect. Two-way ANOVA for self-grooming after social contact revealed a significant treatment effect on day 1 ($F_{(1,28)}=26.97$, $p < 0.0001$) and day 20 ($F_{(1,21)}=9.415$, $p < 0.01$), genotype effect only on day 1 ($F_{(1,28)}=26.97$, $p < 0.0001$) and 'treatment x genotype' interaction effect on day 1 ($F_{(1,28)}=41.16$, $p < 0.0001$), day 3 ($F_{(1,28)}=31.55$, $p < 0.0001$) and day 20 ($F_{(1,21)}=26.46$, $p < 0.0001$) (Figure 3.11C).

Non-social control parameters of self-grooming and rearing behaviours were also measured. Self-grooming did not show any significant differences between the groups and two-way ANOVA did not reveal any treatment or genotype effect in all three assessment days (Figure 3.11D). Rearing behaviour was increased in the *Fmr1* KO saline-treated animals on day 1, as compared with WT saline-treated (mean diff.= -14.88, $p < 0.05$). This effect was reversed by acute OXT treatment (mean diff.= 17.88, $p < 0.01$). Two-way ANOVA revealed only on day 1 a treatment ($F_{(1,28)}=12.39$, $p < 0.01$) and genotype effect ($F_{(1,28)}=6.769$, $p < 0.05$) but no 'treatment x genotype' interaction effect ($F_{(1,28)}=3.08$, ns) (Figure 3.11E).

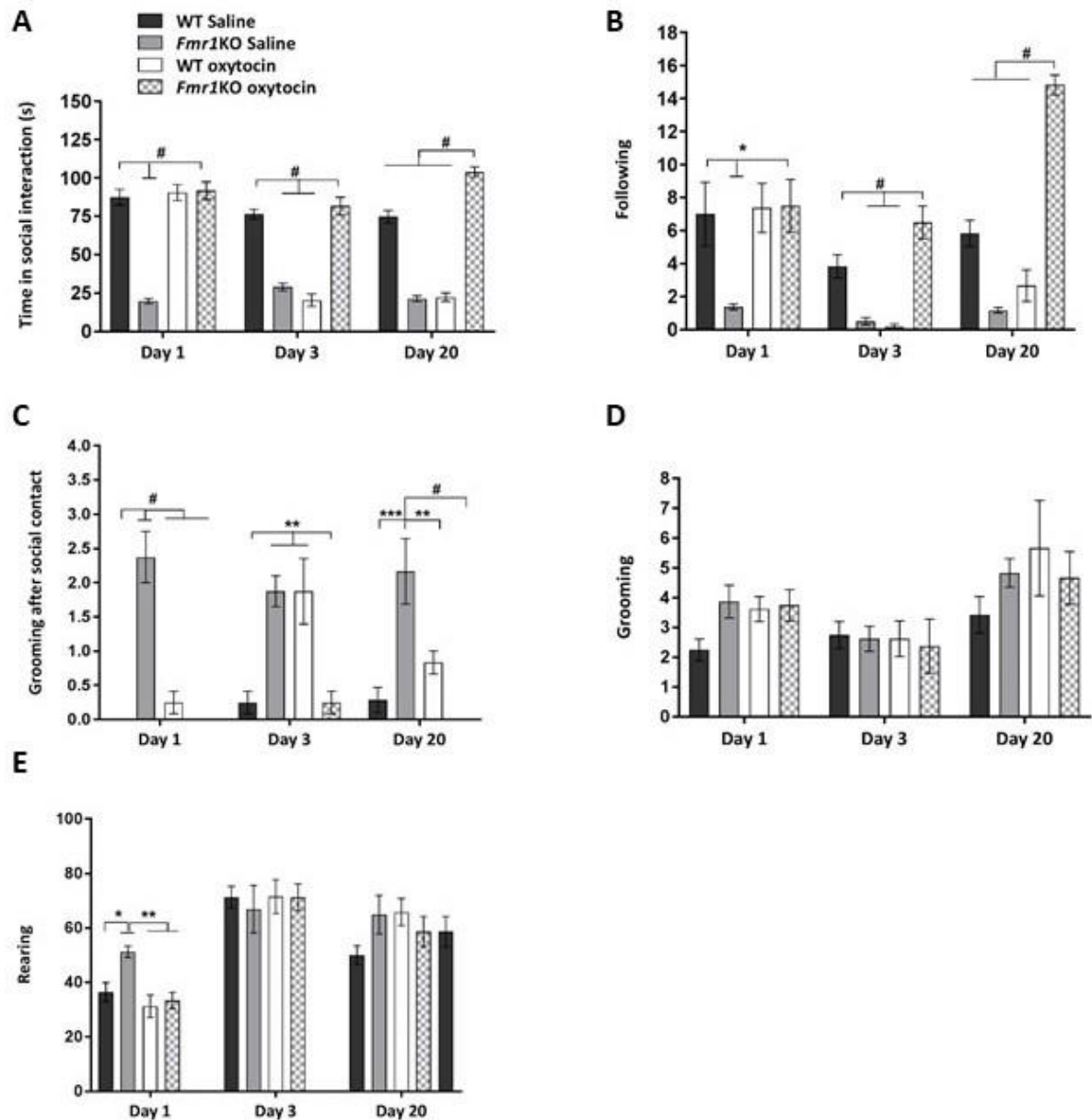


Figure 3.11: *Fmr1* KO show core symptoms of autism including impaired social interactions and stereotypies after social contact, which are reversed after acute, sub-chronic and chronic intranasal OXT treatment.

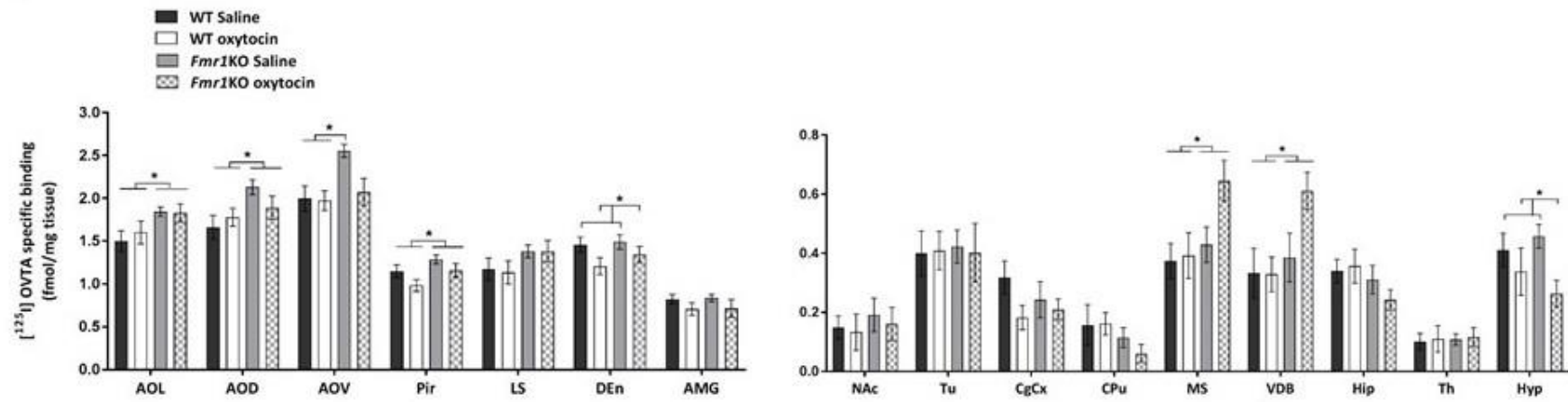
Social behaviours assessed following experiment task were measuring A) duration of time spent in social interactions B) frequency of occurrence of followings and C) grooming after social contact. One following is counted from the time the experimental mouse starts following a 'stimulus' mouse, until the time the experimental mouse changes direction in order to continue following the 'stimulus' mouse. Non-social stereotypic behaviour assessed by measuring frequency of occurrence of D) self-grooming and E) rearing. Two-way ANOVA for factors 'genotype' and 'treatment', followed by Holm-Sidak post hoc analysis was applied on each of the social interaction assessment days. All data are represented as mean $\pm$ SEM. Statistical significance indicated as $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{\#}$ ($n = 6$ per genotype, 3 females and 3 males).

3.4.2. Receptor binding of [¹²⁵I] OVTA, [3H] MPEP and [3H] MK801 by quantitative autoradiography in WT and *Fmr1* KO mice brains treated chronically with OXT or saline

3.4.2.1. Quantitative analysis of [¹²⁵I] OVTA binding

Qualitative analysis of OXTR binding in brains of WT saline-treated mice were similar to OXT-treated WT and OXT-treated *Fmr1* KO mice. High levels of OXTR binding (1.560–2.809 fmol/mg) were detected within the olfactory nuclei including the AOL, AOV and the AOD, moderate levels (1.024–1.549 fmol/mg) in the DEn and the Pir, and low levels (0.018–0.828 fmol/mg) in the CPu and Hip in both OXT and saline-treated groups (Figure 3.12A). Overall, our data demonstrate high levels of OXTR in the brains of the *Fmr1* KO mice compared with the WT controls in cortical and non-cortical brain regions such as the olfactory nuclei (AOL, AOD, AOV), the Pir, MS and VDB. Two-way ANOVA followed by Sidak's multiple comparisons post hoc test indicated a genotype effect as detected in the AOL ($F_{(1,28)}=7.05$, $p<0.05$), AOD ($F_{(1,28)}=6.023$, $p<0.05$), AOV ($F_{(1,28)}=6.59$, $p<0.05$), MS ($F_{(1,27)}=5.218$, $p<0.05$), VDB ($F_{(1,27)}=5.318$, $p<0.05$), Pir ($F_{(1,28)}=5.125$, $p<0.05$), and a treatment effect as observed in the DEn ($F_{(1,28)}=4.683$, $p<0.05$), hypothalamus ($F_{(1,26)}=5.019$, $p<0.05$) and the Pir ($F_{(1,28)}=4.413$, $p<0.05$), but no significant 'treatment x genotype' interaction was found in any of the regions analysed.

A



B

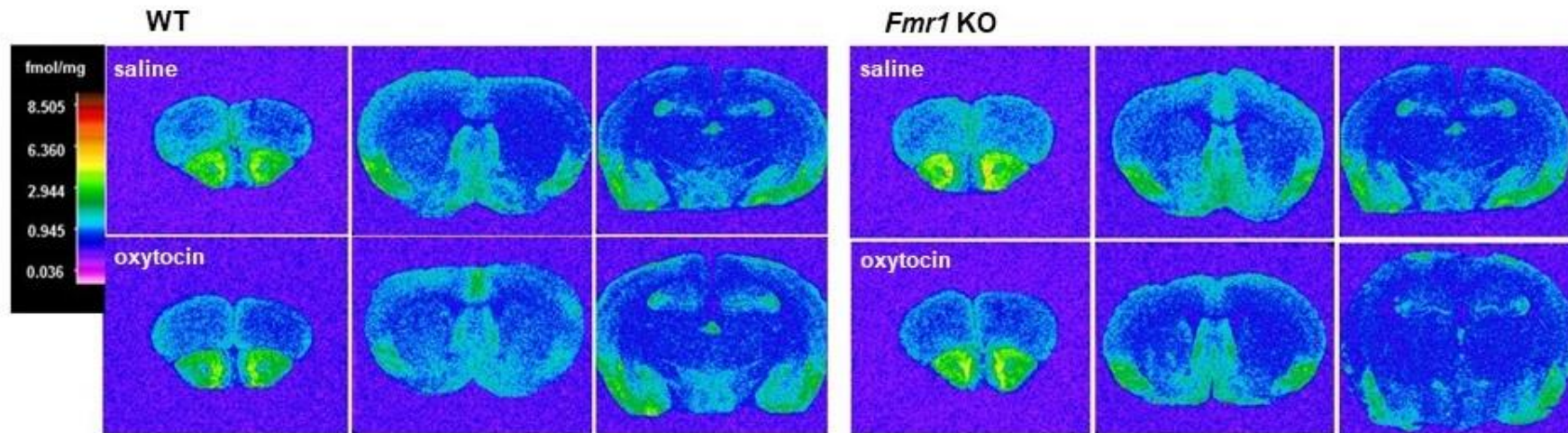


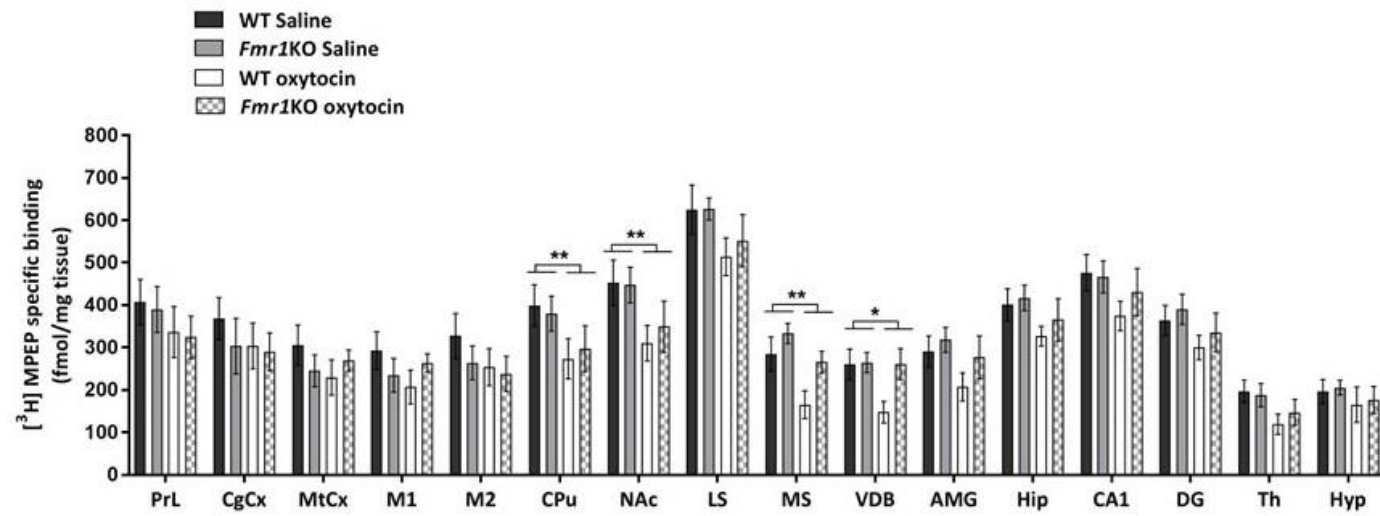
Figure 3.12: OXTR binding.

A) Quantification of OXTR binding levels in brains regions from the *Fmr1* KO and WT mice chronically treated with intranasal OXT. Average bilateral regional OXTR density analysis defined by [¹²⁵I] OVTA specific binding (fmol/mg tissue) in WT and *Fmr1* KO mice treated with saline or OXT. Data are expressed as mean ± SEM, n=5-8 per group, p<0.05*. B) Representative images of adjacent coronal brain section from complete mapping of the brain at the level of the olfactory nuclei (Bregma 3.08) (first columns), medial septum (Bregma 0.74) (second columns) and hypothalamus (Bregma -1.46) (third columns). The colour bar illustrates a pseudocolour interpretation of black and white image density calibrated in fmol/mg of tissue equivalent. Top row; Saline-treated and bottom row; OXT-treated groups. For abbreviations see p.17-26.

3.4.2.2. Quantitative analysis of [3H] MPEP binding

Qualitative analysis of mGluR5 binding revealed similar distribution of receptors in the groups analysed. High levels of mGluR5 binding (798.8–381.3 fmol/mg) were detected within the LS, moderate levels (455.7–188.3 fmol/mg) in the MS and the VDB, and low levels (137.4–51.8 fmol/mg) in the Th and Hyp. Decrease in mGluR5 binding was observed in OXT treated groups and was irrespective of genotype in some regions analysed. Two-way ANOVA followed by Sidak's multiple comparisons *post hoc* test indicated significant treatment effect in the striatum including the NAc ($F_{(1,23)}=5.585$, $p<0.05$), the CPu ($F_{(1,23)}=4.51$, $p<0.05$), the MS ($F_{(1,24)}=9.169$, $p<0.01$) and VDB ($F_{(1,24)}=4.516$, $p<0.05$). No significant 'genotype x treatment' interaction effect was observed in any of the regions analysed (see Figure 3.13A).

A



B

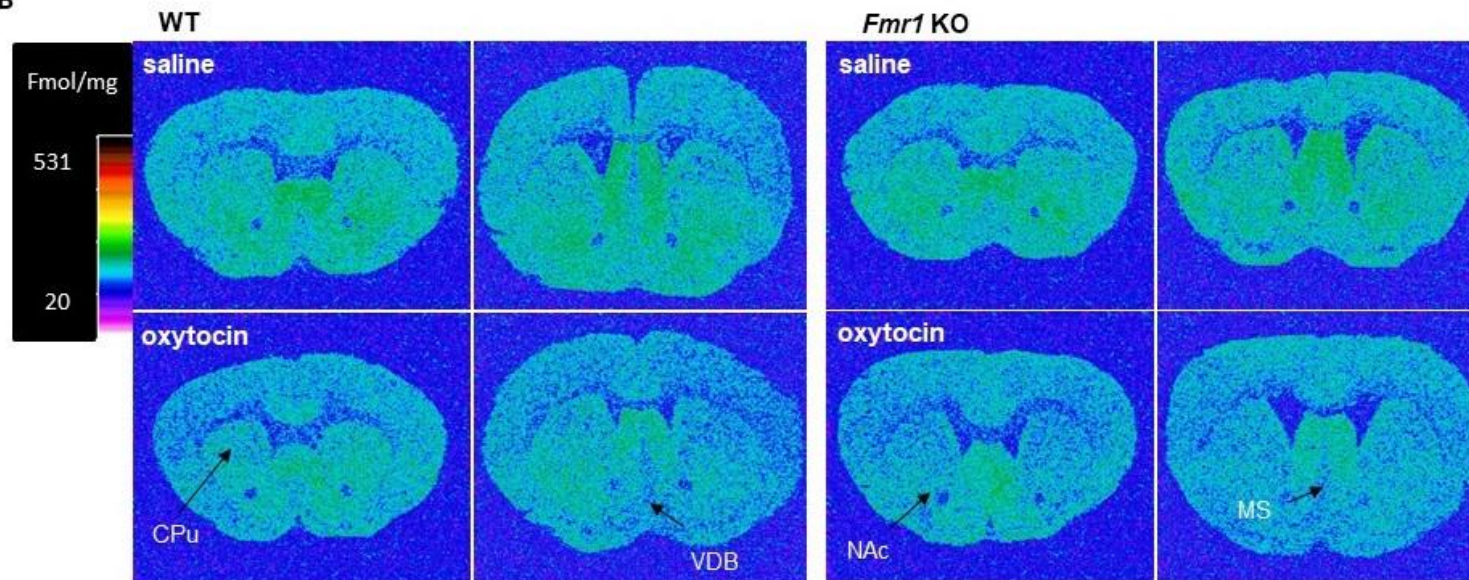


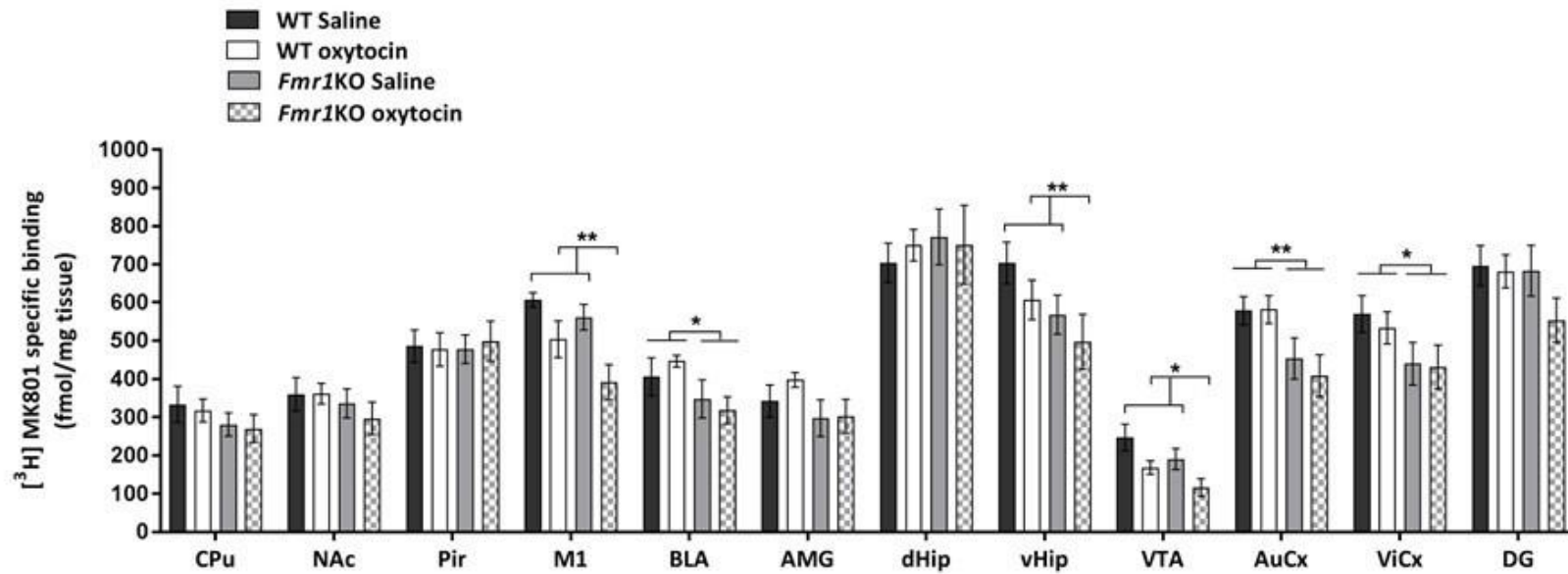
Figure 3.13: mGluR5 binding

A) Quantification of mGluR5 binding levels in brains from the *Fmr1* KO and WT mice chronically treated with intranasal OXT. Average bilateral regional mGluR5 density analysis defined by [³H] MPEP specific binding (fmol/mg tissue) in WT and *Fmr1* KO mice treated with saline or OXT. Data are expressed as mean ± SEM, n=4-8 per group, p<0.05*; p<0.01** B) Representative images of adjacent coronal brain sections from complete mapping of the brain at the level of the NAc (Bregma 1.74) (first column), MS (Bregma 0.74) (second column) and Hyp (Bregma -1.46) (third column). The colour bar illustrates a pseudocolour interpretation of black and white image density calibrated in fmol/mg of tissue equivalent. Top row; Saline-treated and bottom row; OXT-treated groups. For abbreviations see p.17-26.

3.4.2.3. Quantitative analysis of [3H] MK801 binding

Emerging evidence show that dysfunction of NMDAR at excitatory synapses is associated with ASD. Animal models of ASD display NMDAR dysfunction, and repairing this deficit rescues ASD-like behaviours. Therefore, following our data on OXTR binding levels, we also analysed [3H] (+)MK801 binding in the brains of *Fmr1* KO mice treated either with saline or OXT to investigate NMDAR binding levels in the brains of these mice. Qualitative analysis of NMDAR binding revealed similar distribution of receptors in the groups analysed. High levels of NMDAR binding (707.8–1229.9 fmol/mg) were detected within the DG, dHip and AuCx, moderate levels (363.6–640.6 fmol/mg) in the BLA and the Pir, and low levels (55.6–351.5 fmol/mg) in the CPu, NAc and VTA (Figure 3.14A). Overall, our data demonstrate that the density of NMDAR was less abundant in the brains of the *Fmr1* KO mice compared with their WT littermates in cortical brain regions including the motor (M1), auditory (AuCx) and visual cortices (ViCx) and the non-cortical region, the basolateral amygdaloid nuclei (BLA). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test indicated a genotype effect in the AuCx ($F_{(1,23)}=10.61$, $p<0.01$), ViCx ($F_{(1,22)}=5.308$, $p<0.05$) and the BLA ($F_{(1,24)}=5.401$, $p<0.05$), and a treatment effect in brain regions such as the ventral hippocampus (vHip) ($F_{(1,23)}=4.904$, $p<0.01$), the VTA ($F_{(1,16)}=7.644$, $p<0.05$) and the M1 region ($F_{(1,22)}=11.13$, $p<0.01$) (Figure 3.14A). Chronic OXT decreases NMDAR binding in the aforementioned regions irrespective of genotype. No significant 'genotype x treatment' interaction effect was observed in any of the regions analysed.

A



B

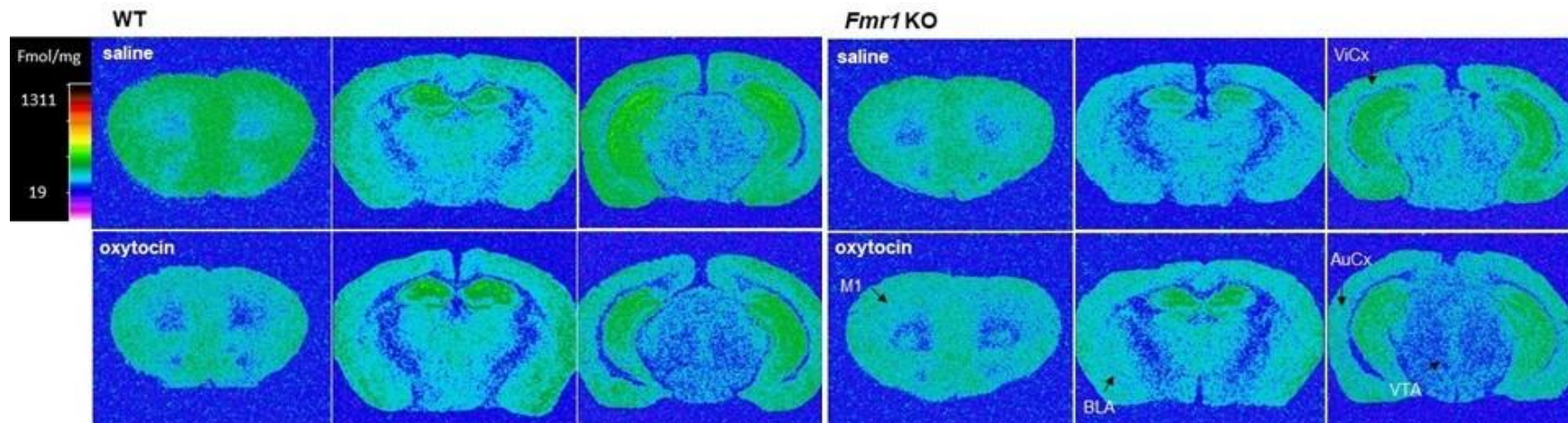


Figure 3.14: NMDAR binding

A) Regional distribution and quantification of NMDAR binding levels in brains from the *Fmr1* KO and WT mice chronically treated with intranasal OXT. Average bilateral regional NMDAR density defined by [³H] MK801 specific binding (fmol/mg tissue) in WT and *Fmr1* KO mice treated with vehicle or OXT. Data are expressed as mean $\pm$ SEM, n=4-8 per group, p<0.05*, p<0.01**. B) Representative images of adjacent coronal brain sections from complete mapping of the brain at the level of the NAc (Bregma 1.74) (first column), AMG (Bregma -1.46) (second column) and VTA (Bregma -1.46) (third column). The colour bar illustrates a pseudocolour interpretation of black and white image density calibrated in fmol/mg of tissue equivalent. Top row; Saline-treated and bottom row; OXT-treated groups. For abbreviations see p.17-26.

3.4.2.4. Quantitative Real Time PCR analysis for several genes of interest in WT and *Fmr1* KO mice brains chronically treated with OXT or saline

For the qRT-PCR experiments, we focused on two brain regions, that are majorly implicated in the pathophysiology of ASD, the central amygdaloid nuclei (CeA) and the caudate putamen (CPu).

3.4.2.5. Real-time Quantitative PCR analysis in the CPu

Transcription of several candidate genes for ASD were changed in the *Fmr1* KO mice treated with saline or OXT in the CPu, a brain region critical for motor and reward function. One sample t-test was used to analyse the transcript levels of the candidate genes in the brains of the WT and *Fmr1* KO mice treated with OXT or saline. Several genes displayed significant changes in their mRNA expression including *Avpr1a*, *Avpr1b*, *OXT* and *OXTr*. Transcripts of *Avpr1a* gene were found significantly (~2 fold) down-regulated in the *Fmr1* KO saline-treated vs. WT saline-treated group ($p < 0.05$), while there was a significant up-regulation (~2 fold) in the *Fmr1* KO OXT vs. *Fmr1* KO saline ($p < 0.05$). Similarly to that outcome, *Avpr1b* transcript levels were significantly up-regulated (~3.2 fold) in the *Fmr1* KO OXT vs. *Fmr1* KO saline group ($p < 0.001$). *Avp* and *Avpr1a* demonstrate an opposing pattern across all four groups screened for, however, *Avp* transcripts did not show any significant changes between groups ($p > 0.05$). mRNA *Oxt* levels were found globally down-regulated across all four groups, with a significant decrease (~4 fold) marked in the *Fmr1* KO saline-treated vs. WT saline treated group ($p < 0.01$). *OXTr* and *Oprm1* mRNA did not show any significant difference, however, those two genes demonstrated an opposing expression pattern for their transcripts. Both *Pdyn* and *Penk* mRNA levels demonstrate a significant down-regulation (1-1.5 fold) in the *Fmr1* KO saline vs. WT saline treated group ($p < 0.05$),

whereas *Penk* transcripts were also significantly up-regulated (~1.2 fold) in the *Fmr1* KO OXT vs. *Fmr1* KO saline group ($p<0.05$). *Grm4* and *Grm8* transcripts reveal a similar expression profile with a marked decrease in the *Fmr1* KO saline vs. WT saline ($p<0.01$, $p<0.0001$ for *Grm4* and *Grm8*, respectively) (see Figure 3.15). Additionally, the fold change differences of additional genes implicated in ASD were assessed, as illustrated on Table 3.3.

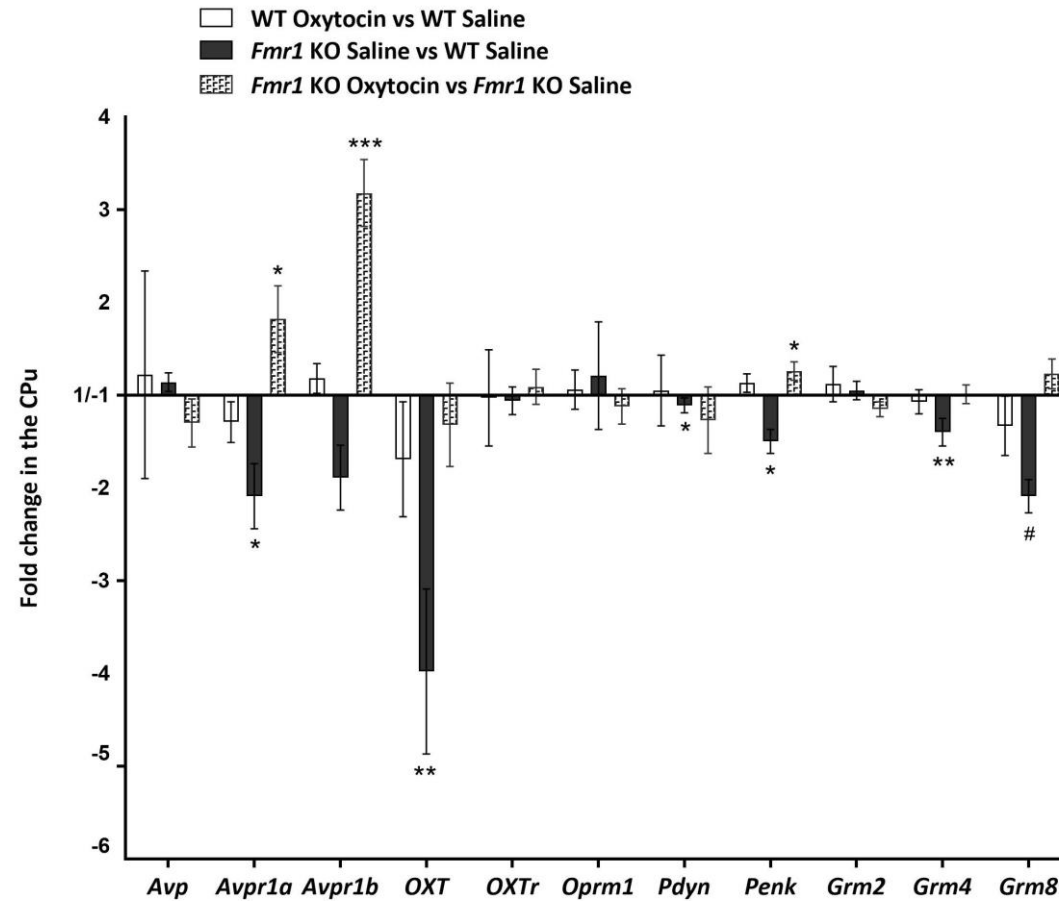


Figure 3.15: *Fmr1* KO and WT mice treated with OXT or saline display transcriptional alterations within several systems: the oxytocin and vasopressin neuropeptides, the endogenous opioid and glutamatergic system in the CPu.

Brain-region specific mRNA extracted from tissues of WT and *Fmr1* KO mice treated with OXT or saline (n=3-4/genotype/ treatment group) for the CPu brain regions. Data analysis was performed using a comparative C_t method. Significance of qRT-PCR results was assessed after transformation using a one sample *t*-test ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{\#}$).

Table 3.3: Fold change differences of gene expression levels in the CPu of WT and *Fmr1* KO mice treated with OXT or saline.

Gene	<i>Fmr1</i> KO Sal vs WT Sal			WT OXT vs WT Sal			<i>Fmr1</i> KO OXT vs <i>Fmr1</i> KO Sal		
	Mean	SEM	P value	Mean	SEM	p value	Mean	SEM	p value
<i>Arc</i>	-2.32	0.56	0.045	1.21	0.15	0.171	2.04	0.26	0.002
<i>B3galnt1</i>	-2.14	9.67	0.226	1.12	0.08	0.155	1.46	0.18	0.035
<i>Bdnf</i>	-3.02	1.61	0.022	-1.23	1.15	0.644	-1.02	0.74	0.333
<i>Cck</i>	-2.11	0.50	0.246	-1.33	0.39	0.872	1.24	0.27	0.166
<i>Cckra</i>	-1.54	0.31	0.143	-1.22	0.20	0.544	1.68	0.17	0.005
<i>Cckrb</i>	-1.78	0.30	0.048	-1.04	0.13	0.419	-1.30	0.27	0.240
<i>C-fos</i>	-1.12	0.02	0.001	-1.00	0.07	0.805	1.15	0.04	0.008
<i>Col6a4</i>	-1.03	0.12	0.457	1.41	0.21	0.01	-1.15	0.12	0.072
<i>Cyp26b1</i>	-1.18	0.11	0.070	1.09	0.10	0.846	-1.00	0.10	0.724
<i>Eif2s3y</i>	-1.19	4.64	0.192	-1.20	1.79	0.075	-4.55	2.85	0.079
<i>Esrrb</i>	1.08	0.12	0.902	1.22	0.48	0.415	1.18	0.10	0.158
<i>Galr1</i>	1.08	0.04	0.164	1.00	0.11	0.421	-1.04	0.04	0.413
<i>Galr2</i>	-1.27	0.13	0.035	-1.05	0.18	0.781	1.24	0.09	0.081
<i>Gh</i>	-3.72	1.41	0.038	1.12	2.14	0.463	2.59	0.75	0.033
<i>Gpr68</i>	-1.40	0.12	0.002	-1.18	0.07	0.143	-1.05	0.05	0.887
<i>Insrr</i>	-1.27	0.07	0.005	1.05	0.11	0.148	1.18	0.10	0.083
<i>Medag</i>	1.00	5.10	0.280	1.42	0.23	0.029	-1.06	0.13	0.961
<i>Ndn</i>	-1.06	0.30	0.117	1.13	0.07	0.137	1.69	0.24	0.012
<i>Neurod6</i>	1.67	0.23	0.058	1.18	0.09	0.054	-1.18	6.82	0.283
<i>Rxfp1</i>	1.43	0.49	0.305	-1.12	0.81	0.580	-1.57	0.22	0.058
<i>Htr6</i>	-1.17	0.12	0.536	1.03	0.13	0.589	-1.07	0.18	0.935
<i>Shisa6</i>	1.02	0.28	0.683	1.03	0.34	0.454	-1.56	0.36	0.193
<i>slc17a7</i>	-2.41	1.41	0.268	-1.40	0.68	0.626	-1.10	0.63	0.607
<i>Syt17</i>	-1.12	1.13	0.917	1.10	0.70	0.616	-1.48	0.47	0.918
<i>Vdr</i>	-1.42	0.21	0.132	-1.02	0.21	0.615	1.35	0.09	0.018
<i>Vip</i>	1.20	0.34	0.832	1.15	0.24	0.114	-1.53	0.18	0.041
<i>Vipr1</i>	-1.39	0.07	0.000	1.07	0.10	0.717	-1.18	0.10	0.058
<i>Vipr2</i>	-1.06	0.09	0.313	1.03	0.09	0.626	1.10	0.10	0.596
<i>Wnk4</i>	-1.90	0.12	0.000	-1.01	0.19	0.375	-1.01	0.09	0.415

Data are expressed as mean $\pm$ SEM. n=3-4 /genotype/ treatment group. Details of genes with significant regulation is highlighted in bold; p<0.05. For abbreviations see p.17-26.

3.4.2.6. Real-time Quantitative PCR analysis in the CeA

In order to investigate the expression levels of several candidate genes, we also examined transcription in another targeted brain region, the CeA, which is important in the emotional processing. *Avp* transcript levels were significantly decreased (~1.8 fold) in the *Fmr1* KO OXT-treated mice vs. *Fmr1* KO saline-treated control animals ($p < 0.05$). Interestingly, mRNA expression of the *Avpr1b* gene demonstrates a global increase (0.1 fold) in the expression across the *Fmr1* KO and WT OXT or saline treated groups though not statistically significant ($p > 0.05$), while the two genes, *Avp* and *Avpr1a*, represent an opposing expression profile across the four groups, similar in expression profile respective to the CPu brain region. The transcript levels of *Oxtr* and *Oxt* ($p < 0.01$ and $p < 0.05$, respectively) were found to be significantly lower in the *Fmr1* KO OXT-treated mice vs. *Fmr1* KO saline-treated control animals, whereas the latter also showed significant decrease in the *Fmr1* KO OXT-treated mice vs. WT Saline-treated ($p < 0.05$). A similar pattern of expression was found for *Oxt*, *Oxtr* and *Oprm1* however, there was no marked significant difference across all four groups for the latter. *Penk* mRNA was found significantly up-regulated (~1.3 fold) in the *Fmr1* KO saline vs. WT saline treated group ($p < 0.0001$). OXT also significantly increased *Penk* mRNA levels in the WT mice ($p < 0.01$). *Grm4* transcripts were overall down-regulated and a significant decrease of approximately 1.5 fold was observed in both the *Fmr1* KO saline vs. WT saline-treated group ($p < 0.05$) and the *Fmr1* KO OXT vs. WT saline-treated group ($p < 0.01$). *Grm8* mRNA was found up-regulated in the WT saline treated animals ($p < 0.01$) (see Figure 3.16). Similarly to the CPu analysis, the fold change differences of the same list of genes were assessed also in the CeA, as illustrated on Table 3.4.

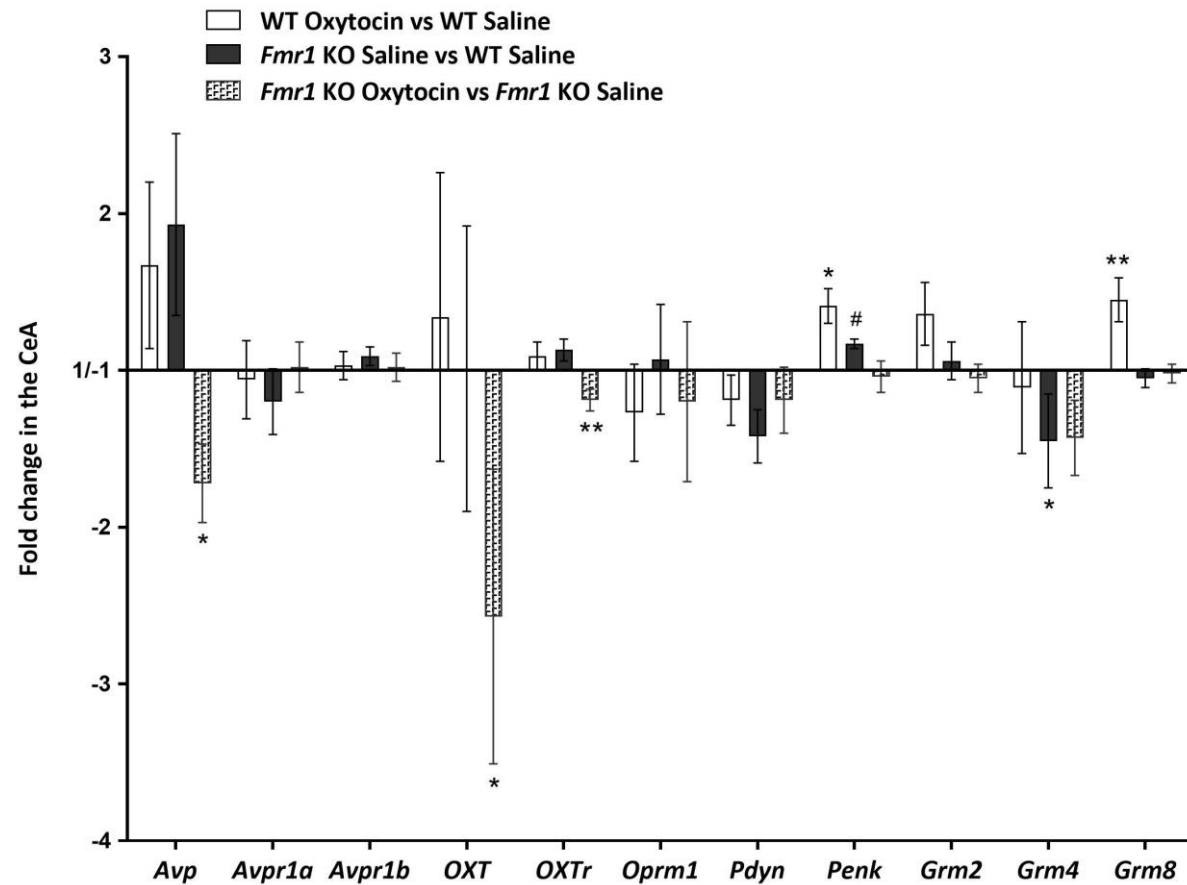


Figure 3.16: *Fmr1* KO and WT mice treated with OXT or saline display transcriptional alterations within several systems: the oxytocin and vasopressin neuropeptides, the endogenous opioid and glutamatergic system in the CeA.

Brain-region specific mRNA extracted from tissues of WT and *Fmr1* KO mice treated with OXT or saline (n=3-4 per group) for the CPu brain regions. Data analysis was performed using a comparative C_t method. Significance of qRT-PCR results was assessed after transformation using a one sample t -test ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.001^{\#}$)

Table 3.4: Fold change differences of gene expression levels in the CeA of WT and *Fmr1* KO mice treated with OXT or saline.

Gene	<i>Fmr1</i> KO Sal vs WT Sal			WT OXT vs WT Sal			<i>Fmr1</i> KO OXT vs <i>Fmr1</i> KO Sal		
	Mean	SEM	p value	Mean	SEM	p value	Mean	SEM	p value
<i>Arc</i>	-1.19	0.13	0.013	-1.03	0.08	0.928	-1.38	0.11	0.117
<i>B3galnt1</i>	-1.99	8.27	0.304	1.18	0.07	0.011	1.11	4.84	0.381
<i>Bdnf</i>	1.18	0.06	0.026	1.36	0.15	0.098	-1.23	0.10	0.066
<i>Cck</i>	1.03	0.08	0.396	1.26	0.10	0.022	-1.17	0.06	0.033
<i>Cckra</i>	1.04	0.39	0.410	1.16	0.33	0.289	1.11	0.17	0.514
<i>Cckrb</i>	-1.05	0.09	0.369	1.03	0.19	0.345	-1.19	0.13	0.093
<i>C-fos</i>	1.23	0.09	0.047	1.29	0.20	0.271	-1.02	0.11	0.504
<i>Col6a4</i>	1.90	0.31	0.062	1.96	0.66	0.048	-1.48	0.45	0.052
<i>Cyp26b1</i>	-1.34	0.08	0.001	1.16	0.49	0.614	-1.76	0.21	0.002
<i>Eif2s3y</i>	-1.03	1.32	0.085	-4.30	1.71	0.033	1.21	1.51	0.110
<i>Esrrb</i>	-1.47	0.30	0.555	1.11	0.15	0.291	-1.05	0.25	0.683
<i>Galr1</i>	1.12	0.12	0.410	1.04	0.07	0.107	1.05	0.17	0.472
<i>Galr2</i>	1.03	0.06	0.782	-1.04	0.17	0.699	-1.06	0.11	0.303
<i>Gh</i>	-1.54	0.34	0.159	1.65	1.34	0.060	1.55	0.12	0.005
<i>Gpr68</i>	-1.12	0.06	0.286	1.09	0.15	0.504	-1.00	0.13	0.756
<i>Insrr</i>	1.00	0.28	0.280	1.01	0.26	0.804	-1.64	0.24	0.088
<i>Medag</i>	1.01	0.18	0.820	1.59	0.09	0.000	-1.30	0.79	0.379
<i>Ndn</i>	2.07	0.27	0.009	1.24	0.10	0.033	-1.21	0.18	0.059
<i>Neurod6</i>	1.12	0.14	0.860	1.02	0.16	0.244	1.04	0.10	0.865
<i>Rxfp1</i>	1.36	0.40	0.676	1.02	0.18	0.618	-1.70	0.28	0.009
<i>Htr6</i>	-1.21	0.12	0.143	-1.11	0.12	0.711	-1.20	0.11	0.177
<i>Shisa6</i>	-1.20	0.06	0.036	1.14	0.11	0.119	1.19	0.17	0.351
<i>slc17a7</i>	-1.01	0.07	0.439	1.08	0.11	0.740	-1.24	0.04	0.000
<i>Syt17</i>	1.02	0.07	0.542	1.20	0.19	0.758	-1.17	0.14	0.163
<i>Vdr</i>	-1.25	0.46	0.191	1.18	0.27	0.895	-1.03	0.26	0.896
<i>Vip</i>	-1.09	0.05	0.284	1.17	0.12	0.610	-1.12	0.08	0.136
<i>Vipr1</i>	-1.05	0.03	0.356	1.04	0.15	0.456	-1.29	0.09	0.120
<i>Vipr2</i>	-1.05	0.14	0.996	-1.06	0.18	0.605	-1.18	0.13	0.812
<i>Wnk4</i>	-1.30	0.14	0.033	1.00	0.58	0.407	-1.21	0.15	0.203

Data are expressed as mean $\pm$ SEM (n=3-4 /genotype/treatment group). Details of genes with significant regulation is highlighted in bold; p<0.05. For abbreviations see p.17-26.

3.4.3. Battery of behavioural paradigms assessing core symptoms of autism and comorbidity symptoms in the *Fmr1* KO and WT mice treated with chronic OXT or saline for 39 days

3.4.3.1. Three-chamber sociability test and motor stereotypies outcomes

Sociability was assessed with the three-chamber sociability paradigm in the *Fmr1* KO and WT male and female mice population (n=8, 4 females; 4 males) chronically treated with OXT or saline on days 34 and 35 of chronic administration (Timeline diagram on Figure 3.6). Three-way ANOVA for factors 'genotype', 'treatment' and 'preference' followed by Holm Sidak *post hoc* analysis revealed a significant 'preference' ($F_{(1,56)}=15.61$, $p<0.001$), 'preference x genotype' effect ($F_{(1,56)}=16.49$, $p<0.001$) and 'preference x treatment x genotype' effect ($F_{(1,56)}=14.73$, $p<0.001$) for the time spent around cage. Overall, our data demonstrate that *Fmr1* KO mice treated with saline spent significantly less time around the cage with the stranger mouse than the cage containing the toy (mean diff.= -21.25, $p<0.05$ mouse vs. toy) compared with WT mice that spent the majority of their time around the 'social cage' (Figure 3.17A). OXT treatment reversed this social impairment in *Fmr1* KO mice as illustrated by the increase in time spent around the cage with the stranger mouse vs. the toy cage (mean diff.= 20.29, $p<0.05$ mouse vs. toy), as illustrated on Figure 3.17A. Motor stereotypies were assessed on days 26 and 27 of OXT administration by measuring the number of rearing, grooming, circling, head shakes as well as total time spent digging, as they were recorded under real-time observation during a 10-min live session (Figure 3.17B_{i-v}). Motor stereotypies as displayed by increased number of circling (mean diff.= -4.917, $p<0.001$ vs. WT saline) and head shakes events (mean diff.= -4.625, $p<0.001$ vs. WT saline), and reduced time spent digging (mean diff.= 108.6, $p<0.001$ vs. WT saline) were observed in the saline-treated mutant mice as compared with the saline WT animals. Two-way ANOVA followed by Holm Sidak's multiple comparisons revealed a significant treatment

($F_{(1,30)}=6.2$, $p<0.05$) and 'genotype x treatment' interaction effect ($F_{(1,30)}=5.768$, $p<0.05$) in rearing behaviour, and a significant genotype effect in both grooming ($F_{(1,30)}=5.972$, $p<0.05$) and circling behaviour ($F_{(1,30)}=20.03$, $p<0.001$). Head shakes showed significant genotype ($F_{(1,30)}=10.18$, $p<0.01$), treatment ($F_{(1,30)}=5.529$, $p<0.05$) and interaction ($F_{(1,30)}=9.14$, $p<0.01$) effects. Time spent digging also revealed a significant interaction effect ($F_{(1,30)}=9.104$, $p<0.01$) and genotype effect ($F_{(1,30)}=12.13$, $p<0.01$). Interestingly, OXT treatment in the *Fmr1* KO mice did not seem to reduce those stereotypic behaviours, whereas OXT treatment in the WT mice indicated increased rearing behaviour (mean diff.= -20.78, $p<0.01$ vs. WT saline), head shakes (mean diff.= -4, $p<0.01$ vs. WT saline), as well as, decreased total time spent digging (mean diff.= 70.07, $p<0.05$ vs. WT saline).

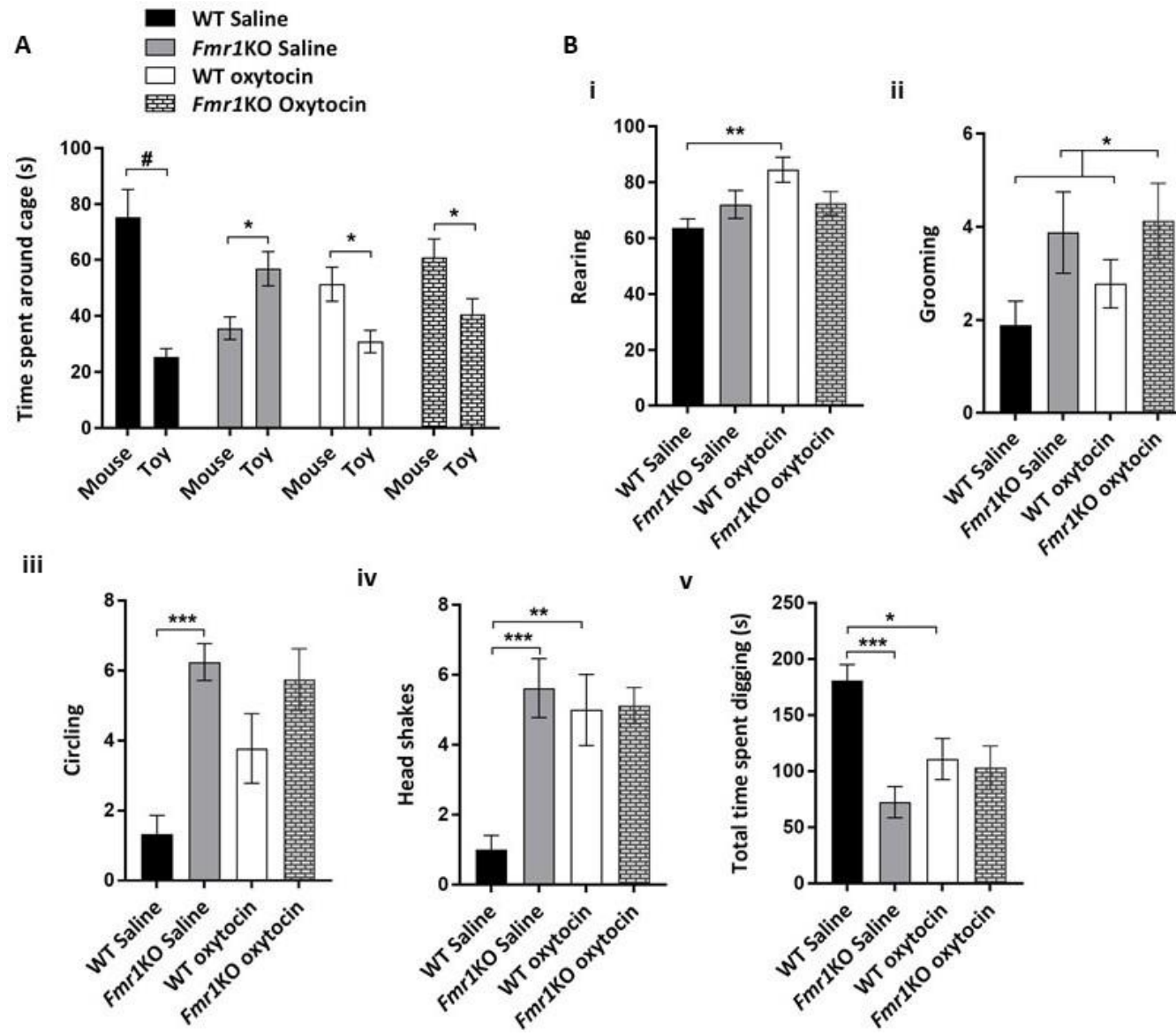


Figure 3.17: Effect of chronic oxytocin on core symptoms of ASD assessed by 3-chamber sociability test and motor stereotypies assessment in cohort 2 (OXT-or saline treatment over 39 days).

A) Three chamber-sociability (Crawley's) test assessed social behaviour after measuring time spent in close contact with the mouse (nose and paw contacts) as compared to time spent in contact with the toy. Three-way *ANOVA* for factors 'preference', 'genotype' and 'treatment' was applied, followed by Holm Sidak post hoc. Data are represented as mean $\pm$ SEM (n=8). B_{i-v}) Non-social stereotypic behaviour was assessed by measuring frequency of occurrence of i) rearing, ii) self-grooming, iii) circling, iv) head shakes and v) time spent digging. Two-way *ANOVA* for factors 'genotype' and 'treatment', followed by Holm-Sidak post hoc analysis was applied on each of the behaviours observed. All data are represented as mean $\pm$ SEM (n=8-9). Statistical significance indicated as $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^\#$.

