The role of oxytocin in drug addiction

by

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BSc (Hons)

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The Faculty of Health and Medical Sciences

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Panos Zanos
ABSTRACT

There is mounting evidence that the neuropeptide oxytocin is a possible candidate for the treatment of drug addiction. Recently, it has been demonstrated that oxytocin can reduce methamphetamine self-administration, conditioned place preference and reinstatement of methamphetamine-seeking in rodents. Similarly, it has been shown that oxytocin has inhibitory effects on sensitisation, tolerance and self-administration of opioids and cocaine. However the mechanisms underlying the action of oxytocin on the addictive processes remain unknown.

This work aimed to identify an association between the oxytocinergic system and the emergence of emotional impairment (i.e. anxiety- and depressive-like behaviours and social deficits) during opioid withdrawal. It also aimed to elucidate whether an acute administration of the oxytocin analogue carbetocin is able to reverse this emotional impairment. Furthermore, this project investigated the effects of chronic opioid and psychostimulant administration and withdrawal on the central oxytocin receptor and peptide levels via quantitative receptor autoradiography and/or enzyme-linked immunosorbent assay, respectively.

Profound emotional deficits occurred following prolonged morphine withdrawal, associated with a hypo-oxytocinergic state in the hypothalamus and a rebound up-regulation of the oxytocin receptor in the amygdala in a mouse model. Moreover, acute administration of carbetocin was able to completely reverse this enhanced depressive-like, anxiety-like and impaired social behaviour. Similarly, it was shown that chronic administration of cocaine and methamphetamine-induced marked up-regulation of
oxytocin receptor binding in the amygdala, which in the case of cocaine persisted following protracted withdrawal.

Collectively, the results described in this thesis highlight a common dysregulation of the oxytocinergic system induced by chronic opioid and psychostimulant exposure and abstinence. This dysregulation may at least partly underlie the emotional consequences of drug addiction. Additionally, this is the first study to support the oxytocinergic system as a novel target for the treatment of the emotional consequences of opioid abstinence. Since anxiety, mood and social impairment constitute motivational triggers to relapse during abstinence it is anticipated that an oxytocin-based pharmacotherapy may be able to assist with relapse prevention.
PUBLICATIONS

Papers


Wright, R.S., Zanos, P., Yoo, J.H., Winsky-Sommerer, R., Hourani, S., Kitchen, I., Bailey, A. Complete abolition of stereotypic rearing and mGluR5 up-regulation in chronically methamphetamine- but not cocaine-treated A2A receptor knockout mice. Neuropsychopharmacology (In preparation).

Zanos, P., Georgiou, P., Kitchen, I., Winsky-Sommerer, R., Bailey, A. Persistent brain-region-specific up-regulation of the vasopressin (V1aR) and oxytocin receptor following chronic morphine and cocaine administration and withdrawal in mice. Addiction Biology (In preparation).

Peer-reviewed published abstracts

(http://www.pa2online.org/abstracts/vol10issue4abst136p.pdf)

Non peer-reviewed published abstracts

Zanos, P., Alshehri, M., Sahabandu, T., Winsky-Sommerer, R., Kitchen, I., Bailey, A. 2011. Persistent brain region-specific upregulation of vasopressin (V1aR) and oxytocin receptors following chronic intermittent escalating-dose morphine administration in mice. Pharmacological Reports, 63(1)

Zanos, P., Alshehri, M., Sahabandu, T., Winsky-Sommerer, R., Kitchen, I., Bailey, A. 2011. Persistent brain region-specific upregulation of vasopressin (V1aR) and oxytocin receptors following chronic intermittent escalating-dose morphine administration in mice 9th World Congress in Neurohypophysial Hormones.

Conference presentations

- National/International Conferences

10th World congress on Neurohypophyséal Hormones – WCNH, Bristol, U.K. 2013. ‘The oxytocin analogue carbetocin reverses the negative emotional symptoms induced by protracted opioid Abstinence’ (poster presentation)

European Opioid Conference, Guildford, U.K. 2013. ‘Carbetocin reverses the behavioural phenotype induced by opioid abstinence’ (poster presentation)

British Pharmacological Society (BPS) winter meeting, London, U.K. 2012. ‘The oxytocin analogue carbetocin reverses impaired emotional-like behaviour during prolonged abstinence from chronic morphine treatment’ (oral presentation)

University Global Partnership Network Workshop: Genes and Behaviour, Guildford, Surrey, U.K. 2012. ‘Carbetocin reverses emotional impairment during prolonged morphine withdrawal’ (oral presentation)
British Pharmacological Society (BPS) winter meeting, London, U.K. 2011. ‘Persistent brain region-specific up-regulation of the V1a receptor following chronic cocaine and morphine administration and withdrawal in mice’ (oral presentation)

9th World congress on Neurohypophyseal Hormones – WCNH, Boston, U.S. 2011. ‘Persistent brain region-specific upregulation of vasopressin (V1aR) and oxytocin receptors in chronic intermittent morphine administration in mice’ (poster presentation)

European Opioid Conference, Krakow, Poland. 2011. ‘Persistent brain region-specific upregulation of vasopressin (V1aR) and oxytocin receptors in chronic intermittent escalating-dose morphine administration in mice’ (poster presentation)

- University Conferences

Faculty of Health and Medical Sciences Festival of research Conference, University of Surrey, Guildford, Surrey, U.K. 2013. ‘Impaired emotional-like behaviour is reversed by carbetocin during protracted withdrawal from chronic morphine administration’ (poster presentation)

Postgraduate Research Conference (PGR), University of Surrey, Guildford, Surrey, U.K. 2013. ‘Oxytocin: Is the “love” hormone a novel treatment for drug addiction?’ (oral presentation)

Faculty of Health and Medical Sciences Festival of research Conference, University of Surrey, Guildford, Surrey, U.K. 2012. ‘Impaired emotional-like behaviours during prolonged abstinence from chronic morphine treatment: involvement of the oxytocinergic system and therapeutic implications’ (oral presentation)

Sleep Chronobiology Neurodisorders (SCN) meeting, University of Surrey, Guildford, Surrey, U.K. 2012. ‘Effects of chronic morphine administration and withdrawal on the endogenous oxytocinergic system’ (poster presentation)