3.4.3.2. Anxiety assessed by marble burying and novelty suppressed feeding paradigms and learning and memory assessment by novel object recognition paradigm

Fmr1 KO mice were also assessed for secondary symptoms of autism including anxiety and cognitive impairment. Marble burying and novelty suppressed feeding tasks were applied on day 21 and day 37 of chronic OXT administration, respectively, for evaluating anxiety in WT and mutant mice following chronic intranasal administration of saline or OXT (Timeline diagram on Figure 3.6). Two-way ANOVA for factors 'genotype' and 'treatment', followed by Holm Sidak *post hoc* analysis revealed a genotype effect ($F_{(1,30)}=5.813$, $p<0.05$), but it did not show any treatment effect ($F_{(1,30)}=0.033$, ns) for the marble burying task. Saline-treated *Fmr1* KO mice showed increased marble burying behaviour, indicative of increased anxiety, as shown in Figure 3.18A and, OXT treatment did not rescue this phenotype (mean diff.= 1.375, ns vs. *Fmr1* KO saline). In the novelty suppressed feeding paradigm, when measuring feeding latency in the saline-treated *Fmr1* KO mice, it was revealed that there was an increase in food latency (mean diff.= -125.1, $p<0.05$ WT saline vs. *Fmr1* KO saline), indicative of increased anxiety levels observed in the mutant mice and that effect was reversed after OXT treatment (mean diff.= -57.27, ns WT saline vs. *Fmr1* KO OXT). Two-way ANOVA for factors 'genotype' and 'treatment', followed by Holm Sidak *post hoc* analysis indicated a significant interaction effect ($F_{(1,30)}=13.25$, $p<0.01$), with no effects of treatment ($F_{(1,30)}=1.66$, ns) or genotype ($F_{(1,30)}=0.4972$, ns). In particular, OXT increased feeding latency in WT mice to levels similar to saline-treated *Fmr1* KO mice (mean diff.= -142.1, $p<0.01$ WT OXT vs. WT saline) (Figure 3.18B), indicating that chronic OXT increases anxiety in WT mice. Cognitive function was also evaluated in the *Fmr1* KO mice with the novel object recognition paradigm on days 28 and 29 of the treatment's timeline. As described, phase 1 was the habituation phase in which the mice were introduced to the

objects for the first time, followed by phase 2, where one of the objects used in phase 1 was displaced, and finally phase 3 in which a novel object was introduced for the first time. Measurement of the sniffing duration ratio was used for the second and third phase of the task assessing recognition of spatial change (location-dependent) and novelty (object-dependent), respectively (Figure 3.18C). A two-way ANOVA following Holm Sidak's multiple comparisons *post hoc* analysis revealed a significant interaction effect ($F_{(1,30)}=4.913$, $p<0.05$), treatment ($F_{(1,30)}=4.686$, $p<0.05$) and genotype ($F_{(1,30)}=11.65$, $p<0.01$) effect for recognition of spatial change (location-dependent). Interestingly, WT OXT-treated mice showed a significant increase in sniffing duration ratio compared with WT saline-treated (mean diff.= -25.19, $p<0.05$), indicating increased spatial recognition (Figure 3.18C). OXT had no effect on *Fmr1* KO mice and there was no difference in sniffing ratio between WT saline-treated and *Fmr1* KO mice saline treated. Two-way ANOVA did not show any significant effect for novelty (object-dependent).

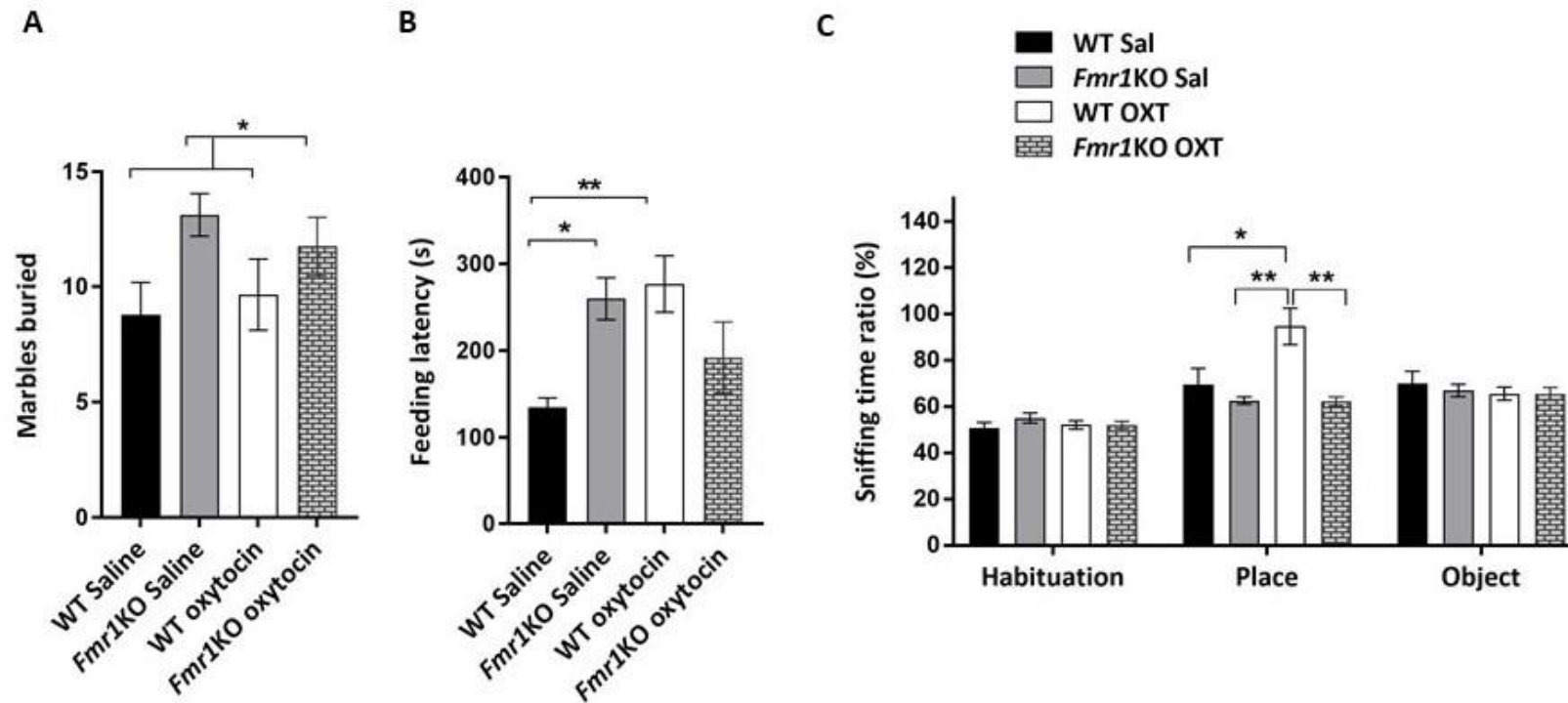


Figure 3.18: Effect of chronic oxytocin on comorbid symptoms of ASD in *Fmr1* KO and WT mice.

Fmr1 KO and WT mice were treated with saline or OXT for 39 days were assessed with the novel object recognition test for cognitive impairment and with the marbles burying and novelty suppressed feeding test for anxiety. A) Novel object recognition assessing learning and memory function for spatial (hippocampal-dependent memory) and novelty (object-dependent) preferences. Three-way ANOVA for factors 'preference', 'genotype' and 'treatment' was applied. Data are represented as mean ± SEM (n=8). B-C) Comorbid anxiety was evaluated by marble burying and novelty-suppressed feeding paradigms. Data from marble burying and novelty suppressed feeding were analysed by two-way ANOVA followed by Holm-Sidak's multiple comparisons. Data are represented as mean ± SEM (n=8-9). Significance is noted as $p < 0.05^*$, $p < 0.01^{**}$.

3.4.4. Discussion

3.4.5. Chronic and acute intranasal OXT administration in the *Fmr1* KO mice reverses social impairment and motor stereotypies in an *Fmr1* KO model of ASD

This present study is the first one to report the beneficial prosocial effects of chronic administration of OXT in the *Fmr1* KO mouse model of autism. This evidence will add insight to the current research on OXT's prosocial effects and ASD by validating these effects in another well-characterised mouse model of autism. Our results demonstrate that *Fmr1* KO show core symptoms of autism including impaired social interactions and stereotypies. Social interactions impairment is reversed after acute, sub-chronic and chronic intranasal OXT treatment (Figure 3.11). Increased manifestation of other motor stereotypies such as self-grooming, circling and number of head shakes were increased in the *Fmr1* KO mice; however, OXT treatment did not reverse these behaviours (Figure 3.17). Interestingly, detrimental effects of OXT was evident in the WT mice treated sub-chronically and chronically, but not acutely, as demonstrated by the impaired social interactions in the social interaction test (Figure 3.11), as well as increased motor stereotypies, as manifested by increased head shakes, rearing (Figure 3.11) and reduced time spent digging (Figure 3.17).

Acute and chronic OXT administration in the *Fmr1* KO mice was sufficient to relieve social deficits, while improving social behaviour suggesting that OXT treatment has beneficial effects in relieving core symptoms of autism. In line with previous studies (Spencer *et al.*, 2005; McNaughton *et al.*, 2008; Dahlhaus and El-Husseini, 2010), *Fmr1* KO mice displayed deficits in their social interactions as illustrated by decreased time spent in social contact (nose and paw contact) with an unfamiliar mouse as well as in the occurrence of followings. Three-chamber sociability test has also confirmed this after showing that these mutants demonstrate increased preference of the cage containing the toy over the mouse cage

(McNaughton *et al.*, 2008; Dahlhaus and El-Husseini, 2010). These effects were reversed after acute intranasal OXT administration (day 1) and persisted over a period of 20 days in a direct social interaction paradigm. Interestingly, WT mice treated sub-chronically and chronically, but not acutely, with OXT displayed reduced sociability, as illustrated on Day 3 of social interaction assessment, which persisted until Day 20 of OXT administration. Previous work on WT mice treated acutely and chronically with intranasal OXT have demonstrated decreased social interactions when assessed with unfamiliar same sex mice in a similar paradigm (Huang *et al.*, 2014). This is only partially in line with our findings as our data failed to show this impairment on Day 1 of social interaction assessment following acute OXT administration (Figure 3.11). Collectively, the above strongly suggest that although chronic OXT treatment has deleterious effects on social behaviour in healthy, wild-type subjects, it shows remarkably persistent beneficial effects in *Fmr1* KO.

A separate cohort, in which the mutants were tested in a three-chamber sociability test over a 39-OXT administration period managed to replicate the above findings (Figure 3.17A). In particular, *Fmr1* KO mice displayed reduced preference in interacting with the stranger mouse, in agreement with previous reports (Sethna *et al.*, 2017) and our results described over the 20-days paradigm. Chronic OXT administration in the *Fmr1* KO mice was able to reverse that, as it increased social encounters with the stranger mouse as compared to the toy. In contrast to our findings with the social interaction test (20-days intranasal OXT administration-cohort 1), WT OXT-treated mice did not show impairment in this test, demonstrated by increased preference of exploring the mouse cage over the toy cage. Thus, these results might argue for differences between the two tests used, rather than the significant social impairment observed in the WT mice following prolonged OXT administration. There may be various reasons for this discrepancy. During this separate cohort, the *Fmr1* KO mice were exposed to a battery of behavioural tests and the animals might have exhibited carry-over effects due to their repeated examination; therefore, the

three-chamber sociability test just partially failed to confirm our previous findings. Results using the three-chambered social approach with *Fmr1* KO mice to evaluate their sociability have not been consistent. Several groups have reported normal sociability in the *Fmr1* KO mice, preferring to explore the novel mouse over the novel object (McNaughton *et al.*, 2008; Liu and Smith, 2009; Liu, Chuang and Smith, 2011; Pietropaolo *et al.*, 2011; Sorensen *et al.*, 2015), while others suggest that the sociability of these mutants is impaired, exhibiting preference for an object over a novel mouse (Dahlhaus and El-Husseini, 2010) with marked decrease in the sniffing duration exploring the novel mouse as compared to WT controls (McNaughton *et al.*, 2008). Differences in time of day of tests, housing conditions, age of the animals tested, characteristics of 'stranger' mice used and, most importantly, changes in the experimental paradigms likely explain the disparate results. Collectively, the above confirm our previously reported finding that social approach is diminished in *Fmr1* KO mice. However, it is not consistent with the evidence showing that prolonged OXT administration in WT healthy animals has detrimental effects in their social behaviour. This might be explained due to paradigms testing social behaviour with different approaches. While three-chamber sociability measures the time spent around the cage containing the novel mouse (social/non-social stimulus), the social interaction test measures direct social contact (nose and paw contact) with another novel mouse.

Non-social behaviours in the *Fmr1* KO and WT mice acutely, sub-chronically and chronically treated with OXT or saline were also evaluated during the social interaction paradigm (used as control paradigm in social context) (Figure 3.11) and in a dedicated paradigm observing motor stereotypies on single caged mice in a non-social context (Figure 3.17). The behaviours assessed during the direct social interaction include rearing, self-grooming and self-grooming after social contact behaviours. OXT intranasal infusion has been associated with reduction of repetitive behaviours in patients with ASD (Hollander *et al.*, 2003), therefore we anticipated reproducing this effect in the *Fmr1* KO mice. Acute and chronic OXT

administration in the *Fmr1* KO mice decreased the aversiveness of social contacts by showing decreased self-grooming after social contact (social behaviour) in our paradigm, a behaviour which was significantly increased in the mutant mice demonstrating that OXT can eliminate social aversion in mice characterised by ASD-like phenotype. However, measurements of non-social behaviour (self-grooming and rearing), did not show any treatment or genotype effect. Motor stereotypies were assessed on a separate cohort of the 39-day OXT administration paradigm, in which stereotypic behaviours of circling, head shakes, self-grooming, rearing and time spent digging were monitored. Interestingly, OXT treatment did not exhibit beneficial effects on stereotypy manifested by any of these behaviours in the *Fmr1* KO mice. On the contrary, chronic OXT induced stereotypy as manifested by increasing head shakes and rearing in WT mice. The discrepancy in the motor stereotypy results might indicate that prolonged OXT administration (over 39 days) demolishes the efficacy of OXT, establishing treatment resistance in the *Fmr1* KO autistic mice, at least for stereotypy-type behaviour. On the other hand, overstimulation of a 'healthy' oxytocinergic brain system, with abnormal endogenous OXT levels, might result in induction of stereotypical behaviour in WT mice; these await validation with additional experiments. Ultimately, these pave the way for evaluating an oxytocin administration timeline that can potentially benefit translational studies.

3.4.6. Chronic and acute intranasal OXT administration in the *Fmr1* KO mice has beneficial effects on comorbid symptoms of autism

ASD also display several comorbid symptoms including anxiety and cognitive impairment. The behavioural tests we utilised to evaluate anxiety in *Fmr1* KO mice challenged different aspects of anxiety. For timeline diagrams see Figures 3.5 and 3.6. Our results demonstrate that in the marble burying task, *Fmr1* KO mice buried more marbles than WT did, indicating increased anxiety-induced stereotypies (Albelda and Joel, 2012), and OXT treatment in the

mutated mice did not affect this anxiogenic phenotype (Figure 3.18A). OXT is well known for its anxiolytic properties (as reviewed by Neumann and Slattery (2016)), and also induces stereotypies (Insel *et al.*, 1999), thus we anticipated to observe decreased anxiety-induced stereotypies in the mutants following OXT administration. However, this was not the case suggesting a lack of OXT efficacy on the anxiety comorbidity symptoms of ASD in these mice. On the other hand, the novelty suppressed feeding test assesses conflict anxiety by challenging approach/avoidance behaviour (Aupperle and Paulus, 2010). The latency to feed in a novel environment was longer in the mutant mice representing increased anxiety (Figure 3.18B), which is in line with previous work utilising the same paradigm in a mouse model of ASD with CNVs (at human orthologue 15q11-13) (Tamada *et al.*, 2010). Also, reduced feeding latency was observed in the mutants following OXT administration demonstrated as no difference between the mutants and the WT, which suggests that this parameter is normalized and OXT was indeed anxiolytic in this paradigm. Overall, our results show discrepancy between the two tests, suggesting that chronic OXT treatment in the *Fmr1* KO model does affect comorbidity in symptoms associated with ASD in the context of conflict anxiety, but not in anxiety-induced stereotypies. Interestingly, OXT increased feeding latency in WT mice to levels similar to those of the *Fmr1* KO, indicating increase anxiety following chronic OXT administration in WT mice, which is in line with the anxiogenic-like phenotype in the literature (Peters *et al.*, 2014). These suggest that prolonged overstimulation of a 'healthy' oxytocinergic brain system has detrimental effects not only in the sociability domain but also in the anxiety domain in people with intact emotionality. Our data agree with most studies of the *Fmr1* KO mouse model reporting increased anxiety-like behaviour in the marble burying test (Spencer *et al.*, 2011; Westmark *et al.*, 2011; Veeraragavan *et al.*, 2012; Gholizadeh *et al.*, 2014; Sethna *et al.*, 2017) as well as in the novelty suppressed feeding paradigm.

The novel object recognition task assesses the ability to distinguish between familiar and novel objects indicated by more time spent exploring a novel object over a familiar object, which is a dentate gyrus-dependent task (Otto and Eichenbaum, 1992; Knight, 1996; Dolan and Fletcher, 1997; Lisman *et al.*, 1999; Hunsaker and Kesner, 2008). Previous reports have shown that memory assessed by novel object recognition task is impaired in *Fmr1* KO mice (Ventura *et al.*, 2004; Pacey *et al.*, 2011; Bhattacharya *et al.*, 2012; King and Jope, 2013), which is in contrast with our findings where we found no differences between genotypes when assessed with this protocol. Differences in the age of the animals tested, housing conditions and different experimental protocols used are likely to explain the disparate results. During the object phase (phase 3), in which the object used during habituation (phase 1) was replaced by a novel object, mice spent more time exploring the novel object than the familiar object (>60% sniffing time ratio) and this was irrespective of genotype and treatment, clearly indicating that both WT and mutants exhibit intact memory performance with normal levels of response to novelty (Figure 3.18C). During the place phase (phase 2), in which the object which had remained in the arena since habituation was displaced, WT OXT-treated mice displayed significantly more time sniffing the displaced object as compared to all groups, indicating improved memory for object location. Collectively, these suggest that memory remains intact in the *Fmr1* KO mice, however OXT-treatment in WT animals seem to improve memory for spatial location. This is in line with findings in human studies, supporting that OXT administration can improve memory in healthy controls (Ferrier *et al.*, 1980; Bruins *et al.*, 1992) but not in others, which show instead selective amnesic effects (Fehm-Wolfsdorf *et al.*, 1984; Geenen *et al.*, 1988; Heinrichs *et al.*, 2004).

3.4.7. Receptor binding by quantitative autoradiography in brains of *Fmr1* KO mice after chronic and acute intranasal OXT administration

3.4.7.1. Oxytocin receptor binding

To our knowledge, this is the first study investigating OXTR binding levels in the *Fmr1* KO autistic mouse model treated with OXT. Our data demonstrate that inactivation of the *Fmr1* gene modified brain region specific OXTR binding. Our findings report significant genotype effect in the olfactory and septal nuclei and the piriform (Pir) and a significant treatment effect found in the dorsal endopiriform nucleus (Den) and the hypothalamus (Hyp) (Figure 3.12). The up-regulation of OXTR binding in the *Fmr1* KO mice observed in the olfactory nuclei and the piriform cortex could be in response to the hypo-oxytocinergic state of the brain in these mutants (see results from chapter 2, section 2.4.2). Several studies have reported concomitant reduced levels of OXT in the hypothalamus, the main site of OXT synthesis in the brain with an increase in OXTR binding in several regions where OXT-producing neurons project (i.e. the olfactory nuclei, piriform cortex, septum, hypothalamus) (Gimpl and Fahrenholz, 2001; Zanos *et al.*, 2014), suggesting the existence of compensatory mechanisms to the reduced oxytocinergic tone. We showed that *Fmr1* KO mice have decreased OXT mRNA levels in the striatum (Figure 3.15) that could induce compensatory up-regulation of OXTR, which is in line with previous studies (Lee *et al.*, 2005; Zanos *et al.*, 2014). This might reflect mechanistically rapid turn-over of clathrin-mediated internalization of OXTR targeting the receptors to recycle back to the plasma membrane. OXTRs are located in many brain regions including hypothalamus, olfactory nuclei, amygdala and hippocampus (Gould and Zingg, 2003; Zanos *et al.*, 2014) in which they have been implicated in the regulation of social behaviours (Young *et al.*, 1997; Lim and Young, 2006). It has been previously shown that the oxytocinergic system is characterised by plasticity-induced neurochemical mechanisms, which facilitate neuroadaptations following a

range of stimuli (Lipschitz *et al.*, 2005; Goyon *et al.*, 2017). These might suggest that the mechanism underlining the regulation of OXTR in *Fmr1* KO mice is likely to be at the receptor protein level via increased recycling to the membrane and decrease degradation of receptor rather than, increased mRNA *Oxtr* levels. No change in *Oxtr* mRNA levels was observed in the *Fmr1* KO vs. WT.

With regards to chronic OXT treatment on OXTR binding in WT and *Fmr1* KO brains, chronic OXT treatment resulted in reduced OXTR in the Hyp and DEn regions irrespective of genotype, which is in line with previous studies (Huang *et al.*, 2014; Peters *et al.*, 2014), who also reported down-regulation of OXTR following 15 days ICV infusion of OXT (10 ng/h) or chronic intranasal OXT (0.15,0.3IU), respectively, suggesting potential compensatory neuroadaptation of the OXTR system after prolonged OXT administration. It is highly unlikely that the decrease of OXTR in response to OXT treatment explains the social deficits observed (Figure 3.11) in WT mice, as OXTR is down-regulated in both WT and mutants, but decreased social interaction following OXT treatment was only evident in WT mice.

3.4.7.2. Metabotropic glutamate receptor 5 binding

Additionally, we investigated mGluR5 binding levels in the brains of *Fmr1* KO and WT mice after chronic OXT or saline administration (Figure 3.13). mGluR5 antagonism is a promising treatment target for ASD symptoms and our data paradoxically demonstrate that mGluR5 binding levels were found reduced in selected brain regions of both WT and *Fmr1* KO animals following OXT-treatment in comparison with their respective saline-treated groups. Interestingly, in the *Fmr1* mutant mice, enhanced mGluR5 activity has been linked to symptoms of ASD (Nakamoto *et al.*, 2007), and systemic administration of the mGluR5 antagonist, MPEP, normalized seizures, pre-pulse inhibition, and dendritic spine densities (Yan *et al.*, 2005; Dolen *et al.*, 2007; de Vrij *et al.*, 2008).

Notably, our data reported significant treatment effect in the septal nuclei including the MS and VDB, and the striatum such as the NAc and the CPu. Interestingly, selective septal deletion of mGluR5 was reported to abolish sociability but did not affect preference for social novelty, while deletion of OXTR did not affect sociability in young adult mice but significantly impaired preference for social novelty (Mesic *et al.*, 2015). Interestingly, mGluR5 are abundant at septal nuclei showing strong Oxtr signals (Guzmán *et al.*, 2013), however, there is no significant colocalization of mGluR5 and Oxtr suggesting that different neuronal populations of the septum have distinct contributions in the regulation of sociability (Mesic *et al.*, 2015). It is unlikely that the decrease of mGluR5 in the striatum of OXT treated animals in our study may underline the observed alterations of social behaviours in WT and *Fmr1* KO mice following chronic OXT treatment as, while mGluR5 is down-regulated in both genotypes, chronic OXT treatment induces opposite behavioural effects in the two genotypes with respect to social interaction behaviour. Nonetheless, the OXT-induced decrease in mGluR5 appears to be more profound in the MS and VDB in WT mice than in *Fmr1* KO mice. It is impossible to conclude from this study alone whether this brain region specific OXT-induced down-regulation of mGluR5 in WT mice may play a role in the detrimental effect of chronic OXT treatment in WT mice, but given the role of both those regions in social behaviours and emotionality (Goldstein, 1974), this hypothesis is indeed intriguing. In the present study, no change in mGluR5 levels were observed in saline treated *Fmr1* KO vs. WT, suggesting that any changes in mGluR5 activity in *Fmr1* KO mice are not due to alteration in mGluR5 protein numbers.

3.4.7.3. N-methyl-D-aspartate receptor binding

NMDARs are also identified as potential targets for the treatment of ASD symptoms. Therefore, we also measured [³H] MK801 binding in the brains of *Fmr1* KO or WT mice treated either with saline or OXT to investigate NMDAR binding levels in the brains of these

mice (Figure 3.14). Overall, our data demonstrate that the density of NMDAR was less abundant in some of the brain regions of the *Fmr1* KO mice compared with their WT conspecifics, specifically in cortical brain regions including the M1, AuCx and ViCx. Interestingly, NMDAR disruption recapitulated clinical endophenotypes of ASD including N1 latency delay of auditory-evoked response (Gandal *et al.*, 2012), which is supportive of our data clearly showing decrease in NMDAR detected in the AuCx of the *Fmr1* KO mice. This finding appears to be supportive to the NMDAR hypofunction notion in the ASD symptomatology, which has been suggested by others (Gandal *et al.*, 2012). Hence this might suggest that decreased NMDAR levels observed in the *Fmr1* KO mice might contribute to the social deficits observed or more widely to the ASD-symptomatology in these mutants, as previously shown in the *Shank2* null autistic mouse model (Won *et al.*, 2012). Moreover, we found a significant treatment effect in the M1, the ventral hippocampus (vHip) and the VTA irrespective of genotype. Indeed, a decrease of NMDA receptors was observed following chronic OXT administration in these regions. Interestingly, it has been also reported that treatment with OXT after exposure to NMDA antagonist MK-801 was able to re-establish MK-801-induced social interaction deficits in zebrafish (Zimmermann *et al.*, 2016), which may suggest that OXT treatment can exhibit beneficial effects against social deficits in the *Fmr1* KO mice via NMDAR-mediated mechanisms. In addition, it has been shown that OXT can restore sociability in models of social deficit characterised by decreased NMDA levels as described from previous studies (Ferguson *et al.*, 2001; P. R. Lee *et al.*, 2005; Teng *et al.*, 2013). It is unlikely that the differential behavioural effects of chronic OXT administration in WT and *Fmr1* KO are driven by these alterations of NMDA receptors as down-regulation of NMDA receptors was observed in both genotypes, which does not tie with the differences in behavioural outcomes in the two genotypes.

3.4.8. Altered transcription of several candidate genes of autism in the *Fmr1* KO mice after chronic intranasal OXT administration

Multiple genes have been associated with the etiopathology of autism and we quantified transcription of a selection of these collected after RNA sequencing analysis (data not shown) in the *Fmr1* KO and WT control mice treated with OXT or saline. We examined two brain regions, the CPu and the CeA, which are implicated in motor/reward and emotional processes respectively (Figures 3.15 and 3.16). Transcriptional levels were modified for genes coding for the endogenous opioid peptides and their receptors, the neuropeptides, *Oxt* and *Avp* and their receptors, glutamatergic receptors, as well as several others including GPCRs (*Gpr68*), neurohormone (*Gh*), neurotrophic factor (*Bdnf*), plasticity protein (*Arc*) and 5-HT receptor (*Htr6*). For timeline diagram of different cohorts, see Figure 3.5 and 3.6.

3.4.8.1. Regulation of the endogenous opioids in the CPu and CeA

Pdyn and *Penk* transcript levels in the CPu were significantly down-regulated in the *Fmr1* KO Saline vs. the WT saline control group (Figure 3.15). Results from *Penk* expression pattern are in line with data obtained from a different cohort of WT and *Fmr1* KO mice, whereas *Pdyn* transcription data are in contrast (see chapter 2, Figure 2.5). In the latter cohort, unlike the former (i.e. cohort used in this chapter), the animals were not handled and did not undergo any behavioural testing or treatment, thus, gene expression was tested at basal level with no prior 45-minute exposure to social interaction. This might suggest that *Pdyn* may be influenced by these environmental parameters. Indeed, it has been shown that *Pdyn* immunoreactivity is increased upon various stressors including ethanol intake and forced-swim test (McLaughlin *et al.*, 2003; Chung *et al.*, 2014; Anderson *et al.*, 2016). CNV genetic polymorphism detected in the *Pdyn* gene, characterised by segmental duplication, has been associated with ASD and its clinical features are suggestive of a chromosomal syndrome (Kaminsky *et al.*, 2011). Therefore, the detected down-regulation of *Pdyn*

transcripts in the CPu region in the *Fmr1* KO mice might be linked to ASD pathology. Interestingly, the endogenous κ opioid peptides derived from *Pdyn* encode for the dysphoric, anxiogenic, and cognitive disrupting responses to behavioural stress exposure (Bruchas *et al.*, 2010; Carroll and Carlezon, 2013). Whether this might be linked to the anxiogenic-like phenotype that is observed in the *Fmr1* KO mice (Figure 3.18) requires further investigation.

Interestingly, a genetic polymorphism of gain in function on the *PENK* gene in human was found to be protective over social deficits (Troisi *et al.*, 2011). In line with this, our data demonstrate that transcripts of the *Penk* gene are found significantly down-regulated in the CPu, a key region for behaviours relevant to autism (Langen *et al.*, 2009) including sensorimotor gating and social interaction sensitivity (Wyers *et al.*, 1968; Peeke and Herz, 1971; Swerdlow *et al.*, 2001). Whether we can speculate that down-regulation of *Penk* transcripts in the *Fmr1* KO mice is linked to the observed social interaction deficits (Figures 3.11A and 3.17A) requires further investigation. Prior investigation at a protein level is essential, as excess synthesis of proteins has been detected in the FXS model (Gross and Bassell, 2012). Notably, *Penk* transcripts are found significantly up-regulated in the *Fmr1* KO OXT-treated vs. *Fmr1* KO saline-treated mice, which suggests that OXT treatment may attempt to rescue the down-regulated expression of the *Penk* gene in these mutants. Whether this may underline the beneficial effects of OXT in reversing the social deficits in ASD warrants further investigation but the evidence pointing towards effect of *Penk* on social deficit (Troisi *et al.*, 2011) is particularly intriguing.

The amygdala has been long involved with socio-emotional disturbances in ASD (Zalla and Sperduti, 2013), therefore, CeA amygdaloid nuclei is one of our targeted regions for investigation. In the CeA, *Penk* mRNA expression was overall up-regulated following OXT administration in the WT mice (WT OXT vs. WT saline) and the mutants (*Fmr1* KO OXT vs. WT saline) (Figure 3.16). Markedly, this suggests that OXT acts as a positive regulator of *Penk* in WT and *Fmr1* KO mice in the CeA region. These are in line with evidence

supporting the opioid excess theory in autism, as discussed in chapter 2. Whether these changes in *Penk* mRNA regulation by OXT in the two genotypes are driving the differential effects of chronic OXT administration on behaviour cannot be determined by this study alone, but warrants further investigation.

Despite alterations observed in the endogenous peptide mRNA levels, no OXT induced or genotype changes were observed in *Oprm1* mRNA levels. This seemingly suggests a lack of interaction between OXT and MOR at least at mRNA level. Nonetheless, there is emerging evidence to suggest the presence of an interaction between MOR and OXTR. For instance, a profound down-regulation of *Oxt* mRNA and up-regulation of OXTR binding was observed in *Oprm1*^{-/-} KO mice (Becker *et al.*, 2014; Georgiou *et al.*, 2015) suggesting that the presence of such an interaction may be at receptor protein level rather than mRNA.

3.4.8.2. Regulation of the oxytocin and vasopressin in the CPu and CeA

As discussed previously in Chapter 2, the neuropeptides OXT and AVP have been involved in the aetiology of autism. Our data demonstrated that there were interesting links between the expression levels of the neuropeptides along with their receptors, and OXT treatment in the CPu. OXT treatment seems to have either a positive or negative correlation with the expression of certain genes, as it rescues or negatively regulates the expression of transcripts in both brain regions studied.

As illustrated in Figure 3.15, in the CPu *Oxt* mRNA expression was globally down-regulated with marked 4.5-fold significant decrease observed in the *Fmr1* KO animals, strongly suggesting dysregulation of the endogenous oxytocinergic system. Considering the evidence in *Oxt* KO mice pointing towards a socially impaired phenotype (Winslow and Insel, 2002), we suggest that this OXT deficit might explain the social behaviour deficits observed in these mutants (Figure 3.11 and 3.17). Intriguingly, chronic OXT administration had no effect on *Oxt* mRNA levels in WT mice or *Fmr1* KO mice. In conclusion, the results from this study

suggest that the endogenous oxytocinergic system is impaired in the *Fmr1* KO mice and restoration of this system through chronic intranasal administration of OXT benefits sociability behaviour, which is impaired in these mutants.

In addition, the mRNA expression levels of *Avpr1a* and the *Avpr1b* in the CPu (Figure 3.15) region, show marked down-regulation in the *Fmr1* KO saline-treated mice, while up-regulation of *Avpr1b* is observed in the mutants treated with OXT, suggesting that prolonged OXT administration rescues the expression of these transcripts. This might further suggest that the beneficial effects of exogenous OXT in reversing the social deficits in ASD could be induced also by physiological compensatory mechanisms acting via the vasopressinergic system.

Interestingly, while in the CPu there are no genotype or OXT treatment induced effects with respect to *Oxtr* mRNA levels, in the CeA, a brain region characterised as the integrative hub of anxiety (Gilpin *et al.*, 2015), OXT treatment seems to suppress the *Oxt* and *Oxtr* transcription levels in the mutant mice but not WT. The suppressing of *Oxtr* levels is unlikely to have any behavioural significance as it does not translate to changes in OXTR binding. Nonetheless, whether the genotype differences in OXT treatment-induced *Oxt* mRNA regulation has any behavioural significance remains to be investigated.

Lower levels of *Avpr1a* mRNA were observed in *Fmr1* KO mice vs. WT in the CPu, suggesting a dysregulation of the AVP system in the *Fmr1* KO model of ASD. In line with this observation, autistic children with the lowest AVP levels in the blood displayed severe social impairments, demonstrating a positive correlation between blood AVP concentrations and social behaviour (Carson *et al.*, 2015). Interestingly, chronic OXT administration significantly increases transcription levels of *Avpr1a* and *Avpr1b* in the *Fmr1* KO but not in WT mice, in the CPu and decreases *Avp* mRNA expression levels in the CeA (Figure 3.16). Whether this up-regulation of these components of the vasopressinergic system in the *Fmr1* KO mice following chronic OXT administration underpins, at least partially the prosocial effect of OXT in these mice requires further investigation. Interestingly chronic OXT administration has

been shown to increase Avpr1a binding in the LS of mice (Huang *et al.* 2014), which might reinforces our suggestion, given the role of this region in social behaviour.

3.4.8.3. Regulation of glutamatergic system and other genes in the CPu and CeA

As discussed in Chapter 2, a dysfunctional glutamatergic system has been reported in ASD with coexistence of both hypo- and hyper-function in different forms of ASD and mGluRs antagonists are considered as potential therapeutic targets (Carlson, 2012; Chen *et al.*, 2014). Our data demonstrated a global decrease of expression of *Grm4* and *Gmr8* transcripts in the *Fmr1* KO mice in both the CPu (Figure 3.15) and CeA (Figure 3.16) brain regions, which fits with our data showing decreased NMDAR binding in several regions in the *Fmr1* KO mice (Figure 3.14). These data from transcriptional analysis are in support of the hypo-glutamatergic theory in autism, however, investigation at protein level and functional assays will be needed for validation. Our data also revealed that in the CPu, OXT treatment appears to rescue the expression of *Grm4* and *Gmr8* transcripts, while in the CeA OXT increased transcription levels of *Gmr8* in WT and had no effect on *Grm4* transcripts. Interestingly, a partial duplication of *GRM8* has been identified in an individual with autism (Serajee *et al.*, 2003) suggesting that increased *Grm8* expression may be associated with ASD symptoms. Whether such a mechanism is involved in the detrimental effect of chronic OXT administration in WT mice remains to be investigated.

Additionally, we looked into transcription of genes of interest (Table 3.3 and 3.4) that were found differentially regulated in the CPu and CeA brain regions. However, we will only highlight the transcriptional changes on a few of these genes including the *Bdnf* and *Gh* genes. Our data revealed association between differentially regulated *Bdnf* transcripts in the *Fmr1* KO mice and OXT treatment. In particular, *Bdnf* mRNA levels were found to be dramatically down-regulated in the mutants in the CPu region, while OXT treatment does not

rescue its expression (Table 3.3). Several studies have reported dysregulation of Bdnf levels in autism (as reviewed by Ricci *et al.* (2013)). In agreement with our findings, lower serum BDNF levels were associated with impairment in children with autism (Kasarpalkar *et al.*, 2014). In addition, our data reveal reduced transcription of *Gh* (~ 3.7 fold) in the *Fmr1* KO mice in the CPu, which is rescued following OXT treatment (Table 3.3). The growth hormone (*Gh*), has been also implicated in the neurobiology of autism, and it is shown that elevated levels of growth-related hormone in blood have been detected in children with ASD (Mills *et al.*, 2007). Although the findings are in the opposite direction with the aforementioned study, nonetheless, it is supported that there is a link between dysregulation of growth hormone in individuals with autism and the regulatory effect of OXT in restoring the expression of that gene.

3.5. Concluding remarks

In conclusion, we demonstrated that intranasal OXT treatment reverses core symptoms of ASD in the *Fmr1* KO mouse and these beneficial effects persist over time. In particular, our data shows complete restoration of social interactions following acute, sub-chronic and chronic OXT treatment in these mutants, which is characterised by OXT, opioid peptide and mGluR mRNA and glutamate receptor density deficits in the CPu. We also show that chronic OXT restores some of those deficits in *Fmr1* mice (e.g. *Avp1a*, *penk*, *Grm* deficits). On second note, OXT reversed the anxiogenic-like phenotype of *Fmr1* KO mice in a paradigm assessing conflict anxiety, highlighting the therapeutic potential of OXT for treatment of comorbid anxiety associated with ASD. Interestingly, in WT mice sub-chronic and chronic, but not acute, OXT administration induces a social deficit phenotype accompanied by increased motor stereotypies, and anxiety reminiscent of ASD, suggesting a detrimental effect of chronic OXT administration in healthy subjects with intact oxytocinergic and other CNS systems. This is accompanied by a profound OXT-induced dysregulation in several

gene transcripts including *Grm8*, *Penk*. Overall, this study provides important information on the impact and safety of acute and chronic OXT treatment in ASD and healthy subjects, and points towards possible underlining mechanisms.

4. Chapter 4: Effect of social defeat stress on nAChRs and D₂ receptors: An autoradiography study

4.1. Introduction

4.1.1. Depression

One of the psychiatric conditions characterised by social deficits is depression, a serious mood disorder. Depression is a multifactorial disorder caused by a combination of genetics and environment, including psychosocial factors. Psychosocial factors include prolonged exposure to negative life events such as societal negative impact resulting from unemployment, poverty (Weich and Lewis, 1998) as well as adverse familial events such as poor parental care or loss resulting in low self-esteem and emotional instability (Avagianou and Zafiropoulou, 2008; Parsons *et al.*, 2010). For detailed information on the aetiology of depression, see section 1.3.2. One of the major causes of depression is chronic exposure to social stress, and notably bullying, which is a classical form of it, as characterised by Gullander and colleagues (2014).

Depression is characterised by several neuroendocrine, neurotransmitter and neuroanatomical abnormalities, some of which are discussed in the section 1.3.3. Although a great deal of progress has been done in the understanding of the neurobiology of depression, there are still gaps in our knowledge. Current pharmacotherapy is often problematic, and some patients are resistant to it. As a result, better treatment is needed, especially with regards to depression caused by chronic exposure to social defeat (SD) type of stress. To address this need, better understanding of the neurobiology underlining depression, and specifically SD, is needed in order to identify novel targets that may aid in successful treatment of the disorder.

Receptor systems such as nicotinic Acetylcholine receptors (nAChRs) are involved in a variety of neurobiological mechanisms implicated in the pathophysiology of depression. They modulate DA function, influence HPA axis activity and they play a role in cholinergic

neurotransmission (reviewed in (Philip *et al.*, 2010)), which are all mechanisms underlining depression. Nonetheless, little is known about the mechanisms that underline neuroadaptations in this system and contribute to the development of the disease state. In an attempt to refine our understanding of the nAChR system in SD related to depression, this chapter will focus on further investigating the involvement of nAChRs in the development of social stress-induced depression.

4.1.2. Nicotinic receptors: pharmacology, localisation and function

Acetylcholine receptors (AChRs) are ligand-activated neurotransmitter receptors activated by the endogenous neurotransmitter acetylcholine (ACh). There are two types of AChRs: the metabotropic muscarinic receptors (mAChRs), which are membrane GPCRs, and the ionotropic nicotinic receptors, which will gate the channel to favour ion influx that further induces a cellular response upon stimulation by ACh. mAChRs have a slow activation and the subtypes present (M1-M5) facilitate activation of second messengers of PLC, IP3, cAMP and free calcium, which further alter cellular homeostasis. On the other hand, nAChRs, which is our focus in this chapter, are ion channel proteins that are activated by nicotine and open to allow diffusion of cations (Albuquerque *et al.*, 1995; Gotti and Clementi, 2004; Eglén, 2005; Dani and Bertrand, 2007).

Two major findings contributed to the discovery of nAChRs; primarily, the electric organ of the *Torpedo* fish, which is rich in muscle expressing nAChRs and secondly the discovery of the snake toxin α -Bungarotoxin, which binds to muscle-type nAChRs. In particular, the starting point for the nAChR purification was upon discovery of the electric organ of the *Torpedo* fish, as this organ comprises approximately 40% of the protein and it produces an electric pulse to capture preys (Sobel *et al.*, 1979; Kistler and Stroud, 1981). Later, α -bungarotoxin extracted from venom of krait snake, *Bungarus multicinctus*, was shown to bind with high affinity to muscle type nAChRs to inhibit their function and promote

neuromuscular paralysis (Albuquerque *et al.*, 1974; Fertuck and Salpeter, 1974; Barnard *et al.*, 1977; Lee, 1979).

There are subdivisions of nAChRs that include muscle or neuronal subtypes depending on their expression site. Our interest in this project focuses on the neuronal nAChRs, which are widely distributed in the brain. Neuronal nAChRs are organised in pentamers, which is a structure consisting of five protein subunits joined around a central ion channel. In mammals, they are assembled either as homo- or hetero-pentamers that are formed from a pool of nine α -like subunits ($\alpha 2 - \alpha 10$) and three β -subunits ($\beta 2 - \beta 4$). Their differential subtypes exhibit distinct pharmacological and physiological profiles, and anatomical distribution in the brain (Lloyd and Williams, 2000; Albuquerque *et al.*, 2009). The different subunits of the nAChRs determine their specialized properties and functions. The mammalian high affinity nicotine binding receptor consists of at least $\alpha 4$ and $\beta 2$ subunits (Flores *et al.*, 1996; McCallum *et al.*, 2006).

Several toxins target the nAChR system as it plays fundamental role in regulating important life functions such as muscle contraction, autonomic nervous system function and function of defensive measure against predation (Daly, 2005). Nicotine, the active ingredient in tobacco, is the most well-known nAChR targeting agent produced by a family of plants (*Solanaceae*) and primarily used as a potent natural pesticide acting against predation (Eastham *et al.*, 1998). Other toxins that target nAChRs exhibit selectivity for receptor subtype and are produced by plants, fungi, bacteria and animals (Albuquerque *et al.*, 2009). A selective nAChR antagonist that binds with high affinity to the $\alpha 7$ subunit is α -bungarotoxin; the $\alpha 7$ receptor subtype has distinguished function from other neuronal nAChRs (Rangwala *et al.*, 1997; Drisdell and Green, 2000). Another nicotinic agonist producing toxic effects is epibatidine, an alkaloid extracted from the skin of the Ecuadorian tree, *Epipeditobates tricolor* (Dukat and Glennon, 2003; Yogeeswari *et al.*, 2006). Epibatidine binds with high affinity to the $\alpha 4\beta 2^*$ nAChR as well as to other nAChRs targets with various affinities. According to

Lukas *et al.* (1999), an asterisk following the different subtypes is employed to denote that one or more additional subunits might also be present. Several products of epibatidine are valuable pharmaceutical agents for treating numerous diseases such as Alzheimer's disease (Dwoskin and Crooks, 2001; Xiao *et al.*, 2004; Albuquerque *et al.*, 2009).

There are several types of nAChRs in mammals with various subunit compositions according to their location in the body, of which nine α -subunits and three β -subunits are expressed in the brain. The different nAChR subunit combinations have distinct localisation, pharmacological and kinetic properties (Gotti *et al.*, 2006a; Grady *et al.*, 2009). The various subtypes of nAChRs are broadly distributed in the brain. α_7 and $\alpha_4\beta_2$ nAChRs assemble into homo- and hetero-pentamers, respectively, and they are distributed throughout the brain and the spinal cord. In the brain they are found in several brain regions, as illustrated in figure 4.1, including the olfactory bulb, cortex, hippocampus, medial habenula (MHb), hypothalamus, interpeduncular nucleus (lpu), substantia nigra, ventral tegmental area, amygdala and the cerebellum. The $\alpha_4\beta_2$ nAChRs are also specifically found in the raphe nuclei, thalamus and the striatum brain regions, while the α_7 are also located in the pineal gland (Jensen *et al.*, 2005; Taly *et al.*, 2009).

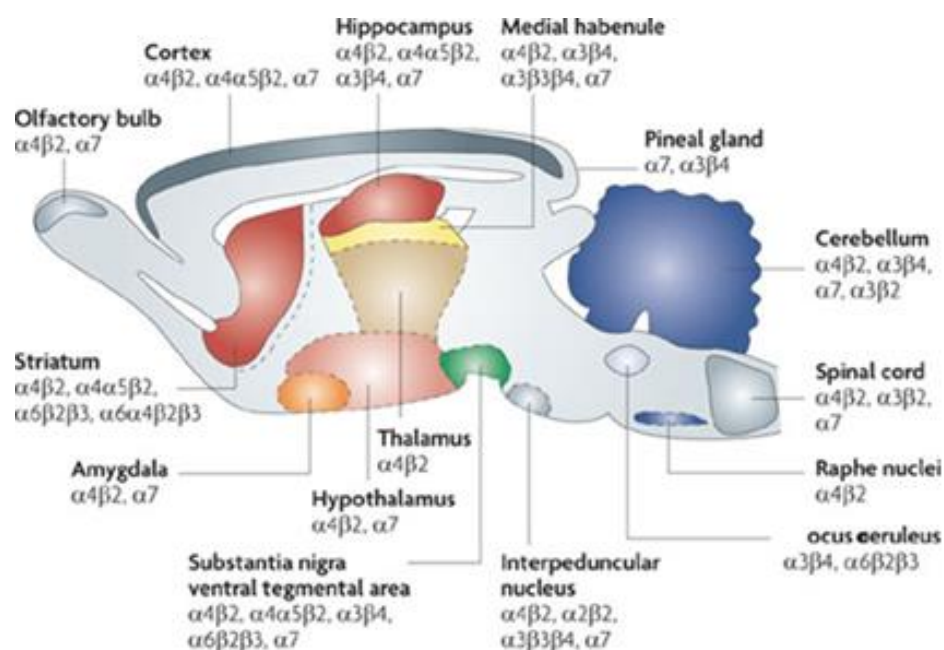


Figure 4.1: Representation of nAChRs subtypes distribution in the brain.

Representation of nAChRs subtypes distribution throughout the brain as described in detail in text. Adapted from Taly *et al.* (2009).

Nicotinic receptors located in the brain are expressed and function both pre-, post- and extra-synaptically (Jones and Yakel, 1997; Wonnacott, 1997; Jones *et al.*, 1999; Gotti *et al.*, 2006b; Colombo *et al.*, 2013). The nAChRs are found in neuronal cell bodies, dendrites and axon terminals and thus modulate the release of other neurotransmitters, hence strongly influencing the surrounding neuronal circuitry (Toth, 1996). In particular, nicotine binds pre-synaptically to nAChRs to facilitate the release of neurotransmitters such as ACh, DA, NA, GABA, 5-HT and glutamate, which then mediate several behavioural responses including reward, dependence, cognition, anxiety, memory (Picciotto *et al.*, 2000). The primary nAChRs in the CNS are $\alpha 7$ and $\beta 2^*$ ($\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$, $\alpha 4\alpha 6\beta 2^*$) with $\alpha 3$ also present but less prevalent than the $\alpha 7$ or $\alpha 4^*$ nAChRs (Jensen *et al.*, 2005; Tapia *et al.*, 2006; Brown *et al.*, 2007; Albuquerque *et al.*, 2009; Kuryatov and Lindstrom, 2011). Preclinical studies have shown that $\alpha 7^*$ nAChRs are implicated in important cognitive functions including learning, memory and attention. Notably, it has been shown that absence of $\alpha 7$ nAChR in the

hippocampus results in significant learning and memory, and attention impairments, as demonstrated in pharmacological inhibition, as well as, genetic studies with knock-out animals (Levin *et al.*, 2002, 2009; Fernandes *et al.*, 2006; Nott and Levin, 2006; Young *et al.*, 2007). These receptors are also implicated in a variety of diseases of cognitive impairment including Alzheimer's disease, ADHD and schizophrenia (Levin, 2012). The $\alpha_4 \beta_2$ nAChRs have been linked to behaviours associated with drug abuse and they are also involved in pain modulation (Changeux *et al.*, 1999; Watkins *et al.*, 1999; Damaj, 2007). For an extensive review on nAChR and their use as therapeutic targets, see review by Dineley *et al.* (2015).

4.1.3. Nicotinic receptors in depression

Nicotine and smoking have been reported to regulate mood via activation of nAChRs in both humans and animals. Various studies support that nicotine-contained in tobacco modulates neuronal systems that further regulate mood (as reviewed in Picciotto *et al.* (2015)). Interestingly, incidence rate of smoking is 50% higher in depressed individuals than the general population (Glassman *et al.*, 1990; Diwan *et al.*, 1998). The relationship between smoking and depression is bidirectional. Depression is thought to be a risk factor for maintaining smoking behaviour and depressed people find it much harder to stop smoking with a higher relapse rate (Glassman *et al.*, 1990; Breslau *et al.*, 1991; Killen *et al.*, 2003). New evidence is emerging suggesting that exposure to nicotine through tobacco smoking is a risk factor for depression.

Nicotine binds with high affinity to nAChRs, activates intracellular responses and long-lasting desensitization occurs upon prolonged exposure (Buccafusco *et al.*, 2009). This further exerts its reinforcing properties in the CNS and autonomic ganglia (Picciotto, 1998). It has been observed that nicotine's patches have beneficial effects for depressed patients; it improves mood in the short-term and increases REM sleep in non-smokers (Salín-Pascual

et al., 1995), while smoking cessation exacerbates symptoms of depression (Glassman *et al.*, 1990). Notably, chronic nicotine administration elicits antidepressant-like effects in rats, assessed by learned helplessness (Semba *et al.*, 1998) and forced swim test (Djurić *et al.*, 1999; Tizabi *et al.*, 1999). Chronic nicotine administration (delivered through nicotine patches) desensitizes rather than activates nAChRs (Reitstetter *et al.*, 1999) as a functional antagonism (Quick and Lester, 2002) suggesting that blockade of nAChRs rather than activation of these receptors may underline this antidepressant effect in mice.

Janowsky and colleagues (1972) have reported over three decades ago that increased levels of ACh might lead to depression. Early observations also supported this hypothesis by showing that organophosphate poisoning in humans, which causes excess ACh levels throughout the brain and body by inhibiting acetylcholinesterase activity, result in depression-like symptoms. Additionally, higher rates of depression seem to appear in orchardists that work with these substances (Gershon and Shaw, 1961). Later on, it was reported that administration of physostigmine, a reversible acetylcholinesterase inhibitor, in human subjects affected with an underlying disorder, increased symptoms of depression and reduced mania (Janowsky *et al.*, 1974). Also, when the same substance was infused in humans not diagnosed with a neuropsychiatric disorder, symptoms of depression were reported (Risch *et al.*, 1981). These provide relatively strong evidence, which support the hypothesis that depression is associated with cholinergic overdrive, while nicotine administration decreases depressive symptoms (Salín-Pascual *et al.*, 1995). The notion that blockade of nAChRs might have antidepressant effects is further supported by studies demonstrating that administration of a non-selective nAChRs antagonist, mecamylamine, displayed decreased symptoms of depression in patients suffering from Tourette's syndrome (Salín-Pascual *et al.* 2000; Mihailescu and Drucker-Colín, 2000) and displayed antidepressant-like properties in naïve mice (Caldarone *et al.*, 2004; Rabenstein *et al.* 2006). Animals studies have revealed that deletion or blockade of specific nAChRs have

antidepressant properties. β_2^* KO mice are characterised by antidepressant-like phenotypes (Caldarone *et al.*, 2004). Amitriptyline, a classical antidepressant had no effect on these animals strongly reinforcing the notion that β_2^* nAChRs are involved in the function of a classical antidepressant. Cytisine, a partial agonist with high affinity for $\alpha_4\beta_2^*$ nAChRs, has been shown to have antidepressant-like properties, via a mechanism involving blockade of $\alpha_4\beta_2^*$ nAChRs and subsequent reduction of neuronal activity in the basolateral amygdaloid nuclei (BLA) (Mineur *et al.* 2007). There is evidence suggesting a key involvement of nAChRs system in the mechanism of action of certain antidepressants. Indeed, it has been shown that clomipramine, a tricyclic antidepressant inhibits the activation of $\alpha_4\beta_2$ nAChRs, which are commonly expressed in the CNS (López-Valdés *et al.* 2002), further supporting the antidepressant effect of nAChR antagonism. Additionally, bupropion, a classic antidepressant and smoking cessation aid, as well as phencyclidine, inhibit nAChRs *in vitro* and *in vivo* in a non-competitive pattern (Fryer and Lukas, 1999; Alkondon and Albuquerque, 2005). Additional studies on rat hippocampal slices have reported that antidepressants which work via monoamine reuptake blockade are able to inhibit nAChRs in the CNS (Hennings *et al.*, 1999). Fluoxetine, a selective 5-HT reuptake inhibitor commonly used to treat depressive-like symptoms exhibits nAChRs antagonistic properties in the CNS at low concentrations (Hennings *et al.* 1997) further supporting nAChRs antagonism as one of the mechanism underlining commonly used antidepressants effect.

Altogether these data reinforce the notion that blockade of nAChRs might have an antidepressant property in their own right, but may also be contributing to the antidepressant effects of various medications, thus implicating nAChRs in the pathophysiology of depression. Nonetheless, the neurobiology underlining this involvement remains unclear. In this chapter, we will focus on understanding the impact of chronic defeat stress, which is characterised by social aversion and anhedonia-induced by repeated exposure to negative social cue on key CNS systems. As discussed in section 1.3.4, chronic SD can lead to

behavioural abnormalities induced by stress and it is believed to capture features of depressive-like state.

4.1.4. 'Social defeat' as a model for depression

The SD paradigm uses social conflict between members of the same species to produce emotional and psychological stress, which is illustrated by submissive, supine posture, frequent vocalisation of distress and freezing behaviour (Blanchard and Blanchard, 1977). The defeat experience is a physical but also a psychogenic stressor, as it involves both physical exposure (i.e. attacks) but also time that the resident is in visual contact with the intruder without allowing physical harm, as described in methods section 4.3.1.2. Such repeated exposure to SD without prior habituation, generates persistent emotional stress in order to examine social subordination (Tidey and Miczek, 1997), providing ethological validity to the mouse model. Preclinical studies in rats demonstrated that repeated exposure to SD induces long-term changes in behaviour and neurochemistry. In particular, it induced significantly decreased locomotion and exploratory activity (Meerlo *et al.*, 1996; Koolhaas *et al.*, 1997; Tidey and Miczek, 1997), increased anxiety and submissive behaviour (Ruis *et al.*, 1999; Crawford *et al.*, 2013), and reduced sexual behaviour and aggression (Meerlo *et al.*, 1996). Furthermore, there is substantial evidence to suggest that VTA dopaminergic system plays a key role in the behavioural manifestation of SD-related depression (Barik *et al.*, 2013).

In humans, only a subset of depressive-like symptoms can be objectively studied in rodents including social avoidance, anhedonia, locomotor and metabolic changes. It has been demonstrated that 4-7 days of repeated exposure to SD induces decreased mobility in the social avoidance and the forced swim test (Berton *et al.*, 1998; Hollis *et al.*, 2010), between 5-10 consecutive days of defeat generate physiological impairments in cardiac and autonomic circadian rhythms, thermoregulation and immune function (Tornatzky and Miczek,

1993; Stefanski, 2000; Sgoifo *et al.*, 2002; Hayashida *et al.*, 2010) and after 5 weeks of SD anhedonia-like behaviours are induced, which is a core symptom of depression in humans (Rygula *et al.*, 2005). According to studies in rodents, exposure to chronic, repeated physical stresses induced depressive-like behaviour (Hollis *et al.*, 2013) and provided evidence for dysregulation of HPA axis (Hill *et al.*, 2012), which were reversible after chronic treatment with traditional antidepressant (Willner, 2005). While these data strengthen the relationship between stress and depression, such stressors are often criticized as artificial and that they are not representative of true nature of social stress exposure in humans (Kessler, 1997; Björkqvist, 2001; Almeida *et al.*, 2002). SD stimulates increased dopaminergic activity in the VTA (Cao *et al.*, 2010), which drives social avoidance and, might further promote alertness in threatening circumstances, as in the case of SD. Interestingly, pharmacological or optogenetic inhibition of dopaminergic activity in the VTA has beneficial effects, as it reverses SD-induced aversion and anhedonia (Barik *et al.*, 2013; Chaudhury *et al.*, 2013). These suggest that the increase in dopaminergic activity is essential for the following behavioural manifestation of stress outcomes (Barik *et al.*, 2013; Chaudhury *et al.*, 2013; Friedman *et al.*, 2014; Marinelli and McCutcheon, 2014).

With respect to the nAChRs system, evidence from pharmacological and molecular genetic studies have shown an inverse relationship between acetylcholinesterase activity and depression. Decreased hippocampal acetylcholinesterase activity, increased anxiety and depression-like behaviours, and decreased resilience to repeated stress in a SD paradigm (Mineur *et al.*, 2013) were reported, suggesting an association between increased acetylcholinesterase activity and depression in a model of chronic SD. Taken together, it is suggested that abnormalities in the cholinergic system may be critical for the aetiology of depression. However, the precise mechanisms underlying the role of nAChRs in depression are not well-understood.

Interestingly, recent evidence in mice suggested that stress and nicotine induce long-lasting cellular adaptations within the VTA dopaminergic system that is regulated by marked remodelling of nicotinic systems, which further cause increased activity of the VTA dopaminergic neurons, and social avoidance behaviour (Morel *et al.*, 2017). Indeed, Morel *et al.* 2017 demonstrated that blockade of β_2^* or α_7^* nAChRs prevented, the development and expression of social-stress-induced neuroadaptations, respectively in a model of chronic social defeat stress (CSS) in mice. Together, these data point towards the hypothesis that chronic SD may lead to profound neuroadaptations of the nAChR in the dopaminergic system. Therefore, we attempted to map alterations that might occur in the density of the α_4 β_2^* and α_7^* nAChRs and D_2 receptors in brains from naive mice that have undergone chronic SD.

4.2. Hypothesis and aims

4.2.1. Hypothesis

Emerging evidence suggest that nicotine is associated with depressive-like behaviour and vice versa, potentially by modulating dopaminergic and nicotinic receptor systems. Hence, we hypothesize that chronic SD exposure will affect nAChRs and D₂R binding in the brains of naïve mice.

4.2.2. Aims

The present study aims are to determine the effects of the exposure to chronic SD on nAChRs and D₂R binding in mice which may at least partially explain the neurobiological mechanisms that underline depressive-like behaviour.

4.3. Materials and Methods

4.3.1. Methods

4.3.1.1. Animals

Adult male mice (C57BL/6J, 2 - 4 months old) were group-housed under standard animal housing conditions, at 22 °C, between 55% to 65% humidity, with a 12:12 hour light/dark cycle (7 am/7 pm) and had *ad libitum* access to water and food. Animals care and the experimental protocols used were approved by the French Ministère de l'Agriculture et de la Forêt (87-848) and the European Directive 86/609/CEE of 11/24/1986 for care of laboratory animals. Mice were separated in 2 groups; group 1 underwent SD stress protocol whilst group 2 did not. Both groups were evaluated for social interaction with the use of social interaction tests during the morning hours (09.00-12.00h) as described in section 4.3.1.2. Mice were culled by cervical dislocation before rapid removal of the brains followed by freezing in isopentane (-20°C) for 30 sec and stored at -80°C. These procedures were performed at the lab of Dr. Barik, following shipping of the brains at the University of Surrey where I processed them for autoradiographic receptor binding experiments.

4.3.1.2. Social defeat stress paradigm

The chronic SD stress paradigm was performed according to established protocols (Barik *et al.*, 2013; Morel *et al.*, 2017). C57BL/6J mice (n=10) were subjected to 10 consecutive days of SD. Each defeat consisted of 5 min of physical interactions between the resident mouse, CD1 (6 month of age), and the intruder, experimental mouse. Following this, the intruder remained for 24h in the resident's home cage that was partitioned in half by perforated transparent polycarbonate, which allowed visual, auditory, and olfactory communication whilst preventing tactile contact; see diagram below (Figure 4.2). Each experimental mouse

faced a novel CD1 each day. The social defeat test was carried out daily through the 10th day of the experimental protocol. Undefeated mice were handled and rotated daily, and housed 2 per cage with the polycarbonate partition separating the cage in two halves. This group of mice were not exposed to an intruder. Animals were daily inspected for health conditions, and they were immediately removed from the experiment if severe wounds were detected.

Social interaction was performed in SD and non-SD mice, 24 h after the last defeat (day 11) in a low luminosity environment (20 lux). Undefeated and defeated mice were introduced for 150s in an open-field (40cmx40cmx25cm) containing an empty perforated polycarbonate box ('no target' condition). Immediately after this, mice were rapidly removed and an unfamiliar CD1 mouse was placed in the box ('target' condition) and mice were re-exposed to the open-field for another 150 s. The time spent in the interaction zone surrounding the polycarbonate box was recorded and scored by an experimenter blinded to the treatments (see Figure 4.3), and it was used as an index of social interaction. Social interaction in mice subjected to chronic SD stress were compared with undefeated mice (NoSD).

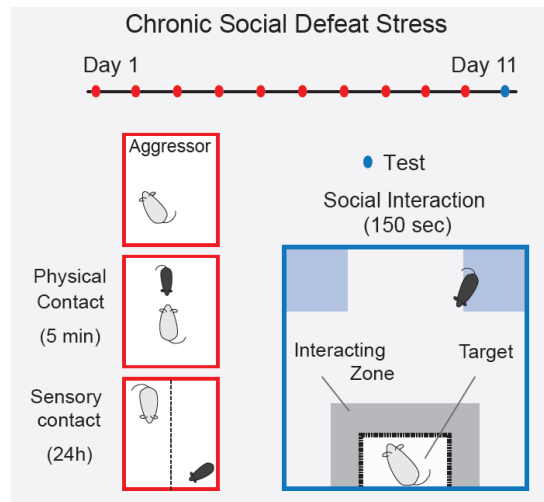


Figure 4.2: Schematic of the chronic social defeat paradigm.

Mice ($n=10$) were subjected to 10 consecutive days of SD. Each defeat lasted for 5 min, in which the experimental mouse (the intruder) was free to have physical interactions with the CD1 resident mouse. Next, the experimental mouse remained for 24h in the resident's home cage. The cage was partitioned in half by a perforated transparent polycarbonate, so mice could maintain visual, auditory, and olfactory communication but not physical contact. For the social interaction assessment on day 11, undefeated (NoSD) and defeated (SD) mice were introduced for 150s in an open-field containing an empty perforated polycarbonate box ('no target' condition). Immediately after this, mice were rapidly removed and an unfamiliar CD1 mouse was placed in the box ('target' condition). Mice were re-exposed to the open-field for another 150s. Experiments were performed by Dr. Barik and colleagues (Laboratory of Excellence (Labex) Biological Psychiatry Laboratory, Paris, France).

4.3.1.3. Preparation of mice brain sections

Twenty μm coronal sections were cut at 300 μm intervals from rostral to caudal levels at -21°C temperature, following thaw-mounting onto gelatin-coated ice-cold microscope slides and were processed for autoradiography. Adjacent sections ($n=10$) were cut for determination of total and NSB binding. Sections were stored at -20°C prior to radioligand binding.

4.3.1.4. Quantitative autoradiographic receptor binding

Quantitative autoradiography is a useful tool for mapping possible receptors that might be involved on the neurochemistry of chronic SD mice and might underline partially the

neurobiology of depressive-like behaviour. Quantitative autoradiography was performed on sections of mice exposed to CSS and mice not exposed to CSS, for $\alpha_4 \beta_2^*$, α_7^* nACh receptors and D₂Rs with cytosine-sensitive [¹²⁵I] epibatidine, [¹²⁵I] α -bungarotoxin and [³H] raclopride, respectively. On the day of the experiment, sections were thawed and processed according to established protocols (Orr-Urtreger *et al.*, 1997; Whiteaker *et al.*, 2000; Léna *et al.*, 2004; Metaxas *et al.*, 2010) with minor modifications. Multiple adjacent sections from both groups were processed together in a paired binding protocol.

$\alpha_4 \beta_2^*$ nAChR binding: sections were pre-incubated for 10 min in 50 mM Tris-HCl buffer containing 120 mM NaCl, 5mM KCl, 2.5 mM CaCl₂ and 1 mM MgCl₂, pH 7.4 at room temperature to remove endogenous ligands. Binding was carried out in 50mM Tris-HCl pre-incubation buffer, pH 7.4 at room temperature, in the presence of 100 pM [¹²⁵I] epibatidine (specific activity 2200 Ci/mmol) for 2 hours. This radioactive ligand retains the high affinity, low non-specific binding of [³H]-epibatidine, while labelling of multiple nAChR subtypes and providing increased specific activity (2200 Ci mmol) and increased detection efficiency ($\approx 80\text{--}85\%$ for ¹²⁵I). This combination should theoretically boost signals by approximately 100-fold (Dávila-García *et al.*, 1997). Two series of adjacent sections were also used from each mouse to measure total [¹²⁵I] epibatidine binding (no competing ligand) and [¹²⁵I] epibatidine binding in the presence of 20 nM cytosine. Cytosine is a partial agonist that blocks additional sites of the $\alpha_4 \beta_2^*$ subtype nAChR, which are distinguished by their relatively low cytosine affinity to differentiate between cytosine-resistant and cytosine-sensitive [¹²⁵I] epibatidine binding sites (Whiteaker *et al.*, 2000). An additional set of adjacent sections were used to determine NSB in the presence of 300 μ M nicotine hydrogen tartrate salt for 2 hours. Incubations were terminated after rinsing slides twice in ice cold (4°C) 50 mM tris-HCl buffer, pH 7.4 for 10 min and a final rapid rinse in ice-cold RO water to remove unbound ligand.

α_7^* nAChR binding: Slides were pre-incubated for 30 min in 50 mM Tris-HCl buffer containing 1% w/v bovine serum albumin, pH 7.4 at room temperature. Pre-incubation of

NSB slides included 1 mM nicotine hydrogen tartrate salt. Binding was carried out in the same buffer in the presence of 3 nM [^{125} I] α -bungarotoxin (specific activity 88.2 Ci/mmol) for 2 hours. NSB was determined in adjacent sections in the presence of 300 μ M nicotine hydrogen tartrate salt (a cold displacer for [^{125} I] α -bungarotoxin) for 2 hours. Incubations were terminated after rinsing slides three times in ice-cold (4°C) 50mM Tris-HCl buffer (not containing BSA), pH 7.4 for 10 min and a final rapid rinse in ice-cold RO water.

D₂R binding: slides were pre-incubated for 20 min in 50 mM Tris-HCl pre-incubation buffer, containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂ and 1 mM MgCl₂, pH 7.4 at room temperature. The slides were then incubated in pre-incubation buffer in the presence of 4 nM [^{3} H] raclopride (specific activity 70 Ci/mmol) for 60 min. NSB was determined in the presence of 10 μ M sulpiride. Incubation was terminated by rapid rinses (6 x 1 min) in ice cold 50mM Tris-HCl buffer, pH 7.4 and a final rapid rinse in ice-cold RO water.

4.3.1.5. Film exposure and development

Following binding, sections were rapidly dried under cold air for 2 hours and dried for up to 7 days using anhydrous calcium sulphate. Adjacent total and non-specific labelled sections were apposed to Kodak Biomax MR-1 film alongside autoradiographic microscale standards of known concentration to permit the conversion of the optical densities of silver grains in autoradiograms to specific activity of tissue-bound ligand to femtomoles per milligram (fmol/mg) of tissue. [^{125} I] epibatidine and [^{125} I] α -bungarotoxin bound sections were exposed to film for 24 hours and 7 days, respectively, with a set of ^{14} C microscale standards which had been cross calibrated to iodinated standards (Miller and Zahniser, 1987). [^{3} H] D₂R-bound sections were exposed to film with ^{3} H microscale standards for a period of 6 weeks.

For development, [^{125} I]- and [^{3} H]- sensitive films were covered with an aqueous solution of 50 % v/v Kodak D19 developer for 2 min. The reaction was stopped by 30 sec rinse in RO water containing few drops of glacial acetic acid. Images were fixed by submersion in Kodak

rapid fix solution for 3 min. Films were then rinsed in RO water and dried overnight in a fume cupboard.

4.3.1.6. Quantitative analysis

Films were analysed by video-based densitometry using an MCID image analyser (Image Research, Canada). In brief, fmol/mg tissue equivalents for receptor binding were derived from either ^3H or ^{14}C microscale standards and the relationship between tissue reactivity and optical density was calculated using MCID software, with appropriate adjustments to allow for radioactive decay of both the standards and the radioligands. Specific receptor binding was derived by subtraction of NSB from total binding for α_7^* nAChRs, and D_2Rs . For $\alpha_4 \beta_2^*$ nAChRs, cytisine-resistant epibatidine binding was subtracted from total epibatidine binding to give cytisine-sensitive epibatidine binding, which represents $\alpha_4 \beta_2^*$ nAChR binding. For each region, quantified measures were taken from both left and right hemispheres, therefore receptor binding represents a duplicate determination for each brain region and the n values listed refer to the number of animals analysed. The following structures were analysed by sampling 5-20 times with a box tool (cortex (8x8 mm), olfactory tubercle (6x6 mm) and hippocampus (5x5 mm)). All other regions were analysed by free-hand drawing. Brain structures were identified by reference to the mouse atlas of Franklin and Paxinos (2001).

4.3.1.7. Statistical analysis

Social interaction after social defeat

For assessing time in interaction zone in the presence and absence of novel interacting mouse, we measured the social interaction behaviour, a unilateral student paired t-test with Bonferroni multiple comparison correction was used, to determine the differences in time

spent in interacting zones between groups (No Target vs Target) in No SD and SD groups. The statistical significance was set at $p < 0.05$. Data are presented as mean $\pm$ SEM.

Autoradiographic receptor binding

All data are presented as mean $\pm$ SEM and were analysed using GraphPad Prism 7. Two-way ANOVA for factors "Social defeat" and "Brain Region" were used for comparison of specific binding levels between groups. Statistical significance was defined as $p < 0.05$. Sidak's multiple comparisons test was carried out to investigate differences in radioligand binding experiments between the groups in individual regions.

4.3.2. Materials

[^{125} I] epibatidine (2200 Ci/mmol), [^{125} I] α -bungarotoxin (88.2 Ci/mmol) and [^{3} H] raclopride (70 Ci/mmol) radioligands were purchased from PerkinElmer Life sciences (MA, USA), cytosine and sulpiride from Tocris Bioscience (Bristol, UK), nicotine hydrogen tartrate salt, Tris-HCl, NaCl, KCl, CaCl_2 , BSA, MgCl_2 were purchased from Sigma-Aldrich (Poole, UK) and anhydrous calcium sulphate from BDH Chemicals (Poole, UK). Brains were sectioned in a Microtome cryostat HM305E (Zeiss Hyrax C 25, Carl Zeiss Microimaging GmbH, Germany).

4.4. Results

4.4.1. Chronic social defeated and non-defeated mice assessed for social interaction

Social interaction in mice subjected to chronic SD stress was compared with control-NoSD group of mice following 10 day of SD stress. SD group displayed social avoidance demonstrated by decreased time spent in the interacting zone (~ 20 sec) as compared with NoSD (~ 35-65 sec). It was also displayed that SD mice spent identical time in the interacting zone both when the 'target' mouse was present or not, whereas the control-NoSD spent significantly increased time in the interacting zone when 'target' mouse was present as compared to the empty cage. These experiments were performed by Dr. Barik's group in Labex, France. Together these results show a decrease in social interaction in SD mice compared with non-SD mice clearly indicating social avoidance.

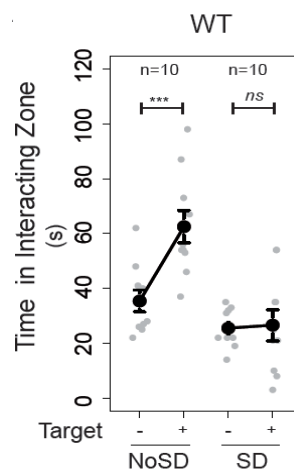


Figure 4.3: Social interaction in mice subjected to chronic social defeat (SD) stress compared to non-social (NoSD) defeat.

'Target' corresponds to the presence (+) or absence (-) of a novel interacting mouse. Defeated group displayed social avoidance.

4.4.2. Effect of chronic social defeat on $\alpha_4\beta_2^*$ nAChRs binding in mice

Extensive brain mapping of epibatidine binding sites by autoradiography revealed no global alteration of epibatidine binding after SD exposure. Cytisine-sensitive [^{125} I] epibatidine binding was used to determine $\alpha_4\beta_2^*$ binding in mice that have or have not undergone SD (Figures 4.4 and 4.5). High levels of $\alpha_4\beta_2^*$ binding (31.9– 25.67 fmol/mg) were detected within the Th and the medial geniculate (MG) regions, moderate levels (24.04 – 17.38 fmol/mg) in the VTA and the CPu, and low levels (12.38 – 1.43 fmol/mg) in the fr and the Pir in both groups. Our data demonstrate that there was no significant difference between the $\alpha_4\beta_2^*$ binding levels in the brains of the SD mice compared with the controls. Although two-way ANOVA revealed a significant effect for $\alpha_4\beta_2^*$ binding sites on 'brain region' ($F_{(33, 605)} = 16.29$, $p < 0.0001$), it did not show any significant effect of 'social defeat' factor ($F_{(1, 605)} = 0.5101$, ns) and 'social defeat x brain region' interaction effect ($F_{(33, 605)} = 0.5373$, ns). There were no significant alterations in total [^{125} I] epibatidine binding, cytisine-sensitive (i.e. $\alpha_4\beta_2^*$) or cytisine resistant binding in any of the regions analysed (Figure 4.5).

Total epibatidine binding sites are widely distributed in the brain and display high binding levels (36.4–21.9 fmol/mg) in brain regions such as the Th, VDB and fr, medium binding levels (20.7–15.6 fmol/mg) in regions such as the NAc, CPu and low levels (13.7–5.4 fmol/mg) in the AMG and Hip. Cytisine-resistant binding sites are also widely distributed in the brain, although in lower binding levels (12.5–4.3 fmol/mg) than epibatidine sensitive binding, in brain regions such as the NAc, CPu and the VTA (Figure 4.5). High levels of cytisine resistant [^{125} I] epibatidine binding (40–33 fmol/mg) were found in the lpu, MHb and the fr, moderate levels (14.3–8.2 fmol/mg) within the MG and the VDB, and low levels (5.1–0.7 fmol/mg) in the rest regions studied. The quantification of the density levels of [^{125} I] epibatidine in the brains analysed was in relation to the ^{14}C standards used, and NSB was indistinguishable from background levels. Two-way ANOVA did not reveal any significant

effect for the factors 'social defeat' and 'social defeat x brain region' interaction in total [¹²⁵I] epibatidine, cytisine sensitive [¹²⁵I] epibatidine and cytisine-resistant [¹²⁵I] epibatidine binding.

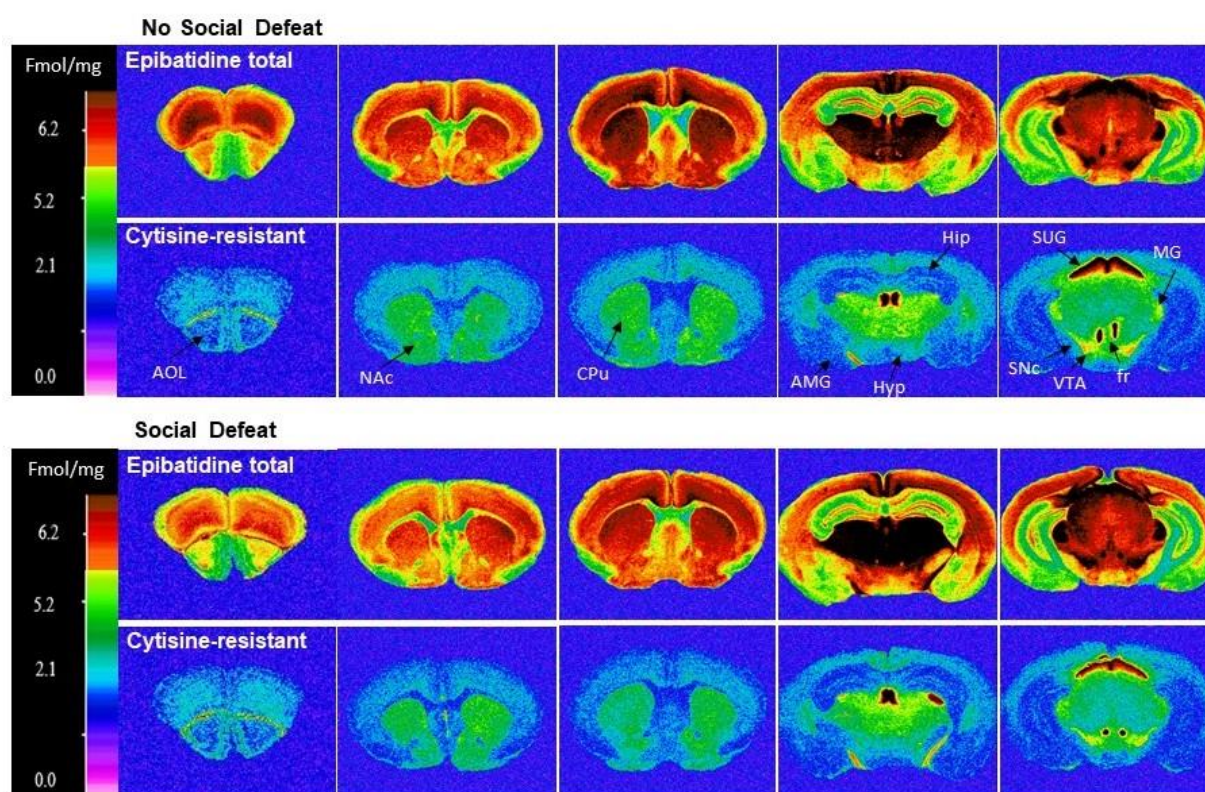


Figure 4.4: Representative autoradiograms of $\alpha_4 \beta_2^*$ nAChRs binding in control non-social defeated (NoSD) and social defeated (SD) mice.

Representative images of total [¹²⁵I] epibatidine (rows 1,3) and cytisine-resistant [¹²⁵I] epibatidine (rows 2,4) binding in coronal adjacent brain sections of wild-type mice that have either undergone SD stress or not. Complete mapping of the brain illustrated above, starting at the level of the olfactory nuclei (Bregma 3.08, column 1), NAc (Bregma 1.10 column 2), CPu (Bregma 0.74 column 3), Th (Bregma -1.46 column 4) and the VTA (Bregma -3.40 column 5). The colour bar illustrates a pseudocolour interpretation of black and white film images. Top two rows; from control (NoSD) mice sections and, bottom two rows; SD mice sections. Non-specific binding was equal to background levels (image not shown). For abbreviations see p.17-26.

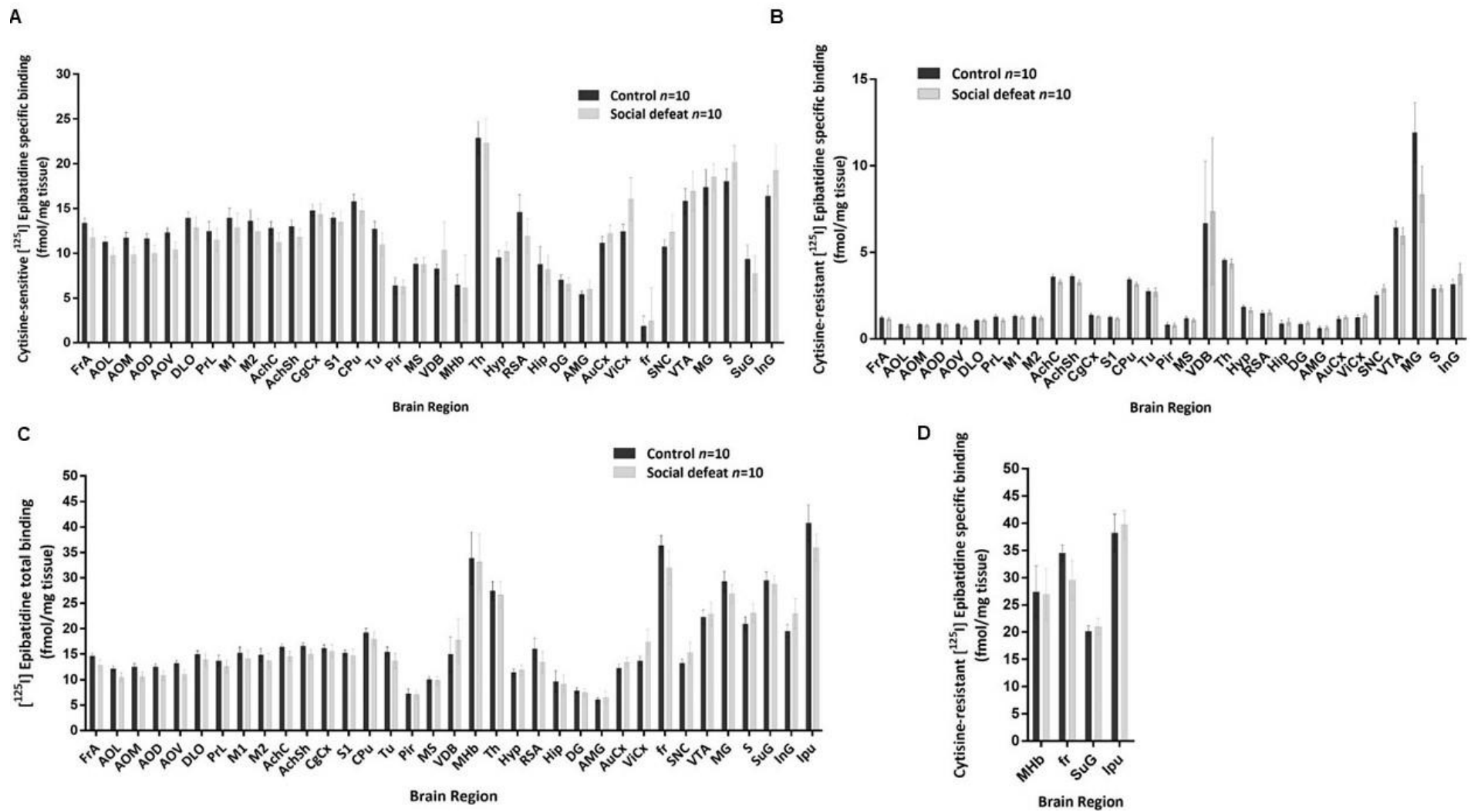


Figure 4.5: Quantification of $\alpha_4 \beta_2^*$ nAChRs binding in control (NoSD) and SD mice.

Cortical and non-cortical regions from brain sections of NoSD and SD mice were bound with [^{125} I] epibatidine alone or in the presence of cytisine. A) Cytisine sensitive [^{125} I] epibatidine binding. Cytisine-sensitive binding was determined by subtracting cytisine-resistant binding from total epibatidine binding. B) Cytisine resistant [^{125} I] epibatidine binding C) Total [^{125} I] epibatidine binding D) Cytisine resistant [^{125} I] epibatidine binding for extra brain regions studied. Data are represented as mean $\pm$ standard error of the mean (n=10). For abbreviations see p.17-26.

4.4.3. Effect of chronic social defeat on α_7^* nAChRs binding in wild-type mice

Extensive brain mapping of α -bungarotoxin binding sites by autoradiography revealed no global alteration of nAChRs numbers after SD. [^{125} I] α -bungarotoxin binding was used to determine α_7 binding in WT mice that have or have not undergone SD (Figures 4.6 and 4.7). High binding was observed in the fr, SUG and DG (37.75–50.05 fmol/mg), moderate levels (3.18–37.04 fmol/mg) within the VLG and the hippocampal CA2, and low levels (19.6– 20.62 fmol/mg) in the AcbC and the AcbSh in both groups. Two-way ANOVA revealed a significant ‘social defeat’ effect ($F_{(1, 582)} = 3.869$, $p < 0.05$) and significant brain region effect ($F_{(32, 582)} = 235.4$, $p < 0.001$) but no significant effect was seen for ‘social defeat x brain region’ interaction ($F_{(32, 582)} = 0.6403$, ns). *Post-hoc* Sidak’s multiple comparisons analysis revealed no significant difference between the two groups in any brain region analysed (Figure 4.7). The quantification of the density levels of [^{125} I] bungarotoxin in the brains analysed was in relation to the ^{14} C standards used.

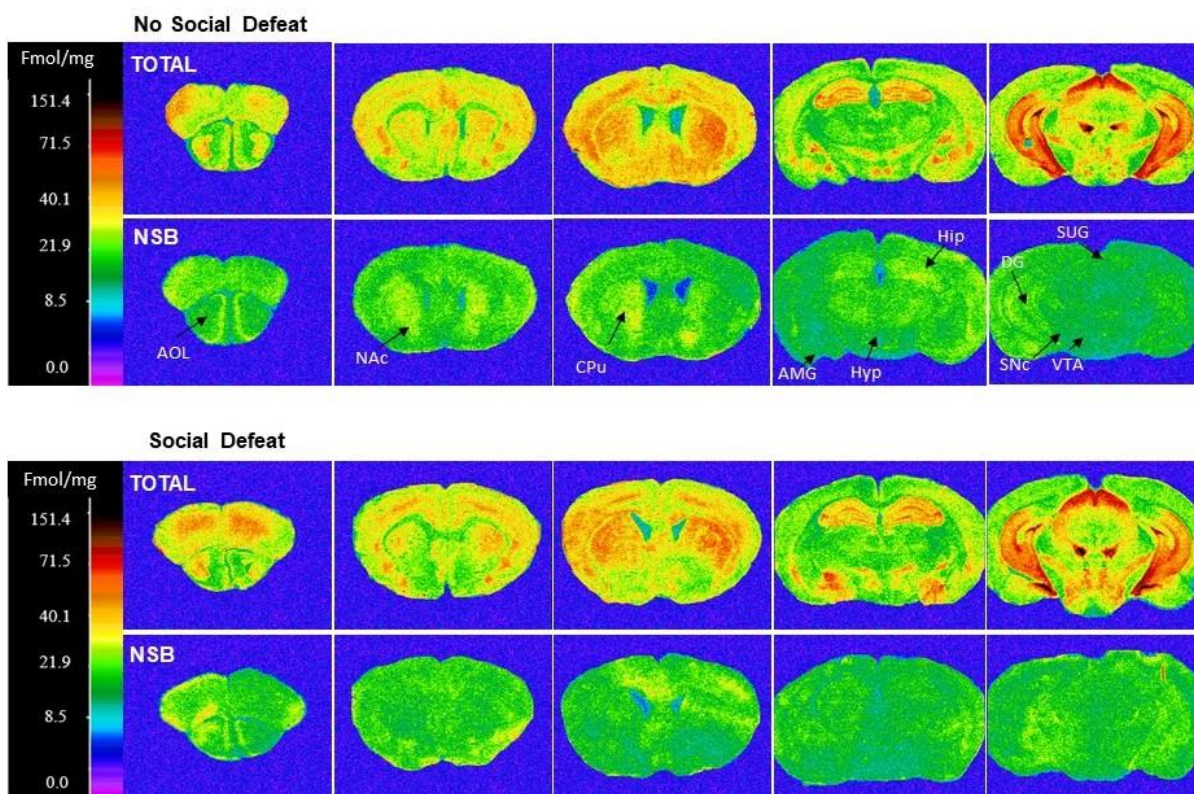


Figure 4.6: Representative autoradiograms of α_7^* nAChRs binding in control non-social defeated (NoSD) and social defeated (SD) mice.

Representative images of total and non-specific [^{125} I] α -bungarotoxin binding in coronal sections from NoSD and SD mice brains. Adjacent sections were incubated for 3 h with 3 nM of [^{125} I] α -bungarotoxin alone for determining the total binding (Total rows) or in the presence of 300 μ M nicotine hydrogen tartrate for determining the NSB row. Complete mapping of the brain illustrated above, starting at the level of the olfactory nuclei (Bregma 3.08), NAc (Bregma 1.10), CPu (Bregma 0.74), Hyp (Bregma -1.46) and the VTA (Bregma -3.40). Top two rows; from NoSD mice sections and, bottom two rows; SD mice sections. For abbreviations see p.17-26.

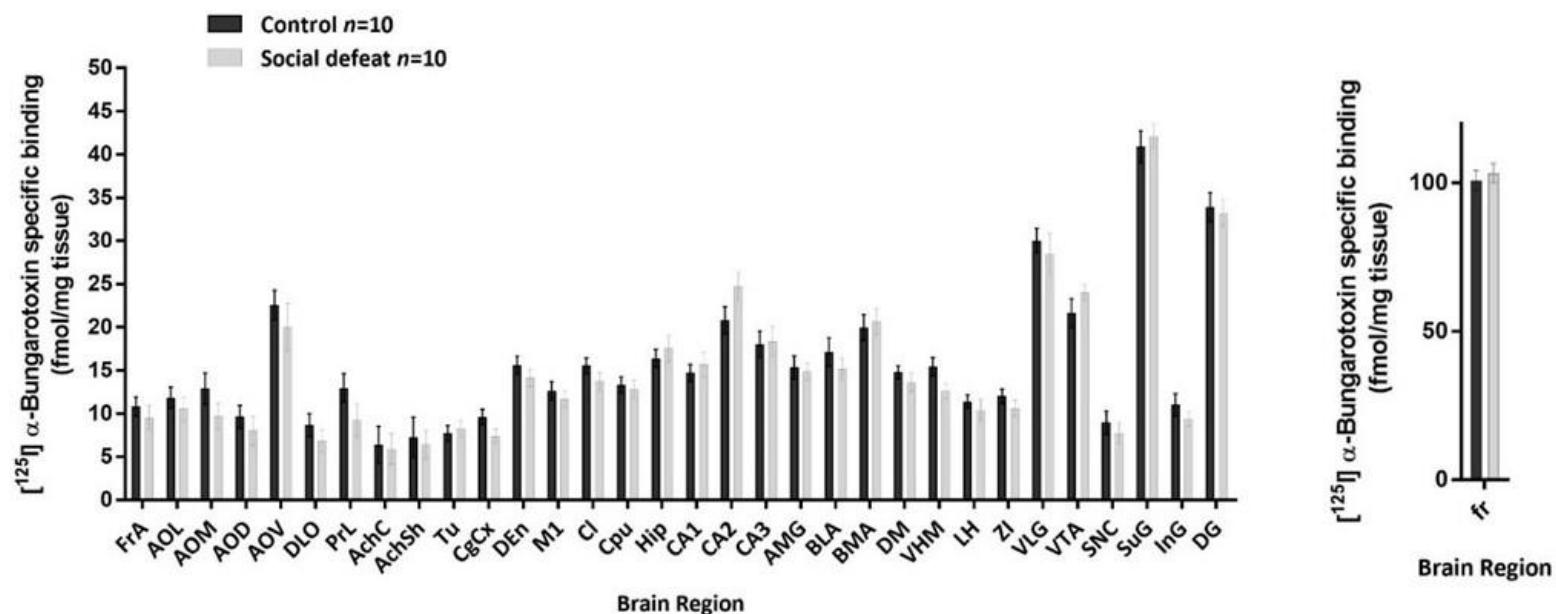


Figure 4.7: Quantitative autoradiographic analysis of α_7^* nAChRs binding in control (NoSD) and social defeated (SD) mice determined by [^{125} I] α -bungarotoxin binding.

Cortical and non-cortical regions from brain sections of NoSD and SD mice were bound with [^{125} I] α -bungarotoxin. Data are represented as mean $\pm$ standard error of the mean (n=10). For abbreviations see p.17-26.

4.4.4. Effect of chronic social defeat on D₂R binding in wild-type mice

[³H] raclopride binding was used to determine D₂R binding levels in the brains of WT mice, which have or have not been exposed to chronic SD (Figure 4.8). High binding was found in the CPu subregions (100.44– 124.30 fmol/mg) and low levels were detected in the NAc subdivisions and the Tu (32– 82.64 fmol/mg). [³H] raclopride binding levels were overall down-regulated in the brains of socially defeated mice in comparison to the non-defeated group. Two-way ANOVA revealed a significant difference on ‘social defeat’ ($F_{(1, 108)} = 17.28$, $p < 0.05$) and ‘brain region’ effect ($F_{(5, 108)} = 92.39$, $p < 0.0001$), but not significant ‘social defeat x brain region’ interaction effect ($F_{(5, 108)} = 0.0475$, ns). *Post-hoc* analysis by Sidak’s multiple comparisons demonstrated that there was no significant difference between the two groups in any brain region analysed (Figure 4.8). NSB was indistinguishable from background level.

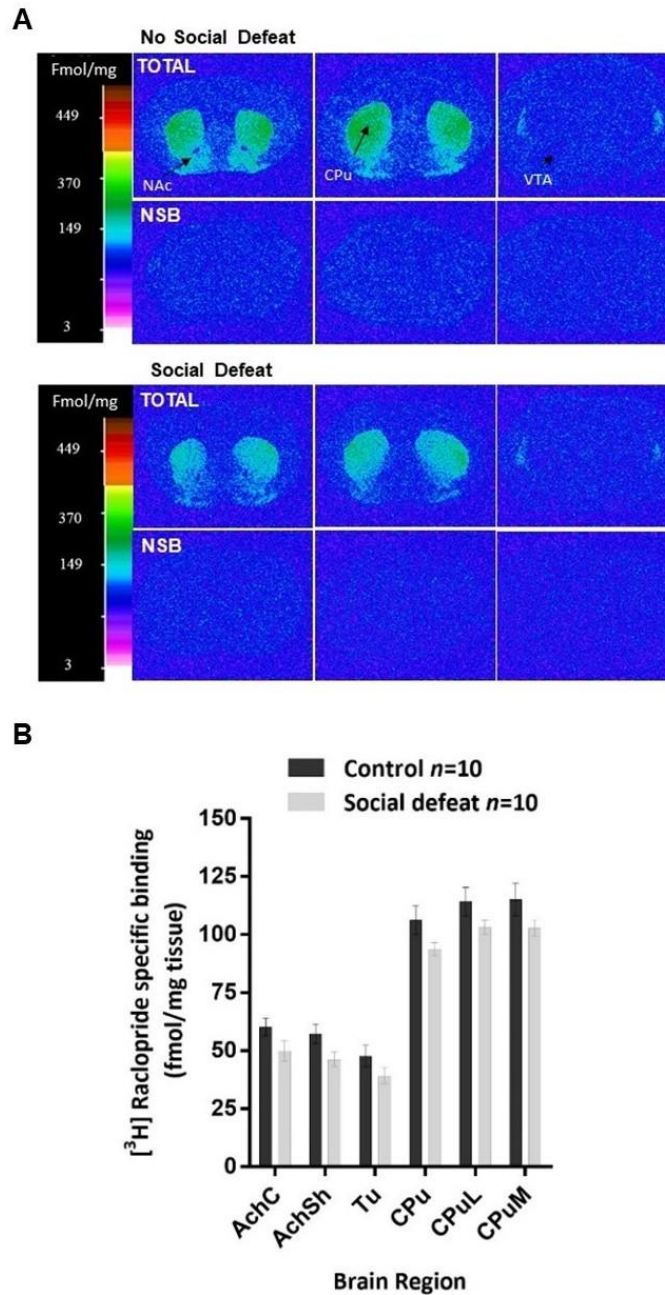


Figure 4.8: Representative autoradiograms of D_2R binding in control non-social defeated (NoSD) and social defeated (SD) mice.

A) Representative images of total and non-specific $[^3\text{H}]$ raclopride binding in coronal sections from control (NoSD) and SD mice brains. The pictures illustrated above start at the level of NAc (Bregma 1.10), CPu (Bregma 0.74) and the VTA (Bregma -3.40). The colour bar indicates a pseudo colour interpretation of black and white image density, calibrated in fmol/mg of tissue equivalent. Top two rows; from NoSD mice sections and, bottom two rows; SD mice sections. B) Quantitative autoradiographic $[^3\text{H}]$ raclopride D_2R binding in NoSD and SD mice. Data are represented as mean $\pm$ standard error of the mean ($n=10$). For abbreviations see p.17-26.

4.5. Discussion

This present study aimed to investigate the effect of chronic SD, a more reliable translational model of depression characterised by repeated exposure to chronic social stress on D₂R, α_4 β_2^* and α_7^* nAChR binding. The rationale for this investigation was based on evidence arising from the study by Morel and colleagues (2017), demonstrating that α_4 β_2^* and α_7^* play a key regulatory role in the interplay between stress and nicotine, at the cellular and behavioural level. Mice following chronic exposure to SD demonstrated behavioural and cellular maladaptations.

With regards to behavioural changes, socially defeated mice displayed a marked social avoidance behaviour following 10-day chronic exposure to SD, as demonstrated by reduced time spent in the interacting zone as compared with the time of NoSD mice (Figure 4.3). Our findings are in line with previous studies demonstrating that SD repeated daily for 10 days resulted in increased anxiety and social aversion (Krishnan and Nestler, 2008). In fact, a single aggression in naïve mice resulted after 45 min in a significant increase in circulating glucocorticoids (Barik *et al.*, 2013). It has been shown that stressors, which induce glucocorticoid increase can change DA-releasing neurons excitability in the midbrain and concomitantly affect DA release (Marinelli, 2007). These neurons project from the VTA to limbic and cortical structures to subserve regulation of motor control, reward and emotions (Marinelli *et al.*, 2006; Dalley and Everitt, 2009; Valenti *et al.*, 2011). Furthermore, they also participate in the modulation of anxiety-like behaviours and cognitive functions (Zweifel *et al.*, 2011). These together suggest that chronic SD stress drives social avoidance behaviour possibly by increasing mesocorticolimbic neuronal activity. Our results support the use of the psychosocial stress paradigm in mice as a reliable translational model to mimic impairment experienced by people exposed to chronic psychosocial stress. Indeed chronic exposure to social stress and, notably bullying, in humans is one of the major causes of depression (Gullander *et al.*, 2014).

In addition to the behavioural outcomes, important cellular adaptations also occurred. After global mapping of [¹²⁵I] epibatidine, cytosine-sensitive [¹²⁵I] epibatidine and cytosine resistant [¹²⁵I] epibatidine binding in both NoSD and SD mice brains, we showed that there were no observed significant changes in numbers of $\alpha_4 \beta_2^*$ binding, total [¹²⁵I] epibatidine and cytosine-resistant [¹²⁵I] epibatidine sites between the two groups in any of the regions analysed (Figure 4.5). This is not in accordance to a recent study by Morel *et al.* (2017), who demonstrated a very small but statistically significant increase in epibatidine binding sites on synaptic fractions dissected from the VTA of SD mice, suggesting an upregulation of $\alpha_4 \beta_2^*$ nAChR in dopaminergic rich brain regions. Additionally, in the same study, they showed an increase in hemicholinium binding sites (a marker of choline transport and, hence, of endogenous cholinergic transmission) in SD mice, indicating increased cholinergic tone in the VTA. Thus, the increase of cholinergic tone, which would subsequently desensitise $\alpha_4 \beta_2^*$ receptors may explain the upregulation of $\alpha_4 \beta_2^*$ receptors in the VTA. This finding is in line with other studies, in which increased cholinergic tone is assumed to explain the lower β_2^* nAChR availability in depressed patients (Saricicek *et al.*, 2012). Interestingly, Morel *et al.*, 2017 showed that SD increased VTA frequency and burst activity of VTA dopaminergic neurons, clearly suggesting VTA dopaminergic mechanism underlining social avoidance behaviour. Intriguingly, while administration of a single dose of nicotine in naive (NoSD) mice enhanced firing and bursting activity of dopaminergic neurons in the VTA, SD mice exhibited no such response to nicotine suggesting potential neuroadaptations in the nAChR system in these mice. Taken all together, these suggest that SD modifies spontaneous activity of DA neurons, their sensitivity to nicotine and it increases the cholinergic tone in the VTA (Morel *et al.*, 2017). Finally, functional and behavioural evidence confirmed a key involvement for $\alpha_4 \beta_2^*$ nAChRs in SD behaviour. More specifically, blockade of $\alpha_4 \beta_2^*$ with chronic treatment with the selective antagonist, dihydro- β -erythroidine, reversed social avoidance clearly demonstrating that $\alpha_4 \beta_2^*$ nAChRs are involved in the development of social avoidance, thus they are potential risk factors for depression. With respect to our present data showing a lack

of $\alpha_4 \beta_2^*$ nAChR alterations in binding in SD mice, we can conclude that any alterations in nicotine sensitivity observed in SD mice is not due to alteration in $\alpha_4 \beta_2^*$ nAChR levels but potentially due to changes in $\alpha_4 \beta_2^*$ functional activity. The reason why no change in $\alpha_4 \beta_2^*$ nAChR binding was observed in the VTA of SD mice, despite the upregulation reported by Morel *et al.*, 2017, could be due to differences in technique, which was used to measure the receptor levels. Nonetheless, even Morel *et al.* 2017, only found a modest (3-5%) increase in $\alpha_4 \beta_2^*$ receptor proteins in SD vs. NoSD mice.

It has been previously shown that α_7 nAChR KO mice exhibited depression-like phenotype as demonstrated by the forced swim paradigm, (Rygula *et al.*, 2005) and by the sucrose preference test (Zhang *et al.*, 2016), which assesses anhedonia, a core symptom of depression in humans. Thus, we investigated the effect of chronic social stress on α_7^* nAChR binding. After global mapping of [^{125} I] α -bungarotoxin binding in both NoSD and SD mice brains, a significant 'social defeat' effect was found on α_7^* binding sites, but post hoc analysis did not show any differences in α_7^* nAChR levels in any regions analysed (Figure 4.7). This is in agreement with Morel *et al.* (2017) who demonstrated no change in α -bungarotoxin binding sites on synaptic fractions dissected from the VTA of SD mice. Interestingly, the same group showed that chronic administration of α_7^* nAChRs antagonist, methyllycaconitine, did not manage to reverse social avoidance in SD mice. However, acute administration of the potent antagonist prevented SD-induced social aversion (Morel *et al.*, 2017), suggesting potential neuroadaptations in the nAChRs system following prolonged overstimulation of α_7^* nAChRs. Altogether, these strongly support that α_7^* nAChRs are involved in the development of social avoidance and, hence, α_7 activation with nicotine may be considered as a risk factor for depression. Indeed, Morel *et al.* (2017) found that α_7 nAChRs activation following intraperitoneal and intra-VTA injection of an α_7^* nAChRs selective positive allosteric modulator (PNU-120596), showed potentiation of stress sensitization and exacerbation of SD. These were followed by concomitant increase in the

VTA dopaminergic firing that ultimately reinforced our hypothesis. Thus, it is suggested that α_7^* nAChRs antagonist may be a potentially novel target for the treatment of depression. With regards to our data showing lack of alterations of α_7^* nAChR binding in brain regions of SD animals, we conclude that any alterations in α_7^* nAChR sensitivity implicated with the aforementioned behavioural studies by Morel *et al.*, (2017), are not due to alterations in α_7^* nAChR density.

Interestingly, significant treatment effect was detected on the D₂R binding displaying an overall region decrease in the SD mice as compared to the NoSD animals (Figure 4.8). This is in line with previous findings, which have shown that reduced D₂R binding in the limbic forebrain is responsible for chronic mild stress-induced anhedonia (Papp *et al.*, 1994). This is also in agreement with data from depressed patients (Larisch *et al.*, 1997; D'Aquila *et al.*, 2000; Gershon, Vishne and Grunhaus, 2007; Lee *et al.*, 2010), showing a DA dysregulation in depression. As discussed before, Morel *et al.*, (2017) showed hyperactivity of VTA dopaminergic neurons in mice that have undergone the same chronic SD protocol. It is possible that this surge of VTA dopaminergic activity may have triggered a compensatory down-regulation of D₂R observed in SD mice in our study. Whether this decrease in D₂R may at least partly mediate the social avoidance behaviour in these SD animals cannot be concluded by this study.

In conclusion, although our results do not demonstrate significant changes in nAChR density in brains of SD mice, there is emerging functional electrophysiological and behavioural evidence to suggest the presence of profound neuroadaptations in these systems. These neuroadaptations potentially contribute to a complex nicotine-stress bidirectional interplay via DA cell activity, which is likely to have a major impact on mood regulation. Our data is supportive of D₂R dysregulation, but shows that any neuroadaptations of the nAChR system in SD is not due to changes in α_7^* and $\alpha_4\beta_2^*$ nAChR density in the brain.

5. Chapter 5: General discussion

5.1. General discussion

This thesis is focused on investigating the neurobiology underlining certain neuropsychopathological conditions characterised by impairment in the social domain. To this end, ASD and depression caused by chronic social stress, were explored. ASD is a neurodevelopmental condition, which includes a spectrum of symptoms characterised by impairment in the social domain and it is often accompanied by other debilitating disorders such as, social anxiety and depression (Williams *et al.*, 2010). The aetiology of these pathologies remains unclear and advances in the understanding of its neurobiological underpinnings are of critical need. This thesis investigated neurobiological systems including the oxytocinergic and the opioid systems that are involved in ASD and comorbidity. Furthermore, the project underlines potential targets for the development of novel therapeutics. For this purpose, we employed mice modelling ASD, the *Fmr1* KO. OXT is a promising treatment, with shown efficacy for the treatment of core symptomatology for ASD. Notably, it has been demonstrated that long-term OXT treatment has efficacy for young men with high-functioning ASD on sociality and communication with a complete absence of adverse effects (Kosaka *et al.*, 2016). Our study provides further evidence by showing that prolonged OXT treatment reverses sociability deficits in the *Fmr1* KO mouse. This study is the first to investigate acute and prolonged OXT treatment effects in this model of ASD, further reinforcing the literature on OXT's efficacy in treating ASD core symptomatology. Nevertheless, our findings demonstrated that OXT has detrimental effects on sociability in wild-type mice, which raises safety concerns of OXT administration in an intact and healthy oxytocinergic system. Our contribution to this field of research provides us with a wider understanding of how we perceive autism and brings in potentials for novel targets that will pave the way for the establishment of novel therapeutics.

Furthermore, a second part of this thesis aimed to shed light in the neurobiology underpinning social defeat in response to repeated social stress by assessing the effect of chronic SD

stress on nicotinic and D₂R density. Although there was no effect detected on $\alpha_4\beta_2^*$ and α_7^* nAChR density in the brain, emerging functional electrophysiological and behavioural evidence (Morel *et al.*, 2017) demonstrated the presence of profound neuroadaptations in these systems in the same SD model used. These results suggest a complex nicotine-stress interplay in the development of social stress-induced depressive disorders and may pave the way for the development of novel targets for stress-related psychiatric disorders.

This chapter will focus on key novel contributions that this work has made on the understanding of the neurobiology of disorders characterised by social impairment in the social domain and the efficacy of potential pharmacotherapy used for their treatment.

5.1.1. Efficacy of long-term oxytocin administration

In the last decade, novel approaches in the field of therapeutics have set new grounds for the treatment of autism. Neuropeptides administered via the intranasal route have been shown to be able to cross the BBB and be delivered directly to the brain in humans (Born *et al.*, 2002) exerting behavioural and functional effects (Born *et al.*, 2002; Pedersen *et al.*, 2013; Aoki *et al.*, 2014; Anagnostou *et al.*, 2014). Ever since this major break-through, the utility of the intranasal route of peptide administration is frequent in clinical trials for the treatment of ASD. Nevertheless, there are points of controversy in respect of this topic amongst basic science researchers, which have been extensively discussed in this thesis. Our findings support that prolonged OXT treatment abolishes sociability defects and comorbid anxiety in yet another model of ASD, the *Fmr1* KO mice, but not stereotypical behaviour in this paradigm, in line with other studies (Penagarikano *et al.*, 2015). First, our results established and confirmed intranasal OXT as a promising pharmacotherapeutic in the *Fmr1* model of ASD, which is a novel model assessing OXT treatment effects. *Fmr1* KO is a model of Fragile X syndrome (FXS) which is characterised by mental retardation and ASD symptoms (Devitt *et al.*, 2015). Currently, there is no available treatment for core ASD

symptoms in patients with FXS including social deficits and stereotypies. However, associated behavioural symptoms of ASD such as irritability, aggression, self-injurious behaviour and severe tantrums are treated with risperidone and aripiprazole (Siegel and Beaulieu, 2012; Spencer *et al.*, 2013), which their efficacy is variable and exhibit significant side effects. Our data supports intranasal OXT administration as a potent pharmacotherapy at least for the treatment of social impairment and comorbid anxiety in patients with FXS. Secondly, this is one of the few studies, which have assessed the chronic effect of OXT in a mouse model of ASD. Majority of the studies in the literature focused on the acute or subchronic effect of OXT. However, it is of paramount importance to establish that not only the acute but also the chronic OXT administration is effective in reversing core symptoms of ASD. The oxytocinergic system is highly plastic and long-term administration of a drug can induce homeostatic compensatory neuroadaptations, which may limit or abolish the beneficial effect of the drug or even cause side effects. Nonetheless, in our ASD model that displays a marked deficit in oxytocin transcription, chronic OXT administration still is as effective as given acutely. This has important implications in support of the safe use of chronic OXT treatment at least in ASD patients.