Postgraduate Research Conference (PGR), University of Surrey, Guildford, Surrey, U.K. 2012. ‘Persistent brain region-specific up-regulation of the V1a receptor following chronic cocaine and morphine administration and withdrawal in mice’ (poster presentation)
Faculty of Health and Medical Sciences Festival of research Conference, University of Surrey, Guildford, Surrey, U.K. 2011. ‘Persistent brain region-specific upregulation of vasopressin (V1aR) and oxytocin receptors in chronic intermittent escalating-dose morphine administration in mice’ (poster presentation)
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxyDA</td>
</tr>
<tr>
<td>A$_{2A}$</td>
<td>Adenosine A$_{2A}$ receptor</td>
</tr>
<tr>
<td>A$_{2A}$$^{-/-}$</td>
<td>A$_{2A}$ receptor knockout</td>
</tr>
<tr>
<td>Acb</td>
<td>Nucleus accumbens</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Amy</td>
<td>Amygdala</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOL</td>
<td>Anterior olfactory nucleus-lateral</td>
</tr>
<tr>
<td>AOM</td>
<td>Anterior olfactory nucleus-medial</td>
</tr>
<tr>
<td>AOV</td>
<td>Anterior olfactory nucleus-ventral</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus of hypothalamus</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral amygdala</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CA2</td>
<td>CA2 region of hippocampus</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CA3</td>
<td>CA3 region of hippocampus</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CBT</td>
<td>Carbetocin</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CeA</td>
<td>Central nucleus of amygdala</td>
</tr>
<tr>
<td>Cg Cx</td>
<td>Cingulate cortex</td>
</tr>
<tr>
<td>CHRE</td>
<td>Composite hormone response element</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate putamen</td>
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<tr>
<td>CRF</td>
<td>Corticotropin-releasing factor</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>D₂</td>
<td>Dopamine D₂ receptor</td>
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<td>DAG</td>
<td>1,2-diacylglycerol</td>
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<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DOPr</td>
<td>δ-opioid receptor</td>
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<tr>
<td>DR</td>
<td>Dorsal raphe nucleus</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus-maze</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FCx</td>
<td>Frontal cortex</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>FST</td>
<td>Forced-swim test</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GHB</td>
<td>γ-hydroxybutyric acid</td>
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<td>GKB</td>
<td>Glycyl-lysyl-arginine</td>
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<td>Globus pallidus</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>HAB</td>
<td>High-anxiety behaviour</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>Hip</td>
<td>Hippocampus</td>
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<td>Hypothalamic-pituitary-adrenal</td>
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<td>Homovanillic acid</td>
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<td>Hyp</td>
<td>Hypothalamus</td>
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<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
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<td>i.p.</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>IP3</td>
<td>Inositol triphosphate</td>
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<td>KOPr</td>
<td>κ-opioid receptor</td>
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<tr>
<td>LAB</td>
<td>Low-anxiety behaviour</td>
</tr>
<tr>
<td>LS</td>
<td>Lateral septum</td>
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<tr>
<td>LSD</td>
<td>Lysergic acid diethylamide</td>
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<td>MAP</td>
<td>Methamphetamine</td>
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<tr>
<td>MDMA</td>
<td>3,4-methylenedioxy-N-methylamphetamine</td>
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<tr>
<td>ME</td>
<td>Median eminence</td>
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<td>Magnesium chloride</td>
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<td>Mor</td>
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<td>mPFC</td>
<td>Medial prefrontal cortex</td>
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<td>Medial preoptic area</td>
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<td>Messenger RNA</td>
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<td>Medial septum</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NL</td>
<td>Neural lobe of the pituitary</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive-compulsive disorder</td>
</tr>
<tr>
<td>OFC</td>
<td>Orbitofrontal cortex</td>
</tr>
<tr>
<td>OT</td>
<td>Oxytocin</td>
</tr>
<tr>
<td>OT^-/-</td>
<td>Oxytocin knockout</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxytocin receptor</td>
</tr>
<tr>
<td>OTR^-/-</td>
<td>Oxytocin receptor knockout</td>
</tr>
<tr>
<td>OTR^FB/FB</td>
<td>Conditional forebrain oxytocin receptor knockout</td>
</tr>
<tr>
<td>OVTA</td>
<td>Ornithine vasotocin</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>Pir</td>
<td>Piriform cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PPI</td>
<td>Pre-pulse inhibition</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of Hypothalamus</td>
</tr>
<tr>
<td>RP</td>
<td>Relapse prevention</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Sal</td>
<td>Saline</td>
</tr>
<tr>
<td>SC</td>
<td>Spinal cord</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SN</td>
<td>Substantia nigra</td>
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<tr>
<td>Sol</td>
<td>Solitary tract nucleus</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus of hypothalamus</td>
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<tr>
<td>SSRI</td>
<td>Serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>STh</td>
<td>Subthalamic nucleus</td>
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<tr>
<td>Th</td>
<td>Thalamus</td>
</tr>
<tr>
<td>TST</td>
<td>Tail suspension test</td>
</tr>
<tr>
<td>Tu</td>
<td>Olfactory tubercule</td>
</tr>
<tr>
<td>VDB</td>
<td>Vertical limb of the diagonal band of Broca</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial nucleus of hypothalamus</td>
</tr>
<tr>
<td>VP</td>
<td>Ventral pallidum</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<tr>
<td>WAY-267464</td>
<td>N-[[4-[(4,10-Dihydro-1-methylpyrazolo[3,4-[1,5]benzodiazepin-5(1H)-yl]carbonyl]-2-methylphenyl]methyl]-4-[(3,5-dihydroxyphenyl)methyl]-1-piperazinecarboxamide dihydrochloride</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Στην αγαπημένη μου οικογένεια...
CHAPTER 1

General Introduction
1.1 Drug addiction in the United Kingdom

Drug addiction is a chronic relapsing brain disorder which has deleterious consequences on an individual’s physical and psychological health. Substance dependence has been characterised according to three different criteria: (1) compulsivity to obtain the drug despite the negative consequences, (2) loss of control upon drug administration and (3) emergence of negative physical and emotional symptoms following drug cessation (American Psychiatric Association, 2013).

Addiction to drugs of abuse has serious societal and economic consequences through criminality, decreased productivity and increased healthcare costs (see Schumacher et al., 2002). It is predominantly observed in the young adult population (16-24 year old) in the United Kingdom (U.K.). It has been reported that in 2012, 19.3% of the young adults had used an illicit drug (Home office, 2013). Class A drugs (e.g. lysergic acid diethylamide (LSD), cocaine, 3,4-methylenedioxy-\textit{N}- methamphetamine (MDMA), methamphetamine (MAP), heroin) are considered to be the most addictive and harmful drugs for the user (Smith and Flatley, 2011) while during the last year 6.3% of the youth population has abused Class A drugs – equating to around 0.4 million people in England (Home Office, 2013).

A serious consequence of abuse of illicit drugs is criminality (e.g. theft, burglary), with an associated annual cost for heroin and cocaine users estimated to reach £15 billion in the U.K. (Home Office, 2013). The U.K. government spends almost £15.4 billion per year tackling illicit substance use (National Audit Office, 2010). Importantly, the cost of healthcare services over the life-span of just one illicit drug user is estimated at £35,000, increasing to £480,000 per user when the crime-related cost is included (National Institute for Health and Clinical Excellence., 2009).
Drug abuse is also considered as a common cause of premature mortality, with illicit drug poisoning (overdose) accounting for approximately 12.5% of total deaths among people between 20-40 years old (Office of National Statistics, 2012). In fact, the U.K. has the fifth highest drug-related mortality rate in Europe according to the European Monitoring Centre for Drugs and Drug Addiction (2012) report.

1.2 Neurobiology of drug addiction

1.2.1 Addiction cycle

Addiction can be characterised as a cycle of neurochemical and psychological changes that bring about a shift from the impulsive use of a drug to the compulsive use (see Koob and Le Moal, 2008); also see Figure 1.1. Acute administration of all drugs of abuse activates the reward pathway of the brain, thus inducing hedonic effects that positively reinforce the user to repeat drug administration. Upon repeated use of the drug, neuroadaptive tolerance to the rewarding effects of the drug is developed therefore, an escalation of the dose is needed in order to achieve the same initial pleasurable effects (see Koob and Le Moal, 2001). During this period, positive reinforcement associated with acute drug administration is gradually replaced with negative reinforcement to prevent the emergence of a negative withdrawal syndrome upon drug cessation. Acute withdrawal from drug use causes unpleasant drug-specific physical symptoms that are short-lived. Drug users who have abstained from drug administration over a long period of time are still vulnerable to relapse to drug-seeking, particularly during re-exposure to the drug itself, previously drug-associated environmental cues and after stress-related conditions (see Koob and Le Moal, 2008).
Upon administration, drugs of abuse induce euphoric effects that positively reinforce the user to repeat drug use. Following repeated administration tolerance can be developed, meaning that increased amount of a drug is needed to induce the initial pleasurable effects. Acute positive reinforcement is gradually replaced with negative reinforcement to prevent the emergence of negative withdrawal symptoms following drug cessation. The acute impulsive use of the drug becomes compulsive use. Even after long-term abstinence individuals are still vulnerable to relapse and re-take the drug. Modified from Le Moal and Koob, (2007).

The transition from impulsive (positive reinforcement) to compulsive (negative reinforcement) use of drugs of abuse drives the cyclic nature of drug addiction through three different stages: (1) binge/intoxication, (2) withdrawal/negative affect and (3) preoccupation/anticipation (see Koob and Le Moal, 2008); also see Figure 1.2.
1.2.1.1 Binge/intoxication stage

Upon acute administration all drugs of abuse activate the brain reward systems. The concept of an anatomical reward brain circuit emerged from the findings of Olds and Milner (1954), who demonstrated that rats would perform a task to acquire an electrical stimulation within specific brain regions. This finding was further supported by Phillips and Fibiger (1978) who proposed the existence of an intact dopaminergic (DAergic) system which is responsible for the rewarding effects of electrical brain stimulation. These findings established the basis for the dopamine (DA) theory of addiction.

Two ascending DAergic systems originating from the cell bodies of the ventral tegmental area (VTA) have been demonstrated, comprising the mesocorticolimbic DAergic system: the mesolimbic DAergic pathway innervates several limbic structures including the nucleus accumbens (Acb), ventral pallidum (VP), hippocampus (Hip), lateral septum (LS) and amygdala (Amy); and the mesocortical DAergic pathway innervating the prefrontal (PFC), the orbitofrontal (OFC) and the anterior cingulate cortices (see Feltenstein and See, 2008).