Based on the inconsistency in clinical and preclinical studies regarding the efficacy of OXT in treating ASD symptoms, one can assume that efficacy may depend on factors such as gender or genetic makeup. There is evidence to suggest that individuals with lowest pre-treatment blood OXT concentrations benefit the most from OXT administration (Parker *et al.*, 2017), which is consistent with our study where we demonstrated a beneficial effect of OXT in a model characterised by OXT deficit at least in the striatum. This may have implications for ASD treatment in a personalised medicine approach, where ASD patients would be screened for OXT status; either genetically for OXT polymorphisms or biochemically by measuring OXT plasma levels, before deciding on treatment interventions. Finally, despite the obvious benefits of OXT treatment in the social aspects and comorbid anxiety aspects of

ASD, we did not observe any obvious benefits of OXT in stereotypical characteristics. This is in part consistent with clinical studies, which demonstrated that long-term OXT treatment (24-weeks) has shown efficacy for young men with high-functioning ASD on comprehensive symptom and social-communicative behaviours, but not on secondary symptoms of stereotypic behaviour and comorbidity including anxiety and depressive state (Kosaka *et al.*, 2016).

5.1.2. Oxytocin's safety for general consumption

The feasibility of intranasal OXT administration, as a non-invasive method in combination with its minimal reported side effects, has also its downsides. Off-label OXT has been prescribed by psychologists to treat mild social unease in those who are not suffering from a diagnosed disorder (Kovács and Kéri, 2015). OXT's beneficial effects in social attachment, pair bonding and social behaviour are misused by profit-driven markets as they advertise it to the general public, as an attractive harmless medication that will improve everyday social contacts and restore relationships in couples. Therefore, the downside of it is based on the off-label use of OXT which led to an increased consumption of OXT by the general population (Kovács and Kéri, 2015).

In this respect, concerns have been raised regarding the physiological consequences of prolonged OXT treatment. In fact, OXT has been associated with severe cardiovascular side-effects such as hypotension and tachycardia (Dyer *et al.*, 2011) electrolyte imbalances and adverse effects in the kidneys (Rasmussen, *et al.*, 2004). Also, chronic OXT administration has the potential to cause hormonal disturbances in females at different reproductive phases (i.e. milk ejection, labour induction) due to the peripheral effects of OXT, which suggests that safety precautions should be taken into consideration when OXT is administered to female participants, who might require regular gynecologist monitoring. Interestingly, Kosaka *et al.* (2016) demonstrated that long-term administration of OXT at a

dose smaller than 32 IU per day for 12 weeks does not lead to hormone-related abnormalities in non-pregnant females, which supports that the safety use of OXT might be dose-dependent in women. Also, clinicians propose to use oxytocin in ASD patients in combination with behavioural therapy, as a tool to ease social contact (Hall *et al.*, 2012; Stavropoulos & Carver, 2013).

Our data clearly show that chronic, not acute OXT treatment in a healthy murine oxytocinergic system with no inherent deficits in social interaction and normal endogenous levels of OXT has adverse effects on social behaviour, increases stereotypic behaviour and is anxiogenic, which reminisces the autistic-like phenotype in the *Fmr1* KO mice. The detrimental effects after prolonged overstimulation of a healthy oxytocinergic system are consistent with other studies in mice (Huang *et al.*, 2014; Peters *et al.*, 2014) and further suggest that life-long management of intranasal OXT treatments should be cautiously applied to the human population. According to recent literature, chronic OXT administration has shown negative consequences, which seem to be dependent on context. OXT might increase the sensitivity to salient social stimuli such that exposure to positive or negative experiences can cause exaggerated responses, which may affect mood states (McQuaid *et al.*, 2014). Preliminary results obtained in studies with OXT administration were found to contradict previous work simply suggesting prosocial effects of the peptide (Kosfeld *et al.*, 2005; Rilling *et al.*, 2012; Zak *et al.*, 2007) and highlighting the need for further understanding of the link between aggression and OXT. Higher scores on antisocial personality traits such as anger and interpersonal manipulation were related to increased aggressive behaviour following OXT administration (Alcorn *et al.*, 2015), which is in line with our findings. Similarly, other studies suggest that OXT treatment in healthy individuals with higher levels of social anxiety may impair social cognitive functioning, as demonstrated by decreased performance on a social cognitive task assessing social working memory (Tabak

et al., 2016). In light of these, we suggest that OXT should be legally regulated and prescribed by physicians upon clinical screening and only for a diagnosed disorder.

5.1.3. Progresses in ASD neurobiology

This thesis investigated neurobiological mechanisms underlining the ASD aetiology. Notably, the endogenous opioid system as a potential candidate implicated in the neurobiology of ASD has been largely under-investigated. Despite the preclinical and clinical evidence supporting opioid dysregulation in ASD and the formulation of an opioid theory in ASD over three-decades ago (Panksepp, 1979), more studies need to focus on this matter. Our findings reveal changes on endogenous opioid peptides transcripts in the brain of the autistic, *Fmr1* KO mouse model, but not on the expression of their respective receptors levels.

Also, our data reveal profound deficits in key transcripts of peptides and receptors in the CNS of the *Fmr1* KO model. For instance, NMDAR proteins and transcripts of opioid peptide, oxytocin, metabotropic glutamate and dopamine receptors subtypes are all down-regulated in specific brain regions in the ASD model. Whether these transcript deficits translate to differences in protein levels or neurotransmitter release with respect to neuropeptides, remains to be investigated. Nonetheless, the data clearly suggest a dysregulation of several key CNS systems (opioid, OXT, glutamate and DA) which may further underline potential targets for therapeutic discovery in this paradigm. Notable is the profound oxytocinergic deficit in the CPu of the *Fmr1* KO, which is likely to underline some of the ASD symptoms observed in these mutants. Hence, restoration of OXT levels by OXT administration may be a neurobiological mechanism underlining the beneficial effect of OXT in ASD. This is supported by other studies conducted in the clinic and the laboratory demonstrating that primary deficits in the oxytocinergic system may cause social defects in ASD subjects, and correcting this deficit can restore social behaviour (Andari *et al.*, 2010; Penagarikano *et al.*,

2015; Parker *et al.*, 2017). Nonetheless, it is evident from this study that multiple systems are impaired in ASD, which may explain the complexity and heterogeneity of the disorder.

The neurobiological changes of transcripts that we observed in the *Fmr1* KO mouse brain underscore the importance of investigating changes at the protein level. This brings out the the potential to identify protein targets, which could point towards the development of novel treatment approaches and perhaps incorporate a unified approach that would involve manipulations of several of these systems. Interestingly, a clinical trial is underway using memantine (NMDAR antagonist) to assess treatment response (over 12-weeks) to social responsiveness in ASD individuals, and has successfully progressed onto Phase 3 ([https://clinicaltrials.gov/Identifier No: NCT01972074](https://clinicaltrials.gov/Identifier%20No%3A%20NCT01972074)). These preclinical and clinical findings constitute a starting point in unravelling the mechanistic implications of several neurotransmitter systems in the ASD pathology. This may progress to the identification of key targets for drug development, eventually considered for testing in human clinical trials.

Moreover, we revealed that chronic intranasal OXT differentially regulates many transcripts of key receptor and peptide systems in specific brain regions in WT and *Fmr1* KO mice. Notably, transcripts of *Avp1a*, *Avpr1b* and *Penk* in the CPu and *Oxt*, *Avp*, *Oxtr*, *Penk* and *Grm8* transcripts in the CeA seem to be differentially regulated by chronic OXT in WT vs. *Fmr1* KO. This may explain the differential and opposing effect of chronic intranasal OXT treatment in WT and the ASD model. Interestingly, our findings incorporate changes that occur in the AVPergic, OXTergic and the opioid peptide systems (Penk) at mRNA level that chronic OXT treatment can rescue. It might be that neuronal networks, which incorporate these systems act synergistically in the *Fmr1* KO mouse and can exert their beneficial effects following chronic OXT treatment in this ASD model. Yet, additional experiments are needed to expand on this hypothesis. This information, nonetheless, is of paramount importance for understanding the pharmacological effect of a drug such as OXT in healthy and disease state and may pave the way for designing a more effective treatment.

5.1.4. Progresses in neurobiology of social defeat consequences

Experiments in this thesis also investigated the effects of a mouse model of chronic social defeat on the density of nAChRs $\alpha_4\beta_2^*$, α_7^* and the DA receptors (D_2 Rs) in the brain. Our findings did not show significant changes in nAChR density in brains of social defeat mice but emerging functional electrophysiological and behavioural evidence (Morel *et al.*, 2017) demonstrated the presence of profound neuroadaptations in these systems in the exact SD model. These effects further contribute to a complex nicotine-stress bidirectional interplay via DA cell activity that is likely to have a major impact on mood regulation. Ultimately, by identifying therapeutic targets for mood regulation disorders such as depression with pharmacological interventions, we can potentially restore social interactions suggesting the possibility of normalising specific negative outcomes of traumatic events such as bullying.

Our data is supportive of D_2 R dysregulation but shows that any neuroadaptations of the nAChR system in SD is not due to changes in α_7^* and $\alpha_4\beta_2^*$ nAChR density in the brain. Morel *et al.* (2017) further unravels a complex nicotine-stress bidirectional interplay and identifies α_7^* nAChRs as a promising therapeutic target for stress-related psychiatric disorders. It is further suggested that nicotine can have important impact on mood dysregulation and should be manipulated with caution. This may have implications for smokers or people using nicotine containing e-cigarettes.

5.1.5. Future directions

In this thesis, we investigated different CNS components that are involved in the ASD pathology at transcription level. Despite our findings demonstrating a lack of alterations in MOR binding and transcripts in the *Fmr1* KO ASD model, the *Fmr1* KO mice displayed potent impairment of MOR mediated biochemical effects (see data in Appendix: A-E) clearly suggesting an involvement of the MOR system in autism. Future studies should focus on identifying if alterations in MOR happen at the second messenger level with GTP γ S binding

assays and if changes in release of opioid peptides take place in this ASD model by applying microdialysis in different brain regions. It might be that these changes occur deeper in the signalling cascade, thus primary neuronal cell culture could be employed to study MOR signalling in the *Fmr1*. Approaches of the same type will need to be applied to explore changes in OXT peptide and receptor activity, glutamate receptor activity and vasopressin peptide and receptor activity. These studies will provide a clearer idea if changes in transcripts translate to changes in neurotransmitter release or receptor activity resulting in changes in functional neurotransmission and behaviour. Electrophysiological and optogenetic studies would reveal and refine any functional alterations in these systems in the ASD model.

Our data showed that some comorbid symptoms in ASD were also affected by OXT treatment. Additional experiments should be also used to validate the absence or presence of the beneficial effects of OXT with regards to these. Notably, open field exploration test for assessing anxiety and Y-maze to assess spatial memory in the *Fmr1* KO mice could be used. It should be noted that this thesis focused on anxiety and cognitive impairment as secondary associated symptoms of ASD but there are other associated symptoms, which could also draw our attention, such as increased sensitivity to seizure and aggression that are present in the *Fmr1* KO mice (Frankland *et al.*, 2004; Mineur *et al.*, 2002; Qin, Kang, & Smith, 2005).

Furthermore, it is important to assess males and females separately and consider biological differences, which might characterise each gender exclusively. In this way, variability of the experimental outcomes may be eliminated and reliability in the drug discovery field would be improved. Additionally, side effects would be minimized because a parameter of heterogeneity would be removed furthermore paving the way for more efficacious treatment innovations in these pathologies.

Lastly, translational research studies are crucial to assess if OXT is effective in FXS patients by investigating potential concomitant disruptions in OXT receptors in these patients with ASD-like phenotype. Functional imaging assays such as PET and fMRI could potentially be employed for these assessments.

5.1.6. Limitations

The use of animal models provided us with valuable information that will allow us to furthering our understanding of the neurobiology of ASD, however it must be noted that the use of these is not without their own limitations. ASD is a complex spectrum of disorders with symptoms overlapping with other disorders such as FXS, ADHD, Tourette's syndrome, and unclear aetiology. These clearly create a burden in investigating the neurobiological basis of ASD. Also, it is possible to precisely control experimental variables when using animal models such as environmental context and social cues, however it is not the case in humans. Significant restrictions might be present when attempting to interpret data from analysis of behavioural symptoms from animals to humans. However, according to Baker, 2011 animals can provide about 80% of translational reliability and, that is sufficient in order to test for treatment (Baker, 2011). Also, our study had limitations that did not allow us to provide conclusive statements from our data. The low sample size of (n=3) was not sufficient to provide us with statistical power when assessing for significant changes among the experimental groups. The reason for that limitation was due to time- and cost- effective approaches that we were bound to take. Additionally, the use of males and females in all qRT-PCR experiments might have increased variability in our data and normalise for effects on sex differences, thus any effect that could be sex-dependent and would drive data in opposite directions could have been masked and we are not being able to observe. Finally, due to high costs of the qRT-PCR experiments we only assessed mRNA levels of several candidate genes implicated in ASD, but did not assess changes on their respective proteins.

This has provided us with the limitation of reaching inconclusive statements in regard to interpretation of altered mRNA levels of several genes.

5.1.7. Concluding remarks

Collectively, the results described in this thesis show that social impairment is reversed upon acute and chronic OXT treatment and importantly, prolonged OXT administration maintains efficacy in respect of core symptomatology of ASD. Considering the significant benefits of OXT treatment in an impaired oxytocinergic system, the same effects do not apply in a healthy oxytocinergic system. Given both the negatives and beneficial effects seen after chronic OXT treatment in our experiments, a deeper understanding is provided on the long-term therapeutic use for the treatment of autism. We further provide evidence for the neurobiological basis of ASD, which is implicating dysregulation of opioid, OXT, AVP, glutamate and DA systems in the *Fmr1* KO mouse model, and undoubtedly warrants additional experiments in order to identify underlying mechanisms.

6. References

- aan het Rot, M., Mathew, S. J. and Charney, D. S. (2009) 'Neurobiological mechanisms in major depressive disorder.', *CMAJ: Canadian Medical Association journal = journal de l'Association medicale canadienne*. Canadian Medical Association, 180(3), pp. 305–13.
- Abrahams, B. and Geschwind, D. (2008) 'Advances in autism genetics: on the threshold of a new neurobiology', *Nature Reviews Genetics*, 9(5), pp. 341–355.
- Adams, B. J. and Vogelaar, A. T. (2005) 'Nutritional abnormalities in autism and effects of nutritional supplementation', *ASA's 36th National Conference on Autism Spectrum Disorders*. Nashville, TN.
- Adolphs, R., Gosselin, F., Buchanan, T. W., Tranel, D., Schyns, P. and Damasio, A. R. (2005) 'A mechanism for impaired fear recognition after amygdala damage', *Nature*. Nature Publishing Group, 433(7021), pp. 68–72.
- Adolphs, R., Tranel, D. and Buchanan, T. W. (2005) 'Amygdala damage impairs emotional memory for gist but not details of complex stimuli', *Nature Neuroscience*, 8(4), pp. 512–8.
- Al-Ayadhi, L. Y. (2005) 'Altered oxytocin and vasopressin levels in autistic children in Central Saudi Arabia', *Neurosciences (Riyadh)*, 10(1), pp. 47–50.
- Al-Hasani, R. and Bruchas, M. R. (2011) 'Molecular mechanisms of opioid receptor-dependent signaling and behavior', *Anesthesiology*, 115(6), pp. 1363–1381.
- Alarcón, M., Cantor, R. M., Liu, J., Gilliam, T. C., Geschwind, D. H. and Autism Genetic Research Exchange Consortium, D. H. (2002) 'Evidence for a language quantitative trait locus on chromosome 7q in multiplex autism families.', *American Journal of Human Genetics*, 70(1), pp. 60–71.
- Alasaari, J., Lagus, M., Ollila, H., Toivola, A., Kivimäki, M., Vahtera, J., Kronholm, E., Härmä, M., Puttonen, S. and Paunio, T. (2012) 'Environmental Stress Affects DNA Methylation of a CpG Rich Promoter Region of Serotonin Transporter Gene in a Nurse Cohort', *PLoS ONE*. Edited by L. Chiariotti, 7(9), p. e45813.
- Albelda, N. and Joel, D. (2012) 'Animal models of obsessive-compulsive disorder: Exploring pharmacology and neural substrates', *Neuroscience & Biobehavioral Reviews*, 36(1), pp. 47–63.
- Albuquerque, E. X., Barnard, E. A., Porter, C. W. and Warnick, J. E. (1974) 'The density of acetylcholine receptors and their sensitivity in the postsynaptic membrane of muscle endplates.', *Proceedings of the National Academy of Sciences of the United States of America*, 71(7), pp. 2818–22.
- Albuquerque, E. X., Pereira, E. F., Alkondon, M. and Rogers, S. W. (2009) 'Mammalian nicotinic acetylcholine receptors: from structure to function', *Physiological Reviews*, 89(1), pp. 73–120.
- Albuquerque, E. X., Pereira, E. F., Castro, N. G., Alkondon, M., Reinhardt, S., Schröder, H. and Maelicke, A. (1995) 'Nicotinic receptor function in the mammalian central nervous system.', *Annals of the New York Academy of Sciences*, 757, pp. 48–72.
- Alcorn, J. L., Green, C. E., Schmitz, J., Lane, S. D. and Lane, S. D. (2015) 'Effects of oxytocin on aggressive responding in healthy adult men.', *Behavioural Pharmacology*, 26(8), pp. 798–804.
- Aldred, S., Moore, K. M., Fitzgerald, M. and Waring, R. H. (2003) 'Plasma amino acid levels in children with autism and their families', *Journal of Autism and Developmental Disorders*,

33(1), pp. 93–97.

Alexander, R. D. (1974) 'The Evolution of Social Behavior', *Annual Review of Ecology and Systematics*, 5(1), pp. 325–383.

Alkondon, M. and Albuquerque, E. X. (2005) 'Nicotinic receptor subtypes in rat hippocampal slices are differentially sensitive to desensitization and early in vivo functional up-regulation by nicotine and to block by bupropion.', *The Journal of Pharmacology and Experimental Therapeutics*, 313(2), pp. 740–750.

Allen, J., Fonagy, P. and Bateman, A. (2008) *Mentalizing in clinical practise*. First. Washington: American Psychiatric Press.

Allen, N. B. and Badcock, P. B. T. (2003) 'The Social Risk Hypothesis of Depressed Mood: Evolutionary, Psychosocial, and Neurobiological Perspectives.', *Psychological Bulletin*, 129(6), pp. 887–913.

Almeida, D. M., Wethington, E. and Kessler, R. C. (2002) 'The Daily Inventory of Stressful Events: An Interview-Based Approach for Measuring Daily Stressors', *Assessment*, 9(1), pp. 41–55.

Alvarez, J. A. and Emory, E. (2006) 'Executive Function and the Frontal Lobes: A Meta-Analytic Review', *Neuropsychology Review*, 16(1), pp. 17–42.

Aman, M. G. and Langworthy, K. S. (2000) 'Pharmacotherapy for hyperactivity in children with autism and other pervasive developmental disorders', *Journal of Autism and Developmental Disorders*, 30(5), pp. 451–459.

Amaral, D. G., Schumann, C. M. and Nordahl, C. W. (2008) 'Neuroanatomy of autism', *Trends Neuroscience*, 31(3), pp. 137–145.

American Psychiatric Association (2013) 'Diagnostic and Statistical Manual of Mental disorders: DSM-5', in. Washington, DC: American Psychiatric Publishing.

Amodeo, D. A., Jones, J. H., Sweeney, J. A. and Ragozzino, M. E. (2012) 'Differences in BTBR T+ tf/J and C57BL/6J mice on probabilistic reversal learning and stereotyped behaviors', *Behavioural Brain Research*, 227(1), pp. 64–72.

Anagnostou, E., Soorya, L., Brian, J., Dupuis, A., Mankad, D., Smile, S. and Jacob, S. (2014) 'Intranasal oxytocin in the treatment of autism spectrum disorders: a review of literature and early safety and efficacy data in youth', *Brain Research*, 1580, pp. 188–198.

Anagnostou, E., Soorya, L., Brian, J., Dupuis, A., Mankad, D., Smile, S. and Jacob, S. (2014) 'Intranasal oxytocin in the treatment of autism spectrum disorders: A review of literature and early safety and efficacy data in youth', *Brain Research*, 1580, pp. 188–198.

Anagnostou, E., Soorya, L., Chaplin, W., Bartz, J., Halpern, D., Wasserman, S., Wang, A. T., Pepa, L., Tanel, N., Kushki, A. and Hollander, E. (2012) 'Intranasal oxytocin versus placebo in the treatment of adults with autism spectrum disorders: a randomized controlled trial', *Molecular Autism*, 3(1), p. 16.

Andari, E., Duhamel, J.-R., Zalla, T., Herbrecht, E., Leboyer, M. and Sirigu, A. (2010) 'Promoting social behavior with oxytocin in high-functioning autism spectrum disorders.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(9), pp. 4389–94.

Anderson, R. I., Lopez, M. F. and Becker, H. C. (2016) 'Stress-Induced Enhancement of Ethanol Intake in C57BL/6J Mice with a History of Chronic Ethanol Exposure: Involvement of Kappa Opioid Receptors', *Frontiers in Cellular Neuroscience*. Frontiers, 10, p. 45.

Aoki, Y., Yahata, N., Watanabe, T., Takano, Y., Kawakubo, Y., Kuwabara, H., Iwashiro, N.,

- Natsubori, T., Inoue, H., Suga, M., Takao, H., Sasaki, H., Gono, W., Kunimatsu, A., Kasai, K. and Yamasue, H. (2014) 'Oxytocin improves behavioural and neural deficits in inferring others' social emotions in autism', *Brain*, 137(Pt 11), pp. 3073–3086.
- Arakawa, H., Arakawa, K., Blanchard, D. C. and Blanchard, R. J. (2008) 'A new test paradigm for social recognition evidenced by urinary scent marking behavior in C57BL/6J mice', *Behavioural Brain Research*, 190(1), pp. 97–104.
- Arking, D. E., Cutler, D. J., Brune, C. W., Teslovich, T. M., West, K., Ikeda, M., Rea, A., Guy, M., Lin, S., Cook, E. H. and Chakravarti, A. (2008) 'A Common Genetic Variant in the Neurexin Superfamily Member CNTNAP2 Increases Familial Risk of Autism', *The American Journal of Human Genetics*, 82(1), pp. 160–164.
- Aronson, M., Hagberg, B. and Gillberg, C. (1997) 'Attention deficits and autistic spectrum problems in children exposed to alcohol during gestation: a follow-up study.', *Developmental Medicine and Child Neurology*, 39(9), pp. 583–7.
- Arseneault, L., Bowes, L. and Shakoor, S. (2010) 'Bullying victimization in youths and mental health problems: "Much ado about nothing"?', *Psychological Medicine*, 40(5), p. 717.
- Ascano Jr., M., Mukherjee, N., Bandaru, P., Miller, J. B., Nusbaum, J. D., Corcoran, D. L., Langlois, C., Munschauer, M., Dewell, S., Hafner, M., Williams, Z., Ohler, U. and Tuschl, T. (2012) 'FMRP targets distinct mRNA sequence elements to regulate protein expression', *Nature*, 492(7429), pp. 382–386.
- Ashwood, P. and Van de Water, J. (2004) 'Is autism an autoimmune disease?', *Autoimmunity Reviews*, 3(7–8), pp. 557–562.
- Asperger, H. (1944) 'Die "Autistischen Psychopathen" im Kindesalter', *Archiv für Psychiatrie und Nervenkrankheiten*, 117, pp. 76–136.
- Aupperle, R. L. and Paulus, M. P. (2010) 'Neural systems underlying approach and avoidance in anxiety disorders.', *Dialogues in Clinical Neuroscience*. Les Laboratoires Servier, 12(4), pp. 517–31.
- Avagianou, P.-A. and Zafiropoulou, M. (2008) 'Parental bonding and depression: personality as a mediating factor.', *International Journal of Adolescent Medicine and Health*, 20(3), pp. 261–9.
- Avgustinovich, D. F., Kovalenko, I. L. and Kudryavtseva, N. N. (2005) 'A Model of Anxious Depression: Persistence of Behavioral Pathology', *Neuroscience and Behavioral Physiology*, 35(9), pp. 917–924.
- Bailey, A., Le Couteur, A., Gottesman, I., Bolton, P., Simonoff, E., Yuzda, E. and Rutter, M. (1995) 'Autism as a strongly genetic disorder: evidence from a British twin study', *Psychological Medicine*, 25(1), pp. 63–77.
- Bailey, D. B., Hatton, D. D., Mesibov, G., Ament, N. and Skinner, M. (2000) 'Early development, temperament, and functional impairment in autism and fragile X syndrome.', *Journal of Autism and Developmental Disorders*, 30(1), pp. 49–59.
- Baker, M. (2011) 'Animal models: Inside the minds of mice and men', *Nature*, 475(7354), pp. 123–128.
- Bakermans-Kranenburg, M. J. and van IJzendoorn, M. H. (2013) 'Sniffing around oxytocin: review and meta-analyses of trials in healthy and clinical groups with implications for pharmacotherapy', *Translational Psychiatry*, 3(5), p. e258.
- Bakker, C. E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A. T., Oostra, B. A. and Willemsen, R. (2000) 'Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse', *Experimental Cell Research*,

258(1), pp. 162–170.

Bales, K. L., Perkeybile, A. M., Conley, O. G., Lee, M. H., Guoynes, C. D., Downing, G. M., Yun, C. R., Solomon, M., Jacob, S. and Mendoza, S. P. (2013) 'Chronic Intranasal Oxytocin Causes Long-Term Impairments in Partner Preference Formation in Male Prairie Voles', *Biological Psychiatry*, 74(3), pp. 180–188.

Balin, B. J., Broadwell, R. D., Salzman, M. and El-Kalliny, M. (1986) 'Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey', *Journal of Comparative Neurology*, 251(2), pp. 260–280.

Ballif, B. C., Hornor, S. A., Jenkins, E., Madan-Khetarpal, S., Surti, U., Jackson, K. E., Asamoah, A., Brock, P. L., Gowans, G. C., Conway, R. L., Graham, J. M., Medne, L., Zackai, E. H., Shaikh, T. H., Geoghegan, J., Selzer, R. R., Eis, P. S., Bejjani, B. A. and Shaffer, L. G. (2007) 'Discovery of a previously unrecognized microdeletion syndrome of 16p11.2-p12.2.', *Nature genetics*, 39(9), pp. 1071–3.

Bals-Kubik, R., Ableitner, A., Herz, A. and Shippenberg, T. S. (1993) 'Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats.', *The Journal of Pharmacology and Experimental Therapeutics*, 264(1), pp. 489–95.

Bandelow, B. and Domschke, K. (2015) 'Panic Disorder', in Stein, D. J. and Vythilingum, B. (eds) *Anxiety Disorders and Gender*. Cham, Switzerland: Springer International Publishing.

Bandelow, B. and Michaelis, S. (2015) 'Epidemiology of anxiety disorders in the 21st century.', *Dialogues in Clinical Neuroscience*. Les Laboratoires Servier, 17(3), pp. 327–35.

Bargmann, W. and Scharrer, E. (1951) 'The site of origin of the hormones of the posterior pituitary', *American Scientist*, 39(2), pp. 255–259.

Barik, J., Marti, F., Morel, C., Fernandez, S. P., Lanteri, C., Godeheu, G., Tassin, J. P., Mombereau, C., Faure, P. and Tronche, F. (2013) 'Chronic stress triggers social aversion via glucocorticoid receptor in dopaminergic neurons', *Science*, 339(6117), pp. 332–335.

Barnard, E. A., Coates, V., Dolly, J. O. and Mallick, B. (1977) 'Binding of α 7-bungarotoxin and cholinergic ligands to acetylcholine receptors in the membrane of skeletal muscle', *Cell Biology International Reports*, 1(1), pp. 99–106.

Barnhill, G. P., Tapscott Cook, K., Tebbenkamp, K. and Smith Myles, B. (2002) 'The Effectiveness of Social Skills Intervention Targeting Nonverbal Communication for Adolescents With Asperger Syndrome and Related Pervasive Developmental Delays', *Focus on Autism and Other Developmental Disabilities*. Sage PublicationsSage CA: Los Angeles, CA, 17(2), pp. 112–118.

Baron-Cohen, S., Ashwin, E., Ashwin, C., Tavassoli, T. and Chakrabarti, B. (2009) 'Talent in autism: hyper-systemizing, hyper-attention to detail and sensory hypersensitivity.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. The Royal Society, 364(1522), pp. 1377–83.

Baron-Cohen, S., Leslie, A. M. and Frith, U. (1985) 'Does the autistic child have a theory of mind'?', *Cognition*, 21(1), pp. 37–46.

Baron-Cohen, S., Scahill, V. L., Izaguirre, J., Hornsey, H. and Robertson, M. M. (1999) 'The prevalence of Gilles de la Tourette syndrome in children and adolescents with autism: a large scale study.', *Psychological Medicine*, 29(5), pp. 1151–9.

Bartz, J. A., Zaki, J., Bolger, N., Hollander, E., Ludwig, N. N., Kolevzon, A. and Ochsner, K. N. (2010) 'Oxytocin Selectively Improves Empathic Accuracy', *Psychological Science*, 21(10), pp. 1426–1428.

- Bartz, J. and Hollander, E. (2008) 'Oxytocin and experimental therapeutics in autism spectrum disorders', in *Advances in Vasopressin and Oxytocin — From Genes to Behaviour to Disease*. Elsevier, pp. 451–462.
- Baskerville, T. A. and Douglas, A. J. (2010) 'Dopamine and oxytocin interactions underlying behaviors: potential contributions to behavioral disorders', *CNS Neuroscience & Therapeutics*, 16(3), pp. e92-123.
- Battaglia, A., Doccini, V., Bernardini, L., Novelli, A., Loddo, S., Capalbo, A., Filippi, T. and Carey, J. C. (2013) 'Confirmation of chromosomal microarray as a first-tier clinical diagnostic test for individuals with developmental delay, intellectual disability, autism spectrum disorders and dysmorphic features', *European Journal of Paediatric Neurology*, 17(6), pp. 589–599.
- Baumeister, A. A., Hawkins, M. F. and Uzelac, S. M. (2003) 'The Myth of Reserpine-Induced Depression: Role in the Historical Development of the Monoamine Hypothesis', *Journal of the History of the Neurosciences*, 12(2), pp. 207–220.
- Bauminger, N. (2002) 'The facilitation of social-emotional understanding and social interaction in high-functioning children with autism: intervention outcomes.', *Journal of autism and developmental disorders*, 32(4), pp. 283–98.
- Baxter, A. J., Brugha, T. S., Erskine, H. E., Scheurer, R. W., Vos, T. and Scott, J. G. (2015) 'The epidemiology and global burden of autism spectrum disorders', *Psychological Medicine*, 45(3), pp. 601–613.
- Bear, M. F., Huber, K. M. and Warren, S. T. (2004) 'The mGluR theory of fragile X mental retardation', *Trends in Neurosciences*, 27(7), pp. 370–377.
- Beardsley, P. M., Howard, J. L., Shelton, K. L. and Carroll, F. I. (2005) 'Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats', *Psychopharmacology (Berl)*, 183(1), pp. 118–126.
- Becker, C., Zeau, B., Rivat, C., Blugeot, A., Hamon, M. and Benoliel, J.-J. (2008) 'Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin', *Molecular Psychiatry*, 13(12), pp. 1079–1092.
- Becker, J. A., Clesse, D., Spiegelhalter, C., Schwab, Y., Le Merrer, J. and Kieffer, B. L. (2014) 'Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity', *Neuropsychopharmacology*, 39(9), pp. 2049–2060.
- Beis, D., Holzwarth, K., Flinders, M., Bader, M., Wöhr, M. and Alenina, N. (2015) 'Brain serotonin deficiency leads to social communication deficits in mice.', *Biology Letters*. The Royal Society, 11(3).
- Bellani, M., Calderoni, S., Muratori, F. and Brambilla, P. (2013) 'Brain anatomy of autism spectrum disorders I. Focus on corpus callosum', *Epidemiology and Psychiatric Sciences*, 22, pp. 1–5.
- Belzung, L. S., Vourc'h, P. and Andres, C. (2005) 'Rodent models for autism: A critical review', *Drug Discovery Today: Disease Models*, 2(2), pp. 93–101.
- Benvenuto, A., Moavero, R., Alessandrelli, R., Manzi, B. and Curatolo, P. (2009) 'Syndromic autism: causes and pathogenetic pathways', *World Journal of Pediatrics*, 5(3), pp. 169–176.
- Bernardet, M. and Crusio, W. E. (2006) 'Fmr1 KO mice as a possible model of autistic features', *The Scientific World Journal*, 6, pp. 1164–1176.
- Bernardi, S., Anagnostou, E., Shen, J., Kolevzon, A., Buxbaum, J. D., Hollander, E., Hof, P. R. and Fan, J. (2011) 'In vivo 1H-magnetic resonance spectroscopy study of the attentional

networks in autism', *Brain Research*, 1380, pp. 198–205.

Berry-Kravis, E., Sumis, A., Hervey, C., Nelson, M., Porges, S. W., Weng, N., Weiler, I. J. and Greenough, W. T. (2008) 'Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome', *Journal of Developmental & Behavioral Pediatrics*, 29(4), pp. 293–302.

Berton, O., Aguerre, S., Sarrieau, A., Mormede, P. and Chaouloff, F. (1998) 'Differential effects of social stress on central serotonergic activity and emotional reactivity in Lewis and spontaneously hypertensive rats.', *Neuroscience*, 82(1), pp. 147–59.

Berton, O. and Nestler, E. J. (2006) 'New approaches to antidepressant drug discovery: beyond monoamines', *Nature Reviews Neuroscience*, 7(2), pp. 137–151.

Bethea, T. C. and Sikich, L. (2007) 'Early pharmacological treatment of autism: a rationale for developmental treatment', *Biological Psychiatry*, 61(4), pp. 521–537.

Bhattacharya, A., Kaphzan, H., Alvarez-Dieppa, A. C., Murphy, J. P., Pierre, P. and Klann, E. (2012) 'Genetic removal of p70 S6 kinase 1 corrects molecular, synaptic, and behavioral phenotypes in fragile X syndrome mice', *Neuron*, 76(2), pp. 325–337.

Bicknell, R. J., Chapman, C. and Leng, G. (1985) 'Effects of opioid agonists and antagonists on oxytocin and vasopressin release in vitro', *Neuroendocrinology*, 41(2), pp. 142–148.

Bielsky, I. F., Hu, S. B., Szegda, K. L., Westphal, H. and Young, L. J. (2004) 'Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice', *Neuropsychopharmacology*, 29(3), pp. 483–493.

Björklund, A., Lindvall, O. and Björklund, A. (1984) 'Dopamine-containing systems in the CNS.', in Björklund A, H. T. (ed.) *Handbook of Chemical Neuroanatomy: Classical Transmitter in the Rat*. Amsterdam: Elsevier, pp. 55–122.

Björkqvist, K. (2001) 'Social defeat as a stressor in humans', *Physiology & Behavior*. Elsevier, 73(3), pp. 435–442.

Blanchard, R. J. and Blanchard, D. C. (1977) 'Aggressive behavior in the rat.', *Behavioral biology*, 21(2), pp. 197–224.

Blatt, G. J. and Fatemi, S. H. (2011) 'Alterations in GABAergic Biomarkers in the Autism Brain: Research Findings and Clinical Implications', *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, 294(10), pp. 1646–1652.

Bleuler, E. (1912) 'The theory of schizophrenic negativism', in *The Journal of Nervous and Mental Disease Publishing Company*. New York.

Bloom, F. (1982) *In: The Biochemical Basis of Neuropharmacology.*, Oxford University. Edited by et al. Cooper JR. New York.

Bobée, S., Mariette, E., Tremblay-Leveau, H. and Caston, J. (2000) 'Effects of early midline cerebellar lesion on cognitive and emotional functions in the rat', *Behavioural Brain Research*, 112(1), pp. 107–117.

Bohus, B., Kovacs, G. L. and de Wied, D. (1978) 'Oxytocin, vasopressin and memory: opposite effects on consolidation and retrieval processes', *Brain Research*, 157(2), pp. 414–417.

Van Bokhoven, P., Oomen, C. A., Hoogendijk, W. J. G., Smit, A. B., Lucassen, P. J. and Spijker, S. (2011) 'Reduction in hippocampal neurogenesis after social defeat is long-lasting and responsive to late antidepressant treatment', *European Journal of Neuroscience*, 33(10), pp. 1833–1840.

Born, J., Lange, T., Kern, W., McGregor, G. P., Bickel, U. and Fehm, H. L. (2002) 'Sniffing

neuropeptides: a transnasal approach to the human brain', *Nature Neuroscience*, 5(6), pp. 514–516.

Boso, M., Emanuele, E., Politi, P., Pace, A., Arra, M., Ucelli di Nemi, S. and Barale, F. (2007) 'Reduced Plasma Apelin Levels in Patients with Autistic Spectrum Disorder', *Archives of Medical Research*, 38(1), pp. 70–74.

Bostrom, C. A., Majaess, N. M., Morch, K., White, E., Eadie, B. D. and Christie, B. R. (2015) 'Rescue of NMDAR-dependent synaptic plasticity in Fmr1 knock-out mice', *Cerebral Cortex*, 25(1), pp. 271–279.

Boucher, J. (2012) 'Research Review: Structural language in autistic spectrum disorder - characteristics and causes', *Journal of Child Psychology and Psychiatry*, 53(3), pp. 219–233.

Bouvard, M. P., Leboyer, M., Launay, J. M., Recasens, C., Plumet, M. H., Waller-Perotte, D., Tabuteau, F., Bondoux, D., Dugas, M., Lensing, P. and et al. (1995) 'Low-dose naltrexone effects on plasma chemistries and clinical symptoms in autism: a double-blind, placebo-controlled study', *Psychiatry research*, 58(3), pp. 191–201.

Bowling, N. and Beehr, T. (2006) 'Workplace harassment from the victim's perspective: A theoretical model and meta-analysis.', *Journal of Applied Psychology*, 91(5), pp. 998–1012.

Bradbury, A. F., Smyth, D. G. and Snell, C. R. (1976) 'Biosynthetic origin and receptor conformation of methionine enkephalin', *Nature*, 260(5547), pp. 165–166.

Brady, K. T., Killeen, T. K., Brewerton, T. and Lucerini, S. (2000) 'Comorbidity of psychiatric disorders and posttraumatic stress disorder.', *The Journal of clinical psychiatry*, 61 Suppl 7, pp. 22–32.

Breier, A., Kelsoe, J. R., Kirwin, P. D., Beller, S. A., Wolkowitz, O. M. and Pickar, D. (1988) 'Early parental loss and development of adult psychopathology.', *Archives of general psychiatry*, 45(11), pp. 987–993.

Breslau, N., Kilbey, M. and Andreski, P. (1991) 'Nicotine dependence, major depression, and anxiety in young adults.', *Archives of general psychiatry*, 48(12), pp. 1069–74.

Brielmaier, J., Matteson, P. G., Silverman, J. L., Senerth, J. M., Kelly, S., Genestine, M., Millonig, J. H., DiCicco-Bloom, E. and Crawley, J. N. (2012) 'Autism-Relevant Social Abnormalities and Cognitive Deficits in Engrailed-2 Knockout Mice', *PLoS ONE*. Edited by G. Chapouthier, 7(7), p. e40914.

Britt, J. P. and McGehee, D. S. (2008) 'Presynaptic Opioid and Nicotinic Receptor Modulation of Dopamine Overflow in the Nucleus Accumbens', *Journal of Neuroscience*, 28(7), pp. 1672–1681.

Brodin, E. S. (2007) 'BALB/c mice: low sociability and other phenotypes that may be relevant to autism', *Behavioral Brain Research*, 176(1), pp. 53–65.

Brown, R. W. B., Collins, A. C., Lindstrom, J. M. and Whiteaker, P. (2007) 'Nicotinic $\alpha 5$ subunit deletion locally reduces high-affinity agonist activation without altering nicotinic receptor numbers', *Journal of Neurochemistry*, 103(1), pp. 204–215.

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., Jin, X., Feng, Y., Wilkinson, K. D., Keene, J. D., Darnell, R. B. and Warren, S. T. (2001) 'Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome', *Cell*, 107(4), pp. 477–487.

Bruchas, M. R., Land, B. B. and Chavkin, C. (2010) 'The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors', *Brain Research*, 1314, pp. 44–55.

- Bruijnzeel, A. W. (2009) 'kappa-Opioid receptor signaling and brain reward function.', *Brain Research Reviews*, 62(1), pp. 127–46.
- Bruins, J., Hijman, R. and Van Ree, J. M. (1992) 'Effect of a single dose of des-glycinamide-[Arg8]vasopressin or oxytocin on cognitive processes in young healthy subjects.', *Peptides*, 13(3), pp. 461–8.
- Buccafusco, J. J., Beach, J. W., Terry, A. V and Jr. (2009) 'Desensitization of nicotinic acetylcholine receptors as a strategy for drug development.', *The Journal of pharmacology and experimental therapeutics*. American Society for Pharmacology and Experimental Therapeutics, 328(2), pp. 364–70.
- Buescher, A. V. S., Cidav, Z., Knapp, M., Mandell, D. S., M, M., SC, M. and D, G. (2014) 'Costs of Autism Spectrum Disorders in the United Kingdom and the United States', *JAMA Pediatrics*. Stationery Office, London, England, 168(8), p. 721.
- Buie, T., Campbell, D. B., Fuchs 3rd, G. J., Furuta, G. T., Levy, J., Vandewater, J., Whitaker, A. H., Atkins, D., Bauman, M. L., Beaudet, A. L., Carr, E. G., Gershon, M. D., Hyman, S. L., Jirapinyo, P., Jyonouchi, H., Kooros, K., Kushak, R., Levitt, P., Levy, S. E., Lewis, J. D., Murray, K. F., Natowicz, M. R., Sabra, A., Wershil, B. K., Weston, S. C., Zeltzer, L. and Winter, H. (2010) 'Evaluation, diagnosis, and treatment of gastrointestinal disorders in individuals with ASDs: a consensus report', *Pediatrics*, 125 Suppl, pp. S1-18.
- Burbach, J. P., Luckman, S. M., Murphy, D. and Gainer, H. (2001) 'Gene regulation in the magnocellular hypothalamo-neurohypophysial system', *Physiological Reviews*, 81(3), pp. 1197–1267.
- Butler, M. G., Allen, A., Singh, D. N., Carpenter, N. J. and Hall, B. D. (1988) 'Photoanthropometric analysis of individuals with the fragile X syndrome.', *American Journal of Medical Genetics*, 30(1–2), pp. 165–8.
- Buwalda, B., Geerdink, M., Vidal, J. and Koolhaas, J. M. (2011) 'Social behavior and social stress in adolescence: A focus on animal models', *Neuroscience & Biobehavioral Reviews*, 35(8), pp. 1713–1721.
- Byrne, R. W. and Corp, N. (2004) 'Neocortex size predicts deception rate in primates', *Proceedings of the Royal Society B: Biological Sciences*, 271(1549), pp. 1693–1699.
- Caldarone, B. J., Harrist, A., Cleary, M. A., Beech, R. D., King, S. L. and Picciotto, M. R. (2004) 'High-affinity nicotinic acetylcholine receptors are required for antidepressant effects of amitriptyline on behavior and hippocampal cell proliferation', *Biological Psychiatry*, 56(9), pp. 657–664.
- Caldwell, H. K., Lee, H. J., Macbeth, A. H. and Young 3rd, W. S. (2008) 'Vasopressin: behavioral roles of an "original" neuropeptide', *Progress in Neurobiology*, 84(1), pp. 1–24.
- Campbell, D. B., Datta, D., Jones, S. T., Batey Lee, E., Sutcliffe, J. S., Hammock, E. A. and Levitt, P. (2011) 'Association of oxytocin receptor (OXTR) gene variants with multiple phenotype domains of autism spectrum disorder', *Journal of Neurodevelopmental Disorders*, 3(2), pp. 101–112.
- Campbell, M., Anderson, L. T., Small, A. M., Adams, P., Gonzalez, N. M. and Ernst, M. (1993) 'Naltrexone in autistic children: behavioral symptoms and attentional learning', *Journal of the American Academy of Child and Adolescent Psychiatry*, 32(6), pp. 1283–1291.
- Campbell, M., Overall, J. E., Small, A. M., Sokol, M. S., Spencer, E. K., Adams, P., Foltz, R. L., Monti, K. M., Perry, R., Nobler, M. and et al. (1989) 'Naltrexone in autistic children: an acute open dose range tolerance trial', *Journal of the American Academy of Child and*

Adolescent Psychiatry, 28(2), pp. 200–206.

Can, A., Dao, D. T., Arad, M., Terrillion, C. E., Piantadosi, S. C. and Gould, T. D. (2012) 'The mouse forced swim test.', *Journal of Visualized Experiments*. MyJoVE Corporation, 59, p. e3638.

Can, A., Dao, D. T., Terrillion, C. E., Piantadosi, S. C., Bhat, S. and Gould, T. D. (2012) 'The tail suspension test.', *Journal of Visualized Experiments*. MyJoVE Corporation, 59, p. e3769.

Canales, J. J. and Graybiel, A. M. (2000) 'A measure of striatal function predicts motor stereotypy', *Nature Neuroscience*, 3(4), pp. 377–383.

Cannell, J. J. (2008) 'Autism and vitamin D', *Medical Hypotheses*, 70(4), pp. 750–759.

Cao, J.-L., Covington, H. E., Friedman, A. K., Wilkinson, M. B., Walsh, J. J., Cooper, D. C., Nestler, E. J. and Han, M.-H. (2010) 'Mesolimbic Dopamine Neurons in the Brain Reward Circuit Mediate Susceptibility to Social Defeat and Antidepressant Action', *Journal of Neuroscience*, 30(49), pp. 16453–16458.

Carlezon, W. A., Béguin, C., DiNieri, J. A., Baumann, M. H., Richards, M. R., Todtenkopf, M. S., Rothman, R. B., Ma, Z., Lee, D. Y.-W. and Cohen, B. M. (2006) 'Depressive-Like Effects of the kappa-opioid Receptor Agonist Salvinorin A on Behavior and Neurochemistry in Rats', *Journal of Pharmacology and Experimental Therapeutics*, 316(1), pp. 440–447.

Carlson, G. C. (2012) 'Glutamate receptor dysfunction and drug targets across models of autism spectrum disorders.', *Pharmacology Biochemistry and Behavior*, 100(4), pp. 850–4.

Carlsson, M. L. (1998) 'Hypothesis: Is infantile autism a hypoglutamatergic disorder? Relevance of glutamate – serotonin interactions for pharmacotherapy', *Journal of Neural Transmission*, 105(4), p. 525.

Carroll, F. I. and Carlezon, W. A. (2013) 'Development of κ Opioid Receptor Antagonists', *Journal of Medicinal Chemistry*, 56(6), pp. 2178–2195.

Carson, D. S., Garner, J. P., Hyde, S. A., Libove, R. A., Berquist, S. W., Hornbeak, K. B., Jackson, L. P., Sumiyoshi, R. D., Howerton, C. L., Hannah, S. L., Partap, S., Phillips, J. M., Hardan, A. Y. and Parker, K. J. (2015) 'Arginine Vasopressin Is a Blood-Based Biomarker of Social Functioning in Children with Autism', *PLOS ONE*. Edited by K. Suzuki. Western Psychological Services, 10(7), p. e0132224.

Carter, C., Meckes, L., Pritchard, L., Swensen, S., Wittman, P. P. and Velde, B. (2004) 'The Friendship Club: an after-school program for children with Asperger syndrome.', *Family & Community Health*, 27(2), pp. 143–50.

Carter, C. S. (2003) 'Developmental consequences of oxytocin', *Physiology & Behavior*, 79(3), pp. 383–397.

Carter, C. S., Boone, E. M., Pournajafi-Nazarloo, H. and Bales, K. L. (2009) 'Consequences of early experiences and exposure to oxytocin and vasopressin are sexually dimorphic', *Developmental Neuroscience*, 31(4), pp. 332–341.

Carter, C. S., Pournajafi-Nazarloo, H., Kramer, K. M., Ziegler, T. E., White-Traut, R., Bello, D. and Schwertz, D. (2007) 'Oxytocin: behavioral associations and potential as a salivary biomarker', *Annals of the New York Academy of Sciences*, 1098, pp. 312–322.

Carter, C. S., Williams, J. R., Witt, D. M. and Insel, T. R. (1992) 'Oxytocin and social bonding.', *Annals of the New York Academy of Sciences*, 652, pp. 204–11.

Caspi, A., Hariri, A. R., Holmes, A., Uher, R. and Moffitt, T. E. (2010) 'Genetic Sensitivity to the Environment: The Case of the Serotonin Transporter Gene and Its Implications for Studying Complex Diseases and Traits', *American Journal of Psychiatry*, 167(5), pp. 509–

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A. and Poulton, R. (2003) 'Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene', *Science*, 301(5631), pp. 386–389.

Caston, J., Yon, E., Mellier, D., Godfrey, H. P., Delhay-bouchaud, N. and Mariani, J. (1998) 'An animal model of autism: behavioural studies in the GS guinea-pig', *European Journal of Neuroscience*, 10(8), pp. 2677–2684.

Cazzullo, A. G., Musetti, M. C., Musetti, L., Bajo, S., Sacerdote, P. and Panerai, A. (1999) 'Beta-endorphin levels in peripheral blood mononuclear cells and long-term naltrexone treatment in autistic children', *European Neuropsychopharmacology*, 9(4), pp. 361–366.

Chabout, J., Serreau, P., Ey, E., Bellier, L., Aubin, T., Bourgeron, T. and Granon, S. (2012) 'Adult Male Mice Emit Context-Specific Ultrasonic Vocalizations That Are Modulated by Prior Isolation or Group Rearing Environment', *PLoS ONE*. Edited by G. Casadesus, 7(1), p. e29401.

Chaidez, V., Hansen, R. L. and Hertz-Picciotto, I. (2014) 'Gastrointestinal problems in children with autism, developmental delays or typical development.', *Journal of Autism and Developmental Disorders*, 44(5), pp. 1117–27.

Changeux, J.-P., Marubio, L. M., Arroyo-Jimenez, M. del M., Cordero-Erausquin, M., Léna, C., Novère, N. Le, D'Exaerde, A. de K., Huchet, M. and Damaj, M. I. (1999) 'Reduced antinociception in mice lacking neuronal nicotinic receptor subunits.', *Nature*, 398(6730), pp. 805–810.

Chao, H.-T., Chen, H., Samaco, R. C., Xue, M., Chahrour, M., Yoo, J., Neul, J. L., Gong, S., Lu, H.-C., Heintz, N., Ekker, M., Rubenstein, J. L. R., Noebels, J. L., Rosenmund, C. and Zoghbi, H. Y. (2010) 'Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes', *Nature*, 468(7321), pp. 263–269.

Charles, R., Sakurai, T., Takahashi, N., Elder, G. A., Gama Sosa, M. A., Young, L. J. and Buxbaum, J. D. (2014) 'Introduction of the human AVPR1A gene substantially alters brain receptor expression patterns and enhances aspects of social behavior in transgenic mice', *Disease Models & Mechanisms*, 7(8), pp. 1013–1022.

Chaudhury, D., Walsh, J. J., Friedman, A. K., Juarez, B., Ku, S. M., Koo, J. W., Ferguson, D., Tsai, H. C., Pomeranz, L., Christoffel, D. J., Nectow, A. R., Ekstrand, M., Domingos, A., Mazei-Robison, M. S., Mouzon, E., Lobo, M. K., Neve, R. L., Friedman, J. M., Russo, S. J., Deisseroth, K., Nestler, E. J. and Han, M. H. (2013) 'Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons', *Nature*, 493(7433), pp. 532–536.

Chavez, B., Chavez-Brown, M. and Rey, J. A. (2006) 'Psychiatry: Role of Risperidone in Children with Autism Spectrum Disorder', *Annals of Pharmacotherapy*, 40(5), pp. 909–916.

Cheh, M. A., Millonig, J. H., Roselli, L. M., Ming, X., Jacobsen, E., Kamdar, S. and Wagner, G. C. (2006) 'En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder', *Brain Research*, 1116(1), pp. 166–176.

Chen, J., Yu, S., Fu, Y. and Li, X. (2014) 'Synaptic proteins and receptors defects in autism spectrum disorders.', *Frontiers in Cellular Neuroscience*. Frontiers Media SA, 8, p. 276.

Chevallier, C., Kohls, G., Troiani, V., Brodtkin, E. S. and Schultz, R. T. (2012) 'The social motivation theory of autism', *Trends in Cognitive Sciences*, 16(4), pp. 231–239.

Chez, M. G., Aimonovitch, M., Buchanan, T., Mrazek, S. and Tremb, R. J. (2004) 'Treating autistic spectrum disorders in children: utility of the cholinesterase inhibitor rivastigmine tartrate.', *Journal of Child Neurology*, 19(3), pp. 165–9.