The mesolimbic pathway was postulated as the main pathway of convergence in addictive behaviours (see Nestler, 2005a), since both natural rewards, such as food and sex, and drug-induced reward acutely increase DA release in the Acb (part of the ventral striatum). The reward produced after the acute administration of drugs of abuse is more robust than those yielded by natural reinforcers (see Di Chiara and Bassareo, 2007). All drugs of abuse induce DA release; however, the mechanism of action of each drug is different. For example, psychostimulants, like cocaine and amphetamine, act directly on the Acb whereas opioids act on the VTA to disinhibit DA release (see Pierce and Kumaresan, 2006). The role of the mesolimbic DAergic pathway in the positive
reinforcing effects of drugs of abuse is further confirmed by microdialysis studies having reliably shown that extracellular DA concentrations in the Acb are increased during morphine, cocaine and amphetamine self-administration (Pettit and Justice, 1989, Di Ciano et al., 1995, Pontieri et al., 1995), and self-administration of a number of drugs of abuse is attenuated upon administration of DA receptor antagonists (Woolverton, 1986, Rassnick et al., 1992, Richardson et al., 1994) or neurotoxic 6-hydroxydopamine (6-OHDA) lesioning of the accumbal DAergic pathways (Roberts et al., 1980, Pettit et al., 1984, Zito et al., 1985). In addition to DA, other transmitter systems have been identified as key players in acute reinforcement of drugs of abuse, including the endogenous opioid system (see Koob and Volkow, 2010).

The ventral striatum (i.e. Acb) is predominantly involved in the acute reinforcing effects of drugs of abuse, while in contrast, the dorsal striatum (caudate putamen – CPu) appears to be primarily recruited during the development of compulsive drug-seeking (Everitt et al., 2008). It is thus hypothesised that the transition from impulsive use of drugs of abuse to compulsive use is mediated by a ventral-to-dorsal striatum shift of drug-taking and seeking behaviour (Everitt et al., 2008).

1.2.1.2 Withdrawal/negative affect stage

Increases in brain reward thresholds have been revealed after acute withdrawal from all drugs of abuse, as measured by direct "brain stimulation reward" (Markou and Koob, 1991, Schulteis et al., 1995, Schulteis et al., 1994, Epping-Jordan et al., 1998, Gardner and Vorel, 1998, Paterson et al., 2000); also see Section 1.3. These findings may indicate decreases in the activity of several neurotransmitter systems that are implicated in the positive reinforcing effects of drugs. Indeed, not only has DA been implicated in the positive reinforcing effects of drugs of abuse but it has also been proposed to be
involved during drug withdrawal. For example, acute withdrawal leads to disruptions in basal DAergic transmission, which has been proposed as a possible mechanism underlying drug craving (Weiss et al., 1996, Shen, 2003). Specifically, the opioid withdrawal syndrome has been shown to be associated with a remarkable decrease in DA release in the limbic forebrain of rats (Druhan et al., 2000). Therefore, the mesocorticolimbic DAergic system might also be involved in the negative reinforcing effects of the drugs of abuse (i.e. dysphoria, anxiety and stress). However, it has been shown that DAergic neurons respond to aversive stimuli either by activation, by not responding or by inhibition (Matsumoto and Hikosaka, 2009). For example, accumbal DA transmission was significantly increased under the aversive condition of social defeat stress in rats (Anstrom et al., 2009). Therefore, the exact role of the mesocorticolimbic DAergic system on the withdrawal-associated negative effects remains unclear.

The endogenous opioid system has been also implicated during drug abstinence. Specifically, enhanced sensitivity of opioid receptor transduction mechanisms was observed within the Acb during opioid withdrawal (Stinus et al., 1990). A candidate for the negative effects induced by drug abstinence is the opioid peptide dynorphin, an endogenous ligand for κ-opioid receptors (KOPr) (see Wee and Koob, 2010). Dynorphin levels are increased in the Acb and Amy during opioid and cocaine withdrawal, and this overactivity of the dynorphin system reduces DAergic function which has been shown to induce aversive effects (see Wee and Koob, 2010).

The emotional impairment related to the withdrawal/negative affect stage may also involve neurochemical systems other than the ones involved in the positive reinforcement effects of addictive substances (Koob and Bloom, 1988). Several
functional observations have provided strong evidence for the existence of a common neurocircuitry within the basal forebrain underlying the aversive effects associated with drug withdrawal, termed the "extended amygdala" (see Koob, 2009). This circuitry is comprised of the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and a part of the medial Acb (Heimer and Alheid, 1991).

During withdrawal from drug administration, the stress system, including the hypothalamic-pituitary-adrenal (HPA) axis and extended amygdala, mediated by the corticotropin-releasing factor (CRF), is activated with a common response of increased corticosterone and amygdalar CRF levels (Rivier et al., 1984, Merlo Pich et al., 1995, Koob et al., 1994, Rasmussen et al., 2000, Olive et al., 2002, Delfs et al., 2000); also see Section 1.2.2.1. Acute withdrawal from drugs may also increase the release of noradrenaline (NA) in the BNST (Roy and Pandey, 2002). These results suggest that during the development of dependence, not only alterations in the function of neurotransmitter systems associated with the acute reinforcing effects of drugs such as DA and opioid peptides, but also a recruitment of the anti-reward (e.g. dynorphin) and stress systems occurs (see Koob and Le Moal, 2001).

1.2.1.3 Preoccupation/anticipation stage

The preoccupation/anticipation stage of the addiction cycle has long been hypothesised to be a key element of relapse to drug-taking after abstinence and it is the stage that defines addiction as a chronic relapsing disorder. Although often linked to the construct of craving, drug craving per se has been difficult to investigate in clinical studies (Tiffany et al., 2000) and often does not correlate with relapse. Relapse to drug-seeking following abstinence can occur during re-exposure to the drug itself (drug-induced reinstatement), exposure to drug-associated environmental stimuli (cue-induced
reinstatement) or under stressful conditions (stress-induced reinstatement). Animal models of drug-craving following abstinence have been developed and used to investigate the neurobiological and behavioural mechanisms underlying relapse (see Section 1.3).

Drug-induced reinstatement has been shown to be localised to the VTA / PFC / Acb / VP circuit and to be possibly mediated by glutamate (McFarland and Kalivas, 2001). Specifically, neurotransmitter systems involved in drug-induced reinstatement implicate a glutamatergic projection from the PFC to the Acb that is modulated by DAergic afferents from the VTA innervating the PFC (Krimer et al., 1997). Indeed, inactivation of these structures has been shown to prevent cocaine-primed reinstatement in rodents (McFarland and Kalivas, 2001).

In addition, neuropharmacological and neurobiological studies using animal models indicate the BLA as a critical substrate underlying cue-induced reinstatement of both cocaine- (Ciccocioppo et al., 2001, Kantak et al., 2002, McLaughlin and See, 2003) and opioid-seeking (Fuchs and See, 2002). Specifically, cue-induced reinstatement is hypothesised to involve DA modulation in the BLA (Berglind et al., 2006) and a glutamatergic projection to the Acb from both the BLA and ventral subiculum (Everitt and Wolf, 2002, Vorel et al., 2001, Shalev et al., 2002).

Stress-induced reinstatement of drug-related responding in animal models appears to depend on the activation of both CRF and NA in the extended amygdala (Shaham et al., 2003, Shalev et al., 2002). For example, non-selective CRF antagonist injections into the BNST blocks footshock-induced reinstatement of cocaine-seeking in the self-administration paradigm, whereas intra-BNST injections of CRF itself reinstated cocaine-seeking (Erb et al., 2001, Erb and Stewart, 1999). Similarly, injection of a CRF₁
receptor antagonist into the BNST blocked footshock-induced reinstatement of opioid-seeking in rats (Wang et al., 2006). Moreover, peripheral administration of α₂-adrenoceptor agonists, administered in doses sufficient to inhibit NA release, abolished footshock-induced reinstatement of cocaine-seeking in rats (Erb et al., 2000) and systemic administration of an α₂-adrenoceptor antagonist, yohimbine, reinstated MAP-seeking (Shepard et al., 2004).

Figure 1.2: The three stages of addiction cycle.
Acute administration of a drug of abuse which involves the activation of the mesolimbic DAergic pathway, reflects the binge/intoxication component of the addiction cycle. Repeated administration of the drug brings about a shift towards the dorsal striatum. The withdrawal/negative affect stage is reflected by the activation of the brain stress systems, such as CRF within the extended amygdala. Reinstatement to drug-seeking following abstinence is associated with the preoccupation/anticipation component of the cycle which is primarily modulated by the PFC and BLA. Modified from Koob and Volkow (2010) and Koob and Le Moal (2001).
1.2.2 Addiction and stress systems

Stress has been defined as a non-specific, physiological response of the body characterised by several alterations including the activation of stress systems (Dunn and Berridge, 1990, Rivier and Plotsky, 1986). A key element of drug addiction responses involves a marked activation of hormonal (HPA axis), and brain (i.e. CRF, NA, dynorphin, vasopressin and orexin) stress systems. Brain stress systems are thought to be localised in the extended amygdala and to be involved in the modulation of the negative emotional state that drives the shift from impulsivity to compulsivity. Since part of this thesis investigates the effects of drugs on the HPA axis, the reader is directed to reviews by (Koob, 2008; 2009) for further information on the role of the brain stress systems in addiction.

1.2.2.1 Hypothalamic-pituitary adrenal axis

Exposure to stress activates the HPA axis (see Figure 1.3) by stimulating a cascade of events involving the release of CRF from the paraventricular nucleus (PVN) of the hypothalamus (Hyp) (Vale et al., 1981, Rivier and Vale, 1983). CRF acts at the level of the anterior pituitary by binding to CRF$_1$ receptors on corticotrope cells, thereby inducing the synthesis of proopiomelanocortin (POMC). Processing of POMC leads to the production of adrenocorticotrophic hormone (ACTH), which is released into the systemic circulation, and by acting on ACTH receptors into the adrenal cortex stimulates glucocorticoid (cortisol in humans and corticosterone in rodents) synthesis and release into the circulatory system to modulate physiological events.

The HPA axis is finely tuned via a negative feedback mechanism by which glucocorticoids inhibit excessive HPA axis activation by reducing the synthesis and release of ACTH and CRF (see Whitnall, 1993). Vasopressin peptide, which is co-
expressed with CRF in neurons of the PVN, is also regulated in response to stress and acts synergistically with CRF to stimulate ACTH release (see Whitnall, 1993). For further information on the HPA axis the reader is directed to reviews by Smith and Vale (2006) and de Kloet et al., (2005).

**Figure 1.3: Schematic representation of the Hypothalamic-Pituitary-Adrenal axis.**
In response to stress, neural inputs from the central nervous system converge on the paraventricular nucleus (PVN) of the hypothalamus and induce the synthesis and release of corticotropin-releasing factor (CRF), which in turn stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH stimulates the synthesis of glucocorticoids. Glucocorticoids negatively feedback to the pituitary and hypothalamus to reduce the synthesis and release of ACTH and CRF, respectively. Modified from Turnbull and Rivier (1999).
Alteration of HPA axis activity is thought to be a common neurobiological mechanism underlying several addiction processes (Heinrichs and Koob, 2004, Marinelli and Piazza, 2002, Sinha, 2001). Clinical as well as pre-clinical studies have investigated the effects of opioids and psychostimulants on HPA axis activity (see Table 1).

In rodents, acute morphine elevates HPA axis activity (Nikolarakis et al., 1987), while acute morphine blocks HPA axis activation induced by stress (Zhou et al., 1999b), indicating a counter-regulatory role of opioids on the stress response. Serum cortisol levels are significantly decreased in chronic heroin addicts (Rasheed and Tareen, 1995); however, basal HPA axis activity is normalised in steady-state methadone-maintained patients (Kreek et al., 1984). In rats, chronic administration of morphine (Buckingham and Cooper, 1984) as well as steady-state methadone treatment replicated the normalisation of the HPA axis response (Zhou et al., 1996, Leri et al., 2012, Leri et al., 2006, Leri et al., 2009), indicating the development of tolerance to the initial stimulatory effects of morphine. Following acute withdrawal from opioid administration, HPA axis activity is elevated in both humans (Cami et al., 1992, Beam et al., 2001) and animals (Houshyar et al., 2001), and it returns to basal conditions after protracted withdrawal (Houshyar et al., 2001, Cami et al., 1992).

In contrast to the inhibitory effects of opioids on HPA axis activity upon acute administration, cocaine increases HPA axis activity in humans. After a cocaine challenge ACTH levels are lower in cocaine-dependent than in occasional cocaine users, suggesting that chronic cocaine abuse induces tolerance to its initial stimulatory effects (Mendelson et al., 1998); however, both groups had significantly elevated ACTH levels compared to controls. Cocaine-addicted patients show persistent hyper-activation of the HPA axis following both acute (Contoreggi et al., 2003, Buydens-
Branchey et al., 2002) and protracted drug abstinence (Wilkins et al., 2005); animal models of cocaine addiction confirmed these clinical data (see Table 1.1).

Similarly to cocaine, acute administration of amphetamine in rats induces a marked activation of HPA axis activity (Swerdlow et al., 1993, Knych and Eisenberg, 1979). In addition, protracted withdrawal from chronic amphetamine administration is followed by increased basal ACTH and corticosterone levels in rodents (Russig et al., 2003).

Acute MAP administration induces activation of the HPA axis in both humans (Fehm et al., 1984) and animals (Szumlinski et al., 2001). This hyperresponsiveness of the HPA axis persists following repeated administration of MAP in rats (Grace et al., 2008). In contrast with the sustained hyper-activation of the HPA axis following protracted withdrawal from cocaine and amphetamine, MAP abstinence has not been shown to alter basal cortisol and ACTH levels in humans (Zorick et al., 2011). Since the DAergic system is considered as a modulator of HPA axis activity (Van Craenenbroeck et al., 2005, Radley et al., 2009, Asanuma et al., 2003), these discrepancies may be attributable to the magnitude of DA release induced by these drugs during withdrawal. Although there is evidence for differential influence of abstinence from these drugs of abuse on the extracellular DA levels within the striatum in rodents (Zhang et al., 2001), the exact mechanism underpinning the differences in HPA axis activity after protracted withdrawal from psychostimulants remain unknown and needs further elucidation.