- Chez, M. G., Burton, Q., Dowling, T., Chang, M., Khanna, P. and Kramer, C. (2007) 'Memantine as Adjunctive Therapy in Children Diagnosed With Autistic Spectrum Disorders: An Observation of Initial Clinical Response and Maintenance Tolerability', *Journal of Child Neurology*, 22(5), pp. 574–579.
- Di Chiara, G. and Imperato, A. (1988) 'Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats', *Journal of Pharmacology and Experimental Therapeutics*, 244(3), pp. 1067–1080.
- Chmielewski, W. X. and Beste, C. (2015) 'Action control processes in autism spectrum disorder--insights from a neurobiological and neuroanatomical perspective', *Prog Neurobiol*, 124, pp. 49–83.
- Choleris, E., Gustafsson, J. A., Korach, K. S., Muglia, L. J., Pfaff, D. W. and Ogawa, S. (2003) 'An estrogen-dependent four-gene micronet regulating social recognition: a study with oxytocin and estrogen receptor-alpha and -beta knockout mice', *Proceedings of the National Academy of Sciences of the United States of America*, 100(10), pp. 6192–6197.
- Chow, H. S., Chen, Z. and Matsuura, G. T. (1999) 'Direct transport of cocaine from the nasal cavity to the brain following intranasal cocaine administration in rats', *Journal of Pharmaceutical Sciences*, 88(8), pp. 754–758.
- Chung, S., Kim, H. J., Kim, H. J., Choi, S. H., Cho, J. H., Cho, Y. H., Kim, D.-H. and Shin, K. H. (2014) 'Desipramine and citalopram attenuate pretest swim-induced increases in prodynorphin immunoreactivity in the dorsal bed nucleus of the stria terminalis and the lateral division of the central nucleus of the amygdala in the forced swimming test', *Neuropeptides*, 48(5), pp. 273–280.
- Cinque, C., Pondiki, S., Oddi, D., Di Certo, M. G., Marinelli, S., Troisi, A., Moles, A. and D'Amato, F. R. (2012) 'Modeling socially anhedonic syndromes: genetic and pharmacological manipulation of opioid neurotransmission in mice', *Translational Psychiatry*, 2, p. e155.
- Coghlan, S., Horder, J., Inkster, B., Mendez, M. A., Murphy, D. G. and Nutt, D. J. (2012) 'GABA system dysfunction in autism and related disorders: From synapse to symptoms', *Neuroscience & Biobehavioral Reviews*, 36(9), pp. 2044–2055.
- Colombo, S. F., Mazzo, F., Pistillo, F. and Gotti, C. (2013) 'Biogenesis, trafficking and up-regulation of nicotinic ACh receptors', *Biochemical Pharmacology*, 86(8), pp. 1063–1073.
- Comery, T. A., Harris, J. B., Willems, P. J., Oostra, B. A., Irwin, S. A., Weiler, I. J. and Greenough, W. T. (1997) 'Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits', *Proceedings of the National Academy of Sciences of the United States of America*, 94(10), pp. 5401–5404.
- Condren, R. M., O'Neill, A., Ryan, M. C. M., Barrett, P. and Thakore, J. H. (2002) 'HPA axis response to a psychological stressor in generalised social phobia', *Psychoneuroendocrinology*, 27(6), pp. 693–703.
- Conti, F., Sertic, S., Reversi, A. and Chini, B. (2009) 'Intracellular trafficking of the human oxytocin receptor: evidence of receptor recycling via a Rab4/Rab5 "short cycle"', *American Journal of Physiology - Endocrinology and Metabolism*, 296(3), pp. E532–42.
- Courchesne, E., Campbell, K. and Solso, S. (2011) 'Brain growth across the life span in autism: Age-specific changes in anatomical pathology', *Brain Research*, 1380, pp. 138–145.
- Courchesne, E., Karns, C. M., Davis, H. R., Ziccardi, R., Carper, R. A., Tigue, Z. D., Chisum, H. J., Moses, P., Pierce, K., Lord, C., Lincoln, A. J., Pizzo, S., Schreibman, L., Haas, R. H., Akshoomoff, N. A. and Courchesne, R. Y. (2001) 'Unusual brain growth patterns in early life

in patients with autistic disorder: an MRI study', *Neurology*, 57(2), pp. 245–254.

Courchesne, E., Redcay, E. and Kennedy, D. P. (2004) 'The autistic brain: birth through adulthood', *Current Opinion in Neurology*, 17(4), pp. 489–496.

Crawford, L. K., Rahman, S. F. and Beck, S. G. (2013) 'Social Stress Alters Inhibitory Synaptic Input to Distinct Subpopulations of Raphe Serotonin Neurons', *ACS Chemical Neuroscience*. American Chemical Society, 4(1), pp. 200–209.

Crawley, J. N. (1985) 'Exploratory behavior models of anxiety in mice.', *Neuroscience and Biobehavioral Reviews*, 9(1), pp. 37–44.

Crawley, J. N. (2004) 'Designing mouse behavioral tasks relevant to autistic-like behaviors', *Mental Retardation and Developmental Disabilities Research Reviews*, 10(4), pp. 248–258.

Cryan, J. F. and Sweeney, F. F. (2011) 'The age of anxiety: role of animal models of anxiolytic action in drug discovery', *British Journal of Pharmacology*, 164(4), pp. 1129–1161.

Cummings, C., Caporino, N. and Kendall, P. (2014) 'Comorbidity of anxiety and depression in children and adolescents: 20 years after.', *Psychological Bulletin*, 140(3), pp. 816–845.

Curatolo, P. and Bombardieri, R. (2007) 'Tuberous sclerosis', in *Handbook of Clinical Neurology*, pp. 129–151.

Curenton, S. M., Justice, L. M., A., R. M., G., B. K., D., C. and S., S. C. (2004) 'African American and Caucasian Preschoolers' Use of Decontextualized Language', *Language Speech and Hearing Services in Schools*. Paul H. Brookes, Baltimore, 35(3), p. 240.

Curtis, J. and Wang, Z. (2005) 'Ventral tegmental area involvement in pair bonding in male prairie voles', *Physiology & Behavior*, 86(3), pp. 338–346.

Czéh, B., Michaelis, T., Watanabe, T., Frahm, J., de Biurrun, G., van Kampen, M., Bartolomucci, A. and Fuchs, E. (2001) 'Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(22), pp. 12796–801.

Czéh, B., Müller-Keuker, J. I. H., Rygula, R., Abumaria, N., Hiemke, C., Domenici, E. and Fuchs, E. (2007) 'Chronic Social Stress Inhibits Cell Proliferation in the Adult Medial Prefrontal Cortex: Hemispheric Asymmetry and Reversal by Fluoxetine Treatment', *Neuropsychopharmacology*, 32(7), pp. 1490–1503.

D'Aquila, P. S., Collu, M., Gessa, G. L. and Serra, G. (2000) 'The role of dopamine in the mechanism of action of antidepressant drugs.', *European Journal of Pharmacology*, 405(1–3), pp. 365–73.

Dadds, M. R., MacDonald, E., Cauchi, A., Williams, K., Levy, F. and Brennan, J. (2014) 'Nasal oxytocin for social deficits in childhood autism: A randomized controlled trial', *Journal of Autism and Developmental Disorders*, 44(3), pp. 521–531.

Dahlhaus, R. and El-Husseini, A. (2010) 'Altered neuroligin expression is involved in social deficits in a mouse model of the fragile X syndrome', *Behavioural Brain Research*, 208(1), pp. 96–105.

Dalley, J. W. and Everitt, B. J. (2009) 'Dopamine receptors in the learning, memory and drug reward circuitry', *Seminars in Cell & Developmental Biology*, 20(4), pp. 403–410.

Daly, J. W. (2005) 'Nicotinic agonists, antagonists, and modulators from natural sources', *Cellular and Molecular Neurobiology*, pp. 513–552.

Damaj, M. I. (2007) 'Nicotinic regulation of calcium/calmodulin-dependent protein kinase II activation in the spinal cord.', *The Journal of pharmacology and experimental therapeutics*.

American Society for Pharmacology and Experimental Therapeutics, 320(1), pp. 244–9.

Dani, J. a and Bertrand, D. (2007) 'Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system.', *Annual Review of Pharmacology and Toxicology*, 47, pp. 699–729.

Darnell, J. C., Van Driesche, S. J., Zhang, C., Hung, K. Y., Mele, A., Fraser, C. E., Stone, E. F., Chen, C., Fak, J. J., Chi, S. W., Licatalosi, D. D., Richter, J. D. and Darnell, R. B. (2011) 'FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism', *Cell*, 146(2), pp. 247–261.

Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T. and Darnell, R. B. (2001) 'Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function', *Cell*, 107(4), pp. 489–499.

Davids, J. R., Hagerman, R. J. and Eilert, R. E. (1990) 'Orthopaedic aspects of fragile-X syndrome.', *The Journal of Bone and Joint Surgery. American Volume*, 72(6), pp. 889–96.

Davidson, R. J., Putnam, K. M. and Larson, C. L. (2000) 'Dysfunction in the neural circuitry of emotion regulation--a possible prelude to violence.', *Science (New York, N.Y.)*, 289(5479), pp. 591–4.

Davies, D. L. and Shepherd, M. (1955) 'Reserpine in the treatment of anxious and depressed patients', *The Lancet*. Elsevier, 266(6881), pp. 117–120.

Dávila-García, M. I., Musachio, J. L., Perry, D. C., Xiao, Y., Horti, A., London, E. D., Dannals, R. F. and Kellar, K. J. (1997) '[125I]IPH, an epibatidine analog, binds with high affinity to neuronal nicotinic cholinergic receptors.', *The Journal of Pharmacology and Experimental Therapeutics*, 282(1), pp. 445–51.

Davis, E., Fennoy, I., Laraque, D., Kanem, N., Brown, G. and Mitchell, J. (1992) 'Autism and developmental abnormalities in children with perinatal cocaine exposure.', *Journal of the National Medical Association*. National Medical Association, 84(4), pp. 315–9.

Davis, L. L., Clark, D. M., Kramer, G. L., Moeller, F. G. and Petty, F. (1999) 'D-fenfluramine challenge in posttraumatic stress disorder.', *Biological Psychiatry*, 45(7), pp. 928–30.

Dayanithi, G., Sabatier, N. and Widmer, H. (2000) 'Intracellular calcium signalling in magnocellular neurones of the rat supraoptic nucleus: understanding the autoregulatory mechanisms.', *Experimental Physiology*, 85 Spec No, p. 75S–84S.

Demyttenaere, K., Bruffaerts, R., Posada-Villa, J., Gasquet, I., Kovess, V., Lepine, J. P., Angermeyer, M. C., Bernert, S., de Girolamo, G., Morosini, P., Polidori, G., Kikkawa, T., Kawakami, N., Ono, Y., Takeshima, T., Uda, H., Karam, E. G., Fayyad, J. A., Karam, A. N., Mneimneh, Z. N., Medina-Mora, M. E., Borges, G., Lara, C., de Graaf, R., Ormel, J., Gureje, O., Shen, Y., Huang, Y., Zhang, M., Alonso, J., Haro, J. M., Vilagut, G., Bromet, E. J., Gluzman, S., Webb, C., Kessler, R. C., Merikangas, K. R., Anthony, J. C., Von Korff, M. R., Wang, P. S., Brugha, T. S., Aguilar-Gaxiola, S., Lee, S., Heeringa, S., Pennell, B.-E., Zaslavsky, A. M., Ustun, T. B., Chatterji, S. and WHO World Mental Health Survey Consortium (2004) 'Prevalence, severity, and unmet need for treatment of mental disorders in the World Health Organization World Mental Health Surveys.', *JAMA*, 291(21), pp. 2581–90.

Devitt, N. M., Gallagher, L. and Reilly, R. B. (2015) 'Autism Spectrum Disorder (ASD) and Fragile X Syndrome (FXS): Two Overlapping Disorders Reviewed through Electroencephalography-What Can be Interpreted from the Available Information?', *Brain Sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 5(2), pp. 92–117.

Devost, D., Wrzal, P. and Zingg, H. H. (2008) 'Oxytocin receptor signalling', *Progress in*

Brain Research, 170, pp. 167–176.

DeVries, A. C., Young 3rd, W. S. and Nelson, R. J. (1997) 'Reduced aggressive behaviour in mice with targeted disruption of the oxytocin gene', *Journal of Neuroendocrinology*, 9(5), pp. 363–368.

Devys, D., Lutz, Y., Rouyer, N., Bellocq, J. P. and Mandel, J. L. (1993) 'The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation', *Nature Genetics*, 4(4), pp. 335–340.

Diaz, A., Ruiz, F., Florez, J., Pazos, A. and Hurle, M. A. (1995) 'Regulation of dihydropyridine-sensitive Ca^{++} channels during opioid tolerance and supersensitivity in rats', *Journal of Pharmacology and Experimental Therapeutics*, 274(3), pp. 1538–1544.

DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S. R., Schmitz, C., Schultz, R. T., Crawley, J. and Young, L. J. (2006) 'The developmental neurobiology of autism spectrum disorder', *Journal of Neuroscience*, 26(26), pp. 6897–6906.

Diergaarde, L., Gerrits, M. A., Brouwers, J. P. and van Ree, J. M. (2005) 'Early amygdala damage disrupts performance on medial prefrontal cortex-related tasks but spares spatial learning and memory in the rat', *Neuroscience*, 130(3), pp. 581–590.

Dineley, K. T., Pandya, A. A. and Yakel, J. L. (2015) 'Nicotinic ACh receptors as therapeutic targets in CNS disorders.', *Trends in Pharmacological Sciences*, 36(2), pp. 96–108.

Diwan, A., Castine, M., Pomerleau, C. S., Meador-Woodruff, J. H. and Dalack, G. W. (1998) 'Differential prevalence of cigarette smoking in patients with schizophrenic vs mood disorders', *Schizophrenia Research*, 33(1), pp. 113–118.

Djurić, V. J., Dunn, E., Overstreet, D. H., Dragomir, A. and Steiner, M. (1999) 'Antidepressant Effect of Ingested Nicotine in Female Rats of Flinders Resistant and Sensitive Lines', *Physiology & Behavior*, 67(4), pp. 533–537.

Dodge, K. A. (1993) 'Social-Cognitive Mechanisms in the Development of Conduct Disorder and Depression', *Annual Review of Psychology*, 44(1), pp. 559–584.

Dolan, R. J. and Fletcher, P. C. (1997) 'Dissociating prefrontal and hippocampal function in episodic memory encoding', *Nature*. Nature Publishing Group, 388(6642), pp. 582–585.

Dolen, G., Osterweil, E., Rao, B. S. S., Smith, G. B., Auerbach, B. D., Chattarji, S., Bear, M. F., Dölen, G., Osterweil, E., Rao, B. S. S., Smith, G. B., Auerbach, B. D., Chattarji, S. and Bear, M. F. (2007) 'Correction of fragile X syndrome in mice', *Neuron*, 56(6).

Domes, G., Heinrichs, M., Glascher, J., Buchel, C., Braus, D. F. and Herpertz, S. C. (2007) 'Oxytocin attenuates amygdala responses to emotional faces regardless of valence', *Biological Psychiatry*, 62(10), pp. 1187–1190.

Domes, G., Heinrichs, M., Michel, A., Berger, C. and Herpertz, S. C. (2007) 'Oxytocin improves "mind-reading" in humans', *Biological Psychiatry*, 61(6), pp. 731–733.

Domes, G., Lischke, A., Berger, C., Grossmann, A., Hauenstein, K., Heinrichs, M. and Herpertz, S. C. (2010) 'Effects of intranasal oxytocin on emotional face processing in women', *Psychoneuroendocrinology*, 35(1), pp. 83–93.

Domes, G., Steiner, A., Porges, S. W. and Heinrichs, M. (2013) 'Oxytocin differentially modulates eye gaze to naturalistic social signals of happiness and anger', *Psychoneuroendocrinology*, 38(7), pp. 1198–1202.

Donaldson, Z. R. and Young, L. J. (2008) 'Oxytocin, vasopressin, and the neurogenetics of sociality', *Science*, 322(5903), pp. 900–904.

Doty and Bromley (2007) 'Cranial nerve I: olfactory nerve', in Goetz, C. (ed.) *Textbook of*

Clinical Neurology. 3rd edn. Philadelphia: W.B. Saunders, pp. 99–112.

Doyle-Thomas, K. A. R., Duerden, E. G., Taylor, M. J., Lerch, J. P., Soorya, L. V., Wang, A. T., Fan, J., Hollander, E. and Anagnostou, E. (2013) 'Effects of age and symptomatology on cortical thickness in autism spectrum disorders', *Research in Autism Spectrum Disorders*, 7(1), pp. 141–150.

Drago, F., Pedersen, C. A., Caldwell, J. D. and Prange Jr., A. J. (1986) 'Oxytocin potently enhances novelty-induced grooming behavior in the rat', *Brain Research*, 368(2), pp. 287–295.

De Dreu, C. K. (2012) 'Oxytocin modulates cooperation within and competition between groups: an integrative review and research agenda', *Hormones and Behavior*, 61(3), pp. 419–428.

Drisdel, R. C. and Green, W. N. (2000) 'Neuronal alpha-bungarotoxin receptors are alpha7 subunit homomers.', *The Journal of neuroscience: the official journal of the Society for Neuroscience*, 20(1), pp. 133–9.

Duerden, E. G., Mak-Fan, K. M., Taylor, M. J. and Roberts, S. W. (2012) 'Regional differences in grey and white matter in children and adults with autism spectrum disorders: an activation likelihood estimate (ALE) meta-analysis', *Autism Research*, 5(1), pp. 49–66.

Dukat, M. and Glennon, R. A. (2003) 'Epibatidine: Impact on nicotinic receptor research', *Cellular and Molecular Neurobiology*, 23(3), pp. 365–378.

Dunbar, R. I. (2009) 'The social brain hypothesis and its implications for social evolution', *Annals of Human Biology*, 36(5), pp. 562–572.

Dunbar, R. I. M. (1992) 'Neocortex size as a constraint on group size in primates', *Journal of Human Evolution*, 22(6), pp. 469–493.

Dunbar, R. I. M. (1998) 'The Social Brain Hypothesis', *Evolutionary Anthropology*, 6(5), pp. 178–190.

Dunbar, R. I. M. and Shultz, S. (2007) 'Evolution in the Social Brain', *Science*, 317(5843), pp. 1344–1347.

Dunn, A. J. and Berridge, C. W. (1990) 'Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses?', *Brain Research Reviews*, 15(2), pp. 71–100.

Durand, C. M., Betancur, C., Boeckers, T. M., Bockmann, J., Chaste, P., Fauchereau, F., Nygren, G., Rastam, M., Gillberg, I. C., Anckarsäter, H., Sponheim, E., Goubran-Botros, H., Delorme, R., Chabane, N., Mouren-Simeoni, M.-C., de Mas, P., Bieth, E., Rogé, B., Héron, D., Burglen, L., Gillberg, C., Leboyer, M. and Bourgeron, T. (2007) 'Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders.', *Nature Genetics*. In press, 39(1), pp. 25–7.

Dwoskin, L. P. and Crooks, P. a (2001) 'Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery.', *The Journal of Pharmacology and Experimental Therapeutics*, 298(2), pp. 395–402.

Dyer, R. A., Butwick, A. J. and Carvalho, B. (2011) 'Oxytocin for labour and caesarean delivery: implications for the anaesthesiologist', *Current Opinion in Anesthesiology*, 24(3), pp. 255–261.

Dykens, E. M., Sutcliffe, J. S. and Levitt, P. (2004) 'Autism and 15q11-q13 disorders: Behavioral, genetic, and pathophysiological issues', *Mental Retardation and Developmental Disabilities Research Reviews*, 10(4), pp. 284–291.

- Eastham, H. M., Lind, R. J., Eastlake, J. L., Clarke, B. S., Towner, P., Reynolds, S. E., Wolstenholme, A. J. and Wonnacott, S. (1998) 'Characterization of a nicotinic acetylcholine receptor from the insect *Manduca sexta*', *European Journal of Neuroscience*, 10(3), pp. 879–889.
- Eberhart, D. E., Malter, H. E., Feng, Y. and Warren, S. T. (1996) 'The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals', *Human Molecular Genetics*, 5(8), pp. 1083–1091.
- Ebstein, R. P., Israel, S., Lerer, E., Uzefovsky, F., Shalev, I., Gritsenko, I., Riebold, M., Salomon, S. and Yirmiya, N. (2009) 'Arginine vasopressin and oxytocin modulate human social behavior', *Annals of the New York Academy of Sciences*, 1167, pp. 87–102.
- Ecker, C., Ginestet, C., Feng, Y., Johnston, P., Lombardo, M. V., Lai, M. C., Suckling, J., Palaniyappan, L., Daly, E., Murphy, C. M., Williams, S. C., Bullmore, E. T., Baron-Cohen, S., Brammer, M., Murphy, D. G. and Consortium, M. A. (2013) 'Brain surface anatomy in adults with autism: the relationship between surface area, cortical thickness, and autistic symptoms', *JAMA Psychiatry*, 70(1), pp. 59–70.
- Edbauer, D., Neilson, J. R., Foster, K. A., Wang, C. F., Seeburg, D. P., Batterton, M. N., Tada, T., Dolan, B. M., Sharp, P. A. and Sheng, M. (2010) 'Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132', *Neuron*, 65(3), pp. 373–384.
- Egashira, N., Tanoue, A., Matsuda, T., Koushi, E., Harada, S., Takano, Y., Tsujimoto, G., Mishima, K., Iwasaki, K. and Fujiwara, M. (2007) 'Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice', *Behavioural Brain Research*, 178(1), pp. 123–127.
- Eglen, R. M. (2005) 'Muscarinic Receptor Subtype Pharmacology and Physiology', *Progress in Medicinal Chemistry*, pp. 105–136.
- Eichler, E. E. and Zimmerman, A. W. (2008) 'A Hot Spot of Genetic Instability in Autism', *New England Journal of Medicine*, 358(7), pp. 737–739.
- Einarsen, S. (2000) 'Harassment and bullying at work: A review of the scandinavian approach', *Aggression and Violent Behavior*, 5(4), pp. 379–401.
- Einarsen, S. and Skogstad, A. (1996) 'Bullying at work: Epidemiological findings in public and private organizations', *European Journal of Work and Organizational Psychology*. Taylor & Francis Group, 5(2), pp. 185–201.
- Elinoff, M., Chafouleas, S. and Sassu, K. (2004) 'Bullying: considerations for defining and intervening in school settings', *Psychology in the Schools*, 41, pp. 887–897.
- Emmerson, P. J., Liu, M. R., Woods, J. H. and Medzihradsky, F. (1994) 'Binding affinity and selectivity of opioids at mu, delta and kappa receptors in monkey brain membranes', *Journal of Pharmacology and Experimental Therapeutics*, 271(3), pp. 1630–1637.
- Engel, K., Bandelow, B., Gruber, O. and Wedekind, D. (2009) 'Neuroimaging in anxiety disorders', *Journal of Neural Transmission*, 116(6), pp. 703–716.
- Engelmann, M., Ebner, K., Landgraf, R., Holsboer, F. and Wotjak, C. T. (1999) 'Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats', *Journal of Neuroendocrinology*, 11(11), pp. 867–872.
- Engelmann, M. and Landgraf, R. (1994) 'Microdialysis administration of vasopressin into the septum improves social recognition in Brattleboro rats', *Physiology & Behavior*, 55(1), pp. 145–149.
- Epperson, C. N., McDougale, C. J. and Price, L. H. (1996) 'Intranasal oxytocin in obsessive-

compulsive disorder', *Biological Psychiatry*, 40(6), pp. 547–549.

Erickson, C. A., Posey, D. J., Stigler, K. A., Mullett, J., Katschke, A. R. and McDougale, C. J. (2007) 'A retrospective study of memantine in children and adolescents with pervasive developmental disorders', *Psychopharmacology*, 191(1), pp. 141–147.

Erickson, C. A., Veenstra-Vanderweele, J. M., Melmed, R. D., McCracken, J. T., Ginsberg, L. D., Sikich, L., Scahill, L., Cherubini, M., Zarevics, P., Walton-Bowen, K., Carpenter, R. L., Bear, M. F., Wang, P. P. and King, B. H. (2014) 'STX209 (Arbaclofen) for Autism Spectrum Disorders: An 8-Week Open-Label Study', *Journal of Autism and Developmental Disorders*, 44(4), pp. 958–964.

Ermisch, A., Barth, T., Ruhle, H. J., Skopkova, J., Hrbas, P. and Landgraf, R. (1985) 'On the blood-brain barrier to peptides: accumulation of labelled vasopressin, DesGlyNH₂-vasopressin and oxytocin by brain regions', *Endocrinol Exp*, 19(1), pp. 29–37.

Ernst, M., Zametkin, A. J., Matochik, J. A., Pascualvaca, D. and Cohen, R. M. (1997) 'Low medial prefrontal dopaminergic activity in autistic children', *Lancet*, 350(9078), p. 638.

Eronen, M., Angermeyer, M. C. and Schulze, B. (1998) 'The psychiatric epidemiology of violent behaviour.', *Social Psychiatry and Psychiatric Epidemiology*, 33 Suppl 1, pp. S13-23.

Estes, A., Shaw, D. W., Sparks, B. F., Friedman, S., Giedd, J. N., Dawson, G., Bryan, M. and Dager, S. R. (2011) 'Basal ganglia morphometry and repetitive behavior in young children with autism spectrum disorder', *Autism Research*, 4(3), pp. 212–220.

Faravelli, C., Lo Sauro, C., Godini, L., Lelli, L., Benni, L., Pietrini, F., Lazzeretti, L., Talamba, G. A., Fioravanti, G. and Ricca, V. (2012) 'Childhood stressful events, HPA axis and anxiety disorders.', *World Journal of Psychiatry*. Baishideng Publishing Group Inc, 2(1), pp. 13–25.

Farmer, A. E. and McGuffin, P. (2003) 'Humiliation, loss and other types of life events and difficulties: a comparison of depressed subjects, healthy controls and their siblings.', *Psychological Medicine*, 33(7), pp. 1169–75.

Fatemi, S. H. (2008) 'The hyperglutamatergic hypothesis of autism', *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 32(3), p. 911.

Fatemi, S. H., Folsom, T. D., Reutiman, T. J. and Thuras, P. D. (2009) 'Expression of GABA(B) receptors is altered in brains of subjects with autism', *Cerebellum*, 8(1), pp. 64–69.

Fava, M. (1998) 'Depression with anger attacks.', *The Journal of Clinical Psychiatry*, 59 Suppl 1, pp. 18–22.

Fehm-Wolfsdorf, G., Born, J., Voigt, K. and Fehm HL. (1984) 'Human memory and neurohypophyseal hormones: opposite effects of vasopressin and oxytocin. - PubMed - NCBI', *Psychoneuroendocrinology*, 9(3), pp. 285–92.

Feifel, D., Macdonald, K., Cobb, P. and Minassian, A. (2012) 'Adjunctive intranasal oxytocin improves verbal memory in people with schizophrenia', *Schizophrenia Research*, 139(1–3), pp. 207–210.

Feifel, D., Macdonald, K., Nguyen, A., Cobb, P., Warlan, H., Galangue, B., Minassian, A., Becker, O., Cooper, J., Perry, W., Lefebvre, M., Gonzales, J. and Hadley, A. (2010) 'Adjunctive intranasal oxytocin reduces symptoms in schizophrenia patients', *Biological Psychiatry*, 68(7), pp. 678–680.

Feingold, D., Weiser, M., Rehm, J. and Lev-Ran, S. (2015) 'The association between cannabis use and mood disorders: A longitudinal study', *Journal of Affective Disorders*, 172, pp. 211–218.

Ferguson, J. N., Aldag, J. M., Insel, T. R. and Young, L. J. (2001) 'Oxytocin in the medial

amygdala is essential for social recognition in the mouse', *Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 21(20), pp. 8278–8285.

Ferguson, J. N., Young, L. J., Hearn, E. F., Matzuk, M. M., Insel, T. R. and Winslow, J. T. (2000) 'Social amnesia in mice lacking the oxytocin gene', *Nature Genetics*, 25(3), pp. 284–288.

Fernandes, C., Hoyle, E., Dempster, E., Schalkwyk, L. C. and Collier, D. A. (2006) 'Performance deficit of $\alpha 7$ nicotinic receptor knockout mice in a delayed matching-to-place task suggests a mild impairment of working/episodic-like memory', *Genes, Brain and Behavior*, 5(6), pp. 433–440.

Fernández-Guasti, A., Fiedler, J., Herrera, L. and Handa, R. (2012) 'Sex, Stress, and Mood Disorders: At the Intersection of Adrenal and Gonadal Hormones', *Hormone and Metabolic Research*, 44(8), pp. 607–618.

Ferrier, B. M., Kennett, D. J. and Devlin, M. C. (1980) 'Influence of oxytocin on human memory processes.', *Life sciences*, 27(24), pp. 2311–7.

Fertuck, H. C. and Salpeter, M. M. (1974) 'Localization of acetylcholine receptor by 125I-labeled alpha-bungarotoxin binding at mouse motor endplates.', *Proceedings of the National Academy of Sciences of the United States of America*, 71(4), pp. 1376–8.

Fett, A.-K. J., Viechtbauer, W., Dominguez, M.-G., Penn, D. L., van Os, J. and Krabbendam, L. (2011) 'The relationship between neurocognition and social cognition with functional outcomes in schizophrenia: A meta-analysis', *Neuroscience & Biobehavioral Reviews*, 35(3), pp. 573–588.

Fichtner, C. G., Arora, R. C., O'Connor, F. L. and Crayton, J. W. (1994) 'Platelet paroxetine binding and fluoxetine pharmacotherapy in posttraumatic stress disorder: preliminary observations on a possible predictor of clinical treatment response.', *Life Sciences*, 54(3), p. PL39-44.

Filipek, P. A., Juranek, J., Nguyen, M. T., Cummings, C. and Gargus, J. J. (2004) 'Relative carnitine deficiency in autism.', *Journal of Autism and Developmental Disorders*, 34(6), pp. 615–23.

Fineberg, N. A., Haddad, P. M., Carpenter, L., Gannon, B., Sharpe, R., Young, A. H., Joyce, E., Rowe, J., Wellsted, D., Nutt, D. J. and Sahakian, B. J. (2013) 'The size, burden and cost of disorders of the brain in the UK.', *Journal of Psychopharmacology (Oxford, England)*. SAGE Publications, 27(9), pp. 761–70.

Finlay, B. L. and Darlington, R. B. (1995) 'Linked regularities in the development and evolution of mammalian brains.', *Science (New York, N.Y.)*, 268(5217), pp. 1578–84.

Fish, E. W., Krouse, M. C., Stringfield, S. J., Diberto, J. F., Robinson, J. E. and Malanga, C. J. (2013) 'Changes in sensitivity of reward and motor behavior to dopaminergic, glutamatergic, and cholinergic drugs in a mouse model of fragile X syndrome', *PLoS One*, 8(10), p. e77896.

Flinn, M. V., Quinlan, R. J., Decker, S. A., Turner, M. T. and England, B. G. (1996) 'Male-female differences in effects of parental absence on glucocorticoid stress response', *Human Nature*, 7(2), pp. 125–162.

Flores, C. M., DeCamp, R. M., Kilo, S., Rogers, S. W. and Hargreaves, K. M. (1996) 'Neuronal nicotinic receptor expression in sensory neurons of the rat trigeminal ganglion: demonstration of $\alpha 3\beta 4$, a novel subtype in the mammalian nervous system.', *Journal of Neuroscience: The Official Journal of The Society for Neuroscience*, 16(24), pp. 7892–7901.

Folstein, S. and Rutter, M. (1977) 'Infantile autism: a genetic study of 21 twin pairs', *Journal*

of Child Psychology and Psychiatry, 18(4), pp. 297–321.

Fombonne, E. (2009) 'Epidemiology of Pervasive Developmental Disorders', *Pediatric Research*, 65(6), pp. 591–598.

Fournier, K. A., Hass, C. J., Naik, S. K., Lodha, N. and Cauraugh, J. H. (2010) 'Motor Coordination in Autism Spectrum Disorders: A Synthesis and Meta-Analysis', *Journal of Autism and Developmental Disorders*, 40(10), pp. 1227–1240.

Francis, S. M., Kim, S.-J., Kistner-Griffin, E., Guter, S., Cook, E. H. and Jacob, S. (2016) 'ASD and Genetic Associations with Receptors for Oxytocin and Vasopressin—AVPR1A, AVPR1B, and OXTR', *Frontiers in Neuroscience*, 10, p. 516.

Frankland, P. W., Wang, Y., Rosner, B., Shimizu, T., Balleine, B. W., Dykens, E. M., Ornitz, E. M. and Silva, A. J. (2004) 'Sensorimotor gating abnormalities in young males with fragile X syndrome and Fmr1-knockout mice', *Molecular Psychiatry*, 9(4), pp. 417–425.

Frazier, T. W. and Hardan, A. Y. (2009) 'A meta-analysis of the corpus callosum in autism', *Biological Psychiatry*, 66(10), pp. 935–941.

Freeman, S. M., Inoue, K., Smith, A. L., Goodman, M. M. and Young, L. J. (2014) 'The neuroanatomical distribution of oxytocin receptor binding and mRNA in the male rhesus macaque (*Macaca mulatta*)', *Psychoneuroendocrinology*, 45, pp. 128–141.

Friedman, A. K., Walsh, J. J., Juarez, B., Ku, S. M., Chaudhury, D., Wang, J., Li, X., Dietz, D. M., Pan, N., Vialou, V. F., Neve, R. L., Yue, Z. and Han, M.-H. (2014) 'Enhancing Depression Mechanisms in Midbrain Dopamine Neurons Achieves Homeostatic Resilience', *Science*, 344(6181), pp. 313–319.

Frith, C. D. and Frith, U. (2006) 'The Neural Basis of Mentalizing', *Neuron*, 50(4), pp. 531–534.

Fryer, J. and Lukas, R. (1999) 'Noncompetitive functional inhibition at diverse, human nicotinic acetylcholine receptor subtypes by bupropion, phencyclidine, and ibogaine', *Journal of Pharmacology and Experimental Therapeutics*, 288(1), pp. 88–92.

Fuchikami, M., Morinobu, S., Segawa, M., Okamoto, Y., Yamawaki, S., Ozaki, N., Inoue, T., Kusumi, I., Koyama, T., Tsuchiyama, K. and Terao, T. (2011) 'DNA Methylation Profiles of the Brain-Derived Neurotrophic Factor (BDNF) Gene as a Potent Diagnostic Biomarker in Major Depression', *PLoS ONE*. Edited by K. Hashimoto, 6(8), p. e23881.

Fuchs, R. A., Eaddy, J. L., Su, Z.-I. and Bell, G. H. (2007) 'Interactions of the basolateral amygdala with the dorsal hippocampus and dorsomedial prefrontal cortex regulate drug context-induced reinstatement of cocaine-seeking in rats', *European Journal of Neuroscience*, 26(2), pp. 487–498.

Fujiwara, T., Morisaki, N., Honda, Y., Sampei, M. and Tani, Y. (2016) 'Chemicals, Nutrition, and Autism Spectrum Disorder: A Mini-Review.', *Frontiers in Neuroscience*. Frontiers Media SA, 10, p. 174.

Furlan, P. M., DeMartinis, N., Schweizer, E., Rickels, K. and Lucki, I. (2001) 'Abnormal salivary cortisol levels in social phobic patients in response to acute psychological but not physical stress.', *Biological Psychiatry*, 50(4), pp. 254–9.

Furmark, T., Tillfors, M., Garpenstrand, H., Marteinsdottir, I., Långström, B., Oreland, L. and Fredrikson, M. (2004) 'Serotonin transporter polymorphism related to amygdala excitability and symptom severity in patients with social phobia', *Neuroscience Letters*, 362(3), pp. 189–192.

Gadow, K., Devincent, C., Pomeroy, J. and Azizian, A. (2005) 'Comparison of DSM-IV symptoms in elementary school-age children with PDD versus clinic and community

samples', *Autism*, 9(4), pp. 392–415.

Galynker, I. I., Yaseen, Z. S., Katz, C., Zhang, X., Jennings-Donovan, G., Dashnaw, S., Hirsch, J., Mayberg, H., Cohen, L. J. and Winston, A. (2012) 'Distinct but overlapping neural networks subserve depression and insecure attachment', *Social Cognitive and Affective Neuroscience*, 7(8), pp. 896–908.

Gámez-Guadix, M., Orue, I., Smith, P. and Calvete, E. (2013) 'Longitudinal and Reciprocal Relations of Cyberbullying With Depression, Substance Use, and Problematic Internet Use Among Adolescents', *Journal of Adolescent Health*, 53(4), pp. 446–452.

Gandal, M. J., Anderson, R. L., Billingslea, E. N., Carlson, G. C., Roberts, T. P. L. and Siegel, S. J. (2012) 'Mice with reduced NMDA receptor expression: more consistent with autism than schizophrenia?', *Genes, Brain and Behavior*, 11(6), pp. 740–50.

Garber, K. B., Visootsak, J. and Warren, S. T. (2008) 'Fragile X syndrome', *European Journal of Human Genetics*, 16(6), pp. 666–672.

Gardiner, S. M. and Bennett, T. (1983) 'The cardiovascular and renal responses to short-term isolation in Brattleboro rats', *Clinical Science*, 64(4), pp. 377–382.

Gavériaux-Ruff, C. and Kieffer, B. L. (2002) 'Opioid receptor genes inactivated in mice: the highlights.', *Neuropeptides*, 36(2–3), pp. 62–71.

Geenen, V., Adam, F., Baro, V., Mantanus, H., Anseau, M., Timsit-Berthier, M. and Legros, J. J. (1988) 'Inhibitory influence of oxytocin infusion on contingent negative variation and some memory tasks in normal men.', *Psychoneuroendocrinology*, 13(5), pp. 367–75.

Georgiou, P., Zanos, P., Ehteramy, M., Hourani, S., Kitchen, I., Maldonado, R. and Bailey, A. (2015) 'Differential regulation of mGlu5 R and MOPr by priming- and cue-induced reinstatement of cocaine-seeking behaviour in mice', *Addiction Biology*, 20(5), pp. 902–912.

Gerhard, R. (2013) 'Sensory Systems: The Coupling between Brain and Environment', in *The Long Evolution of Brains and Minds*. Springer Netherlands, pp. 165–192.

Gershon, A. A., Vishne, T. and Grunhaus, L. (2007) 'Dopamine D2-Like Receptors and the Antidepressant Response', *Biological Psychiatry*, 61(2), pp. 145–153.

Gershon, S. and Shaw, F. H. (1961) 'Psychiatric sequelae of chronic exposure to organophosphorus insecticides', *Lancet*, pp. 1371–1374.

Gharani, N., Benayed, R., Mancuso, V., Brzustowicz, L. M. and Millonig, J. H. (2004) 'Association of the homeobox transcription factor, ENGRAILED 2, 3, with autism spectrum disorder', *Molecular Psychiatry*, 9(5), pp. 474–484.

Ghaziuddin, M., Ghaziuddin, N. and Greden, J. (2002) 'Depression in persons with autism: implications for research and clinical care.', *Journal of Autism and Developmental Disorders*, 32(4), pp. 299–306.

Gholizadeh, S., Arsenault, J., Xuan, I. C., Pacey, L. K. and Hampson, D. R. (2014) 'Reduced phenotypic severity following adeno-associated virus-mediated Fmr1 gene delivery in fragile X mice', *Neuropsychopharmacology*, 39(13), pp. 3100–3111.

Gillberg, C., Terenius, L., Hagberg, B., Witt-Engerstrom, I. and Eriksson, I. (1990) 'CSF beta-endorphins in childhood neuropsychiatric disorders', *Brain & Development*, 12(1), pp. 88–92.

Gillberg, C., Terenius, L. and Lonnerholm, G. (1985) 'Endorphin activity in childhood psychosis. Spinal fluid levels in 24 cases', *Archives of General Psychiatry*, 42(8), pp. 780–783.

Gillman, P. K. (2007) 'Tricyclic antidepressant pharmacology and therapeutic drug interactions updated', *British Journal of Pharmacology*. Blackwell Publishing Ltd, 151(6), pp.

Gilpin, N. W., Herman, M. A. and Roberto, M. (2015) 'The central amygdala as an integrative hub for anxiety and alcohol use disorders.', *Biological Psychiatry*, 77(10), pp. 859–69.

Gimpl, G. and Fahrenholz, F. (2001) 'The oxytocin receptor system: structure, function, and regulation', *Physiological Reviews*, 81(2), pp. 629–683.

Gladding, C. M., Fitzjohn, S. M. and Molnár, E. (2009) 'Metabotropic glutamate receptor-mediated long-term depression: molecular mechanisms.', *Pharmacological Reviews*. American Society for Pharmacology and Experimental Therapeutics, 61(4), pp. 395–412.

Glassman, A. H., Helzer, J. E., Covey, L. S., Cottler, L. B., Stetner, F., Tipp, J. E. and Johnson, J. (1990) 'Smoking, smoking cessation, and major depression.', *JAMA*, 264(12), pp. 1546–9.

Gogolla, N., LeBlanc, J. J., Quast, K. B., Südhof, T. C., Fagiolini, M. and Hensch, T. K. (2009) 'Common circuit defect of excitatory-inhibitory balance in mouse models of autism', *Journal of Neurodevelopmental Disorders*, 1(2), pp. 172–181.

Goldman, M. B., Gomes, A. M., Carter, C. S. and Lee, R. (2011) 'Divergent effects of two different doses of intranasal oxytocin on facial affect discrimination in schizophrenic patients with and without polydipsia', *Psychopharmacology*, 216(1), pp. 101–110.

Goldman, N., Glej, D. A., Lin, Y.-H. and Weinstein, M. (2010) 'The serotonin transporter polymorphism (5-HTTLPR): allelic variation and links with depressive symptoms', *Depression and Anxiety*, 27(3), pp. 260–269.

Goldstein, M. L. (1974) 'The effect of septal and amygdaloid lesions on the duration of emotionality in the white rat', *Bulletin of the Psychonomic Society*, 4(3), pp. 163–165.

Gong, Z.-L., Luo, C.-M., Wang, L., Shen, L., Wei, F., Tong, R.-J. and Liu, Y. (2013) 'Serum 25-hydroxyvitamin D levels in Chinese children with autism spectrum disorders', *NeuroReport*, 25(1), p. 1.

Gordon, I., Jack, A., Pretzsch, C. M., Vander Wyk, B., Leckman, J. F., Feldman, R. and Pelphrey, K. A. (2016) 'Intranasal Oxytocin Enhances Connectivity in the Neural Circuitry Supporting Social Motivation and Social Perception in Children with Autism', *Scientific Reports*, 6, p. 35054.

Gordon, I., Vander Wyk, B. C., Bennett, R. H., Cordeaux, C., Lucas, M. V., Eilbott, J. A., Zagoory-Sharon, O., Leckman, J. F., Feldman, R. and Pelphrey, K. A. (2013) 'Oxytocin enhances brain function in children with autism.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(52), pp. 20953–8.

Gotti, C. and Clementi, F. (2004) 'Neuronal nicotinic receptors: From structure to pathology', *Progress in Neurobiology*, pp. 363–396.

Gotti, C., Zoli, M. and Clementi, F. (2006) 'Brain nicotinic acetylcholine receptors: native subtypes and their relevance', *Trends in Pharmacological Sciences*, 27(9), pp. 482–491.

Gould, B. R. and Zingg, H. H. (2003) 'Mapping oxytocin receptor gene expression in the mouse brain and mammary gland using an oxytocin receptor-LacZ reporter mouse', *Neuroscience*, 122(1), pp. 155–167.

Goyon, S., Althammer, F., Da Silva Gouveia, M., Eliava, M., Poisbeau, P., Grinevich, V. and Charlet, A. (2017) 'The unexpected level of plasticity of the oxytocinergic system', *European Neuropsychopharmacology*, 27, pp. S26–S27.

Grady, S. R., Moretti, M., Zoli, M., Marks, M. J., Zanardi, A., Pucci, L., Clementi, F. and Gotti, C. (2009) 'Rodent Habenulo-Interpeduncular Pathway Expresses a Large Variety of

Uncommon nAChR Subtypes, But Only the $\alpha 4$ and $\alpha 3 \beta 4$ Subtypes Mediate Acetylcholine Release', *Journal of Neuroscience*, 29(7), pp. 2272–2282.

Green, D., Charman, T., Pickles, A., Chandler, S., Loukas, T., Simonnof, E. and Baird, G. (2009) 'Impairment in movement skills of children with autistic spectrum disorders', *Developmental Medicine & Child Neurology*, 51(4), pp. 311–316.

Green, L., Fein, D., Modahl, C., Feinstein, C., Waterhouse, L. and Morris, M. (2001) 'Oxytocin and autistic disorder: alterations in peptide forms', *Biological Psychiatry*, 50(8), pp. 609–613.

Gregory, S. G., Connelly, J. J., Towers, A. J., Johnson, J., Biscocho, D., Markunas, C. A., Lintas, C., Abramson, R. K., Wright, H. H., Ellis, P., Langford, C. F., Worley, G., Delong, G. R., Murphy, S. K., Cuccaro, M. L., Persico, A. and Pericak-Vance, M. A. (2009) 'Genomic and epigenetic evidence for oxytocin receptor deficiency in autism', *BMC Medicine*, 7, p. 62.

Greimel, E., Nehrkorn, B., Schulte-Rüther, M., Fink, G. R., Nickl-Jockschat, T., Herpertz-Dahlmann, B., Konrad, K. and Eickhoff, S. B. (2013) 'Changes in grey matter development in autism spectrum disorder', *Brain Structure and Function*, 218(4), pp. 929–942.

Grinevich, V., Knobloch-Bollmann, H. S., Eliava, M., Busnelli, M. and Chini, B. (2016) 'Assembling the Puzzle: Pathways of Oxytocin Signaling in the Brain', *Biological Psychiatry*, 79(3), pp. 155–164.

Gross, C. and Bassell, G. J. (2012) 'Excess protein synthesis in FXS patient lymphoblastoid cells can be rescued with a p110 β -selective inhibitor.', *Molecular Medicine (Cambridge, Mass.)*. The Feinstein Institute for Medical Research, 18(1), pp. 336–45.

Gross, C., Hoffmann, A., Bassell, G. J. and Berry-Kravis, E. M. (2015) 'Therapeutic Strategies in Fragile X Syndrome: From Bench to Bedside and Back', *Neurotherapeutics*, 12(3), pp. 584–608.

Gross, C., Nakamoto, M., Yao, X., Chan, C. B., Yim, S. Y., Ye, K., Warren, S. T. and Bassell, G. J. (2010) 'Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile X syndrome', *Journal of Neuroscience*, 30(32), pp. 10624–10638.

Grundschober, Roche and Pharmaceutical, R. (2015) 'Vasopressin blocker improves social deficits in rat autism model', in *2015 Society for Neuroscience Annual Meeting in Chicago*. Spectrum.

Gruss and Braun (2004) 'Age- and region-specific imbalances of basal amino acids and monoamine metabolism in limbic regions of female Fmr1 knock-out mice', *Neurochemistry International*, 45(1), pp. 81–88.

Guastella, A. J., Einfeld, S. L., Gray, K. M., Rinehart, N. J., Tonge, B. J., Lambert, T. J. and Hickie, I. B. (2010) 'Intranasal oxytocin improves emotion recognition for youth with autism spectrum disorders', *Biological Psychiatry*, 67(7), pp. 692–694.

Guastella, A. J., Hickie, I. B., McGuinness, M. M., Otis, M., Woods, E. A., Disinger, H. M., Chan, H. K., Chen, T. F. and Banati, R. B. (2013) 'Recommendations for the standardisation of oxytocin nasal administration and guidelines for its reporting in human research', *Psychoneuroendocrinology*, 38(5), pp. 612–625.

Guastella, A. J., Mitchell, P. B. and Dadds, M. R. (2008) 'Oxytocin increases gaze to the eye region of human faces', *Biological Psychiatry*, 63(1), pp. 3–5.

Gubellini, P., Pisani, A., Centonze, D., Bernardi, G. and Calabresi, P. (2004) 'Metabotropic glutamate receptors and striatal synaptic plasticity: implications for neurological diseases', *Progress in Neurobiology*, 74(5), pp. 271–300.

- Gullander, M., Hogh, A., Hansen, Å., Persson, R., Rugulies, R., Kolstad, H., Thomsen, J., Willert, M., Grynderup, M., Mors, O. and Bonde, J. (2014) 'Exposure to Workplace Bullying and Risk of Depression', *Journal of Occupational and Environmental Medicine*, 56(12), pp. 1258–1265.
- Gunaydin, L. A., Grosenick, L., Finkelstein, J. C., Kauvar, I. V., Fenno, L. E., Adhikari, A., Lammel, S., Mirzabekov, J. J., Airan, R. D., Zalocusky, K. A., Tye, K. M., Anikeeva, P., Malenka, R. C. and Deisseroth, K. (2014) 'Natural neural projection dynamics underlying social behavior', *Cell*, 157(7), pp. 1535–1551.
- Guzmán, Y. F., Tronson, N. C., Jovasevic, V., Sato, K., Guedea, A. L., Mizukami, H., Nishimori, K. and Radulovic, J. (2013) 'Fear-enhancing effects of septal oxytocin receptors', *Nature Neuroscience*, 16(9), pp. 1185–1187.
- Haber, S. N., Kim, K. S., Mailly, P. and Calzavara, R. (2006) 'Reward-related cortical inputs define a large striatal region in primates that interface with associative cortical connections, providing a substrate for incentive-based learning', *Journal of Neuroscience*, 26(32), pp. 8368–8376.
- Haber, S. N. and Knutson, B. (2010) 'The reward circuit: linking primate anatomy and human imaging', *Neuropsychopharmacology*, 35(1), pp. 4–26.
- Hadjikhani, N., Joseph, R. M., Snyder, J. and Tager-Flusberg, H. (2005) 'Anatomical Differences in the Mirror Neuron System and Social Cognition Network in Autism', *Cerebral Cortex*, 16(9), pp. 1276–1282.
- Hagerman, R. J. and Hagerman, P. J. (2002) 'Fragile X syndrome: diagnosis, treatment, and research'. Johns Hopkins University Press, 39(10), p. 540.
- Halgren, C., Kjaergaard, S., Bak, M., Hansen, C., El-Schich, Z., Anderson, C. M., Henriksen, K. F., Hjalgrim, H., Kirchhoff, M., Bijlsma, E. K., Nielsen, M., den Hollander, N. S., Ruivenkamp, C. A., Isidor, B., Le Caignec, C., Zannolli, R., Mucciolo, M., Renieri, A., Mari, F., Anderlid, B. M., Andrieux, J., Dieux, A., Tommerup, N. and Bache, I. (2012) 'Corpus callosum abnormalities, intellectual disability, speech impairment, and autism in patients with haploinsufficiency of ARID1B', *Clinical Genetics*, 82(3), pp. 248–255.
- Hall, S. S., Lightbody, A. A., McCarthy, B. E., Parker, K. J. and Reiss, A. L. (2012) 'Effects of intranasal oxytocin on social anxiety in males with fragile X syndrome', *Psychoneuroendocrinology*, 37(4), pp. 509–518.
- Hallmayer, J., Cleveland, S., Torres, A., Phillips, J., Cohen, B., Torigoe, T., Miller, J., Fedele, A., Collins, J., Smith, K., Lotspeich, L., Croen, L. A., Ozonoff, S., Lajonchere, C., Grether, J. K. and Risch, N. (2011) 'Genetic heritability and shared environmental factors among twin pairs with autism', *Archives of General Psychiatry*, 68(11), pp. 1095–1102.
- Harkness, K. L. and Monroe, S. M. (2006) 'Severe melancholic depression is more vulnerable than non-melancholic depression to minor precipitating life events', *Journal of Affective Disorders*, 91(2–3), pp. 257–263.
- Hasler, G. (2010) 'Pathophysiology of depression: do we have any solid evidence of interest to clinicians?', *World Psychiatry: Official Journal of the World Psychiatric Association (WPA)*. World Psychiatric Association, 9(3), pp. 155–61.
- Hayashida, S., Oka, T., Mera, T. and Tsuji, S. (2010) 'Repeated social defeat stress induces chronic hyperthermia in rats', *Physiology & Behavior*, 101(1), pp. 124–131.
- Hazlett, H. C., Poe, M., Gerig, G., Smith, R. G., Provenzale, J., Ross, A., Gilmore, J. and Piven, J. (2005) 'Magnetic resonance imaging and head circumference study of brain size in autism: birth through age 2 years', *Archives of General Psychiatry*, 62(12), pp. 1366–1376.

- Head, A. M., McGillivray, J. A. and Stokes, M. A. (2014) 'Gender differences in emotionality and sociability in children with autism spectrum disorders', *Molecular Autism*, 5(1), p. 19.
- Heim, C. and Nemeroff, C. B. (2001) 'The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies.', *Biological Psychiatry*, 49(12), pp. 1023–39.
- Heinrichs, M., Meinlschmidt, G., Wippich, W., Ehler, U. and Hellhammer, D. H. (2004) 'Selective amnesic effects of oxytocin on human memory', *Physiology & Behavior*, 83(1), pp. 31–38.
- Heller, H. and Zaimis, E. J. (1949) 'The antidiuretic and oxytocic hormones in the posterior pituitary glands of newborn infants and adults', *Journal of Physiology*, 109(1–2), pp. 162–169.
- Hennessey, M. B. and Moorman, L. (1989) 'Factors influencing cortisol and behavioral responses to maternal separation in guinea pigs.', *Behavioral Neuroscience*, 103(2), pp. 378–85.
- Hennings, E. C., Kiss, J. P. and Vizi, E. S. (1997) 'Nicotinic acetylcholine receptor antagonist effect of fluoxetine in rat hippocampal slices.', *Brain Research*, 759(2), pp. 292–4.
- Hennings, E. C. P., Kiss, J. P., De Oliveira, K., Toth, P. T. and Vizi, E. S. (1999) 'Nicotinic acetylcholine receptor antagonistic activity of monoamine uptake blockers in rat hippocampal slices', *Journal of Neurochemistry*, 73(3), pp. 1043–1050.
- Herman, B. H. and Panksepp, J. (1978) 'Effects of morphine and naloxone on separation distress and approach attachment: evidence for opiate mediation of social affect', *Pharmacology Biochemistry and Behavior*, 9(2), pp. 213–220.
- Hettema, J. M., Neale, M. C. and Kendler, K. S. (2001) 'A Review and Meta-Analysis of the Genetic Epidemiology of Anxiety Disorders', *American Journal of Psychiatry*, 158(10), pp. 1568–1578.
- Hettema, J. M., Neale, M. C., Myers, J. M., Prescott, C. A. and Kendler, K. S. (2006) 'A Population-Based Twin Study of the Relationship Between Neuroticism and Internalizing Disorders', *American Journal of Psychiatry*, 163(5), pp. 857–864.
- Hill, C. A. (1987) 'Affiliation motivation: people who need people ... but in different ways', *Journal of Personality and Social Psychology*, 52(5), pp. 1008–1018.
- Hill, M. N., Helleman, K. G. C., Verma, P., Gorzalka, B. B. and Weinberg, J. (2012) 'Neurobiology of chronic mild stress: parallels to major depression.', *Neuroscience and Biobehavioral Reviews*, 36(9), pp. 2085–117.
- Hirschfeld, R. M. (2000) 'History and evolution of the monoamine hypothesis of depression.', *The Journal of clinical psychiatry*, 61 Suppl 6, pp. 4–6.
- Hofvander, B., Delorme, R., Chaste, P., Nydén, A., Wentz, E., Ståhlberg, O., Herbrecht, E., Stopin, A., Anckarsäter, H., Gillberg, C., Råstam, M. and Leboyer, M. (2009) 'Psychiatric and psychosocial problems in adults with normal-intelligence autism spectrum disorders', *BMC Psychiatry*, 9(1), p. 35.
- Hollander, E., Bartz, J., Chaplin, W., Phillips, A., Sumner, J., Soorya, L., Anagnostou, E. and Wasserman, S. (2007) 'Oxytocin increases retention of social cognition in autism', *Biological Psychiatry*, 61(4), pp. 498–503.
- Hollander, E., Novotny, S., Hanratty, M., Yaffe, R., DeCaria, C. M., Aronowitz, B. R. and Mosovich, S. (2003) 'Oxytocin infusion reduces repetitive behaviors in adults with autistic and Asperger's disorders', *Neuropsychopharmacology*, 28(1), pp. 193–198.