Taken together, these results indicate that opioid and psychostimulant administration results in a profound dysregulation of HPA activation, which in the case of psychostimulants, persists following protracted withdrawal; thus, it might constitute a candidate mechanism to trigger relapse after abstinence.
<table>
<thead>
<tr>
<th>Addictive substance</th>
<th>Administration paradigm</th>
<th>Subject</th>
<th>Effects on HPA axis</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Morphine            | **Acute** 5 mg/kg, i.p. | Male Sprague-Dawley rats (300-400 g) housed in groups | ↑ plasma ACTH levels  
↑ CRF content in Hyp | Nikolarakis et al., 1987 |
|                     | **Acute** 30 mg orally   | Humans (males and females 22-48 years old) | ↓ plasma ACTH levels  
↓ plasma cortisol levels | Allolio et al., 1987 |
|                     | **Chronic** 0.2 mg/kg, i.p., 8 days | Male Sprague-Dawley rats (75-100 g) housed in groups | ↔ plasma ACTH levels  
↔ plasma corticosterone levels  
↓ CRF content in the ME | Buckingham and Cooper, 1984 |
<p>|                     | <strong>Acute spontaneous withdrawal</strong> 2 x 10-100 mg/kg, s.c./ 16 days, then 12h withdrawal | Male Sprague-Dawley rats (250-350 g) housed individually | ↑ plasma corticosterone levels | Houshyar et al., 2001 |
|                     | <strong>Protracted spontaneous withdrawal</strong> 2 x 10-100 mg/kg, s.c./ 16 days then 16 days withdrawal | Male Sprague-Dawley rats (250-350 g) housed individually | ↔ plasma corticosterone levels | Houshyar et al., 2001 |</p>
<table>
<thead>
<tr>
<th>Naloxone-precipitated withdrawal</th>
<th>Male Wistar rats (220-2540 g) housed in groups</th>
<th>↑ plasma corticosterone levels ↑ CRF mRNA in PVN</th>
<th>Nunez et al., 2007</th>
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<tr>
<td><strong>Heroin</strong></td>
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<tr>
<td><strong>Chronic</strong></td>
<td>Humans (males, 20-50 years old)</td>
<td>↓ serum cortisol levels</td>
<td>Rasheed and Tareen, 1995</td>
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<tr>
<td><strong>Acute withdrawal</strong></td>
<td>Humans (males and females 19-47 years old)</td>
<td>↑ salivary cortisol levels</td>
<td>Bearn et al., 2001</td>
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<td><strong>Acute withdrawal</strong></td>
<td>Humans (males 21-32 years old)</td>
<td>↑ salivary cortisol levels</td>
<td>Cami et al., 1992</td>
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<td><strong>Protracted withdrawal</strong></td>
<td>Humans (males and females 22-44 years old)</td>
<td>↑ plasma cortisol levels (nocturnal) ↔ plasma cortisol levels (diurnal)</td>
<td>Li et al., 2008b</td>
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<tr>
<td><strong>lofexidine-assisted heroin detoxification</strong></td>
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<td><strong>lofexidine-assisted heroin detoxification</strong></td>
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<td><strong>6 days of clonidine or guanfacine- assisted detoxification</strong></td>
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<tr>
<td><strong>lofexidine-assisted heroin detoxification followed by 10-15 days withdrawal</strong></td>
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<tr>
<td>Protracted withdrawal</td>
<td>Humans (males 21-32 years old)</td>
<td>→ plasma cortisol levels</td>
<td>Cami et al., 1992</td>
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<td>Cocaine</td>
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<td>Acute 15 mg/kg, i.p.</td>
<td>Male Sprague-Dawley rats (150-200 g) housed in groups</td>
<td>↑ ACTH release in the anterior pituitary ↑ plasma corticosterone levels</td>
<td>Borowsky and Kuhn, 1991</td>
</tr>
<tr>
<td>Acute 5 mg/kg, i.v.</td>
<td>Male Sprague-Dawley rats (200-250 g) housed in groups</td>
<td>↑ CRF mRNA in PVN</td>
<td>Rivier and Lee, 1994</td>
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<td>Acute 7.5 mg/kg, i.p.</td>
<td>Male Sprague-Dawley rats (180-220 g) housed in groups</td>
<td>↓ CRF content in Hyp</td>
<td>Sarnyai et al., 1993</td>
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<td>Acute 2 mg/kg, intranasally</td>
<td>Humans (males 23-46 years old)</td>
<td>↑ plasma cortisol levels</td>
<td>Heesch et al., 1995</td>
</tr>
<tr>
<td>Chronic 3 x 15 mg/kg, i.p. for 14 days</td>
<td>Male mice of 129 x C57BL/6J background (4-6 months old) housed individually</td>
<td>↑ plasma ACTH levels ↑ plasma corticosterone levels</td>
<td>Zhou et al., 1999a</td>
</tr>
<tr>
<td>Chronic Intranasal, intravenous or freebase</td>
<td>Humans (males and females 23-36 years old)</td>
<td>↑ plasma ACTH levels</td>
<td>Mendelson et al., 1989</td>
</tr>
<tr>
<td>Condition</td>
<td>Description</td>
<td>Outcome</td>
<td>References</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Chronic</td>
<td>5.6 ± 2.8 (SEM) of years use</td>
<td>↑ plasma cortisol levels</td>
<td>Baumann et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Humans (males and females 21-38 years old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute spontaneous withdrawal</td>
<td>30 mg/kg, i.p. x 14 days then 12h withdrawal</td>
<td>↑ plasma ACTH levels</td>
<td>Mantsch et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Male Sprague-Dawley rats (90 days old) housed in pairs</td>
<td>↑ plasma corticosterone levels</td>
<td></td>
</tr>
<tr>
<td>Acute spontaneous withdrawal</td>
<td>3 x 15-30 mg/kg, i.p. x 14 days, then 1 day withdrawal</td>
<td>↑ plasma ACTH levels</td>
<td>Zhou et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Male Fischer rats (190-220 g) housed individually</td>
<td>↑ plasma corticosterone levels</td>
<td></td>
</tr>
<tr>
<td>Acute withdrawal</td>
<td>1-4 weeks spontaneous withdrawal</td>
<td>↑ plasma cortisol levels</td>
<td>Contoreggi et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Humans (males and females, 28.2 ± 6.1 SD years old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute withdrawal</td>
<td>6 days spontaneous abstinence</td>
<td>↑ plasma cortisol levels</td>
<td>Buydens-Branchey et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Humans (males 38.64±6.05 SD years old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolonged spontaneous withdrawal</td>
<td>3 x 15-30 mg/kg, i.p. x 14 days, then 14 days withdrawal</td>
<td>↑ plasma ACTH levels</td>
<td>Zhou et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Male Fischer rats (190-220 g) housed individually</td>
<td>↑ plasma corticosterone levels</td>
<td></td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>Prolonged withdrawal</td>
<td>Humans (male veterans, mostly Africans-Americans)</td>
<td>↑ plasma cortisol levels</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
<tr>
<td>Acute 0.1 mg/kg, i.p.</td>
<td>Female Sprague-Dawley rats (225-250 g) housed in groups</td>
<td>↑ plasma corticosterone levels</td>
<td>Szumlinski et al., 2001</td>
</tr>
<tr>
<td>Acute 15 mg/kg, i.v.</td>
<td>Humans (males and females 22-30 years old)</td>
<td>↑ plasma cortisol levels</td>
<td>Fehm et al., 1984</td>
</tr>
<tr>
<td>Chronic 4 x 10 mg/kg, s.c. for 5 days</td>
<td>Male and Female Sprague-Dawley rats (postnatal days 11-15) housed in groups</td>
<td>↑ plasma corticosterone levels</td>
<td>Grace et al., 2008</td>
</tr>
<tr>
<td>Prolonged spontaneous withdrawal</td>
<td>Male Fuellinsdorf Wistar rats (6 weeks old) housed in groups</td>
<td>↔ plasma corticosterone levels</td>
<td>Morimasa et al., 1987</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Prolonged spontaneous withdrawal</td>
<td>Humans (males and females dependent individuals 25-45 years old)</td>
<td>↔ plasma cortisol levels ↔ plasma ACTH levels</td>
</tr>
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<td>-------------</td>
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</tr>
<tr>
<td>Acute</td>
<td>1 or 5 mg/kg, s.c.</td>
<td>Male Sprague-Dawley ras (250-350 g) housed in groups</td>
<td>↑ plasma corticosterone levels</td>
</tr>
<tr>
<td>Prolonged spontaneous withdrawal</td>
<td>Chronic: 1x1-5 mg/kg, i.p. over 6 days, Withdrawal: 5 days drug-free in home cages</td>
<td>Male Wistar rats (250-350 g) housed individually</td>
<td>↑ plasma corticosterone levels</td>
</tr>
<tr>
<td>Prolonged spontaneous withdrawal</td>
<td>Chronic: 2 mg/kg, i.p. for 7 days, then 4 mg/kg, i.p. for 7 days, Withdrawal: 2 weeks drug-free in home cages</td>
<td>Male Wistar rats (250-275 g) housed in groups</td>
<td>↑ plasma ACTH levels in response to restraint stress ↑ plasma corticosterone levels in response to restraint stress</td>
</tr>
</tbody>
</table>
| **Amphetamine-induced reinstatement**  
| **Chronic:** 3 x 1-9 mg/kg, i.p. for 3 days and a single 10 mg/kg, i.p. injection on day 4  
| **Withdrawal:** 30 days spontaneous withdrawal  
| **Reinstatement:** 1 mg/kg, i.p. amphetamine injection  | **Male Wistar rats (250-350 g) housed individually**  | ↓ plasma ACTH levels  
|  |  | ↓ plasma corticosterone levels  | **Russig et al., 2006**  

A detailed summary of the effects of opioids and psychostimulant drugs on the HPA axis activity upon acute and chronic administration as well as acute and chronic withdrawal. ↑ increase; ↓ decrease; ↔ no effect; Abbreviations: ACTH, adenocorticotropic hormone; CRF, corticotropin releasing factor; Hyp, hypothalamus; i.p., intraperitoneal; i.v., intravenous; ME, median eminence; mRNA, messenger RNA; s.c., subcutaneous; PVN, paraventricular nucleus of the hypothalamus; SEM, standard error of the mean.
1.3 Behavioural paradigms used in drug addiction research

Understanding of the neurobiological alterations that occur during different phases of the addiction cycle has been mostly derived from the development of animal models (see Koob et al., 2009); also see Table 1. Each of the three stages of the addiction cycle has been assessed in various animal models of drug addiction for the better understanding of the underpinning mechanisms by which drugs of abuse exert their neurochemical and behavioural effects.

Animal models for the binge/intoxication stage of the addiction cycle can be conceptualized as measuring acute drug rewarding effects. Validated models of reward and reinforcement include drug self-administration, conditioned place preference, and decreases in brain reward thresholds. Animal models for the study of the withdrawal/negative affect stage include conditioned place aversion (rather than preference), increased brain reward thresholds, and dependence-induced increases in drug-seeking. Animal models of drug craving (preoccupation/anticipation stage) involve reinstatement of drug-seeking following withdrawal from the drugs, by drug-associated or environmental cues linked to the drug, and by exposure to stressors (Shaham et al., 2003). Since this thesis is focused on the depressive-, anxiety-like and social behaviours related to addiction, for further information on the behavioural paradigms used in drug addiction research the reader is directed to reviews by Heidbreder, (2011) and Lynch et al., (2010).
## Table 1.2: Behavioural paradigms used to study addictive-related behaviours in animal models

<table>
<thead>
<tr>
<th>Stage of the addiction cycle</th>
<th>Behavioural paradigms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binge / Intoxication</strong></td>
<td><strong>Brain stimulation reward</strong></td>
<td>Bain and Kornetsky, 1989, Olds and Milner, 1954</td>
</tr>
<tr>
<td>(Acute effects of drugs of abuse)</td>
<td>Conditioned-place preference</td>
<td>Sanchis-Segura and Spanagel, 2006</td>
</tr>
<tr>
<td></td>
<td>Self-administration (intravenous or oral)</td>
<td>Collins et al., 1984, Hyytia et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Drug discrimination</td>
<td>Falk and Lau, 1995, See et al., 1999</td>
</tr>
<tr>
<td><strong>Withdrawal / Negative Affect</strong></td>
<td><strong>Brain stimulation reward</strong></td>
<td>Epping-Jordan et al., 1998</td>
</tr>
<tr>
<td>(Effects of prolonged administration of drugs of abuse and withdrawal)</td>
<td>Conditioned-place aversion</td>
<td>Funada and Shippenberg, 1996, Hand et al., 1988</td>
</tr>
<tr>
<td></td>
<td>Self-administration</td>
<td>Roberts et al., 1996</td>
</tr>
<tr>
<td><strong>Preoccupation / Anticipation</strong></td>
<td><strong>Self-administration</strong></td>
<td>Stewart and Wise, 1992</td>
</tr>
<tr>
<td>(Reinstatement)</td>
<td>Conditioned-place preference</td>
<td>Mueller and Stewart, 2000</td>
</tr>
</tbody>
</table>

Addiction is often characterised by three different stages; the binge/intoxication, withdrawal/negative affect and preoccupation/anticipation stages. To examine the neurobiological and behavioural effects of each of these stages, several animal models have been developed. Modified from Koob et al., (2009).
1.4 Current pharmacotherapies for drug addiction

The hallmark in the treatment of drug addiction is the prevention of relapse following abstinence. As this thesis concentrates on opioids and psychostimulant drugs of abuse, current pharmacotherapies for nicotine and alcohol addictions will not be discussed.

Despite many years of extensive research, no effective pharmacotherapy currently exists for psychostimulant addiction (see Kampman et al., 2005). Indeed, drugs targeting DAergic transmission have been widely assessed as possible therapeutic interventions for the treatment of addiction to psychostimulant drugs both in pre-clinical and clinical trials. However, in humans the suggested treatments were not effective in reducing the reinforcing effects of the drugs (Haney et al., 2001).

For opioid addiction, current treatment strategies include substitution therapies that use partial opioid agonists including methadone (partial μ-opioid receptor (MOPr) agonist), buprenorphine (partial MOPr and nociceptin receptor agonist and KOPr antagonist) and naltrexone (global opioid receptor antagonist) (see Stein et al., 2012). Methadone is the preferred drug for the treatment of opioid dependence due to its high bioavailability when taken orally; however, this drug has a substantial addiction liability itself making its availability highly controlled, thus reducing compliance (see Connock et al., 2007). Buprenorphine provides another possible treatment for opioid abusers with less euphoric effects than methadone (see Lintzeris et al., 2009). However, the safety of buprenorphine when administered along with other sedative substances such as alcohol is still unclear, and may also lead to death (see Lintzeris et al., 2009). Naltrexone is another medication used in opioid dependence treatment that does not display abuse potential (see Mannelli et al., 2011) and has been shown to reduce opioid use (Giordano et al., 1990). Nevertheless, this treatment has been linked with poor treatment retention.
and low patient compliance, mostly due to several side-effects associated with its use including depression and dysphoria (Crowley et al., 1985, Hollister et al., 1981). Finally, a buprenorphine-naloxone combination (called Suboxone) has been used as another strategy for the management of opioid addiction yielding some positive results in reducing heroin use (Rothman et al., 2000, McKeeganey et al., 2013). Although naloxone has been shown to reduce abuse potential of buprenorphine (Alho et al., 2007), reports of combined buprenorphine/naloxone abuse have been also reported (Robinson et al., 1993).

Recent clinical trials on the treatment of drug addiction have started to utilise vaccines for opioid and psychostimulant addiction with some promising therapeutic efficacy for the reduction of drug craving (see Shen et al., 2012). However, possible side effects and ethical implications may prevent these vaccines from being manufactured. Therefore, it is clear that novel, more effective pharmacotherapies are needed for the treatment of drug addiction, especially substances targeting relapse following abstinence.
1.5 Comorbidity: drug addiction and other mental disorders

Drugs of abuse can cause users to experience several symptoms of other mental disorders, including anxiety, depression and social phobia (see National Institute on Drug Abuse, 2010). Comorbidity between mental and substance use disorders is highly prevalent worldwide. It has been estimated that 45% of the drug-dependent population has a comorbid psychiatric disorder, compared with 12% among the non-dependent population (Farrell et al., 2003). Moreover, drug-related mental disorders accounted for almost 6,640 hospitalised patients during 2012 in the U.K. (Office of National Statistics, 2012).

This comorbidity might be caused by overlapping factors such as several neurobiological alterations in the brain, or genetic influences; however, the exact mechanisms underlying this comorbidity remain poorly understood. Several clinical studies have been conducted and a number of animal models have been developed to study the underlying neuroanatomical and neurochemical substrates of this comorbidity (see Aston-Jones and Harris, 2004, Harris and Gewirtz, 2005, Koob and Le Moal, 2005). As the focus of this thesis involves co-occurring anxiety, depression and social-anxiety behaviours with drug addiction, comorbidity with schizophrenia, bipolar disease and other psychiatric disorders will not be discussed. For more information on these comorbidities, the reader is directed to reviews on this literature by Brown et al., (2012), Batel, (2000), Macdonald and Feifel, (2012) and Altamura, (2007).
1.5.1 Anxiety

Substance use and anxiety disorders are considered two of the most widely prevalent disorders that are often comorbid and poorly treated (DuPont, 1995). This comorbidity is associated with negative outcomes and poor prognoses for the treatment of both disorders. Several clinical studies observed increased anxiety following chronic administration of heroin (Woody et al., 1975), cocaine (Roberts and Horton, 2003, Pettinati et al., 1989), amphetamine (Hohoff et al., 2005), MAP (Glasner-Edwards et al., 2010) and MDMA (Milani et al., 2004). These findings are supported by pre-clinical data showing anxiogenic-like effects in animals upon administration of opioids (Rezayof et al., 2009), cocaine (Basso et al., 1999, Rogerio and Takahashi, 1992), amphetamine (Lin et al., 1999), MAP (Pometlova et al., 2012) and MDMA (Lin et al., 1999), as measured by using the elevated plus-maze (EPM).

The anxiogenic effect of drugs of abuse has been shown to be associated with changes in the release of several brain neurotransmitters. For example, increased glutamate release in the brain was observed following administration of cocaine (Shoji et al., 1997, Reid et al., 1997), amphetamine (Reid et al., 1997), MAP (Abekawa et al., 1994) and MDMA (Anneken and Gudelsky, 2012, Anneken et al., 2012) in rodents. Due to the ability of glutamate to induce anxiogenic-like behaviours it has been hypothesised that this induction of glutamate release may be involved in the anxiogenic effects of drugs of abuse (see Bergink et al., 2004). Nonetheless, opioid administration, acute and chronic, was shown to decrease the release of glutamate in the brain (Okura et al., 2007, Jin et al., 2006, Guo et al., 2005), suggesting that different mechanisms account for the anxiogenic mechanisms of each class of drugs of abuse.
Anxiety profiles have been also observed in individuals abstinent from heroin (Powell and Taylor, 1992, Shi et al., 2009) and cocaine (Roberts and Horton, 2003). In animal studies, spontaneous withdrawal from opioids (Zhang and Schulteis, 2008, Schulteis et al., 1998, Lee et al., 2011), cocaine (El Hage et al., 2012) and MAP (Nawata et al., 2012) has been shown to be accompanied by enhanced anxiety-like behaviour using the EPM (see Table 1.3).

Anxiety-like behaviour during abstinence is considered as a key vulnerability factor for relapse to drug-seeking. The most widely used psychological supportive treatment for anxiety-related behaviours following cessation of drug use is Relapse Prevention (RP) therapy based on Marlatt’s cognitive behavioural model (Marlatt and George, 1984). This programme focuses mainly on helping addicts to develop skills to cope with putative high-risk situations (such as anxiety-related situations) or cues associated with drug administration. However, a meta-analysis on the efficacy of this treatment strategy revealed poor effect on reducing substance use, but it showed high effectiveness for improvement in overall psychosocial adjustment (Irvin et al., 1999).