- Hollander, E., Phillips, A., Chaplin, W., Zagursky, K., Novotny, S., Wasserman, S. and Iyengar, R. (2005) 'A Placebo Controlled Crossover Trial of Liquid Fluoxetine on Repetitive Behaviors in Childhood and Adolescent Autism', *Neuropsychopharmacology*, 30(3), pp. 582–589.
- Hollander, E., Soorya, L., Chaplin, W., Anagnostou, E., Taylor, B. P., Ferretti, C. J., Wasserman, S., Swanson, E. and Settapani, C. (2012) 'A Double-Blind Placebo-Controlled Trial of Fluoxetine for Repetitive Behaviors and Global Severity in Adult Autism Spectrum Disorders', *American Journal of Psychiatry*, 169(3), pp. 292–299.
- Hollis, F., Isgor, C. and Kabbaj, M. (2013) 'The consequences of adolescent chronic unpredictable stress exposure on brain and behavior', *Neuroscience*, 249, pp. 232–241.
- Hollis, F. and Kabbaj, M. (2014) 'Social Defeat as an Animal Model for Depression', *ILAR Journal*, 55(2), pp. 221–232.
- Hollis, F., Wang, H., Dietz, D., Gunjan, A. and Kabbaj, M. (2010) 'The effects of repeated social defeat on long-term depressive-like behavior and short-term histone modifications in the hippocampus in male Sprague-Dawley rats', *Psychopharmacology*, 211(1), pp. 69–77.
- Hornig, M., Weissenbock, H., Horscroft, N. and Lipkin, W. I. (1999) 'An infection-based model of neurodevelopmental damage', *Proceedings of the National Academy of Sciences of the United States*, 96(21), pp. 12102–12107.
- Hossain, F. and Ali, M. (2014) 'Relation between Individual and Society', *Open Journal of Social Sciences*, 2(8), pp. 130–137.
- Howlin, P., Magiati, I. and Charman, T. (2009) 'Systematic Review of Early Intensive Behavioral Interventions for Children With Autism', *American Journal on Intellectual and Developmental Disabilities*, 114(1), p. 23.
- Huang, E. and Reichardt, L. (2001) 'Neurotrophins: Roles in Neuronal Development and Function', *Annual Review of Neuroscience*, 24(1), pp. 677–736.
- Huang, H., Michetti, C., Busnelli, M., Managò, F., Sannino, S., Scheggia, D., Giancardo, L., Sona, D., Murino, V., Chini, B., Scattoni, M. L. and Papaleo, F. (2014) 'Chronic and Acute Intranasal Oxytocin Produce Divergent Social Effects in Mice', *Neuropsychopharmacology*, 39(5), pp. 1102–1114.
- Huber, K. M., Gallagher, S. M., Warren, S. T. and Bear, M. F. (2002) 'Altered synaptic plasticity in a mouse model of fragile X mental retardation', *Proceedings of the National Academy of Sciences of the United States*, 99(11), pp. 7746–7750.
- Huguet, G., Ey, E. and Bourgeron, T. (2013) 'The Genetic Landscapes of Autism Spectrum Disorders', *Annual Review of Genomics and Human Genetics*, 14(1), pp. 191–213.
- Hunsaker, M. R. and Kesner, R. P. (2008) 'Evaluating the differential roles of the dorsal dentate gyrus, dorsal CA3, and dorsal CA1 during a temporal ordering for spatial locations task', *Hippocampus*, 18(9), pp. 955–964.
- Hutcheson, D. M., Matthes, H. W., Valjent, E., Sanchez-Blazquez, P., Rodriguez-Diaz, M., Garzon, J., Kieffer, B. L. and Maldonado, R. (2001) 'Lack of dependence and rewarding effects of deltorphin II in mu-opioid receptor-deficient mice', *European Journal of Neuroscience*, 13(1), pp. 153–161.
- El Idrissi, A., Ding, X. H., Scalia, J., Trenkner, E., Brown, W. T. and Dobkin, C. (2005) 'Decreased GABA(A) receptor expression in the seizure-prone fragile X mouse', *Neuroscience Letters*, 377(3), pp. 141–146.
- Illum, L. (2000) 'Transport of drugs from the nasal cavity to the central nervous system', *European Journal of Pharmaceutical Sciences*, 11(1), pp. 1–18.

Inglebret, E., Jones, C. and Pavel, D. M. (2008) 'Integrating American Indian/Alaska Native Culture Into Shared Storybook Intervention', *Language Speech and Hearing Services in Schools*, 39(4), p. 521.

Insel, T. R. (2010) 'The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior', *Neuron*, 65(6), pp. 768–779.

Insel, T. R. and Hulihan, T. J. (1995) 'A gender-specific mechanism for pair bonding: oxytocin and partner preference formation in monogamous voles', *Behavioral Neuroscience*, 109(4), pp. 782–789.

Insel, T. R., O'Brien, D. J. and Leckman, J. F. (1999) 'Oxytocin, vasopressin, and autism: is there a connection?', *Biological Psychiatry*, 45(2), pp. 145–57.

Iossifov, I., Ronemus, M., Levy, D., Wang, Z., Hakker, I., Rosenbaum, J., Yamrom, B., Lee, Y. H., Narzisi, G., Leotta, A., Kendall, J., Grabowska, E., Ma, B., Marks, S., Rodgers, L., Stepansky, A., Troge, J., Andrews, P., Bekritsky, M., Pradhan, K., Ghiban, E., Kramer, M., Parla, J., Demeter, R., Fulton, L. L., Fulton, R. S., Magrini, V. J., Ye, K., Darnell, J. C., Darnell, R. B., Mardis, E. R., Wilson, R. K., Schatz, M. C., McCombie, W. R. and Wigler, M. (2012) 'De novo gene disruptions in children on the autistic spectrum', *Neuron*, 74(2), pp. 285–299.

Ivell, R. and Richter, D. (1984) 'The gene for the hypothalamic peptide hormone oxytocin is highly expressed in the bovine corpus luteum: biosynthesis, structure and sequence analysis', *EMBO Journal*, 3(10), pp. 2351–2354.

Jacob, S., Brune, C. W., Carter, C. S., Leventhal, B. L., Lord, C. and Cook Jr., E. H. (2007) 'Association of the oxytocin receptor gene (OXTR) in Caucasian children and adolescents with autism', *Neuroscience Letters*, 417(1), pp. 6–9.

Jacob, S., Landeros-Weisenberger, A. and Leckman, J. F. (2009) 'Autism spectrum and obsessive-compulsive disorders: OC behaviors, phenotypes and genetics.', *Autism research: official journal of the International Society for Autism Research*, 2(6), pp. 293–311.

Jacquemont, S., Berry-Kravis, E., Hagerman, R., von Raison, F., Gasparini, F., Apostol, G., Ufer, M., Des Portes, V. and Gomez-Mancilla, B. (2014) 'The challenges of clinical trials in fragile X syndrome', *Psychopharmacology (Berl)*, 231(6), pp. 1237–1250.

Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I. C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C., Bourgeron, T. and Paris Autism Research International Sibpair, S. (2003) 'Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism', *Nature Genetics*, 34(1), pp. 27–29.

Jamot, L., Matthes, H. W. D., Simonin, F., Kieffer, B. L. and Roder, J. C. (2003) 'Differential involvement of the mu and kappa opioid receptors in spatial learning.', *Genes, Brain and Behavior*, 2(2), pp. 80–92.

Janowsky, D. S., el-Yousef, M. K. and Davis, J. M. (1974) 'Acetylcholine and depression.', *Psychosomatic medicine*, 36(3), pp. 248–57.

Janowsky, D. S., el-Yousef, M. K., Davis, J. M. and Sekerke, H. J. (1972) 'A cholinergic-adrenergic hypothesis of mania and depression.', *Lancet (London, England)*, 2(7778), pp. 632–5. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/4116781> (Accessed: 24 May 2017).

Jans, L. A. W., Riedel, W. J., Markus, C. R. and Blokland, A. (2007) 'Serotonergic vulnerability and depression: assumptions, experimental evidence and implications', *Molecular Psychiatry*, 12(6), pp. 522–543.

Jansen, L. M., Gispen-de Wied, C. C., Wiegant, V. M., Westenberg, H. G., Lahuis, B. E. and

- van Engeland, H. (2006) 'Autonomic and neuroendocrine responses to a psychosocial stressor in adults with autistic spectrum disorder', *Journal of Autism and Developmental Disorder*, 36(7), pp. 891–899.
- Jarrett, T. M., McMurray, M. S., Walker, C. H. and Johns, J. M. (2006) 'Cocaine treatment alters oxytocin receptor binding but not mRNA production in postpartum rat dams.', *Neuropeptides*, 40(3), pp. 161–7.
- Jensen, A. A., Frølund, B., Liljefors, T. and Krogsaard-Larsen, P. (2005) 'Neuronal Nicotinic Acetylcholine Receptors: Structural Revelations, Target Identifications, and Therapeutic Inspirations', *Journal of Medicinal Chemistry*, 48(15), pp. 4705–4745.
- Jerison, H. J. (1973) 'Review: Evolution of the Brain and Intelligence: Evolution of the Brain and Intelligence by Harry J. Jerison', *Current Anthropology*, pp. 403–426.
- Jiao, Y., Chen, R., Ke, X., Chu, K., Lu, Z. and Herskovits, E. H. (2010) 'Predictive models of autism spectrum disorder based on brain regional cortical thickness', *NeuroImage*, 50(2), pp. 589–599.
- Jin, D., Liu, H. X., Hirai, H., Torashima, T., Nagai, T., Lopatina, O., Shnyder, N. A., Yamada, K., Noda, M., Seike, T., Fujita, K., Takasawa, S., Yokoyama, S., Koizumi, K., Shiraishi, Y., Tanaka, S., Hashii, M., Yoshihara, T., Higashida, K., Islam, M. S., Yamada, N., Hayashi, K., Noguchi, N., Kato, I., Okamoto, H., Matsushima, A., Salmina, A., Munesue, T., Shimizu, N., Mochida, S., Asano, M. and Higashida, H. (2007) 'CD38 is critical for social behaviour by regulating oxytocin secretion', *Nature*, 446(7131), pp. 41–45.
- Jin, P., Zarnescu, D. C., Ceman, S., Nakamoto, M., Mowrey, J., Jongens, T. A., Nelson, D. L., Moses, K. and Warren, S. T. (2004) 'Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway', *Nature Neuroscience*, 7(2), pp. 113–117.
- Jones, S., Sudweeks, S., Yakel, J. L., Jones, S., Sudweeks, S. and Yakel, J. L. (1999) 'Nicotinic receptors in the brain: correlating physiology with function.', *Trends in Neurosciences*. Elsevier, 22(12), pp. 555–61.
- Jones, S. and Yakel, J. L. (1997) 'Functional nicotinic ACh receptors on interneurons in the rat hippocampus', *The Journal of Physiology*, 504(3), pp. 603–610.
- Juvonen, J. and Graham, S. (2014) 'Bullying in Schools: The Power of Bullies and the Plight of Victims', *Annual Review of Psychology*, 65(1), pp. 159–185.
- Kahne, D., Tudorica, A., Borella, A., Shapiro, L., Johnstone, F., Huang, W. and Whitaker-Azmitia, P. M. (2002) 'Behavioral and magnetic resonance spectroscopic studies in the rat hyperserotonergic model of autism', *Physiology & Behavior*, 75(3), pp. 403–410.
- Kalat, J. W. (1978) 'Speculations on similarities between autism and opiate addiction', *Journal of Autism and Childhood Schizophrenia*, 8(4), pp. 477–479.
- Kalin, N. H., Shelton, S. E. and Lynn, D. E. (1995) 'Opiate systems in mother and infant primates coordinate intimate contact during reunion', *Psychoneuroendocrinology*, 20(7), pp. 735–742.
- Kalogjera, L. and Dzepina, D. (2012) 'Management of Smell Dysfunction', *Current Allergy and Asthma Reports*, 12(2), pp. 154–162.
- Kaminsky, E. B., Kaul, V., Paschall, J., Church, D. M., Bunke, B., Kunig, D., Moreno-De-Luca, D., Moreno-De-Luca, A., Mülle, J. G., Warren, S. T., Richard, G., Compton, J. G., Fuller, A. E., Gliem, T. J., Huang, S., Collinson, M. N., Beal, S. J., Ackley, T., Pickering, D. L., Golden, D. M., Aston, E., Whitby, H., Shetty, S., Rossi, M. R., Rudd, M. K., South, S. T., Brothman, A. R., Sanger, W. G., Iyer, R. K., Crolla, J. A., Thorland, E. C., Aradhya, S.,

- Ledbetter, D. H. and Martin, C. L. (2011) 'An evidence-based approach to establish the functional and clinical significance of copy number variants in intellectual and developmental disabilities', *Genetics in Medicine*, 13(9), pp. 777–784.
- Kang, H., Kim, J., Lee, J., Kim, S., Bae, K., Kim, S., Shin, I., Kim, H., Shin, M. and Yoon, J. (2013) 'BDNF promoter methylation and suicidal behavior in depressive patients', *Journal of Affective Disorders*, 151(2), pp. 679–685.
- Kanner, L. (1943) *Autistic disturbances of affective contact*, *Acta Paedopsychiatrica*.
- Kasarpalkar, N. J., Kothari, S. T. and Dave, U. P. (2014) 'Brain-Derived Neurotrophic Factor in children with Autism Spectrum Disorder.', *Annals of Neurosciences*, 21(4), pp. 129–33.
- Kates, W. R., Ikuta, I. and Burnette, C. P. (2009) 'Gyrification patterns in monozygotic twin pairs varying in discordance for autism', *Autism Research*, 2(5), pp. 267–278.
- Kavaliers, M., Colwell, D. D., Choleris, E., Agmo, A., Muglia, L. J., Ogawa, S. and Pfaff, D. W. (2003) 'Impaired discrimination of and aversion to parasitized male odors by female oxytocin knockout mice', *Genes, Brain and Behavior*, 2(4), pp. 220–230.
- Kawamichi, H., Sugawara, S. K., Hamano, Y. H., Makita, K., Kochiyama, T. and Sadato, N. (2016) 'Increased frequency of social interaction is associated with enjoyment enhancement and reward system activation', *Scientific Reports*, 6, p. 24561.
- Kazdoba, T. M., Leach, P. T., Yang, M., Silverman, J. L., Solomon, M. and Crawley, J. N. (2016) 'Translational Mouse Models of Autism: Advancing Toward Pharmacological Therapeutics.', *Current Topics in Behavioral Neurosciences*, 28, pp. 1–52.
- Kazek, B., Huzarska, M., Grzybowska-Chlebowczyk, U., Kajor, M., Ciupińska-Kajor, M., Woś, H. and Marszał, E. (2010) 'Platelet and intestinal 5-HT_{2A} receptor mRNA in autistic spectrum disorders - results of a pilot study.', *Acta neurobiologiae experimentalis*, 70(2), pp. 232–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20628446>.
- Kendler, K. S., Gardner, C. O. and Prescott, C. A. (2002) 'Toward a Comprehensive Developmental Model for Major Depression in Women', *American Journal of Psychiatry*, 159(7), pp. 1133–1145.
- Kendler, K. S., Gardner, C. O. and Prescott, C. A. (2006) 'Toward a Comprehensive Developmental Model for Major Depression in Men', *American Journal of Psychiatry*, 163(1), pp. 115–124.
- Kendler, K. S., Hettema, J. M., Butera, F., Gardner, C. O. and Prescott, C. A. (2003) 'Life Event Dimensions of Loss, Humiliation, Entrapment, and Danger in the Prediction of Onsets of Major Depression and Generalized Anxiety', *Archives of General Psychiatry*, 60(8), p. 789.
- Kendler, K. S., Karkowski, L. M. and Prescott, C. A. (1999) 'Fears and phobias: reliability and heritability.', *Psychological Medicine*, 29(3), pp. 539–53.
- Kendler, K. S., Kessler, R. C., Walters, E. E., MacLean, C., Neale, M. C., Heath, A. C. and Eaves, L. J. (1995) 'Stressful life events, genetic liability, and onset of an episode of major depression in women', *American Journal of Psychiatry*, 152(6), pp. 833–842.
- Kendrick, K. M., Keverne, E. B., Hinton, M. R. and Goode, J. A. (1991) 'Cerebrospinal fluid and plasma concentrations of oxytocin and vasopressin during parturition and vaginocervical stimulation in the sheep', *Brain Research Bulletin*, 26(5), pp. 803–807.
- Kenny, E. M., Cormican, P., Furlong, S., Heron, E., Kenny, G., Fahey, C., Kelleher, E., Ennis, S., Tropea, D., Anney, R., Corvin, A. P., Donohoe, G., Gallagher, L., Gill, M. and Morris, D. W. (2014) 'Excess of rare novel loss-of-function variants in synaptic genes in schizophrenia and autism spectrum disorders', *Molecular Psychiatry*, 19(8), pp. 872–879.

Kern, J. K. and Jones, A. M. (2006) 'Evidence of Toxicity, Oxidative Stress, and Neuronal Insult in Autism', *Journal of Toxicology and Environmental Health, Part B*, 9(6), pp. 485–499.

Kessler, R. C. (1997) 'THE EFFECTS OF STRESSFUL LIFE EVENTS ON DEPRESSION', *Annual Review of Psychology*, 48(1), pp. 191–214.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R. and Walters, E. E. (2005) 'Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication', *Archives of General Psychiatry*, 62(6), p. 593.

Kessler, R. C., McGonagle, K. A., Zhao, S., Nelson, C. B., Hughes, M., Eshleman, S., Wittchen, H. U. and Kendler, K. S. (1994) 'Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey.', *Archives of General Psychiatry*, 51(1), pp. 8–19. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8279933> (Accessed: 28 June 2017).

Kessler, R. C. and Walters, E. E. (1998) 'Epidemiology of DSM-III-R major depression and minor depression among adolescents and young adults in the national comorbidity survey', *Depression and Anxiety*, 7(1), pp. 3–14.

Khalil, A. M., Faghihi, M. A., Modarresi, F., Brothers, S. P. and Wahlestedt, C. (2008) 'A novel RNA transcript with antiapoptotic function is silenced in fragile X syndrome', *PLoS One*, 3(1), p. e1486.

Kidd, S. A., Lachiewicz, A., Barbouth, D., Blitz, R. K., Delahunty, C., McBrien, D., Visootsak, J. and Berry-Kravis, E. (2014) 'Fragile X syndrome: a review of associated medical problems', *Pediatrics*, 134(5), pp. 995–1005. doi: 10.1542/peds.2013-4301.

Killen, J. D., Fortmann, S. P., Schatzberg, A., Hayward, C. and Varady, A. (2003) 'Onset of major depression during treatment for nicotine dependence.', *Addictive behaviors*, 28(3), pp. 461–70.

Kim, J., Szatmari, P., Bryson, S., Streiner, D. and Wilson, F. (2000) 'The Prevalence of Anxiety and Mood Problems among Children with Autism and Asperger Syndrome', *Autism*, 4(2), pp. 117–132.

Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J. and Okayama, H. (1992) 'Structure and expression of a human oxytocin receptor', *Nature*, 356(6369), pp. 526–529.

King, B. H., Hollander, E., Sikich, L., McCracken, J. T., Scahill, L., Bregman, J. D., Donnelly, C. L., Anagnostou, E., Dukes, K., Sullivan, L., Hirtz, D., Wagner, A., Ritz, L. and STAART Psychopharmacology Network, for the S. P. (2009) 'Lack of efficacy of citalopram in children with autism spectrum disorders and high levels of repetitive behavior: citalopram ineffective in children with autism.', *Archives of General Psychiatry*, 66(6), pp. 583–90.

King, M. K. and Jope, R. S. (2013) 'Lithium treatment alleviates impaired cognition in a mouse model of fragile X syndrome.', *Genes, Brain and Behavior*, 12(7), pp. 723–31.

Kirsch, P., Esslinger, C., Chen, Q., Mier, D., Lis, S., Siddhanti, S., Gruppe, H., Mattay, V. S., Gallhofer, B. and Meyer-Lindenberg, A. (2005) 'Oxytocin modulates neural circuitry for social cognition and fear in humans', *Journal of Neuroscience*, 25(49), pp. 11489–11493.

Kistler, J. and Stroud, R. M. (1981) 'Crystalline arrays of membrane-bound acetylcholine receptor.', *Proceedings of the National Academy of Sciences of the United States of America*, 78(6), pp. 3678–82.

Kitchen, I., Slowe, S. J., Matthes, H. W. and Kieffer, B. (1997) 'Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene', *Brain Research*, 778(1), pp. 73–88.

Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J. C., Pariante, C. M.,

- Pace, T. W. W., Mercer, K. B., Mayberg, H. S., Bradley, B., Nemeroff, C. B., Holsboer, F., Heim, C. M., Ressler, K. J., Rein, T. and Binder, E. B. (2012) 'Allele-specific FKBP5 DNA demethylation mediates gene–childhood trauma interactions', *Nature Neuroscience*, 16(1), pp. 33–41.
- Klug, W., Cummings, M., Spencer, C. and Palladino, M. (2014) *Concepts of Genetics + Modified Masteringgenetics Books a La Carte Edition*. Eleventh. Pearson College Div.
- Knickmeyer, R. C., Wheelwright, S. and Baron-Cohen, S. B. (2008) 'Sex-typical Play: Masculinization/Defeminization in Girls with an Autism Spectrum Condition', *Journal of Autism and Developmental Disorders*, 38(6), pp. 1028–1035.
- Knight, R. T. (1996) 'Contribution of human hippocampal region to novelty detection', *Nature*, 383(6597), pp. 256–259.
- Knowles, P. A., Conner, R. L. and Panksepp, J. (1989) 'Opiate effects on social behavior of juvenile dogs as a function of social deprivation', *Pharmacology Biochemistry and Behavior*, 33(3), pp. 533–537.
- Kočovská, E., Andorsdóttir, G., Weihe, P., Halling, J., Fernell, E., Stóra, T., Biskupstø, R., Gillberg, I. C., Shea, R., Billstedt, E., Bourgeron, T., Minnis, H. and Gillberg, C. (2014) 'Vitamin D in the General Population of Young Adults with Autism in the Faroe Islands', *Journal of Autism and Developmental Disorders*, 44(12), pp. 2996–3005.
- Kohn, S. and Asnis, G. (2003) "Aggression in psychiatric disorders", in Totowa, N. H. P. I. (ed.) *Neurobiology of Aggression: Understanding and Preventing Violence*. Mattson M., p. 135.
- Kolevzon, A., Gross, R. and Reichenberg, A. (2007) 'Prenatal and Perinatal Risk Factors for Autism', *Archives of Pediatrics & Adolescent Medicine*, 161(4), p. 326.
- Kong, A., Frigge, M., Masson, G., Besenbacher, S., Sulem, P., Magnusson, G., Gudjonsson, S., Sigurdsson, A., Jonasdottir, A., Jonasdottir, A., Wong, W., Sigurdsson, G., Walters, G., Steinberg, S., Helgason, H., Thorleifsson, G., Gudbjartsson, D., Helgason, A., Magnusson, O., Thorsteinsdottir, U. and Stefansson, K. (2012) 'Rate of de novo mutations and the importance of father's age to disease risk.', *Nature*, 488(7412), pp. 471–5.
- Koolhaas, J. M., De Boer, S. F., De Rutter, A. J., Meerlo, P. and Sgoifo, A. (1997) 'Social stress in rats and mice.', *Acta physiologica Scandinavica. Supplementum*, 640, pp. 69–72.
- Kosaka, H., Okamoto, Y., Munesue, T., Yamasue, H., Inohara, K., Fujioka, T., Anme, T., Orisaka, M., Ishitobi, M., Jung, M., Fujisawa, T. X., Tanaka, S., Arai, S., Asano, M., Saito, D. N., Sadato, N., Tomoda, A., Omori, M., Sato, M., Okazawa, H., Higashida, H. and Wada, Y. (2016) 'Oxytocin efficacy is modulated by dosage and oxytocin receptor genotype in young adults with high-functioning autism: a 24-week randomized clinical trial', *Translational Psychiatry*, 6(8), p. e872. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/27552585>.
- Kosfeld, M., Heinrichs, M., Zak, P. J., Fischbacher, U. and Fehr, E. (2005) 'Oxytocin increases trust in humans', *Nature*, 435(7042), pp. 673–676.
- Kovács, B. and Kéri, S. (2015) 'Off-label intranasal oxytocin use in adults is associated with increased amygdala-cingulate resting-state connectivity', *European Psychiatry*, 30(4), pp. 542–547.
- Kozniowska, E. and Romaniuk, K. (2008) 'Vasopressin in vascular regulation and water homeostasis in the brain.', *Journal of Physiology and Pharmacology: an official Journal of the Polish Physiological Society*, 59 Suppl 8, pp. 109–16.
- Kranz, T. M., Kopp, M., Waltes, R., Sachse, M., Duketis, E., Jarczok, T. A., Degenhardt, F., Gorgen, K., Meyer, J., Freitag, C. M. and Chiocchetti, A. G. (2016) 'Meta-analysis and

association of two common polymorphisms of the human oxytocin receptor gene in autism spectrum disorder', *Autism Research*, 9(10), pp. 1036–1045.

Kreek, M. J. (1996) 'Opiates, opioids and addiction.', *Molecular Psychiatry*, 1(3), pp. 232–54.

Krishnan, V. and Nestler, E. J. (2008) 'The molecular neurobiology of depression.', *Nature*, 455(7215), pp. 894–902.

Kubota, Y., Kimura, T., Hashimoto, K., Tokugawa, Y., Nobunaga, K., Azuma, C., Saji, F. and Murata, Y. (1996) 'Structure and expression of the mouse oxytocin receptor gene.', *Molecular and Cellular Endocrinology*, 124(1–2), pp. 25–32.

Kudo, H. and Dunbar, R. I. M. (2001) 'Neocortex size and social network size in primates', *Animal Behaviour*, 62(4), pp. 711–722.

Kumar, B., Prakash, A., Sewal, R. K., Medhi, B. and Modi, M. (2012) 'Drug therapy in autism: a present and future perspective.', *Pharmacological reports : PR*, 64(6), pp. 1291–304.

Kumsta, R., Hummel, E., Chen, F. S. and Heinrichs, M. (2013) 'Epigenetic regulation of the oxytocin receptor gene: implications for behavioral neuroscience', *Frontiers in Neuroscience*, 7, p. 83.

Kuryatov, A. and Lindstrom, J. (2011) 'Expression of Functional Human $\alpha 2 \beta 3^*$ Acetylcholine Receptors in *Xenopus laevis* Oocytes Achieved through Subunit Chimeras and Concatamers', *Molecular Pharmacology*, 79(1), pp. 126–140.

Labonté, B., Suderman, M., Maussion, G., Navaro, L., Yerko, V., Mahar, I., Bureau, A., Mechawar, N., Szyf, M., Meaney, M. J. and Turecki, G. (2012) 'Genome-wide Epigenetic Regulation by Early-Life Trauma', *Archives of General Psychiatry*, 69(7), pp. 722–31.

Lachiewicz, A. M. and Dawson, D. V (1994) 'Do young boys with fragile X syndrome have macroorchidism?', *Pediatrics*, 93(6 Pt 1), pp. 992–5.

Ladd, P. D., Smith, L. E., Rabaia, N. A., Moore, J. M., Georges, S. A., Hansen, R. S., Hagerman, R. J., Tassone, F., Tapscott, S. J. and Filippova, G. N. (2007) 'An antisense transcript spanning the CGG repeat region of FMR1 is upregulated in premutation carriers but silenced in full mutation individuals', *Human Molecular Genetics*, 16(24), pp. 3174–3187.

Lai, M.-C., Lombardo, M. V and Baron-Cohen, S. (2014) 'Autism', *The Lancet*, 383(9920), pp. 896–910.

Lake, C. R., Ziegler, M. G. and Murphy, D. L. (1977) 'Increased norepinephrine levels and decreased dopamine-beta-hydroxylase activity in primary autism', *Archives of General Psychiatry*, 34(5), pp. 553–556.

Lambert, O. and Bourin, M. (2002) 'SNRIs: mechanism of action and clinical features', *Expert Review of Neurotherapeutics*, 2(6), pp. 849–858.

Landgraf, R., Ermisch, A. and Hess, J. (1979) 'Indications for a brain uptake of labelled vasopressin and oxytocin and the problem of the blood-brain barrier', *Endokrinologie*, 73(1), pp. 77–81.

Landgraf, R. and Neumann, I. D. (2004) 'Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication', *Frontiers in Neuroendocrinology*, 25(3–4), pp. 150–176.

Langen, M., Schnack, H. G., Nederveen, H., Bos, D., Lahuis, B. E., de Jonge, M. V, van Engeland, H. and Durston, S. (2009) 'Changes in the developmental trajectories of striatum in autism', *Biological Psychiatry*, 66(4), pp. 327–333.

Larisch, R., Klimke, A., Vosberg, H., Löffler, S., Gaebel, W. and Müller-Gärtner, H.-W. (1997)

'In Vivo Evidence for the Involvement of Dopamine-D2Receptors in Striatum and Anterior Cingulate Gyrus in Major Depression', *NeuroImage*, 5(4), pp. 251–260.

Larsson, H. J., Eaton, W. W., Madsen, K. M., Vestergaard, M., Olesen, A. V., Agerbo, E., Schendel, D., Thorsen, P. and Mortensen, P. B. (2005) 'Risk Factors for Autism: Perinatal Factors, Parental Psychiatric History, and Socioeconomic Status', *American Journal of Epidemiology*, 161(10), pp. 916–925.

Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M. P., Raynaud, M., Ronce, N., Lemonnier, E., Calvas, P., Laudier, B., Chelly, J., Fryns, J. P., Ropers, H. H., Hamel, B. C., Andres, C., Barthelemy, C., Moraine, C. and Briault, S. (2004) 'X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family', *The American Journal of Human Genetics*, 74(3), pp. 552–557.

Leach, P. T., Hayes, J., Pride, M., Silverman, J. L. and Crawley, J. N. (2016) 'Normal Performance of Fmr1 Mice on a Touchscreen Delayed Nonmatching to Position Working Memory Task.', *eNeuro. Society for Neuroscience*, 3(1), pp. 143–215.

Leblond, C., Heinrich, J., Delorme, R., Proepper, C., Betancur, C., Huguet, G., Konyukh, M., Chaste, P., Ey, E., Rastam, M., Anckarsäter, H., Nygren, G., Gillberg, I., Melke, J., Toro, R., Regnault, B., Fauchereau, F., Mercati, O., Lemièrre, N., Skuse, D., Poot, K., Holt, R., Monaco, A., Järvelä, I., Kantojärvi, K., Vanhala, R., Curran, S., Collier, D., Bolton, P., Chiocchetti, A., Klauck, S., Poustka, F., Freitag, C., Waltes, R., Kopp, M., Duketis, E., Bacchelli, E., Minopoli, F., Ruta, L., Battaglia, A., Mazzone, L., Maestrini, E., Sequeira, A., Oliveira, B., Vicente, A., Oliveira, G., Pinto, D., Scherer, S., Zelenika, D., Delepine, M., Lathrop, M., Bonneau, D., Guinchat, V., Devillard, F., Assouline, B., Mouren, M., Leboyer, M., Gillberg, C., Boeckers, T. and Bourgeron, T. (2012) 'Genetic and Functional Analyses of SHANK2 Mutations Suggest a Multiple Hit Model of Autism Spectrum Disorders', *PLoS Genetics*, 8(2), p. e1002521.

Leboyer, M., Bouvard, M. P. and Dugas, M. (1988) 'Effects on naltrexone on infantile autism', *Lancet*, 1(8587), p. 715.

Leboyer, M., Bouvard, M. P., Launay, J. M., Tabuteau, F., Waller, D., Dugas, M., Kerdelhue, B., Lensing, P. and Panksepp, J. (1992) 'Brief report: a double-blind study of naltrexone in infantile autism', *Journal of Autism and Developmental Disorders*, 22(2), pp. 309–319.

Leboyer, M., Bouvard, M. P., Recasens, C., Philippe, A., Guilloud-Bataille, M., Bondoux, D., Tabuteau, F., Dugas, M., Panksepp, J., Launay, J. M. and et al. (1994) 'Difference between plasma N- and C-terminally directed beta-endorphin immunoreactivity in infantile autism', *The American Journal of Psychiatry*, 151(12), pp. 1797–1801.

Leboyer, M., Philippe, A., Bouvard, M., Guilloud-Bataille, M., Bondoux, D., Tabuteau, F., Feingold, J., Mouren-Simeoni, M. C. and Launay, J. M. (1999) 'Whole blood serotonin and plasma beta-endorphin in autistic probands and their first-degree relatives', *Biological Psychiatry*, 45(2), pp. 158–163.

Lee, C. Y. (1979) 'Recent advances in chemistry and pharmacology of snake toxins.', *Advances in Cytopharmacology*, 3, pp. 1–16.

Lee, H.-J., Lee, M.-S., Kang, R.-H., Kim, H., Kim, S.-D., Kee, B.-S., Kim, Y. H., Kim, Y.-K., Kim, J. B., Yeon, B. K., Oh, K. S., Oh, B.-H., Yoon, J.-S., Lee, C., Jung, H. Y., Chee, I.-S. and Paik, I. H. (2005) 'Influence of the serotonin transporter promoter gene polymorphism on susceptibility to posttraumatic stress disorder', *Depression and Anxiety*, 21(3), pp. 135–139.

Lee, P. R., Brady, D. L., Shapiro, R. A., Dorsa, D. M. and Koenig, J. I. (2005) 'Social Interaction Deficits Caused by Chronic Phencyclidine Administration are Reversed by

Oxytocin', *Neuropsychopharmacology*, 30(10), pp. 1883–1894.

Lee, S., Jeong, J., Kwak, Y. and Park, S. K. (2010) 'Depression research: where are we now?', *Molecular Brain*, 3(1), p. 8.

Lee, V. H. (1988) 'Enzymatic barriers to peptide and protein absorption', *Critical Reviews in Therapeutic Drug Carrier Systems*, 5(2), pp. 69–97.

Léna, I., Matthes, H., Kieffer, B. and Kitchen, I. (2004) 'Quantitative autoradiography of dopamine receptors in the brains of micro-opioid receptor knockout mice.', *Neuroscience letters*, 356(3), pp. 220–4.

Leng, G. and Ludwig, M. (2008) 'Neurotransmitters and peptides: whispered secrets and public announcements', *The Journal of Physiology*, 586(23), pp. 5625–5632.

Leng, G. and Ludwig, M. (2016a) 'Intranasal Oxytocin: Myths and Delusions', *Biological Psychiatry*, 79(3), pp. 243–250.

Leng, G. and Ludwig, M. (2016b) 'Reply to: Intranasal Oxytocin Mechanisms Can Be Better Understood, but Its Effects on Social Cognition and Behavior Are Not to Be Sniffed At', *Biological Psychiatry*, 79(8), pp. e51–2.

Leng, G. and Sabatier, N. (2017) 'Oxytocin – The Sweet Hormone?', *Trends in Endocrinology & Metabolism*, 28(5), pp. 365–376.

Lerer, E., Levi, S., Salomon, S., Darvasi, A., Yirmiya, N. and Ebstein, R. P. (2008) 'Association between the oxytocin receptor (OXTR) gene and autism: relationship to Vineland Adaptive Behavior Scales and cognition', *Molecular Psychiatry*, 13(10), pp. 980–988.

Lesch, K. P., Bengel, D., Heils, A., Sabol, S. Z., Greenberg, B. D., Petri, S., Benjamin, J., Müller, C. R., Hamer, D. H. and Murphy, D. L. (1996) 'Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region.', *Science (New York, N.Y.)*, 274(5292), pp. 1527–31.

Lesch, K. P. and Mössner, R. (1998) 'Genetically driven variation in serotonin uptake: is there a link to affective spectrum, neurodevelopmental, and neurodegenerative disorders?', *Biological Psychiatry*, 44(3), pp. 179–92.

Levin, E. D. (2012) 'α7-Nicotinic receptors and cognition.', *Current Drug Targets*, 13(5), pp. 602–6.

Levin, E. D., Bradley, A., Addy, N. and Sigurani, N. (2002) 'Hippocampal alpha 7 and alpha 4 beta 2 nicotinic receptors and working memory.', *Neuroscience*, 109(4), pp. 757–65.

Levin, E. D., Petro, A., Rezvani, A. H., Pollard, N., Christopher, N. C., Strauss, M., Avery, J., Nicholson, J. and Rose, J. E. (2009) 'Nicotinic α7- or β2-containing receptor knockout: Effects on radial-arm maze learning and long-term nicotine consumption in mice', *Behavioural Brain Research*, 196(2), pp. 207–213.

Levine, S., Johnson, D. F. and Gonzalez, C. A. (1985) 'Behavioral and hormonal responses to separation in infant rhesus monkeys and mothers.', *Behavioral Neuroscience*, 99(3), pp. 399–410.

Lewis, M. and Kim, S.-J. (2009) 'The pathophysiology of restricted repetitive behavior.', *Journal of Neurodevelopmental Disorders*, 1(2), pp. 114–32.

Leyfer, O. T., Folstein, S. E., Bacalman, S., Davis, N. O., Dinh, E., Morgan, J., Tager-Flusberg, H. and Lainhart, J. E. (2006) 'Comorbid Psychiatric Disorders in Children with Autism: Interview Development and Rates of Disorders', *Journal of Autism and Developmental Disorders*, 36(7), pp. 849–861.

- Li, Y., Field, P. M. and Raisman, G. (2005) 'Olfactory ensheathing cells and olfactory nerve fibroblasts maintain continuous open channels for regrowth of olfactory nerve fibres', *Glia*, 52(3), pp. 245–251.
- Li, Z., Zhang, Y., Ku, L., Wilkinson, K. D., Warren, S. T. and Feng, Y. (2001) 'The fragile X mental retardation protein inhibits translation via interacting with mRNA.', *Nucleic Acids Research*. Oxford University Press, 29(11), pp. 2276–83.
- Liebowitz, M. R. (1983) *The Chemistry of Love*. Little Bro. Boston.
- Lijam, N., Paylor, R., McDonald, M. P., Crawley, J. N., Deng, C. X., Herrup, K., Stevens, K. E., Maccaferri, G., McBain, C. J., Sussman, D. J. and Wynshaw-Boris, A. (1997) 'Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1', *Cell*, 90(5), pp. 895–905.
- Lim, M. M. and Young, L. J. (2006) 'Neuropeptidergic regulation of affiliative behavior and social bonding in animals', *Hormones and Behavior*, 50(4), pp. 506–517.
- Lin, Y. and Wang, S. (2014) 'Prenatal lipopolysaccharide exposure increases depression-like behaviors and reduces hippocampal neurogenesis in adult rats', *Behavioural Brain Research*, 259, pp. 24–34.
- Lipschitz, D. L., Crowley, W. R., Armstrong, W. E. and Bealer, S. L. (2005) 'Neurochemical bases of plasticity in the magnocellular oxytocin system during gestation', *Experimental Neurology*, 196(2), pp. 210–223.
- Lisman, J. E., Berger, T. ., Leung, L. ., McNaughton, B. ., Mercer, E. ., Anderson, D. ., Mayford, M., Kandel, E. . and Tonegawa, S. (1999) 'Relating hippocampal circuitry to function: recall of memory sequences by reciprocal dentate-CA3 interactions.', *Neuron*, 22(2), pp. 233–42.
- Liu, X., Kawamura, Y., Shimada, T., Otowa, T., Koishi, S., Sugiyama, T., Nishida, H., Hashimoto, O., Nakagami, R., Tochigi, M., Umekage, T., Kano, Y., Miyagawa, T., Kato, N., Tokunaga, K. and Sasaki, T. (2010) 'Association of the oxytocin receptor (OXTR) gene polymorphisms with autism spectrum disorder (ASD) in the Japanese population', *Journal of Human Genetics*, 55(3), pp. 137–141.
- Liu, Z.-H., Chuang, D.-M. and Smith, C. B. (2011) 'Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome', *The International Journal of Neuropsychopharmacology*, 14(5), pp. 618–630.
- Liu, Z.-H. and Smith, C. B. (2009) 'Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome.', *Neuroscience Letters*, 454(1), pp. 62–6.
- Lloyd, G. K. and Williams, M. (2000) 'Neuronal Nicotinic Acetylcholine Receptors as Novel Drug Targets', *The Journal of Pharmacology and Experimental Therapeutics*, 2(292), pp. 461–467.
- Loesch, D. Z., Huggins, R. M. and Hagerman, R. J. (2004) 'Phenotypic variation and FMRP levels in fragile X', *Mental Retardation and Developmental Disabilities Research Reviews*, 10(1), pp. 31–41.
- Lohoff, F. W. (2010) 'Overview of the genetics of major depressive disorder.', *Current Psychiatry Reports*, 12(6), pp. 539–46.
- Loomes, R., Hull, L. and Mandy, W. P. L. (2017) 'What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis', *Journal of the American Academy of Child & Adolescent Psychiatry*, 56(6), pp. 466–474.
- LoParo, D. and Waldman, I. D. (2015) 'The oxytocin receptor gene (OXTR) is associated with autism spectrum disorder: a meta-analysis', *Molecular Psychiatry*, 20(5), pp. 640–646.

- López-León, S., Janssens, A. C. J. W., González-Zuloeta Ladd, A. M., Del-Favero, J., Claes, S. J., Oostra, B. A. and van Duijn, C. M. (2008) 'Meta-analyses of genetic studies on major depressive disorder', *Molecular Psychiatry*, 13(8), pp. 772–785.
- López-Valdés, H. E., García-Colunga, J. and Miledi, R. (2002) 'Effects of clomipramine on neuronal nicotinic acetylcholine receptors', *European Journal of Pharmacology*, 444(1–2), pp. 13–19.
- Lopizzo, N., Bocchio C, Plazzotta, G., Tarazi, F., Pariante, C., Riva, M. and Cattaneo, A. (2015) 'Gene-environment interaction in major depression: focus on experience-dependent biological systems.', *Frontiers in Psychiatry*. Frontiers Media SA, 6, p. 68.
- Lord, C. and Bishop, S. L. (2015) 'Recent Advances in Autism Research as Reflected in DSM-5 Criteria for Autism Spectrum Disorder', *Annual Review of Clinical Psychology*, 11, pp. 53–70.
- Lord, C., Rutter, M. and Le Couteur, A. (1994) 'Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders', *Journal of Autism and Developmental Disorders*, 24(5), pp. 659–685.
- Lord, J. A., Waterfield, A. A., Hughes, J. and Kosterlitz, H. W. (1977) 'Endogenous opioid peptides: multiple agonists and receptors', *Nature*, 267(5611), pp. 495–499.
- Loseth, G. E., Ellingsen, D. M. and Leknes, S. (2014) 'State-dependent mu-opioid modulation of social motivation', *Frontiers in Behavioral Neuroscience*, 8, p. 430.
- Love, T. M. (2014) 'Oxytocin, motivation and the role of dopamine', *Pharmacology Biochemistry and Behavior*, 119, pp. 49–60.
- Lu, R., Wang, H., Liang, Z., Ku, L., O'Donnell W, T., Li, W., Warren, S. T. and Feng, Y. (2004) 'The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development', *Proceedings of the National Academy of Sciences of the United States*, 101(42), pp. 15201–15206.
- Lubs, H. A. (1969) 'A marker X chromosome', *American Journal of Human Genetics*, 21(3), pp. 231–244.
- Ludwig, M., Sabatier, N., Bull, P. M., Landgraf, R., Dayanithi, G. and Leng, G. (2002) 'Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites', *Nature*, 418(6893), pp. 85–89.
- Lugnegård, T., Hallerbäck, M. U. and Gillberg, C. (2011) 'Psychiatric comorbidity in young adults with a clinical diagnosis of Asperger syndrome', *Research in Developmental Disabilities*, 32(5), pp. 1910–1917.
- Lukas, R. J., Changeux, J. P., Le Novère, N., Albuquerque, E. X., Balfour, D. J., Berg, D. K., Bertrand, D., Chiappinelli, V. A., Clarke, P. B., Collins, A. C., Dani, J. A., Grady, S. R., Kellar, K. J., Lindstrom, J. M., Marks, M. J., Quik, M., Taylor, P. W. and Wonnacott, S. (1999) 'International Union of Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits.', *Pharmacological Reviews*, 51(2), pp. 397–401.
- Lutz, P. E. and Kieffer, B. L. (2013) 'Opioid receptors: distinct roles in mood disorders', *Trends in Neurosciences*, 36(3), pp. 195–206.
- MacDonald, K. and Feifel, D. (2013) 'Helping oxytocin deliver: considerations in the development of oxytocin-based therapeutics for brain disorders', *Frontiers in Neuroscience*, 7, p. 35.
- Machin, A. J. and Dunbar, R. I. M. (2011) 'The brain opioid theory of social attachment: A review of the evidence', *Behaviour*, 148(9), pp. 985–1025.

- Macneil, B., Lopes, V. and Minnes, P. (2009) 'Anxiety in children and adolescents with Autism Spectrum Disorders', *Research in Autism Spectrum Disorders*, 3, pp. 1–21.
- Mahajan, R., Bernal, M. P., Panzer, R., Whitaker, A., Roberts, W., Handen, B., Hardan, A., Anagnostou, E., Veenstra-VanderWeele, J. and Autism Speaks Autism Treatment Network Psychopharmacology Committee (2012) 'Clinical Practice Pathways for Evaluation and Medication Choice for Attention-Deficit/Hyperactivity Disorder Symptoms in Autism Spectrum Disorders', *Pediatrics*, 130(Supplement 2), pp. S125–S138.
- Maisonneuve, I. M., Archer, S. and Glick, S. D. (1994) 'U50,488, a kappa opioid receptor agonist, attenuates cocaine-induced increases in extracellular dopamine in the nucleus accumbens of rats', *Neuroscience Letters*, 181(1–2), pp. 57–60.
- Malatynska, E. and Knapp, R. J. (2005) 'Dominant–submissive behavior as models of mania and depression', *Neuroscience & Biobehavioral Reviews*, 29(4–5), pp. 715–737.
- Mammen, O., Kolko, D. and Pilkonis, P. (2002) 'Negative affect and parental aggression in child physical abuse.', *Child Abuse & Neglect*, 26(4), pp. 407–24.
- Manning, M., Stoev, S., Chini, B., Durroux, T., Mouillac, B. and Guillon, G. (2008) 'Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents', *Progress in Brain Research*, 170, pp. 473–512.
- Mansour, A., Hoversten, M. T., Taylor, L. P., Watson, S. J. and Akil, H. (1995) 'The cloned mu, delta and kappa receptors and their endogenous ligands: evidence for two opioid peptide recognition cores.', *Brain Research*, 700(1–2), pp. 89–98.
- Manzi, B., Loizzo, A. L., Giana, G. and Curatolo, P. (2008) 'Autism and Metabolic Diseases', *Journal of Child Neurology*, 23(3), pp. 307–314.
- Marinelli, M. (2007) 'Stress and Addiction', in *Stress and Addiction*. M. Al'Absi. Burlington, MA: Elsevier, pp. 41–83.
- Marinelli, M. and McCutcheon, J. E. (2014) 'Heterogeneity of dopamine neuron activity across traits and states', *Neuroscience*, 282, pp. 176–197.
- Marinelli, M., Rudick, C. N., Hu, X.-T. and White, F. J. (2006) 'Excitability of dopamine neurons: modulation and physiological consequences.', *CNS & neurological disorders drug targets*, 5(1), pp. 79–97.
- Marsh, A. A., Yu, H. H., Pine, D. S., Gorodetsky, E. K., Goldman, D. and Blair, R. J. (2012) 'The influence of oxytocin administration on responses to infant faces and potential moderation by OXTR genotype', *Psychopharmacology (Berl)*, 224(4), pp. 469–476.
- Martin-Merino, E., Ruigomez, A., Wallander, M.-A., Johansson, S. and Garcia-Rodriguez, L. A. (2010) 'Prevalence, incidence, morbidity and treatment patterns in a cohort of patients diagnosed with anxiety in UK primary care', *Family Practice*, 27(1), pp. 9–16.
- Martin, A., Scahill, L., Klin, A. and Volkmar, F. (1999) 'Higher-Functioning Pervasive Developmental Disorders: Rates and Patterns of Psychotropic Drug Use', *Journal of the American Academy of Child & Adolescent Psychiatry*, 38(7), pp. 923–931.
- Martin, E. I., Ressler, K. J., Binder, E. and Nemeroff, C. B. (2009) 'The neurobiology of anxiety disorders: brain imaging, genetics, and psychoneuroendocrinology.', *The Psychiatric Clinics of North America*, 32(3), pp. 549–75.
- Martin, W. R., Eades, C. G., Thompson, J. A., Huppler, R. E. and Gilbert, P. E. (1976) 'The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog', *Journal of Pharmacology and Experimental Therapeutics*, 197(3), pp. 517–532.

- Martinez, M., Calvo-Torrent, A. and Pico-Alfonso, M. A. (1998) 'Social defeat and subordination as models of social stress in laboratory rodents: A review', *Aggressive Behavior*, 24(4), pp. 241–256.
- Mashhoon, Y., Wells, A. M. and Kantak, K. M. (2010) 'Interaction of the rostral basolateral amygdala and prelimbic prefrontal cortex in regulating reinstatement of cocaine-seeking behavior', *Pharmacology Biochemistry and Behavior*, 96(3), pp. 347–353.
- Maski, K. P., Jeste, S. S. and Spence, S. J. (2011) 'Common neurological co-morbidities in autism spectrum disorders', *Current Opinion in Pediatrics*, 23(6), pp. 609–615.
- Mathew, S. J. and Ho, S. (2006) 'Etiology and neurobiology of social anxiety disorder.', *The Journal of Clinical Psychiatry*, 67 Suppl 1, pp. 9–13.
- Matson, J. L. and Nebel-Schwalm, M. S. (2007) 'Comorbid psychopathology with autism spectrum disorder in children: An overview', *Research in Developmental Disabilities*, 28(4), pp. 341–352.
- Matthews, G. A., Nieh, E. H., Vander Weele, C. M., Halbert, S. A., Pradhan, R. V., Yosafat, A. S., Glober, G. F., Izadmehr, E. M., Thomas, R. E., Lacy, G. D., Wildes, C. P., Ungless, M. A. and Tye, K. M. (2016) 'Dorsal Raphe Dopamine Neurons Represent the Experience of Social Isolation', *Cell*, 164(4), pp. 617–631.
- Mattila, M.-L., Hurtig, T., Haapsamo, H., Jussila, K., Kuusikko-Gauffin, S., Kielenen, M., Linna, S.-L., Ebeling, H., Bloigu, R., Joskitt, L., Pauls, D. L. and Moilanen, I. (2010) 'Comorbid Psychiatric Disorders Associated with Asperger Syndrome/High-functioning Autism: A Community- and Clinic-based Study', *Journal of Autism and Developmental Disorders*, 40(9), pp. 1080–1093.
- Mattila, M.-L., Kielenen, M., Linna, S.-L., Jussila, K., Ebeling, H., Bloigu, R., Joseph, R. M. and Moilanen, I. (2011) 'Autism Spectrum Disorders According to DSM-IV-TR and Comparison With DSM-5 Draft Criteria: An Epidemiological Study', *Journal of the American Academy of Child & Adolescent Psychiatry*, 50(6), p. 583–592.e11.
- McAlonan, G. M., Cheung, V., Cheung, C., Suckling, J., Lam, G. Y., Tai, K. S., Yip, L., Murphy, D. G. M. and Chua, S. E. (2004) 'Mapping the brain in autism. A voxel-based MRI study of volumetric differences and intercorrelations in autism', *Brain*, 128(2), pp. 268–276.
- McCallum, S. E., Collins, A. C., Paylor, R. and Marks, M. J. (2006) 'Deletion of the beta 2 nicotinic acetylcholine receptor subunit alters development of tolerance to nicotine and eliminates receptor upregulation', in *Psychopharmacology*, pp. 314–327.
- McCracken, J. T., McGough, J., Shah, B., Cronin, P., Hong, D., Aman, M. G., Arnold, L. E., Lindsay, R., Nash, P., Hollway, J., McDougle, C. J., Posey, D., Swiezy, N., Kohn, A., Scahill, L., Martin, A., Koenig, K., Volkmar, F., Carroll, D., Lancor, A., Tierney, E., Ghuman, J., Gonzalez, N. M., Grados, M., Vitiello, B., Ritz, L., Davies, M., Robinson, J., McMahon, D. and Research Units on Pediatric Psychopharmacology Autism Network (2002) 'Risperidone in Children with Autism and Serious Behavioral Problems', *New England Journal of Medicine*, 347(5), pp. 314–321.
- McDougle, C. J., Naylor, S. T., Cohen, D. J., Volkmar, F. R., Heninger, G. R. and Price, L. H. (1996) 'A double-blind, placebo-controlled study of fluvoxamine in adults with autistic disorder.', *Archives of General Psychiatry*, 53(11), pp. 1001–8.
- McDougle, C. J., Scahill, L., Aman, M. G., McCracken, J. T., Tierney, E., Davies, M., Arnold, L. E., Posey, D. J., Martin, A., Ghuman, J. K., Shah, B., Chuang, S. Z., Swiezy, N. B., Gonzalez, N. M., Hollway, J., Koenig, K., McGough, J. J., Ritz, L. and Vitiello, B. (2005) 'Risperidone for the Core Symptom Domains of Autism: Results From the Study by the Autism Network of the Research Units on Pediatric Psychopharmacology', *American Journal*

of *Psychiatry*, 162(6), pp. 1142–1148.

McEwen, B. S. (2005) 'Glucocorticoids, depression, and mood disorders: structural remodeling in the brain', *Metabolism*, 54(5), pp. 20–23.

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonté, B., Szyf, M., Turecki, G. and Meaney, M. J. (2009) 'Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse', *Nature Neuroscience*, 12(3), pp. 342–348.

McLaughlin, J. P., Li, S., Valdez, J., Chavkin, T. A. and Chavkin, C. (2006) 'Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system', *Neuropsychopharmacology*, 31(6), pp. 1241–1248.

McLaughlin, J. P., Marton-Popovici, M. and Chavkin, C. (2003) 'Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses.', *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*, 23(13), pp. 5674–83.

McNaughton, C. H., Moon, J., Strawderman, M. S., Maclean, K. N., Evans, J. and Strupp, B. J. (2008) 'Evidence for social anxiety and impaired social cognition in a mouse model of fragile X syndrome.', *Behavioral Neuroscience*. American Psychological Association, 122(2), pp. 293–300.

McQuaid, R. J., McInnis, O. A., Abizaid, A. and Anisman, H. (2014) 'Making room for oxytocin in understanding depression', *Neuroscience & Biobehavioral Reviews*, 45, pp. 305–322.

Meerlo, P., Overkamp, G. J. F., Benning, M. A., Koolhaas, J. M. and Van Den Hoofdakker, R. H. (1996) 'Long-term changes in open field behaviour following a single social defeat in rats can be reversed by sleep deprivation', *Physiology & Behavior*, 60(1), pp. 115–119.

Meerlo, Overkamp, Daan, Van Den Hoofdakker RH and Koolhaas (1996) 'Changes in Behaviour and Body Weight Following a Single or Double Social Defeat in Rats.', *Stress (Amsterdam, Netherlands)*, 1(1), pp. 21–32.