1.5.2 Depression

Co-occurring drug addiction and depressive-like symptoms are linked with poor treatment prognosis, including high rates of continued substance abuse and mental impairment (Alterman et al., 1996, Brooner et al., 1997, Kosten et al., 1986). It has been hypothesised that patients with depression may take advantage of the acute rewarding effects of the drugs of abuse to relieve their depressive state, which can lead to the development of dependence upon chronic administration (Markou et al., 1998). It is also known that chronic administration of drugs of abuse trigger neuroadaptational changes
in the brain that might contribute to mood disturbances potentially leading to depression.

In humans, depressive symptoms and a subjective emotional distress state are commonly associated with withdrawal from opioids (Ritter, 2002) and cocaine (Dackis et al., 1987, Gawin and Kleber, 1986, Kosten and O'Connor, 2003). Animal models of addiction have confirmed clinical observations concerning increased depressive-like behaviour following withdrawal from drug administration. Specifically, several animal studies have reported increase forced-swim test (FST) immobility time during withdrawal from opioids (Anraku et al., 2001), cocaine (Filip et al., 2006, Perrine et al., 2008) and amphetamine (Cryan et al., 2003), also see Table 1.3. The validity of using these animal models for the elucidation of the neurobiological changes associated with the development of depression during drug abstinence is supported by successful reduction of depressive-related behaviours using antidepressant administration during withdrawal from cocaine (Frankowska et al., 2010) and MDMA (Thompson et al., 2004) in rodents.

Anhedonia, a key symptom of clinical depression, is widely modelled in animal models by using the sucrose preference and intracranial self-stimulation tests and reflect decreased interest in engaging in pleasure-seeking behaviour. Anhedonia-like behaviours have been reported in rodents withdrawn from opioids (Schulteis et al., 1994), cocaine (Markou and Koob, 1992) and MDMA (Galiano et al., 2005, Straiko et al., 2007). There is evidence indicating that antidepressant treatments may be effective in reversing anhedonic effects induced by cocaine (Markou et al., 1992, Scheggi et al., 2011). Similarly, amphetamine withdrawal has been shown to induce reward deficits (Kitanaka et al., 2008) such as decreased sucrose intake (Orsini et al., 2001, Der-
Avakian and Markou, 2010) and elevated brain reward thresholds (Paterson et al., 2000). These impaired behaviours were reversed by treatment with selective antidepressants (Harrison et al., 2001, Markou et al., 2005).

A possible mechanism linking depression and substance use disorders is decreased serotonergic system activity. Indeed, decreased function of the serotonin (5-hydroxytryptamine, 5-HT) system has been implicated in increased vulnerability to the development of depression, stress and social impairment (Albert et al., 2012, Kane et al., 2012). Similarly, this hypo-activity of the serotonergic system is involved in emotional deficits following withdrawal from drugs of abuse; opioid and psychostimulant withdrawal were shown to suppress 5-HT release in the brain (see Kirby et al., 2011). Moreover, social isolation, which has been associated with depressive behaviours, has also been repeatedly shown to induce a hypo-serotonergic state (Muchimapura et al., 2003, Muchimapura et al., 2002, Bickerdike et al., 1993, Fulford and Marsden, 1998). Selective serotonin reuptake inhibitors (SSRIs), which increase serotonergic activity in the brain (see Costagliola et al., 2008), have been used in clinical trials for the treatment of depressive consequences of addiction. However, fluoxetine, an SSRI drug, yielded negative results in the alleviation of the depressive symptoms in a tri-morbid population of depressed cocaine/alcohol abusers (Cornelius et al., 1998) and depressed methadone-maintained opioid patients (Petrakis et al., 1998). Also, several other studies have failed to support the effectiveness of classic antidepressants for the treatment of drug-addicted individuals (e.g. Johnson et al., 1996, Lima et al., 2001, Nunes et al., 2004).
Table 1.3: Effects of protracted withdrawal from drugs of abuse on anxiety- and depressive-like behaviours

<table>
<thead>
<tr>
<th>Addictive substance</th>
<th>Administration paradigm</th>
<th>Animal model</th>
<th>Behavioural paradigm</th>
<th>Behavioural effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>Chronic: Morphine pellet (75 mg/pellet, s.c.) (x 72 hours) Withdrawal: Removal of morphine pellet for 8 hrs</td>
<td>Male Wistar rats (260-280 g) housed in groups</td>
<td>Elevated plus-maze</td>
<td>↓ time in open arms</td>
<td>Schulteis et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Acute administration: 4 injections of 5.6 or 10 mg/kg, s.c. Withdrawal: naloxone 1 mg/kg, s.c. (8 hrs following the last injection)</td>
<td>Male Wistar rats (275-375 g) housed in groups</td>
<td>Elevated plus-maze</td>
<td>↓ %time and % entries in open arms</td>
<td>Zhang and Schulteis, 2008</td>
</tr>
<tr>
<td></td>
<td>Chronic: 2 x 40 mg/kg/day, s.c. (x 5 days) Withdrawal: 72 hrs spontaneous withdrawal</td>
<td>Male Sprague-Dawley rats (260-280 g) housed in groups</td>
<td>Elevated plus-maze</td>
<td>↓ %time and % entries in open arms</td>
<td>Lee et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Chronic: 2 x 20-100 mg/kg, i.p. (x 14)</td>
<td>Male Sprague-Dawley rats (260-</td>
<td>Forced-swim test</td>
<td>↑ immobility time</td>
<td>Anraku et al., 2001</td>
</tr>
<tr>
<td>Treatment</td>
<td>Details</td>
<td>Procedure</td>
<td>Outcome</td>
<td>Reference</td>
<td></td>
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<tr>
<td><strong>Cocaine</strong></td>
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<tr>
<td>Chronic: 3 x 15 mg/kg/day, i.p. (x 14 days)</td>
<td>Male Sprague-Dawley rats (275-375 g) housed in groups</td>
<td>Elevated plus-maze</td>
<td>↓ %time and % entries in open arms</td>
<td>Perrine et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Chronic: 1 x 20 mg/kg/day, i.p. (x 14 days)</td>
<td>Male Sprague-Dawley rats (200-225 g) housed in groups</td>
<td>Elevated plus-maze</td>
<td>↓ head-scanning time</td>
<td>El Hage et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Chronic: 1 x 10 mg/kg/day, i.p. (x 5 days)</td>
<td>Male Wistar rats (220-230 g) housed in groups</td>
<td>Forced-swim test</td>
<td>↑ immobility time</td>
<td>Filip et al., 2006</td>
<td></td>
</tr>
<tr>
<td><strong>Withdrawal</strong></td>
<td></td>
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<tr>
<td>Withdrawal: 3 or 6 days spontaneous withdrawal</td>
<td>310 g) housed in groups</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td>2 x 40 mg/kg/day, s.c. (x 5 days)</td>
<td>Male Sprague-Dawley rats (260-280 g) housed in groups</td>
<td>Forced-swim test</td>
<td>↑ immobility time</td>
<td>Lee et al., 2011</td>
</tr>
</tbody>
</table>
| **Withdrawal:** 10 days spontaneous withdrawal | **Chronic:** 3 x 15 mg/kg/day, i.p. (x 14 days)  
**Withdrawal:** 24 hrs spontaneous withdrawal | Male Sprague-Dawley rats (275-375 g) housed in groups | Forced-swim test | ↑ mean immobility counts | Perrine et al., 2008 |
|-----------------|------------------------------------------------|------------------------------------------------|-----------------|------------------------|----------------------|
| **Methamphetamine**  
**Self-administration:** 0.02mg/0.1ml i.v. infusion over 6s, 2 hr-sessions/day (x 10 days)  
**Withdrawal:** 10 days spontaneous withdrawal | Male Wistar rats (250-350 g) housed in groups, food limited to 15-20 g/day/ body | Elevated plus-maze | ↓ time in open arms | Nawata et al., 2012 |
| **Amphetamine**  
**Chronic:** minipump 5 or 10 mg/kg/day, s.c. (x 6 days)  
**Withdrawal:** 48 and 72 hrs spontaneous withdrawal | Male Wistar rats (175-225 g) housed in groups | Forced-swim test | ↑ immobility time  
↓ climbing time | Cryan et al., 2003 |
<p>| <strong>Chronic:</strong> 1 x 2, or 4 mg/kg, i.p. (x 14 days) | Male Sprague-Dawley rats (230-250 g) housed in | Forced-swim test | ↑ immobility time | Che et al., 2013 |</p>
<table>
<thead>
<tr>
<th>Withdrawal</th>
<th>groups</th>
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</tr>
</thead>
<tbody>
<tr>
<td>14 days spontaneous withdrawal</td>
<td></td>
<td></td>
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</tbody>
</table>

A detailed summary of the anxiety- and depressive-like behaviours induced by protracted abstinence from opioid and psychostimulant administration in rodents. † increase; ‡ decrease; Abbreviations: i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.
1.5.3 Anti-social behaviours

The anti-social consequences of drug addiction are considered important for the development of novel pharmacotherapies for drug addiction treatment. Anti-social behaviours and drug use are linked and comorbidity between these two is common. It has been reported that almost 80% of prisoners have a history of illicit substance use and many are imprisoned for anti-social acts under the influence of an addictive substance (e.g. Dolan et al., 2007).