Melas, P. A., Wei, Y., Wong, C. C. Y., Sjöholm, L. K., Åberg, E., Mill, J., Schalling, M., Forsell, Y. and Lavebratt, C. (2013) 'Genetic and epigenetic associations of MAOA and NR3C1 with depression and childhood adversities', *The International Journal of Neuropsychopharmacology*, 16(7), pp. 1513–1528.

Melis, M. R., Melis, T., Cocco, C., Succu, S., Sanna, F., Pillolla, G., Boi, A., Ferri, G. L. and Argiolas, A. (2007) 'Oxytocin injected into the ventral tegmental area induces penile erection and increases extracellular dopamine in the nucleus accumbens and paraventricular nucleus of the hypothalamus of male rats', *European Journal of Neuroscience*, 26(4), pp. 1026–1035.

Meng, J., Fujita, H., Nagahara, N., Kashiwai, A., Yoshioka, Y. and Funato, M. (1992) 'Two patients with chromosome 6q terminal deletions with breakpoints at q24.3 and q25.3', *American Journal of Medical Genetics Part A*, 43(4), pp. 747–750.

Menke, A. (2013) 'Gene expression: Biomarker of antidepressant therapy?', *International Review of Psychiatry*, 25(5), pp. 579–591.

Menke, A. and Binder, E. (2014) 'Epigenetic alterations in depression and antidepressant treatment.', *Dialogues in Clinical Neuroscience*, 16(3), pp. 395–404.

Mens, W. B., Laczi, F., Tonnaer, J. A., de Kloet, E. R. and van Wimersma Greidanus, T. B. (1983) 'Vasopressin and oxytocin content in cerebrospinal fluid and in various brain areas after administration of histamine and pentylentetrazol', *Pharmacology Biochemistry and Behavior*, 19(4), pp. 587–591.

- Le Merrer, J., Becker, J. A., Befort, K. and Kieffer, B. L. (2009) 'Reward processing by the opioid system in the brain', *Physiological Reviews*, 89(4), pp. 1379–1412.
- Le Merrer, J., Rezai, X., Scherrer, G., Becker, J. A. J. and Kieffer, B. L. (2013) 'Impaired hippocampus-dependent and facilitated striatum-dependent behaviors in mice lacking the δ opioid receptor.', *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*, 38(6), pp. 1050–9.
- Mesic, I., Guzman, Y. F., Guedea, A. L., Jovasevic, V., Corcoran, K. A., Leaderbrand, K., Nishimori, K., Contractor, A. and Radulovic, J. (2015) 'Double Dissociation of the Roles of Metabotropic Glutamate Receptor 5 and Oxytocin Receptor in Discrete Social Behaviors.', *Neuropsychopharmacology*, 40(10), pp. 2337–46.
- Metaxas, A., Bailey, A., Barbano, M., Galeote, L., Maldonado, R. and Kitchen, I. (2010) 'Differential region-specific regulation of $\alpha 4\beta 2^*$ nAChRs by self-administered and non-contingent nicotine in C57BL/6J mice', *Addiction Biology*, 15(4), pp. 464–479.
- Meyer-Lindenberg, A., Domes, G., Kirsch, P. and Heinrichs, M. (2011) 'Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine', *Nature Reviews Neuroscience*, 12(9), pp. 524–538.
- Meyer-Lindenberg, A., Kolachana, B., Gold, B., Olsh, A., Nicodemus, K. K., Mattay, V., Dean, M. and Weinberger, D. R. (2009) 'Genetic variants in AVPR1A linked to autism predict amygdala activation and personality traits in healthy humans', *Molecular Psychiatry*, 14(10), pp. 968–975.
- Miczek, K. A., Maxson, S. C., Fish, E. W. and Faccidomo, S. (2001) 'Aggressive behavioral phenotypes in mice.', *Behavioural Brain Research*, 125(1–2), pp. 167–81.
- Miczek, K., Yap, J., Covington, H. and III (2008) 'Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake.', *Pharmacology & Therapeutics*, 120(2), pp. 102–28.
- Mientjes, E. J., Nieuwenhuizen, I., Kirkpatrick, L., Zu, T., Hoogeveen-Westerveld, M., Severijnen, L., Rifé, M., Willemsen, R., Nelson, D. L. and Oostra, B. A. (2006) 'The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo', *Neurobiology of Disease*, 21(3), pp. 549–555.
- Mihailescu, S. and Drucker-Colín, R. (2000) 'Nicotine and brain disorders.', *Acta pharmacologica Sinica*, 21(2), pp. 97–104.
- Mill, J. and Petronis, A. (2007) 'Molecular studies of major depressive disorder: the epigenetic perspective', *Molecular Psychiatry*, 12(9), pp. 799–814.
- Miller, J. A. and Zahniser, N. R. (1987) 'The use of ^{14}C -labeled tissue paste standards for the calibration of ^{125}I -labeled ligands in quantitative autoradiography.', *Neuroscience Letters*, 81(3), pp. 345–50.
- Miller, J. L., Ashford, J. W., Archer, S. M., Rudy, A. C. and Wermeling, D. P. (2008) 'Comparison of intranasal administration of haloperidol with intravenous and intramuscular administration: a pilot pharmacokinetic study', *Pharmacotherapy*, 28(7), pp. 875–882.
- Miller, M., Bales, K. L., Taylor, S. L., Yoon, J., Hostetler, C. M., Carter, C. S. and Solomon, M. (2013) 'Oxytocin and vasopressin in children and adolescents with autism spectrum disorders: sex differences and associations with symptoms', *Autism Research*, 6(2), pp. 91–102.
- Mills, J. L., Hediger, M. L., Molloy, C. A., Chrousos, G. P., Manning-Courtney, P., Yu, K. F., Brasington, M. and England, L. J. (2007) 'Elevated levels of growth-related hormones in autism and autism spectrum disorder', *Clinical Endocrinology*, 67(2), pp. 230–237.

- Mineur, Y. S., Obayemi, A., Wigstrand, M. B., Fote, G. M., Calarco, C. A., Li, A. M. and Picciotto, M. R. (2013) 'Cholinergic signaling in the hippocampus regulates social stress resilience and anxiety- and depression-like behavior.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(9), pp. 3573–8.
- Mineur, Y. S., Sluyter, F., de Wit, S., Oostra, B. A. and Crusio, W. E. (2002) 'Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse', *Hippocampus*, 12(1), pp. 39–46.
- Mineur, Y. S., Somenzi, O. and Picciotto, M. R. (2007) 'Cytisine, a partial agonist of high-affinity nicotinic acetylcholine receptors, has antidepressant-like properties in male C57BL/6J mice.', *Neuropharmacology*, 52(5), pp. 1256–62.
- Ming, X., Brimacombe, M. and Wagner, G. C. (2007) 'Prevalence of motor impairment in autism spectrum disorders', *Brain and Development*, 29(9), pp. 565–570.
- Modahl, C., Fein, D., Waterhouse, L. and Newton, N. (1992) 'Does oxytocin deficiency mediate social deficits in autism?', *Journal of Autism and Developmental Disorders*, 22(3), pp. 449–451.
- Modahl, C., Green, L., Fein, D., Morris, M., Waterhouse, L., Feinstein, C. and Levin, H. (1998) 'Plasma oxytocin levels in autistic children', *Biological Psychiatry*, 43(4), pp. 270–277.
- Moles, A., Kieffer, B. L. and D'Amato, F. R. (2004) 'Deficit in attachment behavior in mice lacking the mu-opioid receptor gene', *Science*, 304(5679), pp. 1983–1986.
- Monroe, S. M. and Harkness, K. L. (2005) 'Life Stress, the "Kindling" Hypothesis, and the Recurrence of Depression: Considerations From a Life Stress Perspective.', *Psychological Review*, 112(2), pp. 417–445.
- Monte, D., Noble, Turchi, Cummins and Averbeck (2014) 'CSF and Blood Oxytocin Concentration Changes following Intranasal Delivery in Macaque', *PLoS One*, 9(8).
- Moore, S. J., Turnpenny, P., Quinn, A., Glover, S., Lloyd, D. J., Montgomery, T. and Dean, J. C. S. (2000) 'A clinical study of 57 children with fetal anticonvulsant syndromes', *Journal of Medical Genetics*, 37(7), pp. 489–497.
- Morel, C., Fernandez, S. P., Pantouli, F., Meye, F. J., Marti, F., Tolu, S., Parnaudeau, S., Marie, H., Tronche, F., Maskos, U., Moretti, M., Gotti, C., Han, M.-H., Bailey, A., Mameli, M., Barik, J. and Faure, P. (2017) 'Nicotinic receptors mediate stress-nicotine detrimental interplay via dopamine cells' activity', *Molecular Psychiatry*, 10, p. 145.
- Moussavi, S., Chatterji, S., Verdes, E., Tandon, A., Patel, V. and Ustun, B. (2007) 'Depression, chronic diseases, and decrements in health: results from the World Health Surveys', *The Lancet*, 370(9590), pp. 851–858.
- Mulder, E. J., Anderson, G. M., Kema, I. P., de Bildt, A., van Lang, N. D., den Boer, J. A. and Minderaa, R. B. (2004) 'Platelet Serotonin Levels in Pervasive Developmental Disorders and Mental Retardation: Diagnostic Group Differences, Within-Group Distribution, and Behavioral Correlates', *Journal of the American Academy of Child & Adolescent Psychiatry*, 43(4), pp. 491–499.
- Munesue, T., Yokoyama, S., Nakamura, K., Anitha, A., Yamada, K., Hayashi, K., Asaka, T., Liu, H. X., Jin, D., Koizumi, K., Islam, M. S., Huang, J. J., Ma, W. J., Kim, U. H., Kim, S. J., Park, K., Kim, D., Kikuchi, M., Ono, Y., Nakatani, H., Suda, S., Miyachi, T., Hirai, H., Salmina, A., Pichugina, Y. A., Soumarokov, A. A., Takei, N., Mori, N., Tsujii, M., Sugiyama, T., Yagi, K., Yamagishi, M., Sasaki, T., Yamasue, H., Kato, N., Hashimoto, R., Taniike, M., Hayashi, Y., Hamada, J., Suzuki, S., Ooi, A., Noda, M., Kamiyama, Y., Kido, M. A., Lopatina, O., Hashii, M., Amina, S., Malavasi, F., Huang, E. J., Zhang, J., Shimizu, N., Yoshikawa, T.,

Matsushima, A., Minabe, Y. and Higashida, H. (2010) 'Two genetic variants of CD38 in subjects with autism spectrum disorder and controls', *Neuroscience Research*, 67(2), pp. 181–191.

Murphy, G., Sarginson, J., Ryan, H. S., O'Hara, R., Schatzberg, A. F. and Lazzeroni, L. C. (2013) 'BDNF and CREB1 genetic variants interact to affect antidepressant treatment outcomes in geriatric depression', *Pharmacogenetics and Genomics*, 23(6), pp. 301–313.

Murray, C. J. L. (1997) 'BURDEN OF DISEASE A comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020 EDITED BY', *Global Burden of Disease and Injury Series*, pp. 3–35.

Murray, C. J. and Lopez, A. D. (1996) 'Evidence-based health policy--lessons from the Global Burden of Disease Study.', *Science (New York, N.Y.)*, 274(5288), pp. 740–3.

Nadler, J. J., Moy, S. S., Dold, G., Trang, D., Simmons, N., Perez, A., Young, N. B., Barbaro, R. P., Piven, J., Magnuson, T. R. and Crawley, J. N. (2004) 'Automated apparatus for quantitation of social approach behaviors in mice', *Genes Brain and Behavior*, 3(5), pp. 303–314.

Nakamoto, M., Nalavadi, V., Epstein, M. P., Narayanan, U., Bassell, G. J. and Warren, S. T. (2007) 'Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors.', *Proceedings of the National Academy of Sciences of the United States of America*, 104(39), pp. 15537–42.

Nakamura, K., Sekine, Y., Ouchi, Y., Tsujii, M., Yoshikawa, E., Futatsubashi, M., Tsuchiya, K. J., Sugihara, G., Iwata, Y., Suzuki, K., Matsuzaki, H., Suda, S., Sugiyama, T., Takei, N. and Mori, N. (2010) 'Brain serotonin and dopamine transporter bindings in adults with high-functioning autism', *Archives of General Psychiatry*, 67(1), pp. 59–68.

Nelson, E. and Alberts, J. R. (1997) 'Oxytocin-induced paw sucking in infant rats', *Annals of the New York Academy of Sciences*, 807, pp. 543–545.

Nelson, E. E. and Panksepp, J. (1998) 'Brain substrates of infant-mother attachment: contributions of opioids, oxytocin, and norepinephrine', *Neuroscience & Biobehavioral Reviews*, 22(3), pp. 437–452.

Neuhaus, E., Beauchaine, T. P. and Bernier, R. (2010) 'Neurobiological correlates of social functioning in autism', *Clinical Psychology Review*, 30(6), pp. 733–748.

Neumann, I. D., Maloumy, R., Beiderbeck, D. I., Lukas, M. and Landgraf, R. (2013) 'Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice', *Psychoneuroendocrinology*, 38(10), pp. 1985–1993.

Neumann, I. D. and Slattery, D. A. (2016) 'Oxytocin in General Anxiety and Social Fear: A Translational Approach', *Biological Psychiatry*, 79(3), pp. 213–221.

Neumann, I., Veenema, A. and Beiderbeck, D. (2010) 'Aggression and anxiety: social context and neurobiological links.', *Frontiers in Behavioral Neuroscience*, 4, p. 12.

Newschaffer, C. J., Croen, L. A., Daniels, J., Giarelli, E., Grether, J. K., Levy, S. E., Mandell, D. S., Miller, L. A., Pinto-Martin, J., Reaven, J., Reynolds, A. M., Rice, C. E., Schendel, D. and Windham, G. C. (2007) 'The epidemiology of autism spectrum disorders', *Annual Review of Public Health*, 28(1), pp. 235–258.

Nickl-Jockschat, T., Habel, U., Maria Michel, T., Manning, J., Laird, A. R., Fox, P. T., Schneider, F. and Eickhoff, S. B. (2012) 'Brain structure anomalies in autism spectrum disorder-a meta-analysis of VBM studies using anatomic likelihood estimation', *Human Brain Mapping*, 33(6), pp. 1470–1489.

Nicolson, R., Craven-Thuss, B. and Smith, J. (2006) 'A Prospective, Open-Label Trial of

Galantamine in Autistic Disorder', *Journal of Child and Adolescent Psychopharmacology*, 16(5), pp. 621–629.

Niederhofer, H. (2007) 'Glutamate Antagonists Seem to be Slightly Effective in Psychopharmacologic Treatment of Autism', *Journal of Clinical Psychopharmacology*, 27(3), pp. 317–318.

Niederhofer, H., Staffen, W. and Mair, A. (2002) 'Galantamine may be effective in treating autistic disorder.', *BMJ (Clinical research ed.)*, 325(7377), p. 1422.

Nielsen, M., Hetland, J., Matthiesen, S. and Einarsen, S. (2012) 'Longitudinal relationships between workplace bullying and psychological distress', *Scandinavian Journal of Work, Environment & Health*, 38(1), pp. 38–46.

Nielsen, M., Matthiesen, S. and Einarsen, S. (2010) 'The impact of methodological moderators on prevalence rates of workplace bullying. A meta-analysis', *Journal of Occupational and Organizational Psychology*, 83(4), pp. 955–979.

Nielsen, M., Tangen, T., Idsoe, T., Matthiesen, S. and Magerøy, N. (2015) 'Post-traumatic stress disorder as a consequence of bullying at work and at school. A literature review and meta-analysis', *Aggression and Violent Behavior*, 21, pp. 17–24.

Nimchinsky, E. A., Oberlander, A. M. and Svoboda, K. (2001) 'Abnormal development of dendritic spines in FMR1 knock-out mice', *Journal of Neuroscience*, 21(14), pp. 5139–5146.

Noto, V., Harrity, C., Walsh, D. and Marron, K. (2016) 'The impact of FMR1 gene mutations on human reproduction and development: a systematic review', *Journal of Assisted Reproduction and Genetics*, 33(9), pp. 1135–1147.

Nott, A. and Levin, E. D. (2006) 'Dorsal hippocampal $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptors and memory', *Brain Research*, 1081(1), pp. 72–78.

Oddi, D., Crusio, W. E., D'Amato, F. R. and Pietropaolo, S. (2013) 'Monogenic mouse models of social dysfunction: implications for autism', *Behavioral Brain Research*, 251, pp. 75–84.

Ohlsson, B., Truedsson, M., Bengtsson, M., Torstenson, R., Sjolund, K., Bjornsson, E. S. and Simren, M. (2005) 'Effects of long-term treatment with oxytocin in chronic constipation; a double blind, placebo-controlled pilot trial', *Neurogastroenterology & Motility*, 17(5), pp. 697–704.

Olweus, D. (1993) *Bullying at school: What we know and what we can do*, Malden, MA: Black-well Publishing.

Orr-Urtreger, A., Gö Ldner, F. M., Saeki, M., Lorenzo, I., Goldberg, L., De Biasi, M., Dani, J. A., Patrick, J. W. and Beaudet, A. L. (1997) 'Mice Deficient in the $\alpha 7$ Neuronal Nicotinic Acetylcholine Receptor Lack α -Bungarotoxin Binding Sites and Hippocampal Fast Nicotinic Currents', *Journal of Neuroscience*, 17(23), pp. 9165–9171.

Osterweil, E. K., Krueger, D. D., Reinhold, K. and Bear, M. F. (2010) 'Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome', *Journal of Neuroscience*, 30(46), pp. 15616–15627.

Otto, T. and Eichenbaum, H. (1992) 'Neuronal activity in the hippocampus during delayed non-match to sample performance in rats: Evidence for hippocampal processing in recognition memory', *Hippocampus*, 2(3), pp. 323–334.

Owens, M. J. and Nemeroff, C. B. (1991) 'Physiology and pharmacology of corticotropin-releasing factor.', *Pharmacological reviews*, 43(4), pp. 425–73.

Owley, T., Salt, J., Guter, S., Grieve, A., Walton, L., Ayuyao, N., Leventhal, B. L. and Cook,

- E. H. (2006) 'A Prospective, Open-Label Trial of Memantine in the Treatment of Cognitive, Behavioral, and Memory Dysfunction in Pervasive Developmental Disorders', *Journal of Child and Adolescent Psychopharmacology*, 16(5), pp. 517–524.
- Pacey, L. K. K., Doss, L., Cifelli, C., der Kooy, D. van, Heximer, S. P. and Hampson, D. R. (2011) 'Genetic deletion of regulator of G-protein signaling 4 (RGS4) rescues a subset of fragile X related phenotypes in the FMR1 knockout mouse', *Molecular and Cellular Neuroscience*, 46(3), pp. 563–572.
- Pakenham, K. I., Samios, C. and Sofronoff, K. (2005) 'Adjustment in mothers of children with Asperger syndrome', *Autism*, 9(2), pp. 191–212.
- Panksepp, J. (1979) 'The opioid excess theory of autism', *Trends in Neurosciences*, 2(0), pp. 174–177.
- Panksepp, J., Herman, B., Conner, R., Bishop, P. and Scott, J. P. (1978) 'The biology of social attachments: opiates alleviate separation distress', *Biological Psychiatry*, 13(5), pp. 607–618.
- Panksepp, J., Herman, B. H., Vilberg, T., Bishop, P. and DeEsquinazi, F. G. (1980) 'Endogenous opioids and social behavior', *Neuroscience & Biobehavioral Reviews*, 4(4), pp. 473–487.
- Panksepp, J. and Lensing, P. (1991) 'Brief report: a synopsis of an open-trial of naltrexone treatment of autism with four children', *Journal of Autism and Developmental Disorder*, 21(2), pp. 243–249.
- Panksepp, J., Nelson, E. and Bekkedal, M. (1997) 'Brain systems for the mediation of social separation-distress and social-reward. Evolutionary antecedents and neuropeptide intermediaries', *Annals of the New York Academy of Sciences*, 807, pp. 78–100.
- Papp, M., Klimek, V. and Willner, P. (1994) 'Parallel changes in dopamine D2 receptor binding in limbic forebrain associated with chronic mild stress-induced anhedonia and its reversal by imipramine.', *Psychopharmacology*, 115(4), pp. 441–6.
- Paradee, W., Melikian, H. E., Rasmussen, D. L., Kenneson, A., Conn, P. J. and Warren, S. T. (1999) 'Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function', *Neuroscience*, 94(1), pp. 185–192.
- Pardridge, W. M. (2002) 'Drug and gene delivery to the brain: the vascular route', *Neuron*, 36(4), pp. 555–558.
- Pariante, C. M. (2006) 'The glucocorticoid receptor: part of the solution or part of the problem?', *Journal of Psychopharmacology*, 20(4_suppl), pp. 79–84.
- Pariante, C. M. and Lightman, S. L. (2008) 'The HPA axis in major depression: classical theories and new developments', *Trends in Neurosciences*, 31(9), pp. 464–468.
- Paribello, C., Tao, L., Folino, A., Berry-Kravis, E., Tranfaglia, M., Ethell, I. M. and Ethell, D. W. (2010) 'Open-label add-on treatment trial of minocycline in fragile X syndrome', *BMC Neurology*, 10(1), p. 91.
- Parker, K. J., Oztan, O., Libove, R. A., Sumiyoshi, R. D., Jackson, L. P., Karhson, D. S., Summers, J. E., Hinman, K. E., Motonaga, K. S., Phillips, J. M., Carson, D. S., Garner, J. P. and Hardan, A. Y. (2017) 'Intranasal oxytocin treatment for social deficits and biomarkers of response in children with autism.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 114(30), pp. 8119–8124.
- Parsons, C. E., Young, K. S., Murray, L., Stein, A. and Kringelbach, M. L. (2010) 'The functional neuroanatomy of the evolving parent–infant relationship', *Progress in Neurobiology*, 91(3), pp. 220–241.

- Pasini, A. and D'Agati, E. (2009) 'Pathophysiology of NSS in ADHD', *World Journal of Biological Psychiatry*, 10(4 Pt 2), pp. 495–502.
- Pawłowski, B., Lowen, C. B. and Dunbar, R. I. M. (1998) 'Neocortex Size, Social Skills and Mating Success in Primates', *Behaviour*, 135(3), pp. 357–368.
- Paxinos, G. and Franklin, K. B. J. (2001) 'The mouse brain in stereotaxic coordinates'. New York: Academic Press.
- Pearson, D., Loveland, K., Lachar, D., Lane, D., Reddoch, S., Mansour, R. and Cleveland, L. (2006) 'A Comparison of Behavioral and Emotional Functioning in Children and Adolescents with Autistic Disorder and PDD-NOS', *Child Neuropsychology*, 12(4–5), pp. 321–333.
- Peça, J., Feliciano, C., Ting, J. T., Wang, W., Wells, M. F., Venkatraman, T. N., Lascola, C. D., Fu, Z. and Feng, G. (2011) 'Shank3 mutant mice display autistic-like behaviours and striatal dysfunction.', *Nature*, 472(7344), pp. 437–42.
- Pedersen, C. A., Gibson, C. M., Rau, S. W., Salimi, K., Smedley, K. L., Casey, R. L., Leserman, J., Jarskog, L. F. and Penn, D. L. (2011) 'Intranasal oxytocin reduces psychotic symptoms and improves Theory of Mind and social perception in schizophrenia', *Schizophrenia Research*, 132(1), pp. 50–53.
- Pedersen, C. A., Smedley, K. L., Leserman, J., Jarskog, L. F., Rau, S. W., Kampov-Polevoi, A., Casey, R. L., Fender, T. and Garbutt, J. C. (2013) 'Intranasal oxytocin blocks alcohol withdrawal in human subjects', *Alcoholism: Clinical and Experimental Research*, 37(3), pp. 484–489.
- Peeke, H. V and Herz, M. J. (1971) 'Caudate nucleus stimulation retroactively impairs complex maze learning in the rat', *Science*, 173(3991), pp. 80–82.
- Pellissier, L. P., Gandia, J., Laboute, T., Becker, J. A. J., Le Merrer, J., Gandia, J., Laboute, T., Becker, J. A. J. and Le Merrer, J. (2017) 'μ opioid receptor, social behaviour and autism spectrum disorder: reward matters', *British Journal of Pharmacology*. London: INRA UMR-0085, CNRS UMR-7247, University of Tours.
- Pellissier, L. P., Pujol, C. N., Becker, J. A. J. and J, L. M. (2016) *Delta opioid receptors, learning and motivation, Handbook of Experimental Pharmacology*. Edited by S. B. Heidelberg. Springer.
- Penagarikano, O., Lazaro, M. T., Lu, X. H., Gordon, A., Dong, H., Lam, H. A., Peles, E., Maidment, N. T., Murphy, N. P., Yang, X. W., Golshani, P. and Geschwind, D. H. (2015) 'Exogenous and evoked oxytocin restores social behavior in the Cntnap2 mouse model of autism', *Science Translational Medicine*, 7(271), p. 271ra8.
- Peng S.A.; Warren, S.T., J. . R. and Peng S.A.; Warren S.T., J. . R. (2004) 'RNA and microRNAs in fragile X mental retardation', *Nature Cell Biology*, 6(11), pp. 1048–1053.
- Peris, J., MacFadyen, K., Smith, J. A., de Kloet, A. D., Wang, L. and Krause, E. G. (2017) 'Oxytocin receptors are expressed on dopamine and glutamate neurons in the mouse ventral tegmental area that project to nucleus accumbens and other mesolimbic targets', *Journal of Comparative Neurology*, 525(5), pp. 1094–1108.
- Perry, D. W., Marston, G. M., Hinder, S. A. J., Munden, A. C. and Roy, A. (2001) 'The Phenomenology of Depressive Illness in People with a Learning Disability and Autism', *Autism*, 5(3), pp. 265–275.
- Peters, S., Slattery, D. A., Uschold-Schmidt, N., Reber, S. O. and Neumann, I. D. (2014) 'Dose-dependent effects of chronic central infusion of oxytocin on anxiety, oxytocin receptor binding and stress-related parameters in mice', *Psychoneuroendocrinology*, 42, pp. 225–236.

- Petrovic, P., Kalisch, R., Singer, T. and Dolan, R. J. (2008) 'Oxytocin attenuates affective evaluations of conditioned faces and amygdala activity', *Journal of Neuroscience*, 28(26), pp. 6607–6615.
- Pfeffer, C. R., Altemus, M., Heo, M. and Jiang, H. (2007) 'Salivary Cortisol and Psychopathology in Children Bereaved by the September 11, 2001 Terror Attacks', *Biological Psychiatry*, 61(8), pp. 957–965.
- Philip, N. S., Carpenter, L. L., Tyrka, A. R. and Price, L. H. (2010) 'Nicotinic acetylcholine receptors and depression: a review of the preclinical and clinical literature.', *Psychopharmacology*, 212(1), pp. 1–12.
- Picciotto, M. R. (1998) 'Common aspects of the action of nicotine and other drugs of abuse', *Drug and Alcohol Dependence*, 51(1–2), pp. 165–172.
- Picciotto, M. R., Caldarone, B. J., King, S. L. and Zachariou, V. (2000) 'Nicotinic Receptors in the Brain: Links between Molecular Biology and Behavior', *Neuropsychopharmacology*, 22(22), pp. 451–465.
- Picciotto, M. R., Lewis, A. S., van Schalkwyk, G. I. and Mineur, Y. S. (2015) 'Mood and anxiety regulation by nicotinic acetylcholine receptors: A potential pathway to modulate aggression and related behavioral states', *Neuropharmacology*, 96(Pt B), pp. 235–243.
- Pieretti, M., Zhang, F. P., Fu, Y. H., Warren, S. T., Oostra, B. A., Caskey, C. T. and Nelson, D. L. (1991) 'Absence of expression of the FMR-1 gene in fragile X syndrome.', *Cell*, 66(4), pp. 817–22.
- Pietropaolo, S., Guilleminot, A., Martin, B., D'Amato, F. R. and Crusio, W. E. (2011) 'Genetic-background modulation of core and variable autistic-like symptoms in Fmr1 knock-out mice', *PLoS One*, 6(2), p. e17073.
- Pineles, S. L., Avery, R. A. and Liu, G. T. (2010) 'Vitamin B12 Optic Neuropathy in Autism', *Pediatrics*, 126(4), pp. e967–e970.
- Piven, J., Arndt, S., Bailey, J. and Andreasen, N. (1996) 'Regional Brain Enlargement in Autism: A Magnetic Resonance Imaging Study', *Journal of the American Academy of Child & Adolescent Psychiatry*, 35(4), pp. 530–536.
- Pizzarelli, R. and Cherubini, E. (2011) 'Alterations of GABAergic Signaling in Autism Spectrum Disorders', *Neural Plasticity*, 2011, pp. 1–12.
- Pletnikov, M. V, Moran, T. H. and Carbone, K. M. (2002) 'Borna disease virus infection of the neonatal rat: developmental brain injury model of autism spectrum disorders', *Frontiers Bioscience*, 7, pp. d593-607.
- Pliakas, A. M., Carlson, R. R., Neve, R. L., Konradi, C., Nestler, E. J. and Carlezon Jr., W. A. (2001) 'Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens', *Journal of Neuroscience*, 21(18), pp. 7397–7403.
- Pobbe, R. L., Pearson, B. L., Defensor, E. B., Bolivar, V. J., Young 3rd, W. S., Lee, H. J., Blanchard, D. C. and Blanchard, R. J. (2012) 'Oxytocin receptor knockout mice display deficits in the expression of autism-related behaviors', *Hormones and Behavior*, 61(3), pp. 436–444.
- Podhorna, J. and Didriksen, M. (2004) 'The heterozygous reeler mouse: behavioural phenotype', *Behavioral Brain Research*, 153(1), pp. 43–54.
- Pop, A. S., Gomez-Mancilla, B., Neri, G., Willemsen, R. and Gasparini, F. (2014) 'Fragile X syndrome: a preclinical review on metabotropic glutamate receptor 5 (mGluR5) antagonists and drug development', *Psychopharmacology (Berl)*, 231(6), pp. 1217–1226.

- Popik, P. and van Ree, J. M. (1991) 'Oxytocin but not vasopressin facilitates social recognition following injection into the medial preoptic area of the rat brain', *European Neuropsychopharmacology*, 1(4), pp. 555–560.
- Portera-Cailliau, C. (2012) 'Which comes first in fragile X syndrome, dendritic spine dysgenesis or defects in circuit plasticity?', *Neuroscientist*, 18(1), pp. 28–44.
- Posey, D. J., Kem, D. L., Swiezy, N. B., Sweeten, T. L., Wiegand, R. E. and McDougale, C. J. (2004) 'A Pilot Study of d -Cycloserine in Subjects With Autistic Disorder', *American Journal of Psychiatry*, 161(11), pp. 2115–2117.
- Premack, D. and Woodruff, G. (1978) 'Does the chimpanzee have a theory of mind?', *Behavioral and Brain Sciences*, 1(4), p. 515.
- Provencal, N., Suderman, M. J., Guillemin, C., Massart, R., Ruggiero, A., Wang, D., Bennett, A. J., Pierre, P. J., Friedman, D. P., Cote, S. M., Hallett, M., Tremblay, R. E., Suomi, S. J. and Szyf, M. (2012) 'The Signature of Maternal Rearing in the Methylome in Rhesus Macaque Prefrontal Cortex and T Cells', *Journal of Neuroscience*, 32(44), pp. 15626–15642.
- Provenzano, G., Clementi, E., Genovesi, S., Scali, M., Tripathi, P. P., Sgado, P. and Bozzi, Y. (2014) 'GH Dysfunction in Engrailed-2 Knockout Mice, a Model for Autism Spectrum Disorders', *Frontiers in Pediatrics*, 2, p. 92.
- Psychiatric GWAS Consortium Coordinating Committee, Cichon, S., Craddock, N., Daly, M., Faraone, S. V., Gejman, P. V., Kelsoe, J., Lehner, T., Levinson, D. F., Moran, A., Sklar, P. and Sullivan, P. F. (2009) 'Genomewide Association Studies: History, Rationale, and Prospects for Psychiatric Disorders', *American Journal of Psychiatry*, 166(5), pp. 540–556.
- Qin, M., Kang, J. and Smith, C. B. (2005) 'A null mutation for Fmr1 in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior', *Neuroscience*, 135(3), pp. 999–1009.
- Qin, X.-Y., Feng, J.-C., Cao, C., Wu, H.-T., Loh, Y. P. and Cheng, Y. (2016) 'Association of Peripheral Blood Levels of Brain-Derived Neurotrophic Factor With Autism Spectrum Disorder in Children', *JAMA Pediatrics*, 170(11), p. 1079.
- Quick, M. W. and Lester, R. A. J. (2002) 'Desensitization of neuronal nicotinic receptors', *Journal of Neurobiology*, 53(4), pp. 457–478.
- Rabenstein, R. L., Caldarone, B. J. and Picciotto, M. R. (2006) 'The nicotinic antagonist mecamylamine has antidepressant-like effects in wild-type but not $\beta 2$ - or $\alpha 7$ -nicotinic acetylcholine receptor subunit knockout mice', *Psychopharmacology*, 189(3), pp. 395–401.
- Ralph-Williams, R. J., Paulus, M. P., Zhuang, X., Hen, R. and Geyer, M. A. (2003) 'Valproate attenuates hyperactive and perseverative behaviors in mutant mice with a dysregulated dopamine system', *Biological Psychiatry*, 53(4), pp. 352–359.
- Ralph, R. J., Paulus, M. P., Fumagalli, F., Caron, M. G. and Geyer, M. A. (2001) 'Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists', *Journal of Neuroscience*, 21(1), pp. 305–313.
- Rangwala, F., Drisdell, R. C., Rakhilin, S., Ko, E., Atluri, P., Harkins, A. B., Fox, A. P., Salman, S. S. and Green, W. N. (1997) 'Neuronal alpha-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors.', *The Journal of Neuroscience*, 17(21), pp. 8201–12.
- Rasalam, A. D., Hailey, H., Williams, J. H. G., Moore, S. J., Turnpenny, P. D., Lloyd, D. J. and Dean, J. C. S. (2005) 'Characteristics of fetal anticonvulsant syndrome associated autistic disorder.', *Developmental Medicine and Child Neurology*, 47(8), pp. 551–555.

- Rasmussen, M. S., Simonsen, J. A., Sandgaard, N. C. F., Hoilund-Carlsen, P. F. and Bie, P. (2004) 'Effects of oxytocin in normal man during low and high sodium diets', *Acta Physiologica Scandinavica*, 181(2), pp. 247–257.
- Razzoli, M., Roncari, E., Guidi, A., Carboni, L., Arban, R., Gerrard, P. and Bacchi, F. (2006) 'Conditioning properties of social subordination in rats: Behavioral and biochemical correlates of anxiety', *Hormones and Behavior*, 50(2), pp. 245–251.
- Reader, S. M. and Laland, K. N. (2002) 'Social intelligence, innovation, and enhanced brain size in primates.', *Proceedings of the National Academy of Sciences of the United States of America*, 99(7), pp. 4436–4441.
- Reddy, K. S. (2005) 'Cytogenetic abnormalities and fragile-x syndrome in Autism Spectrum Disorder', *BMC Medical Genetics*, 6(1), p. 3.
- Van Ree, J. M., Niesink, R. J., Van Wolfswinkel, L., Ramsey, N. F., Kornet, M. M., Van Furth, W. R., Vanderschuren, L. J., Gerrits, M. A. and Van den Berg, C. L. (2000) 'Endogenous opioids and reward', *European Journal of Pharmacology*, 405(1–3), pp. 89–101.
- Reiersen, A. M. and Todd, R. D. (2008) 'Co-occurrence of ADHD and autism spectrum disorders: phenomenology and treatment.', *Expert Review of Neurotherapeutics*, 8(4), pp. 657–69.
- Reitstetter, R., Lukas, R. J. and Gruener, R. (1999) 'Dependence of nicotinic acetylcholine receptor recovery from desensitization on the duration of agonist exposure.', *The Journal of Pharmacology and Experimental Therapeutics*, 289(2), pp. 656–60.
- Reynolds, I. (2001) '[H](+)MK801 Radioligand Binding Assay at the N-Methyl- D -Aspartate Receptor', in *Current Protocols in Pharmacology*. John Wiley & Sons, Inc., p. Unit 1.20.
- Ricceri, L., Moles, A. and Crawley, J. (2007) 'Behavioral phenotyping of mouse models of neurodevelopmental disorders: relevant social behavior patterns across the life span', *Behavioral and Brain Sciences*, 176(1), pp. 40–52.
- Ricci, S., Businaro, R., Ippoliti, F., Lo Vasco, V. R., Massoni, F., Onofri, E., Troili, G. M., Pontecorvi, V., Morelli, M., Rapp Ricciardi, M. and Archer, T. (2013) 'Altered Cytokine and BDNF Levels in Autism Spectrum Disorder', *Neurotoxicity Research*, 24(4), pp. 491–501.
- Rice, C., Rosanoff, M., Dawson, G., Durkin, M., Croen, L., Singer, A. and Yeargin-AM (2012) 'Evaluating Changes in the Prevalence of the Autism Spectrum Disorders (ASDs)', *Public Health Reviews*, 34(2).
- Rilling, J. K., DeMarco, A. C., Hackett, P. D., Thompson, R., Ditzen, B., Patel, R. and Pagnoni, G. (2012) 'Effects of intranasal oxytocin and vasopressin on cooperative behavior and associated brain activity in men', *Psychoneuroendocrinology*, 37(4), pp. 447–461.
- Rimmele, U., Hediger, K., Heinrichs, M. and Klaver, P. (2009) 'Oxytocin makes a face in memory familiar', *Journal of Neuroscience*, 29(1), pp. 38–42.
- Rinehart, N. J., Bradshaw, J. L., Brereton, A. V and Tonge, B. J. (2001) 'Movement preparation in high-functioning autism and Asperger disorder: a serial choice reaction time task involving motor reprogramming', *Journal of Autism and Developmental Disorders*, 31(1), pp. 79–88.
- Rinehart, N. J., Tonge, B. J., Iansek, R., McGinley, J., Brereton, A. V, Enticott, P. G. and Bradshaw, J. L. (2006) 'Gait function in newly diagnosed children with autism: Cerebellar and basal ganglia related motor disorder', *Developmental Medicine & Child Neurology*, 48(10), pp. 819–824.
- Risch, S. C., Cohen, R. M., Janowsky, D. S., Kalin, N. H., Sitaram, N., Gillin, J. C. and

- Murphy, D. L. (1981) 'Physostigmine induction of depressive symptomatology in normal human subjects.', *Psychiatry research*, 4(1), pp. 89–94.
- Ritvo, E., Freeman, B., Pingree, C., Mason-Brothers, A., Jorde, L., Jenson, W., McMahon, W., Petersen, P., Mo, A. and Ritvo, A. (1989) 'The UCLA-University of Utah epidemiologic survey of autism: recurrence risk estimates and genetic counseling', *American Journal of Psychiatry*, 146(8), pp. 1032–1036.
- Robertson, K. D. and Wolffe, A. P. (2000) 'DNA methylation in health and disease', *Nature Reviews Genetics*, 1(1), pp. 11–19.
- Robinson, E., Lichtenstein, P., Anckarsater, H., Happe, F. and Ronald, A. (2013) 'Examining and interpreting the female protective effect against autistic behavior', *Proceedings of the National Academy of Sciences*, 110(13), pp. 5258–5262.
- Roelofs, K., van Peer, J., Berretty, E., Jong, P. de, Spinhoven, P. and Elzinga, B. M. (2009) 'Hypothalamus–Pituitary–Adrenal Axis Hyperresponsiveness Is Associated with Increased Social Avoidance Behavior in Social Phobia', *Biological Psychiatry*, 65(4), pp. 336–343.
- Rohde, P. (2001) 'The relevance of hierarchies, territories, defeat for depression in humans: hypotheses and clinical predictions', *Journal of Affective Disorders*, 65(3), pp. 221–230.
- Rohde, P., Al., E., Dujon, B., Pollack, D. B., Yuwiler, A., Head, J., Al., E., LeCompte, W. A., Pitcairn, T. and Ricci-Bitti, P. E. (2001) 'The relevance of hierarchies, territories, defeat for depression in humans: hypotheses and clinical predictions.', *Journal of Affective Disorders*, 65(3), pp. 221–30.
- Ross, D. L., Klykylo, W. M. and Hitzemann, R. (1987) 'Reduction of elevated CSF beta-endorphin by fenfluramine in infantile autism', *Pediatric Neurology*, 3(2), pp. 83–86.
- Ross, H. E. and Young, L. J. (2009) 'Oxytocin and the neural mechanisms regulating social cognition and affiliative behavior', *Frontiers in Neuroendocrinology*, 30(4), pp. 534–547.
- Rossignol, D. A. and Frye, R. E. (2012) 'Mitochondrial dysfunction in autism spectrum disorders: a systematic review and meta-analysis', *Molecular Psychiatry*, 17(3), pp. 290–314.
- Roy, A. (1981) 'Role of past loss in depression.', *Archives of General Psychiatry*, 38(3), pp. 301–2.
- Roy, M. A., Neale, M. C., Pedersen, N. L., Mathé, A. A. and Kendler, K. S. (1995) 'A twin study of generalized anxiety disorder and major depression.', *Psychological Medicine*, 25(5), pp. 1037–49.
- Roy, S., Barke, R. A. and Loh, H. H. (1998) 'Mu-opioid receptor-knockout mice: role of mu-opioid receptor in morphine mediated immune functions.', *Brain Research. Molecular Brain Research*, 61(1–2), pp. 190–4.
- Rozen, F., Russo, C., Banville, D. and Zingg, H. H. (1995) 'Structure, characterization, and expression of the rat oxytocin receptor gene.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 92(1), pp. 200–4.
- Ruhé, H. G., Mason, N. S. and Schene, A. H. (2007) 'Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies', *Molecular Psychiatry*, 12(4), pp. 331–359. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17389902> (Accessed: 13 July 2017).
- Ruis, M. A., te Brake, J. H., Buwalda, B., De Boer, S. F., Meerlo, P., Korte, S. M., Blokhuis, H. J., Koolhaas, J. M., Tilders, F. . and Koolhaas, J. . (1999) 'Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat.', *Psychoneuroendocrinology*, 24(3), pp. 285–300.

Russo, A. J. (2013) 'Decreased Epidermal Growth Factor (EGF) Associated with HMGB1 and Increased Hyperactivity in Children with Autism.', *Biomarker Insights*. SAGE Publications, 8, pp. 35–41.

Rutter, M. (2005) 'Aetiology of autism: findings and questions', *Journal of Intellectual Disability Research*, 49(4), pp. 231–238.

Ryan, B. C., Young, N. B., Crawley, J. N., Bodfish, J. W. and Moy, S. S. (2010) 'Social deficits, stereotypy and early emergence of repetitive behavior in the C58/J inbred mouse strain', *Behavioural Brain Research*, 208(1), pp. 178–188.

Rygula, R., Abumaria, N., Flügge, G., Fuchs, E., Rüther, E. and Havemann-Reinecke, U. (2005) 'Anhedonia and motivational deficits in rats: Impact of chronic social stress', *Behavioural Brain Research*, 162(1), pp. 127–134.

Sala, M., Braida, D., Lentini, D., Busnelli, M., Bulgheroni, E., Capurro, V., Finardi, A., Donzelli, A., Pattini, L., Rubino, T., Parolaro, D., Nishimori, K., Parenti, M. and Chini, B. (2011) 'Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism', *Biological Psychiatry*, 69(9), pp. 875–882.

Salín-Pascual, R. J., Alcocer-Castillejos, N. V and Alejo-Galarza, G. (2017) 'Nicotine dependence and psychiatric disorders.', *Revista de investigacion clinica; organo del Hospital de Enfermedades de la Nutricion*, 55(6), pp. 677–93.

Salín-Pascual, R. J., de la Fuente, J. R., Galicia-Polo, L. and Drucker-Colín, R. (1995) 'Effects of transderman nicotine on mood and sleep in nonsmoking major depressed patients.', *Psychopharmacology*, 121(4), pp. 476–9.

Salinger, W. L., Ladrow, P. and Wheeler, C. (2003) 'Behavioral phenotype of the reeler mutant mouse: effects of RELN gene dosage and social isolation', *Behavioral Neuroscience*, 117(6), pp. 1257–1275.

Samochowiec, J., Hajduk, A., Samochowiec, A., Horodnicki, J., Stępień, G., Grzywacz, A. and Kucharska-Mazur, J. (2004) 'Association studies of MAO-A, COMT, and 5-HTT genes polymorphisms in patients with anxiety disorders of the phobic spectrum', *Psychiatry Research*, 128(1), pp. 21–26.

Sanders, S. J., Ercan-Sencicek, A. G., Hus, V., Luo, R., Murtha, M. T., Moreno-De-Luca, D., Chu, S. H., Moreau, M. P., Gupta, A. R., Thomson, S. A., Mason, C. E., Bilguvar, K., Celestino-Soper, P. B., Choi, M., Crawford, E. L., Davis, L., Wright, N. R., Dhodapkar, R. M., DiCola, M., DiLullo, N. M., Fernandez, T. V, Fielding-Singh, V., Fishman, D. O., Frahm, S., Garagaloyan, R., Goh, G. S., Kammela, S., Klei, L., Lowe, J. K., Lund, S. C., McGrew, A. D., Meyer, K. A., Moffat, W. J., Murdoch, J. D., O'Roak, B. J., Ober, G. T., Pottenger, R. S., Raubeson, M. J., Song, Y., Wang, Q., Yaspan, B. L., Yu, T. W., Yurkiewicz, I. R., Beaudet, A. L., Cantor, R. M., Curnall, M., Grice, D. E., Gunel, M., Lifton, R. P., Mane, S. M., Martin, D. M., Shaw, C. A., Sheldon, M., Tischfield, J. A., Walsh, C. A., Morrow, E. M., Ledbetter, D. H., Fombonne, E., Lord, C., Martin, C. L., Brooks, A. I., Sutcliffe, J. S., Cook Jr., E. H., Geschwind, D., Roeder, K., Devlin, B. and State, M. W. (2011) 'Multiple recurrent de novo CNVs, including duplications of the 7q11.23 Williams syndrome region, are strongly associated with autism', *Neuron*, 70(5), pp. 863–885.

Sanders, S. J., Murtha, M. T., Gupta, A. R., Murdoch, J. D., Raubeson, M. J., Willsey, A. J., Ercan-Sencicek, A. G., DiLullo, N. M., Parikshak, N. N., Stein, J. L., Walker, M. F., Ober, G. T., Teran, N. A., Song, Y., El-Fishawy, P., Murtha, R. C., Choi, M., Overton, J. D., Bjornson, R. D., Carriero, N. J., Meyer, K. A., Bilguvar, K., Mane, S. M., Sestan, N., Lifton, R. P., Gunel, M., Roeder, K., Geschwind, D. H., Devlin, B. and State, M. W. (2012) 'De novo mutations revealed by whole-exome sequencing are strongly associated with autism',

Nature, 485(7397), pp. 237–241.

Sandman, C. A. and Kemp, A. S. (2011) 'Opioid Antagonists May Reverse Endogenous Opiate "Dependence" in the Treatment of Self-Injurious Behavior', *Pharmaceuticals*, 4(12), pp. 366–381.

Santoro, M. R., Bray, S. M. and Warren, S. T. (2012) 'Molecular mechanisms of fragile X syndrome: a twenty-year perspective', *Annual Review of Pathology*, 7, pp. 219–245.

Saricicek, A., Esterlis, I., Maloney, K. H., Mineur, Y. S., Ruf, B. M., Muralidharan, A., Chen, J. I., Cosgrove, K. P., Kerestes, R., Ghose, S., Tamminga, C. A., Pittman, B., Bois, F., Tamagnan, G., Seibyl, J., Picciotto, M. R., Staley, J. K. and Bhagwagar, Z. (2012) 'Persistent $\beta 2^*$ -nicotinic acetylcholinergic receptor dysfunction in major depressive disorder.', *American Journal of Psychiatry*, 169(8), pp. 851–9.

Sato, D., Lionel, A. C., Leblond, C. S., Prasad, A., Pinto, D., Walker, S., O'Connor, I., Russell, C., Drmic, I. E., Hamdan, F. F., Michaud, J. L., Endris, V., Roeth, R., Delorme, R., Huguet, G., Leboyer, M., Rastam, M., Gillberg, C., Lathrop, M., Stavropoulos, D. J., Anagnostou, E., Weksberg, R., Fombonne, E., Zwaigenbaum, L., Fernandez, B. A., Roberts, W., Rappold, G. A., Marshall, C. R., Bourgeron, T., Szatmari, P. and Scherer, S. W. (2012) 'SHANK1 Deletions in Males with Autism Spectrum Disorder', *The American Journal of Human Genetics*, 90(5), pp. 879–887.

Scahill, L., Hallett, V., Aman, M. G., McDougle, C. J., Eugene Arnold, L., McCracken, J. T., Tierney, E., Deng, Y., Dziura, J., Vitiello, B. and Research Units on Pediatric Psychopharmacology (RUPP) Autism Network (2013) 'Brief Report: Social Disability in Autism Spectrum Disorder: Results from Research Units on Pediatric Psychopharmacology (RUPP) Autism Network Trials', *Journal of Autism and Developmental Disorders*, 43(3), pp. 739–746.

Scheel, C., Rotarska-Jagiela, A., Schilbach, L., Lehnhardt, F. G., Krug, B., Vogeley, K. and Tepest, R. (2011) 'Imaging derived cortical thickness reduction in high-functioning autism: Key regions and temporal slope', *NeuroImage*, 58(2), pp. 391–400.

Schneider, M. and Koch, M. (2005) 'Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment', *Neuropsychopharmacology*, 30(5), pp. 944–957.

Schneider, T. and Przewlocki, R. (2005) 'Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism', *Neuropsychopharmacology*, 30(1), pp. 80–89.

Schneier, F. R., Liebowitz, M. R., Abi-Dargham, A., Zea-Ponce, Y., Lin, S. H. and Laruelle, M. (2000) 'Low dopamine D(2) receptor binding potential in social phobia', *American Journal of Psychiatry*, 157(3), pp. 457–459.

Schoenfelder, E. N., Sandler, I. N., Wolchik, S. and MacKinnon, D. (2011) 'Quality of Social Relationships and the Development of Depression in Parentally-Bereaved Youth', *Journal of Youth and Adolescence*, 40(1), pp. 85–96.

Schuch, V., Utsumi, D. A., Costa, T. V. M. M., Kulikowski, L. D. and Muszkat, M. (2015) 'Attention Deficit Hyperactivity Disorder in the Light of the Epigenetic Paradigm.', *Frontiers in Psychiatry*, 6, p. 126.

Schwarz, C., Snidman, N. and Kagan, J. (1999) 'Adolescent Social Anxiety as an Outcome of Inhibited Temperament in Childhood', *Journal of the American Academy of Child & Adolescent Psychiatry*, 38(8), pp. 1008–1015.

Sears, L. L., Vest, C., Mohamed, S., Bailey, J., Ranson, B. J. and Piven, J. (1999) 'An MRI study of the basal ganglia in autism.', *Progress in Neuro-psychopharmacology & Biological*

Psychiatry, 23(4), pp. 613–24.

Selye, H. (1936) 'A syndrome produced by diverse nocuous agents', *Nature*, 138(3479), p. 32.

Selye, H. (1941) 'Pharmacological classification of steroid hormones', *Nature*, 148, p. 84.

Semba, J., Matakai, C., Yamada, S., Nankai, M. and Toru, M. (1998) *Antidepressant-like Effects of Chronic Nicotine on Learned Helplessness Paradigm in Rats*, *Biological Psychiatry*.

Serajee, F. J., Zhong, H., Nabi, R. and Huq, A. H. M. M. (2003) 'The metabotropic glutamate receptor 8 gene at 7q31: partial duplication and possible association with autism.', *Journal of Medical Genetics*, 40(4), p. e42.

Sethna, F., Feng, W., Ding, Q., Robison, A. J., Feng, Y. and Wang, H. (2017) 'Enhanced expression of ADCY1 underlies aberrant neuronal signalling and behaviour in a syndromic autism model.', *Nature Communications*, 8, p. 14359.

Sgoifo, A., Pozzato, C., Meerlo, P., Costoli, T., Manghi, M., Stilli, D., Olivetti, G. and Musso, E. (2002) 'Intermittent Exposure to Social Defeat and Open-field Test in Rats: Acute and Long-term Effects on ECG, Body Temperature and Physical Activity', *Stress*, 5(1), pp. 23–35.

Shahrokh, D. K., Zhang, T. Y., Diorio, J., Gratton, A. and Meaney, M. J. (2010) 'Oxytocin-dopamine interactions mediate variations in maternal behavior in the rat', *Endocrinology*, 151(5), pp. 2276–2286.

Sharma, A., Hoeffer, C. A., Takayasu, Y., Miyawaki, T., McBride, S. M., Klann, E. and Zukin, R. S. (2010) 'Dysregulation of mTOR signaling in fragile X syndrome', *Journal of Neuroscience*, 30(2), pp. 694–702.

Shinohe, A., Hashimoto, K., Nakamura, K., Tsujii, M., Iwata, Y., Tsuchiya, K. J., Sekine, Y., Suda, S., Suzuki, K., Sugihara, G., Matsuzaki, H., Minabe, Y., Sugiyama, T., Kawai, M., Iyo, M., Takei, N. and Mori, N. (2006) 'Increased serum levels of glutamate in adult patients with autism', *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 30(8), pp. 1472–1477.

Shippenberg, T. S. and Elmer, G. I. (1998) 'The neurobiology of opiate reinforcement.', *Critical Reviews in Neurobiology*, 12(4), pp. 267–303.

Shippenberg, T. S. and Herz, A. (1986) 'Differential effects of mu and kappa opioid systems on motivational processes', *NIDA Research Monograph*, 75, pp. 563–566.

Shippenberg, T. S., Herz, A., Spanagel, R., Bals-Kubik, R. and Stein, C. (1992) 'Conditioning of opioid reinforcement: neuroanatomical and neurochemical substrates.', *Annals of the New York Academy of Sciences*, 654, pp. 347–56.

Sia, A. T., Lim, Y., Lim, E. C., Goh, R. W., Law, H. Y., Landau, R., Teo, Y. Y. and Tan, E. C. (2008) 'A118G single nucleotide polymorphism of human mu-opioid receptor gene influences pain perception and patient-controlled intravenous morphine consumption after intrathecal morphine for postcesarean analgesia', *Anesthesiology*, 109(3), pp. 520–526.

Siegel, M. and Beaulieu, A. A. (2012) 'Psychotropic Medications in Children with Autism Spectrum Disorders: A Systematic Review and Synthesis for Evidence-Based Practice', *Journal of Autism and Developmental Disorders*, 42(8), pp. 1592–1605.

Silverman, J., Yang, M., Lord, C. and Crawley, J. (2010) 'Behavioural phenotyping assays for mouse models of autism', *Nature Reviews Neuroscience*, 11(7), pp. 490–502.

Simonin, F., Gaveriaux-Ruff, C., Befort, K., Matthes, H., Lannes, B., Micheletti, G., Mattei, M.

- G., Charron, G., Bloch, B. and Kieffer, B. (1995) 'kappa-Opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system', *PNAS USA*, 92(15), pp. 7006–7010.
- Simonoff, E., Pickles, A., Charman, T., Chandler, S., Loucas, T. and Baird, G. (2008) 'Psychiatric Disorders in Children With Autism Spectrum Disorders: Prevalence, Comorbidity, and Associated Factors in a Population-Derived Sample', *Journal of the American Academy of Child & Adolescent Psychiatry*, 47(8), pp. 921–929.
- Slaughter, V. (2015) 'Theory of Mind in Infants and Young Children: A Review', *Australian Psychologist*, 50(3), pp. 169–172.
- Slavich, G. M., Monroe, S. M. and Gotlib, I. H. (2011) 'Early Parental Loss and Depression History: Associations with Recent Life Stress in Major Depressive Disorder', *Journal of Psychiatric Research*, 45(9), p. 1146–1152.
- Slavich, G. M., O'Donovan, A., Epel, E. S. and Kemeny, M. E. (2010) 'Black sheep get the blues: A psychobiological model of social rejection and depression', *Neuroscience & Biobehavioral Reviews*, 35(1), pp. 39–45.
- Smith, T., Watthen Lovaas, N. and Ivar Lovaas, O. (2002) 'Behaviors of children with high-functioning autism when paired with typically developing versus delayed peers: a preliminary study', *Behavioral Interventions*. John Wiley & Sons, Ltd., 17(3), pp. 129–143.
- Sobel, A., Hofler, J., Heidmann, T. and Changeux, J. P. (1979) 'Structural and functional properties of the acetylcholine regulator.', *Advances in Cytopharmacology*, 3, pp. 191–6.
- Sochocky, N. and Milin, R. (2013) 'Second Generation Antipsychotics in Asperger's Disorder and High Functioning Autism: A Systematic Review of the Literature and Effectiveness of Meta-Analysis.', *Current Clinical Pharmacology*, 8(4), pp. 370–379.
- Sofronoff, K., Leslie, A. and Brown, W. (2004) 'Parent Management Training and Asperger Syndrome', *Autism*, 8(3), pp. 301–317.
- Sokolov, O., Kost, N., Andreeva, O., Korneeva, E., Meshavkin, V., Tarakanova, Y., Dadayan, A., Zolotarev, Y., Grachev, S., Mikheeva, I., Varlamov, O. and Zozulya, A. (2014) 'Autistic children display elevated urine levels of bovine casomorphin-7 immunoreactivity', *Peptides*, 56, pp. 68–71.
- Solberg, M. and Olweus, D. (2003) 'Prevalence estimation of school bullying with the Olweus Bully/Victim Questionnaire', *Aggressive Behavior*, 29(3), pp. 239–268.
- Solomon, M., Goodlin-Jones, B. L. and Anders, T. F. (2004) 'A social adjustment enhancement intervention for high functioning autism, Asperger's syndrome, and pervasive developmental disorder NOS.', *Journal of Autism and Developmental Disorders*, 34(6), pp. 649–68.
- Sorensen, E. M., Bertelsen, F., Weikop, P., Skovborg, M. M., Banke, T., Drasbek, K. R. and Scheel-Kruger, J. (2015) 'Hyperactivity and lack of social discrimination in the adolescent Fmr1 knockout mouse', *Behavioral Pharmacology*, 26(8 Spec No), pp. 733–740.
- Southwick, S. M., Bremner, D., Krystal, J. H. and Charney, D. S. (1994) 'Psychobiologic research in post-traumatic stress disorder.', *The Psychiatric Clinics of North America*, 17(2), pp. 251–64.
- Sparks, B. F., Friedman, S. D., Shaw, D. W., Aylward, E. H., Echelard, D., Artru, A. A., Maravilla, K. R., Giedd, J. N., Munson, J., Dawson, G. and Dager, S. R. (2002) 'Brain structural abnormalities in young children with autism spectrum disorder.', *Neurology*, 59(2), pp. 184–92.
- Spencer, C. M., Alekseyenko, O., Hamilton, S. M., Thomas, A. M., Serysheva, E., Yuva-

- Paylor, L. A. and Paylor, R. (2011) 'Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses', *Autism Research*, 4(1), pp. 40–56.
- Spencer, C. M., Alekseyenko, O., Serysheva, E., Yuva-Paylor, L. A. and Paylor, R. (2005) 'Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome', *Genes, Brain and Behavior*, 4(7), pp. 420–430.
- Spencer, D., Marshall, J., Post, B., Kulakodlu, M., Newschaffer, C., Dennen, T., Azocar, F. and Jain, A. (2013) 'Psychotropic Medication Use and Polypharmacy in Children With Autism Spectrum Disorders', *Pediatrics*, 132(5), pp. 833–840.
- Spezio, M. L., Huang, P.-Y. S., Castelli, F. and Adolphs, R. (2007) 'Amygdala Damage Impairs Eye Contact During Conversations with Real People', *Journal of Neuroscience*, 27(15), pp. 3994–3997.
- Spijker, J., Graaf, R., Bijl, R. V., Beekman, A. T. F., Ormel, J. and Nolen, W. A. (2004) 'Functional disability and depression in the general population. Results from the Netherlands Mental Health Survey and Incidence Study (NEMESIS)', *Acta Psychiatrica Scandinavica*, 110(3), pp. 208–214.
- Stanfield, A. C., McIntosh, A. M., Spencer, M. D., Philip, R., Gaur, S., Lawrie, S. M. and Al., E. (2008) 'Towards a neuroanatomy of autism: a systematic review and meta-analysis of structural magnetic resonance imaging studies.', *European psychiatry: the journal of the Association of European Psychiatrists*, 23(4), pp. 289–99.
- Stanford, S. (2017) 'Confusing preclinical (predictive) drug screens with animal “models” of psychiatric disorders, or “disorder-like” behaviour, is undermining confidence in behavioural neuroscience', *Journal of Psychopharmacology*, 31(6), pp. 641–643.
- Starr, M. S. (1996) 'The role of dopamine in epilepsy', *Synapse*, 22(2), pp. 159–194.
- Stassen Berger, K. (2007) 'Update on bullying at school: science forgotten?', *Developmental Review*, 27, pp. 90–126.
- Stavropoulos, K. K. M. and Carver, L. J. (2013) 'Research Review: Social motivation and oxytocin in autism - implications for joint attention development and intervention', *Journal of Child Psychology and Psychiatry*, 54(6), pp. 603–618.
- Van Steensel, F. A., Bögels, S. M. and Perrin, S. (2011) 'Anxiety disorders in children and adolescents with autistic spectrum disorders: a meta-analysis.', *Clinical Child and Family Psychology Review*, 14(3), pp. 302–17.
- Stefanski, V. (2000) 'Social stress in laboratory rats: hormonal responses and immune cell distribution.', *Psychoneuroendocrinology*, 25(4), pp. 389–406.
- Stein, M. B., Stein, D. J., Zalta, A., Al., E., Lahmann, C., Pedrosa, G. and Al., E. (2008) 'Social anxiety disorder.', *Lancet (London, England)*. Marcel Dekker, New York, 371(9618), pp. 1115–25.
- Stoop, R. (2012) 'Neuromodulation by oxytocin and vasopressin.', *Neuron*. Elsevier, 76(1), pp. 142–59.
- Stotz-Ingenlath, G. (2000) 'Epistemological aspects of Eugen Bleuler's conception of Schizophrenia in 1911', *Medicine, Health Care and Philosophy*. Kluwer Academic Publishers, 3(2), pp. 153–159.
- Strambi, M., Longini, M., Hayek, J., Berni, S., Macucci, F., Scalacci, E. and Vezzosi, P. (2006) 'Magnesium Profile in Autism', *Biological Trace Element Research*, 109(2), pp. 97–104.

- Striepens, N., Kendrick, K. M., Hanking, V., Landgraf, R., Wullner, U., Maier, W. and Hurlemann, R. (2013) 'Elevated cerebrospinal fluid and blood concentrations of oxytocin following its intranasal administration in humans', *Scientific Reports*, 3, p. 3440.
- Suderman, M., McGowan, P. O., Sasaki, A., Huang, T. C. T., Hallett, M. T., Meaney, M. J., Turecki, G. and Szyf, M. (2012) 'Conserved epigenetic sensitivity to early life experience in the rat and human hippocampus', *Proceedings of the National Academy of Sciences*, 109(Supplement_2), pp. 17266–17272.
- Sukhodolsky, D., Scahill, L., Gadow, K., Arnold, L., Aman, M., McDougle, C., McCracken, J., Tierney, E., Williams WS, Lecavalier, L. and Vitiello, B. (2008) 'Parent-Rated Anxiety Symptoms in Children with Pervasive Developmental Disorders: Frequency and Association with Core Autism Symptoms and Cognitive Functioning', *Journal of Abnormal Child Psychology*, 36(1), pp. 117–128.
- Sutcliffe, J. S., Nelson, D. L., Zhang, F., Pieretti, M., Caskey, C. T., Saxe, D. and Warren, S. T. (1992) 'DNA methylation represses FMR-1 transcription in fragile X syndrome', *Human Molecular Genetics*, 1(6), pp. 397–400.
- Suzuki, K., Hashimoto, K., Iwata, Y., Nakamura, K., Tsujii, M., Tsuchiya, K. J., Sekine, Y., Suda, S., Sugihara, G., Matsuzaki, H., Sugiyama, T., Kawai, M., Minabe, Y., Takei, N. and Mori, N. (2007) 'Decreased Serum Levels of Epidermal Growth Factor in Adult Subjects with High-Functioning Autism', *Biological Psychiatry*, 62(3), pp. 267–269.
- Swann, A. (2003) 'Neuroreceptor mechanisms of aggression and its treatment.', *The Journal of Clinical Psychiatry*, 64 Suppl 4, pp. 26–35. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12672262> (Accessed: 21 August 2017).
- Swerdlow, N. R., Geyer, M. A. and Braff, D. L. (2001) 'Neural circuit regulation of prepulse inhibition of startle in the rat: current knowledge and future challenges', *Psychopharmacology (Berl)*, 156(2–3), pp. 194–215.
- Szeto, A., McCabe, P. M., Nation, D. A., Tabak, B. A., Rossetti, M. A., McCullough, M. E., Schneiderman, N. and Mendez, A. J. (2011) 'Evaluation of enzyme immunoassay and radioimmunoassay methods for the measurement of plasma oxytocin', *Psychosomatic Medicine*, 73(5), pp. 393–400.
- Tabak, B. A., Meyer, M. L., Dutcher, J. M., Castle, E., Irwin, M. R., Lieberman, M. D. and Eisenberger, N. I. (2016) 'Oxytocin, but not vasopressin, impairs social cognitive ability among individuals with higher levels of social anxiety: a randomized controlled trial', *Social Cognitive and Affective Neuroscience*, 11(8), pp. 1272–1279.
- Tabolacci, E., Palumbo, F., Nobile, V. and Neri, G. (2016) 'Transcriptional Reactivation of the FMR1 Gene. A Possible Approach to the Treatment of the Fragile X Syndrome', *Genes (Basel)*, 7(8).
- Takayanagi, Y., Yoshida, M., Bielsky, I. F., Ross, H. E., Kawamata, M., Onaka, T., Yanagisawa, T., Kimura, T., Matzuk, M. M., Young, L. J. and Nishimori, K. (2005) 'Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice', *PNAS USA*, 102(44), pp. 16096–16101.
- Taly, A., Corringer, P., Guedin, D., Lestage, P. and Changeux, J. (2009) 'Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system', *Nature Reviews Drug Discovery*, 8(9), pp. 733–750.
- Tamada, K., Tomonaga, S., Hatanaka, F., Nakai, N., Takao, K., Miyakawa, T., Nakatani, J., Takumi, T., Inokuchi, K. and Osumi, N. (2010) 'Decreased Exploratory Activity in a Mouse Model of 15q Duplication Syndrome; Implications for Disturbance of Serotonin Signaling', *PLoS ONE*, 5(12), p. e15126.

- Tamanini, F., Willemsen, R., van Unen, L., Bontekoe, C., Galjaard, H., Oostra, B. A. and Hoogeveen, A. T. (1997) 'Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis', *Human Molecular Genetics*, 6(8), pp. 1315–1322.
- Tamura, R., Kitamura, H., Endo, T., Hasegawa, N. and Someya, T. (2010) 'Reduced thalamic volume observed across different subgroups of autism spectrum disorders', *Psychiatry Research*, 184(3), pp. 186–188.
- Tapia, L., Kuryatov, A. and Lindstrom, J. (2006) 'Ca²⁺ Permeability of the (4)3(beta2)2 Stoichiometry Greatly Exceeds That of (4)2(beta2)3 Human Acetylcholine Receptors', *Molecular Pharmacology*, 71(3), pp. 769–776.
- Tarabeux, J., Kebir, O., Gauthier, J., Hamdan, F. F., Xiong, L., Piton, A., Spiegelman, D., Henrion, É., Millet, B., Fathalli, F., Joober, R., Rapoport, J. L., DeLisi, L. E., Fombonne, É., Motttron, L., Forget-Dubois, N., Boivin, M., Michaud, J. L., Drapeau, P., Lafrenière, R. G., Rouleau, G. A. and Krebs, M.-O. (2011) 'Rare mutations in N-methyl-D-aspartate glutamate receptors in autism spectrum disorders and schizophrenia', *Translational Psychiatry*, 1(11), p. e55.
- Tassone, F., Hagerman, R. J., Chamberlain, W. D. and Hagerman, P. J. (2000) 'Transcription of the FMR1 gene in individuals with fragile X syndrome', *American Journal of Medical Genetics*, 97(3), pp. 195–203.
- Tassone, F., Hagerman, R. J., Taylor, A. K., Gane, L. W., Godfrey, T. E. and Hagerman, P. J. (2000) 'Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome', *American Journal of Human Genetics*, 66(1), pp. 6–15.
- Tatsumi, M., Groshan, K., Blakely, R. D. and Richelson, E. (1997) 'Pharmacological profile of antidepressants and related compounds at human monoamine transporters', *European Journal of Pharmacology*, 340(2–3), pp. 249–258.
- Tehrani Doost, M., Ghaeli, P., Nikvarz, N., Alaghband Rad, J. and Alimadadi, A. (2014) 'Effects of risperidone on core symptoms of autistic disorder based on childhood autism rating scale: An open label study', *Indian Journal of Psychological Medicine*, 36(1), p. 66.
- Teng, B. L., Nonneman, R. J., Agster, K. L., Nikolova, V. D., Davis, T. T., Riddick, N. V., Baker, L. K., Pedersen, C. A., Jarstfer, M. B. and Moy, S. S. (2013) 'Prosocial effects of oxytocin in two mouse models of autism spectrum disorders', *Neuropharmacology*, 72, pp. 187–196.
- Terranova, M. L. and Laviola, G. (2005) 'Scoring of Social Interactions and Play in Mice During Adolescence', in *Current Protocols in Toxicology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., p. Unit13.10.
- The Dutch-Belgian Fragile X Consortium (1994) 'Fmr1 knockout mice: a model to study fragile X mental retardation'. *Cell*, pp. 23–33.
- The National Autistic Society (2017) *Autism facts and history*. Available at: <http://www.autism.org.uk/about/what-is/myths-facts-stats.aspx> (Accessed: 22 November 2017).
- Thorne, R. G., Emory, C. R., Ala, T. A. and Frey 2nd, W. H. (1995) 'Quantitative analysis of the olfactory pathway for drug delivery to the brain', *Brain Research*, 692(1–2), pp. 278–282.
- Tidey, J. W. and Miczek, K. A. (1997) 'Acquisition of cocaine self-administration after social stress: role of accumbens dopamine', *Psychopharmacology (Berl)*, 130, pp. 203–212.
- Tizabi, Y., Overstreet, D. H., Rezvani, A. H., Louis, V. A., Clark, E., Janowsky, D. S. and Kling, M. A. (1999) 'Antidepressant effects of nicotine in an animal model of depression', *Psychopharmacology*, 142(2), pp. 193–9.

- Todd, R. D. (1991) 'Fluoxetine in autism.', *American Journal of Psychiatry*, 148(8), p. 1089.
- Todtenkopf, M. S., Marcus, J. F., Portoghese, P. S. and Carlezon, W. A. (2004) 'Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats', *Psychopharmacology*, 172(4), pp. 463–470.
- Tomasiewicz, H. C., Todtenkopf, M. S., Chartoff, E. H., Cohen, B. M. and Carlezon, W. A. (2008) 'The Kappa-Opioid Agonist U69,593 Blocks Cocaine-Induced Enhancement of Brain Stimulation Reward', *Biological Psychiatry*, 64(11), pp. 982–988.
- Tordjman, S., Anderson, G. M., Botbol, M., Brailly-Tabard, S., Perez-Diaz, F., Graignic, R., Carlier, M., Schmit, G., Rolland, A. C., Bonnot, O., Trabado, S., Roubertoux, P. and Bronsard, G. (2009) 'Pain reactivity and plasma beta-endorphin in children and adolescents with autistic disorder', *PLoS One*, 4(8), p. e5289.
- Tornatzky, W. and Miczek, K. A. (1993) 'Long-term impairment of autonomic circadian rhythms after brief intermittent social stress.', *Physiology & Behavior*, 53(5), pp. 983–93.
- Torrecilla, M., Marker, C. L., Cintora, S. C., Stoffel, M., Williams, J. T. and Wickman, K. (2002) 'G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons', *Journal of Neuroscience*, 22(11), pp. 4328–4334.
- Toth, E. (1996) 'Effect of nicotine on the level of extracellular amino acids in the hippocampus of rat.', *Neurochemical Research*, 21(8), pp. 903–7.
- Treffert, D. A. (1970) 'Epidemiology of infantile autism.', *Archives of General Psychiatry*, 22(5), pp. 431–8.
- Trezza, V., Damsteegt, R., Achterberg, E. J. and Vanderschuren, L. J. (2011) 'Nucleus accumbens mu-opioid receptors mediate social reward', *Journal of Neuroscience*, 31(17), pp. 6362–6370.
- Trivedi, M. H., Rush, A. J., Wisniewski, S. R., Nierenberg, A. A., Warden, D., Ritz, L., Norquist, G., Howland, R. H., Lebowitz, B., McGrath, P. J., Shores-Wilson, K., Biggs, M. M., Balasubramani, G. K., Fava, M. and STAR*D Study Team (2006) 'Evaluation of Outcomes With Citalopram for Depression Using Measurement-Based Care in STAR*D: Implications for Clinical Practice', *American Journal of Psychiatry*, 163(1), pp. 28–40.
- Troisi, A., Frazzetto, G., Carola, V., Di Lorenzo, G., Coviello, M., D'Amato, F. R., Moles, A., Siracusano, A. and Gross, C. (2011) 'Social hedonic capacity is associated with the A118G polymorphism of the mu-opioid receptor gene (OPRM1) in adult healthy volunteers and psychiatric patients', *Society for Neuroscience*, 6(1), pp. 88–97.
- Tsai, H. C., Zhang, F., Adamantidis, A., Stuber, G. D., Bonci, A., de Lecea, L. and Deisseroth, K. (2009) 'Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning', *Science*, 324(5930), pp. 1080–1084.
- Tsankova, N. M., Berton, O., Renthal, W., Kumar, A., Neve, R. L. and Nestler, E. J. (2006) 'Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action', *Nature Neuroscience*, 9(4), pp. 519–525.
- Tsankova, N., Renthal, W., Kumar, A. and Nestler, E. (2007) 'Epigenetic regulation in psychiatric disorders', *Nature Reviews Neuroscience*, 8(5), pp. 355–367.
- Tuchman, R. and Rapin, I. (2002) 'Epilepsy in autism', *Lancet Neurology*, 1(6), pp. 352–358.
- Tyrka, A. R., Wier, L. M., Price, L. H., Rikhye, K., Ross, N. S., Anderson, G. M., Wilkinson, C. W. and Carpenter, L. L. (2008) 'Cortisol and ACTH responses to the Dex/CRH Test: Influence of temperament', *Hormones and Behavior*, 53(4), pp. 518–525.

- Uher, R. and McGuffin, P. (2010) 'The moderation by the serotonin transporter gene of environmental adversity in the etiology of depression: 2009 update', *Molecular Psychiatry*, 15(1), pp. 18–22.
- Ullmann, R., Turner, G., Kirchhoff, M., Chen, W., Tonge, B., Rosenberg, C., Field, M., Vianna-Morgante, A. M., Christie, L., Krepischi-Santos, A. C., Banna, L., Brereton, A. V., Hill, A., Bisgaard, A.-M., Müller, I., Hultschig, C., Erdogan, F., Wieczorek, G. and Ropers, H. H. (2007) 'Array CGH identifies reciprocal 16p13.1 duplications and deletions that predispose to autism and/or mental retardation.', *Human Mutation*, 28(7), pp. 674–82.
- Urbano, M., Okwara, L., Manser, P., Hartmann, K., Herndon, A. and Deutsch, S. I. (2014) 'A trial of D-cycloserine to treat stereotypies in older adolescents and young adults with autism spectrum disorder.', *Clinical Neuropsychopharmacology*, 37(3), pp. 69–72.
- Ustün, T. B., Ayuso-Mateos, J. L., Chatterji, S., Mathers, C. and Murray, C. J. L. (2004) 'Global burden of depressive disorders in the year 2000.', *The British journal of psychiatry: the journal of mental science*, 184, pp. 386–92.
- Valenti, O., Lodge, D. J. and Grace, A. A. (2011) 'Aversive Stimuli Alter Ventral Tegmental Area Dopamine Neuron Activity via a Common Action in the Ventral Hippocampus', *Journal of Neuroscience*, 31(11), pp. 4280–4289.
- Vanderschuren, L. J., Achterberg, E. J. and Trezza, V. (2016) 'The neurobiology of social play and its rewarding value in rats', *Neuroscience & Biobehavioral Reviews*, 70, pp. 86–105.
- Vanderschuren, L. J., Niesink, R. J., Spruijt, B. M. and Van Ree, J. M. (1995a) 'Effects of morphine on different aspects of social play in juvenile rats', *Psychopharmacology (Berl)*, 117(2), pp. 225–231.
- Vanderschuren, L. J., Niesink, R. J., Spruijt, B. M. and Van Ree, J. M. (1995b) 'Mu- and kappa-opioid receptor-mediated opioid effects on social play in juvenile rats', *European Journal of Pharmacology*, 276(3), pp. 257–266.
- Veening, J. G. and Olivier, B. (2013) 'Intranasal administration of oxytocin: Behavioral and clinical effects, a review', *Neuroscience & Biobehavioral Reviews*, 37(8), pp. 1445–1465.
- Veeraragavan, S., Graham, D., Bui, N., Yuva-Paylor, L. A., Wess, J. and Paylor, R. (2012) 'Genetic reduction of muscarinic M4 receptor modulates analgesic response and acoustic startle response in a mouse model of fragile X syndrome (FXS)', *Behavioural Brain Research*, 228(1), pp. 1–8.
- Ventura, R., Pascucci, T., Catania, M. V., Musumeci, S. A. and Puglisi-Allegra, S. (2004) 'Object recognition impairment in Fmr1 knockout mice is reversed by amphetamine: involvement of dopamine in the medial prefrontal cortex.', *Behavioural Pharmacology*, 15(5–6), pp. 433–442.
- Verheij, C., Bakker, C. E., de Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A. J., Galjaard, H., Reuser, A. J., Hoogeveen, A. T. and Oostra, B. A. (1993) 'Characterization and localization of the FMR-1 gene product associated with fragile X syndrome', *Nature*, 363(6431), pp. 722–724.
- Vicedomini, J. P., Corwin, J. V and Nonneman, A. J. (1982) 'Role of residual anterior neocortex in recovery from neonatal prefrontal lesions in the rat', *Physiology & Behavior*, 28(5), pp. 797–806.
- Du Vigneaud V., Ressler C., Swan C.J.M., Roberts C.W., Katsoyannis P.G. and Gordon, S. (1953) 'THE SYNTHESIS OF AN OCTAPEPTIDE AMIDE WITH THE HORMONAL ACTIVITY OF OXYTOCIN', *Journal of the American Chemical Society*, 75(19), pp. 4879–

- De Vries, B. B., Wieggers, A. M., Smits, A. P., Mohkamsing, S., Duivenvoorden, H. J., Fryns, J. P., Curfs, L. M., Halley, D. J., Oostra, B. A., van den Ouweland, A. M. and Niermeijer, M. F. (1996) 'Mental status of females with an FMR1 gene full mutation', *The American Journal of Human Genetics*, 58(5), pp. 1025–1032.
- de Vrij, F. M. S., Levenga, J., van der Linde, H. C., Koekkoek, S. K., De Zeeuw, C. I., Nelson, D. L., Oostra, B. A. and Willemsen, R. (2008) 'Rescue of behavioral phenotype and neuronal protrusion morphology in Fmr1 KO mice', *Neurobiology of Disease*, 31(1), pp. 127–132.
- Vuong, C., Van Uum, S. H., O'Dell, L. E., Lutfy, K. and Friedman, T. C. (2010) 'The effects of opioids and opioid analogs on animal and human endocrine systems', *Endocrine Reviews*, 31(1), pp. 98–132.
- De Waal, F. B. (2000) 'Primates--a natural heritage of conflict resolution.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 289(5479), pp. 586–90.
- Wallum, B. J., Taborsky Jr., G. J., Porte Jr., D., Figlewicz, D. P., Jacobson, L., Beard, J. C., Ward, W. K. and Dorsa, D. (1987) 'Cerebrospinal fluid insulin levels increase during intravenous insulin infusions in man', *Journal of Clinical Endocrinology & Metabolism*, 64(1), pp. 190–194.
- Walters, A. S., Barrett, R. P., Feinstein, C., Mercurio, A. and Hole, W. T. (1990) 'A case report of naltrexone treatment of self-injury and social withdrawal in autism', *Journal of Autism and Developmental Disorders*, 20(2), pp. 169–176.
- Walther, D. J. and Bader, M. (2003) 'A unique central tryptophan hydroxylase isoform.', *Biochemical Pharmacology*, 66(9), pp. 1673–80.
- Wang, H., Iacoangeli, A., Popp, S., Muslimov, I. A., Imataka, H., Sonenberg, N., Lomakin, I. B. and Tiedge, H. (2002) 'Dendritic BC1 RNA: functional role in regulation of translation initiation', *Journal of Neuroscience*, 22(23), pp. 10232–10241.
- Wang, H., Wu, L. J., Kim, S. S., Lee, F. J., Gong, B., Toyoda, H., Ren, M., Shang, Y. Z., Xu, H., Liu, F., Zhao, M. G. and Zhuo, M. (2008) 'FMRP acts as a key messenger for dopamine modulation in the forebrain', *Neuron*, 59(4), pp. 634–647.
- Wassink, T. H., Brzustowicz, L. M., Bartlett, C. W. and Szatmari, P. (2004) 'The search for autism disease genes', *Mental Retardation and Developmental Disabilities Research Reviews*, 10(4), pp. 272–283.
- Watkins, S. S., Epping-Jordan, M. P., Koob, G. F. and Markou, A. (1999) 'Blockade of nicotine self-administration with nicotinic antagonists in rats.', *Pharmacology, Biochemistry, and Behavior*, 62(4), pp. 743–51.
- Way, B. M., Taylor, S. E. and Eisenberger, N. I. (2009) 'Variation in the mu-opioid receptor gene (OPRM1) is associated with dispositional and neural sensitivity to social rejection', *PNAS USA*, 106(35), pp. 15079–15084.
- Wee, S. and Koob, G. F. (2010) 'The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse', *Psychopharmacology (Berl)*, 210(2), pp. 121–135.
- Weerts, E. M., Kim, Y. K., Wand, G. S., Dannals, R. F., Lee, J. S., Frost, J. J. and McCaul, M. E. (2008) 'Differences in delta- and mu-opioid receptor blockade measured by positron emission tomography in naltrexone-treated recently abstinent alcohol-dependent subjects', *Neuropsychopharmacology*, 33(3), pp. 653–665.
- Weich, S. and Lewis, G. (1998) 'Poverty, unemployment, and common mental disorders: population based cohort study.', *BMJ (Clinical research ed.)*, 317(7151), pp. 115–9.

- Weiler, I. J., Irwin, S. A., Klintsova, A. Y., Spencer, C. M., Brazelton, A. D., Miyashiro, K., Comery, T. A., Patel, B., Eberwine, J. and Greenough, W. T. (1997) 'Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation', *PNAS USA*, 94(10), pp. 5395–5400.
- Weintraub, K. (2011) 'The prevalence puzzle: Autism counts', *Nature*, 479(7371), pp. 22–24.
- Weisbrot, D., Gadow, K., DeVincent, C. and Pomeroy, J. (2005) 'The Presentation of Anxiety in Children with Pervasive Developmental Disorders', *Journal of Child and Adolescent Psychopharmacology*, 15(3), pp. 477–496.
- Weiss, J. M., Stout, J. C., Aaron, M. F., Quan, N., Owens, M. J., Butler, P. D. and Nemeroff, C. B. (1994) 'Depression and anxiety: role of the locus coeruleus and corticotropin-releasing factor.', *Brain Research Bulletin*, 35(5–6), pp. 561–72.
- Weiss, L. A., Shen, Y., Korn, J. M., Arking, D. E., Miller, D. T., Fossdal, R., Saemundsen, E., Stefansson, H., Ferreira, M. A. R., Green, T., Platt, O. S., Ruderfer, D. M., Walsh, C. A., Altshuler, D., Chakravarti, A., Tanzi, R. E., Stefansson, K., Santangelo, S. L., Gusella, J. F., Sklar, P., Wu, B.-L., Daly, M. J. and Autism Consortium (2008) 'Association between Microdeletion and Microduplication at 16p11.2 and Autism', *New England Journal of Medicine*, 358(7), pp. 667–675.
- Weizman, R., Gil-Ad, I., Dick, J., Tyano, S., Szekely, G. A. and Laron, Z. (1988) 'Low plasma immunoreactive beta-endorphin levels in autism', *Journal of the American Academy of Child and Adolescent Psychiatry*, 27(4), pp. 430–433.
- Weizman, R., Weizman, A., Tyano, S., Szekely, G., Weissman, B. A. and Sarne, Y. (1984) 'Humoral-endorphin blood levels in autistic, schizophrenic and healthy subjects', *Psychopharmacology (Berl)*, 82(4), pp. 368–370.
- Weller, E. B., Weller, R. A., Fristad, M. A. and Bowes, J. M. (1990) 'Dexamethasone suppression test and depressive symptoms in bereaved children: a preliminary report', *The Journal of Neuropsychiatry and Clinical Neurosciences*, 2(4), pp. 418–421.
- Wersinger, S. R., Ginns, E. I., O'Carroll, A. M., Lolait, S. J. and Young 3rd, W. S. (2002) 'Vasopressin V1b receptor knockout reduces aggressive behavior in male mice', *Molecular Psychiatry*, 7(9), pp. 975–984.
- Wersinger, S. R., Kelliher, K. R., Zufall, F., Lolait, S. J., O'Carroll, A. M. and Young 3rd, W. S. (2004) 'Social motivation is reduced in vasopressin 1b receptor null mice despite normal performance in an olfactory discrimination task', *Hormones and Behavior*, 46(5), pp. 638–645.
- Van West, D., Claes, S., Sulon, J. and Deboutte, D. (2008) 'Hypothalamic-pituitary-adrenal reactivity in prepubertal children with social phobia', *Journal of Affective Disorders*, 111(2–3), pp. 281–290.
- Westmark, C. J. and Malter, J. S. (2007) 'FMRP mediates mGluR5-dependent translation of amyloid precursor protein', *PLOS Biology*, 5(3), p. e52.
- Westmark, C. J., Westmark, P. R., O'Riordan, K. J., Ray, B. C., Hervey, C. M., Salamat, M. S., Abozeid, S. H., Stein, K. M., Stodola, L. A., Tranfaglia, M., Burger, C., Berry-Kravis, E. M. and Malter, J. S. (2011) 'Reversal of Fragile X Phenotypes by Manipulation of A β PP/A β Levels in Fmr1KO Mice', *PLoS ONE*. Edited by S. T. Ferreira. Nova Science Publishers, Inc, 6(10), p. e26549.
- Whitaker-Azmitia, P. M. (2005) 'Behavioral and cellular consequences of increasing serotonergic activity during brain development: a role in autism?', *Int J Dev Neurosci*, 23(1), pp. 75–83.

- White, C. M., Ji, S., Cai, H., Maudsley, S. and Martin, B. (2010) 'Therapeutic potential of vasoactive intestinal peptide and its receptors in neurological disorders', *CNS & Neurological Disorders-Drug Targets*, 9(5), pp. 661–666.
- White, G. and Roos, B.-E. (1959) 'IPRONIAZID AND IMIPRAMINE IN CHRONIC DEPRESSION', *The Lancet*, 274(7107), p. 848.
- White, S. W., Oswald, D., Ollendick, T. and Scahill, L. (2009) 'Anxiety in children and adolescents with autism spectrum disorders', *Clinical Psychology Review*, 29(3), pp. 216–229. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19223098> (Accessed: 4 July 2017).
- Whiteaker, P., Jimenez, M., McIntosh, J. M., Collins, A. C. and Marks, M. J. (2000) 'Identification of a novel nicotinic binding site in mouse brain using [(125)I]-epibatidine.', *British Journal of Pharmacology*, 131(4), pp. 729–39.
- Whitehouse, A. J. O., Durkin, K., Jaquet, E. and Ziatas, K. (2009) 'Friendship, loneliness and depression in adolescents with Asperger's Syndrome', *Journal of Adolescence*, 32(2), pp. 309–322.
- Widom, S., DuMont, C., Czaja, K. and J, S. (2007) 'A Prospective Investigation of Major Depressive Disorder and Comorbidity in Abused and Neglected Children Grown Up', *Archives of General Psychiatry*, 64(1), p. 49.
- Williams, K., Wheeler, D. M., Silove, N. and Hazell, P. (2010) 'Selective serotonin reuptake inhibitors (SSRIs) for autism spectrum disorders (ASD)', *Cochrane Database of Systematic Reviews*, 6(4), pp. 1044–1078.
- Willner, P. (2005) 'Chronic Mild Stress (CMS) Revisited: Consistency and Behavioural-Neurobiological Concordance in the Effects of CMS', *Neuropsychobiology*, 52(2), pp. 90–110.
- Van Wimersma Greidanus, T. B. and Maigret, C. (1996) 'The role of limbic vasopressin and oxytocin in social recognition', *Brain Research*, 713(1–2), pp. 153–159.
- Windham, G. C., Zhang, L., Gunier, R., Croen, L. A. and Grether, J. K. (2006) 'Autism spectrum disorders in relation to distribution of hazardous air pollutants in the san francisco bay area', *Environmental Health Perspectives*, 114(9), pp. 1438–1444.
- Winer, E. S., Bryant, J., Bartoszek, G., Rojas, E., Nadorff, M. R. and Kilgore, J. (2017) 'Mapping the relationship between anxiety, anhedonia, and depression', *Journal of Affective Disorders*, 221, pp. 289–296.
- Wing, L. (1992) 'Manifestations of Social Problems in High-Functioning Autistic People', in *High-Functioning Individuals with Autism*. Boston, MA, pp. 129–142.
- Wing, L. and Gould, J. (1979) 'Severe impairments of social interaction and associated abnormalities in children: epidemiology and classification', *Journal of Autism and Developmental Disorders*, 9(1), pp. 11–29.
- Wink, L. K., Plawecki, M. H., Erickson, C. A., Stigler, K. A. and McDougale, C. J. (2010) 'Emerging drugs for the treatment of symptoms associated with autism spectrum disorders.', *Expert Opinion on Emerging Drugs*, 15(3), pp. 481–94.
- Winslow, J. T., Hearn, E. F., Ferguson, J., Young, L. J., Matzuk, M. M. and Insel, T. R. (2000) 'Infant vocalization, adult aggression, and fear behavior of an oxytocin null mutant mouse', *Hormones and Behavior*, 37(2), pp. 145–155.
- Winslow, J. T. and Insel, T. R. (2002) 'The social deficits of the oxytocin knockout mouse', *Neuropeptides*, 36(2–3), pp. 221–229.
- Witten, I. B., Steinberg, E. E., Lee, S. Y., Davidson, T. J., Zalocusky, K. A., Brodsky, M.,

- Yizhar, O., Cho, S. L., Gong, S., Ramakrishnan, C., Stuber, G. D., Tye, K. M., Janak, P. H. and Deisseroth, K. (2011) 'Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement', *Neuron*, 72(5), pp. 721–733.
- Wohr, M., Moles, A., Schwarting, R. K. and D'Amato, F. R. (2011) 'Lack of social exploratory activation in male mu-opioid receptor KO mice in response to playback of female ultrasonic vocalizations', *Society for Neuroscience*, 6(1), pp. 76–87.
- Wolburg, H., Wolburg-Buchholz, K., Sam, H., Horvat, S., Deli, M. A. and Mack, A. F. (2008) 'Epithelial and endothelial barriers in the olfactory region of the nasal cavity of the rat', *Histochemistry and Cell Biology*, 130(1), pp. 127–140.
- Wolterink, G., Daenen, L. E., Dubbeldam, S., Gerrits, M. A., van Rijn, R., Kruse, C. G., Van Der Heijden, J. A. and Van Ree, J. M. (2001) 'Early amygdala damage in the rat as a model for neurodevelopmental psychopathological disorders', *European Neuropsychopharmacology*, 11(1), pp. 51–59.
- Won, H., Lee, H.-R., Gee, H. Y., Mah, W., Kim, J.-I., Lee, J., Ha, S., Chung, C., Jung, E. S., Cho, Y. S., Park, S.-G., Lee, K., Daesoo, K., Bae, Y. C. and Kaang, Bong-Kiun Min, Goo Lee Eunjoon, K. (2012) 'Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function', *Nature*, 486(7402), pp. 261–265.
- Wonnacott, S. (1997) 'Presynaptic nicotinic ACh receptors.', *Trends in Neurosciences*, 20(2), pp. 92–8.
- Wood, J. J., Drahota, A., Sze, K., Har, K., Chiu, A. and Langer, D. A. (2009) 'Cognitive behavioral therapy for anxiety in children with autism spectrum disorders: a randomized, controlled trial.', *Journal of Child Psychology and Psychiatry, and Allied Disciplines*, 50(3), pp. 224–34.
- Wood, J. N. and Grafman, J. (2003) 'Human prefrontal cortex: processing and representational perspectives', *Nature Reviews Neuroscience*, 4(2), pp. 139–147.
- Wright, S. R., Zanos, P., Georgiou, P., Yoo, J.-H., Ledent, C., Hourani, S. M., Kitchen, I., Winsky-Sommerer, R. and Bailey, A. (2016) 'A critical role of striatal A2A R-mGlu5 R interactions in modulating the psychomotor and drug-seeking effects of methamphetamine', *Addiction Biology*, 21(4), pp. 811–825.
- Wu, S., Jia, M., Ruan, Y., Liu, J., Guo, Y., Shuang, M., Gong, X., Zhang, Y., Yang, X. and Zhang, D. (2005) 'Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population', *Biological Psychiatry*, 58(1), pp. 74–77.
- Wyers, E. J., Peeke, H. V., Williston, J. S. and Herz, M. J. (1968) 'Retroactive impairment of passive avoidance learning by stimulation of the caudate nucleus', *Experimental Neurology*, 22(3), pp. 350–366.
- Xiao, Y., Baydyuk, M., Wang, H. (Pearl), Davis, H. E. and Kellar, K. J. (2004) *Pharmacology of the agonist binding sites of rat neuronal nicotinic receptor subtypes expressed in HEK 293 cells*, *Bioorganic & Medicinal Chemistry Letters*.
- Xu, L.-M., Li, J.-R., Huang, Y., Zhao, M., Tang, X. and Wei, L. (2012) 'AutismKB: an evidence-based knowledgebase of autism genetics', *Nucleic Acids Research*, 40(D1), pp. D1016–D1022.
- Xu, Y., Chen, X. T., Luo, M., Tang, Y., Zhang, G., Wu, D., Yang, B., Ruan, D. Y. and Wang, H. L. (2015) 'Multiple epigenetic factors predict the attention deficit/hyperactivity disorder among the Chinese Han children', *Journal of Psychiatric Research*, 64, pp. 40–50.
- Yamamoto, A., Iseki, T., Ochi-Sugiyama, M., Okada, N., Fujita, T. and Muranishi, S. (2001) 'Absorption of water-soluble compounds with different molecular weights and', *Journal of*

Controlled Release, 76(3), pp. 363–374.

Yan, Q. J., Rammal, M., Tranfaglia, M. and Bauchwitz, R. P. (2005) 'Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP', *Neuropharmacology*, 49(7), pp. 1053–1066.

Yang, S. Y., Cho, S.-C., Yoo, H. J., Cho, I. H., Park, M., Yoe, J. and Kim, S. A. (2010) 'Family-based association study of microsatellites in the 5' flanking region of AVPR1A with autism spectrum disorder in the Korean population', *Psychiatry Research*, 178(1), pp. 199–201.

Yogeeswari, P., Sriram, D., Ratan Bal, T. and Thirumurugan, R. (2006) 'Epibatidine and its analogues as nicotinic acetylcholine receptor agonist: an update.', *Natural Product Research*, 20(5), pp. 497–505.

You, J.-S., Hu, S.-Y., Chen, B. and Zhang, H.-G. (2005) 'Serotonin transporter and tryptophan hydroxylase gene polymorphisms in Chinese patients with generalized anxiety disorder.', *Psychiatric Genetics*, 15(1), pp. 7–11.

You, Z. B., Herrera-Marschitz, M. and Terenius, L. (1999) 'Modulation of neurotransmitter release in the basal ganglia of the rat brain by dynorphin peptides', *Journal of Pharmacology and Experimental Therapeutics*, 290(3), pp. 1307–1315.

Young, J. W., Crawford, N., Kelly, J. S., Kerr, L. E., Marston, H. M., Spratt, C., Finlayson, K. and Sharkey, J. (2007) 'Impaired attention is central to the cognitive deficits observed in alpha 7 deficient mice', *European Neuropsychopharmacology*, 17(2), pp. 145–155.

Young, L. J., Winslow, J. T., Wang, Z., Gingrich, B., Guo, Q., Matzuk, M. M. and Insel, T. R. (1997) 'Gene targeting approaches to neuroendocrinology: oxytocin, maternal behavior, and affiliation', *Hormones and Behavior*, 31(3), pp. 221–231.

Yrigollen, C. M., Han, S. S., Kochetkova, A., Babitz, T., Chang, J. T., Volkmar, F. R., Leckman, J. F. and Grigorenko, E. L. (2008) 'Genes controlling affiliative behavior as candidate genes for autism', *Biological Psychiatry*, 63(10), pp. 911–916.

Yu, G. Z., Kaba, H., Okutani, F., Takahashi, S. and Higuchi, T. (1996) 'The olfactory bulb: a critical site of action for oxytocin in the induction of maternal behaviour in the rat', *Neuroscience*, 72(4), pp. 1083–1088.

Zak, P. J., Stanton, A. A. and Ahmadi, S. (2007) 'Oxytocin Increases Generosity in Humans', *PLoS ONE*. Edited by S. Brosnan, 2(11), p. e1128.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., Oostra, B. and Bagni, C. (2003) 'The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses', *Cell*, 112(3), pp. 317–327.

Zalla, T. and Sperduti, M. (2013) 'The amygdala and the relevance detection theory of autism: an evolutionary perspective.', *Frontiers in Human Neuroscience*, 7, p. 894.

Zanos, P., Georgiou, P., Wright, S. R., Hourani, S. M., Kitchen, I., Winsky-Sommerer, R. and Bailey, A. (2014) 'The oxytocin analogue carbetocin prevents emotional impairment and stress-induced reinstatement of opioid-seeking in morphine-abstinent mice', *Neuropsychopharmacology*, 39(4), pp. 855–865.

Zanos, P., Wright, S. R., Georgiou, P., Yoo, J. H., Ledent, C., Hourani, S. M., Kitchen, I., Winsky-Sommerer, R. and Bailey, A. (2014) 'Chronic methamphetamine treatment induces oxytocin receptor up-regulation in the amygdala and hypothalamus via an adenosine A2A receptor-independent mechanism', *Pharmacology Biochemistry and Behavior*, 119, pp. 72–79.

Zhang, G., Zhang, Y., Fast, D. M., Lin, Z. and Steenwyk, R. (2011) 'Ultra sensitive

quantitation of endogenous oxytocin in rat and human plasma using a two-dimensional liquid chromatography-tandem mass spectrometry assay', *Analytical Biochemistry*, 416(1), pp. 45–52.

Zhang, J., Yao, W., Ren, Q., Yang, C., Dong, C., Ma, M., Wu, J. and Hashimoto, K. (2016) *Depression-like phenotype by deletion of $\alpha 7$ nicotinic acetylcholine receptor: Role of BDNF-TrkB in nucleus accumbens*, *Scientific Reports*.

Zhao, J., Goldberg, J., Bremner, J. and Vaccarino, V. (2013) 'Association Between Promoter Methylation of Serotonin Transporter Gene and Depressive Symptoms', *Psychosomatic Medicine*, 75(6), pp. 523–529.

Zhao, M. G., Toyoda, H., Ko, S. W., Ding, H. K., Wu, L. J. and Zhuo, M. (2005) 'Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome', *Journal of Neuroscience*, 25(32), pp. 7385–7392.

Zheng, Z., Zhu, T., Qu, Y. and Mu, D. (2016) 'Blood Glutamate Levels in Autism Spectrum Disorder: A Systematic Review and Meta-Analysis', *PLoS One*, 11(7), p. e0158688.

Zhong, S., Monakhov, M., Mok, H. P., Tong, T., Lai, P. S., Chew, S. H. and Ebstein, R. P. (2012) 'U-shaped relation between plasma oxytocin levels and behavior in the trust game', *PLoS One*, 7(12), p. e51095.

Zhou, M., Rebholz, H., Brocia, C., Warner-Schmidt, J. L., Fienberg, A. A., Nairn, A. C., Greengard, P. and Flajolet, M. (2010) 'Forebrain overexpression of CK1delta leads to down-regulation of dopamine receptors and altered locomotor activity reminiscent of ADHD', *PNAS USA*, 107(9), pp. 4401–6.

Zhu, J., Chen, C., Xue, J. C., Kunapuli, S., DeRiel, J. K. and Liu-Chen, L. Y. (1995) 'Cloning of a human kappa opioid receptor from the brain', *Life Sciences*, 56(9), p. PL201-7.

Zill, P., Baghai, T. C., Zwanzger, P., Schüle, C., Eser, D., Rupprecht, R., Möller, H.-J., Bondy, B. and Ackenheil, M. (2004) 'SNP and haplotype analysis of a novel tryptophan hydroxylase isoform (TPH2) gene provide evidence for association with major depression', *Molecular Psychiatry*, 9(11), pp. 1030–1036.

Zimmermann, F. F., Gaspary, K. V., Siebel, A. M. and Bonan, C. D. (2016) 'Oxytocin reversed MK-801-induced social interaction and aggression deficits in zebrafish', *Behavioural Brain Research*, 311, pp. 368–374.

Zoghbi, H. Y., Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q. and Francke, U. (1999) 'Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2', *Nature Genetics*, 23(2), pp. 185–188.

Zweifel, L. S., Fadok, J. P., Argilli, E., Garelick, M. G., Jones, G. L., Dickerson, T. M. K., Allen, J. M., Mizumori, S. J. Y., Bonci, A. and Palmiter, R. D. (2011) 'Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety', *Nature Neuroscience*, 14(5), pp. 620–626.

7. Appendix

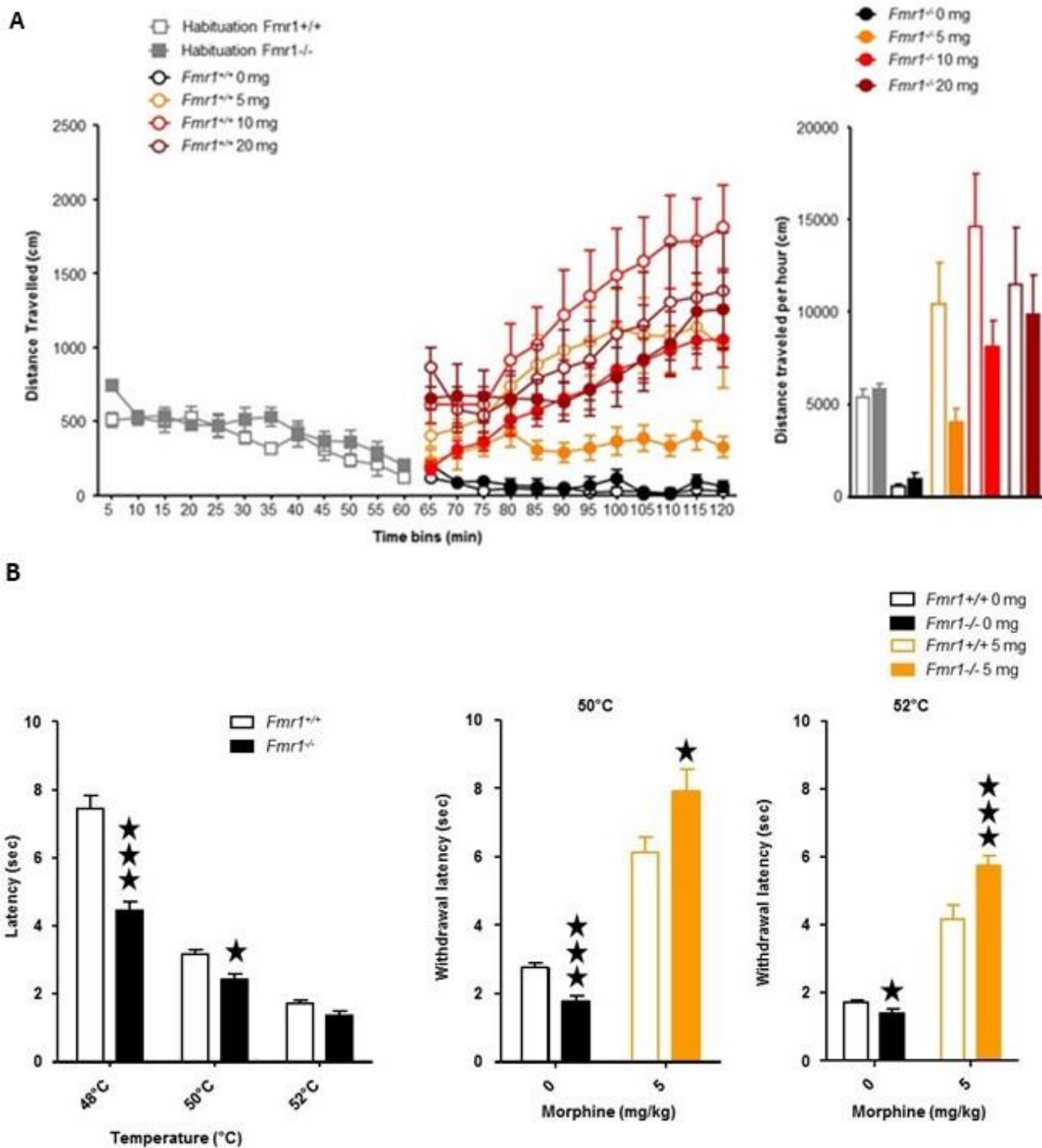


Figure 7.1: Opioid-induced locomotion and analgesia responses in *Fmr1*^{-/-} versus *Fmr1*^{+/+} mice.

A) Morphine-induced locomotion differences between *Fmr1* KO and WT mice. Locomotion was measured by time bins in minutes for WT and *Fmr1* KO mice treated with 5 mg, 10 mg and 20 mg doses of morphine and no morphine B) Morphine-induced analgesia in *Fmr1* KO and WT mice by tail immersion test. WT and *Fmr1* KO mice were treated with 5 mg of morphine or no morphine and nociception responses were monitored by taking tail flick latency measurements in immersion test by using stimulus temperatures of 48°C, 50°C and 52°C. (Behavioral experiments conducted by Dr. Becker and Dr. Le Merrer)

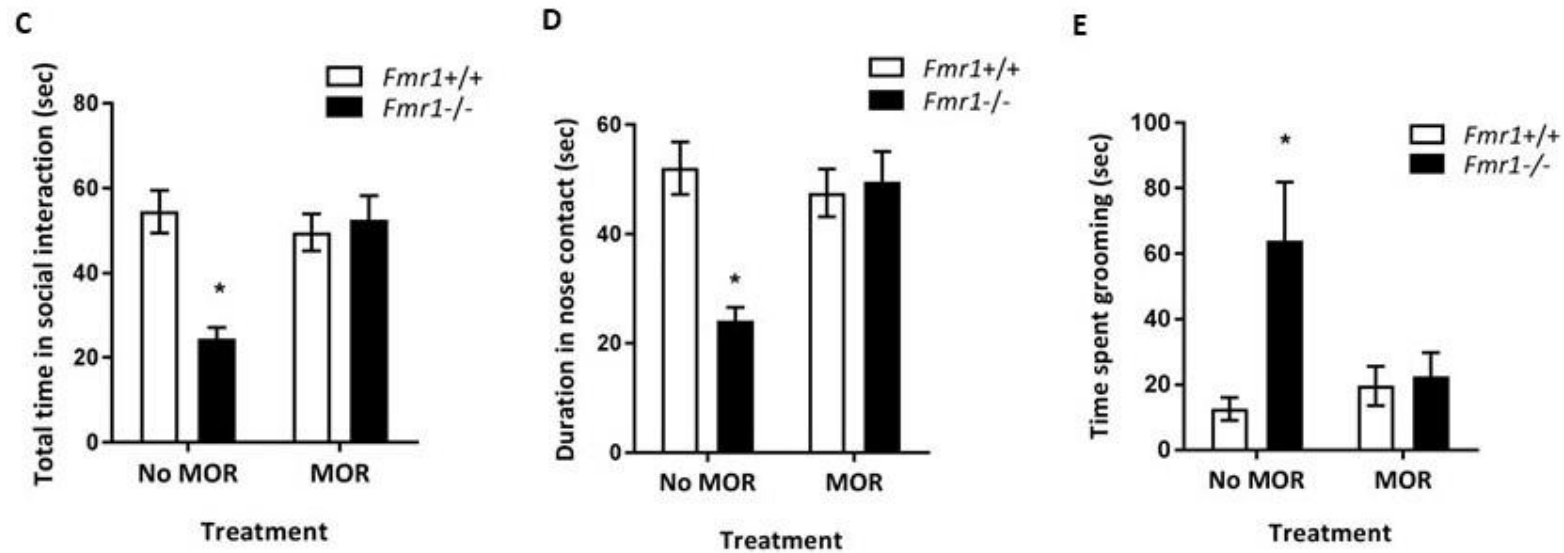


Figure 7.2: Opioid-induced responses in *Fmr1*^{-/-} versus *Fmr1*^{+/+} mice.

Morphine-induced social interactions in *Fmr1* KO and WT mice by direct social interaction paradigm, in which a pair of experimental and stimulus mice are free to interact for 10min. Acute morphine administration of 5 mg in the *Fmr1* KO mice increased the duration of C) social interactions and D) nose contact, and reduced the time spent in grooming behaviour (Behavioral experiments conducted by Dr. Becker and Dr. Le Merrer).