Early clinical studies reported that heroin addicts showed social isolation behaviours, which persisted following abstinence (Tokar et al., 1975), comprising a motivational trigger to relapse and re-take the drug. In parallel, chronic cocaine (Myrick and Brady, 1997), amphetamine (Williams et al., 2000) or MAP (see Homer et al., 2008) induce social phobia in humans. Additionally, cocaine has been found to reduce social interaction in both humans (Brower et al., 1988, Resnick and Resnick, 1984, Weddington, 1993) and animals (Darmani et al., 1990, Hadfield et al., 1982).

Social isolation and social withdrawal behaviours have been proposed to be at least partially mediated via a hypo-DAergic state in the brain. Indeed, hypo-DAergic activity within the striatum has also been observed in social phobia (Schneier et al., 2000). It is also well known that chronic administration of drugs of abuse induces the depletion of DA levels in the striatum (e.g. Wilson et al., 1996). In addition, positron emission tomography (PET) studies in humans have consistently demonstrated decreased striatal DA D₂ receptor levels in heroin, cocaine and MAP addicts (Volkow et al., 1996, Volkow et al., 1997, Wang et al., 1997, Volkow et al., 2001), a finding which has been shown to persist following drug detoxification (see Volkow et al., 2004). Also, decreased D₂ receptor levels are observed following chronic cocaine self-administration.
in monkeys (Moore et al., 1998, Nader et al., 2002) and low $D_2/D_3$ number in the ventral striatum of impulsive rats predicts their vulnerability to escalate cocaine intake (Dalley et al., 2007). Taken together, these findings might suggest that chronic illicit drug use can lead to the development of social phobia via the disruption of the DAergic system and more specifically the $D_2$ receptors. Recently, use of $D_2$ receptor partial agonists, such as aripiprazole, have shown some pre-clinical benefit in reducing MAP self-administration (Wee et al., 2007) and preventing cocaine reinstatement (Feltenstein et al., 2007) in rodents. However, the clinical efficacy of aripiprazole in treating drug addiction is currently unclear (Meini et al., 2011, Haney et al., 2011).

### 1.5.4 Treatment of addiction and comorbid mental disorders

Many drug abusers have been diagnosed with at least one other mental disorder, such as schizophrenia, bipolar disease, depression, anxiety and anti-social behaviours. This comorbidity has been shown to cause poorer drug treatment outcomes and to increase vulnerability to relapse following abstinence from drug use.

It has been shown that medications for the treatment of emotional disorders alone are not likely to completely reduce substance use or help abstinence in dually diagnosed individuals (see Riggs, 2003). Evidence supports the efficiency of psychosocial support in the treatment of drug addiction and the maintenance of abstinence (Koerner, 2010). Accumulating evidence shows a strong interaction between neuronal markers of social behaviour and those of stress and mood regulation (Dabrowska et al., 2011, Debiec, 2005, Di Simplicio et al., 2009, Heinrichs and Gaab, 2007, Windle et al., 2004) and of drug reward/withdrawal (Liu et al., 2011, Young et al., 2011), suggesting that the "social" neuropeptide oxytocin (OT) and its central receptor system may be implicated in the modulation of negative emotional aspects of drug addiction. Therefore, the focus
of this project will be the effects of oxytocin in opioid and psychostimulant addiction processes.
1.6 The oxytocin neuropeptide

Oxytocin is a nine amino-acid peptide (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly) synthesised within several neuronal populations in the brain and peripheral tissue. The OT gene (Figure 1.4) was sequenced in 1984 (Ivell and Richter, 1984b) and was the first peptide hormone to be chemically synthesised in biologically active form (Du Vigneaud et al., 1953). The mouse OT gene, coding for a prepropeptide, consists of two introns and three exons (Hara et al., 1990), similar to those observed in humans (Sausville et al., 1985) and rats (Ivell and Richter, 1984b). The first exon encodes for a translocator signal, the oxytocin peptide, the tripeptide processing signal (GKR) and the first nine residues of neurophysin; the second exon encodes the central part of the neurophysin protein; and the third exon encodes the C-terminus of neurophysin (Hara et al., 1990).

![Diagram of oxytocin gene structure](image)

Figure 1.4: Structure of the oxytocin gene.
(A) Oxytocin gene structure showing exons and introns. The composite hormone response element (CHRE) is given for mouse, rat and humans with their relative positions upstream (-). (B) Domain organisation of the prepropeptide of oxytocin. Enzymes cleave the precursor into fragments, one involving a glycyl-lysyl-arginine (GKR) sequence leaving a carboxamide group at the carboxy-terminus. Modified from Gimp and Fahrenholz, (2001).

---

CH RE

Mouse -174bp GATGACCTTGACC
Rat -168bp GGTGACCTTGACC
Human -164bp GGTGACCTTGACC

---
OT is enzymatically cleaved from the prepropeptide and has functional signalling activity following extracellular release. Neurophysin, a small disulfide-rich protein, is essential for the proper targeting, packaging and storage of OT prior to its release into the bloodstream (Breslow, 1979).

1.6.1 Oxytocin as a neuromodulator in the central nervous system

The neuropeptide OT is primarily synthesised in the magnocellular neurosecretory cells of the supraoptic (SON) and paraventricular (PVN) nuclei of the Hyp (Figure 1.5); it is then transported to the posterior pituitary gland where it is stored in vesicles and is released into the bloodstream to exert its peripheral effects. Additionally, OT is also synthesised in the parvocellular neurons of the PVN which innervates several extrahypothalamic regions in the central nervous system (CNS) including the olfactory nuclei, frontal and cingulate cortices, Acb, LS, vertical limb of the diagonal band of Broca (VDB), Amy, BNST and also the brainstem and spinal cord (Badoer, 2001, Mack et al., 2002, Petersson, 2002, Sofroniew and Weindl, 1978, Tang et al., 1998). OT exerts several central effects through binding to oxytocin receptors (OTR) in these brain areas (Baskerville and Douglas, 2010, Fuxe et al., 2012, Meyer-Lindenberg et al., 2011, Rutherford et al., 2011).
Figure 1.5: Prominent oxytocinergic projections in the brain of rodents.

Oxytocin is primarily synthesised in the magnocellular neurosecretory cells in the supraoptic (SON) and paraventricular (PVN) nuclei; it is then transported to the posterior pituitary gland where it is stored in vesicles and is released into the bloodstream to exert its peripheral effects. Moreover, OT is also synthesised in the parvocellular neurons of the PVN, which innervate several extrahypothalamic regions in the CNS, including the olfactory nuclei, the nucleus accumbens, the lateral septum, the amygdala, bed nucleus of the stria terminalis, and also to the brainstem and spinal cord, to exert its local effects. (* magnocellular neurons; parvocellular neurons)

Abbreviations: Acb, nucleus accumbens; Amy, amygdala; BNST, bed nucleus of stria terminalis; CgCx, cingulate cortex; CPU, caudate-putamen; DR, dorsal raphe nucleus; FCx, frontal cortex; LS, lateral septum; NL, neural lobe of the pituitary; OB, olfactory bulb; PAG, Periaqueductal gray; PVN, paraventricular hypothalamic nucleus; Sol, Solitary tract nucleus; SC, spinal cord; SON, supraoptic hypothalamic nucleus; Th, thalamus; Tu, olfactory tubercle; VDB, vertical limb of the diagonal band of Broca. Figure founded on Baskerville and Douglas, (2010), Fuxe et al., (2012), Meyer-Lindenberg et al., (2011).

Importantly, OT is released from neuronal dendrites and cell bodies in the PVN and SON, where it is considered to impact on a broad network of extra-hypothalamic brain regions via volume transmission (see Landgraf and Neumann, 2004). There is evidence suggesting that OT can be also locally released in several brain regions including the BNST, medial preoptic area (MPOA), and lateral part of the Amy (Young and Gainer, 2003). However, there is continued disagreement as to whether OT released in these extra-hypothalamic brain areas comes from the dendrites of unidentified OT cell bodies or from axonal projections-volume transmission from OT-containing neurons in the
PVN (see Neumann, 2007). There is also some evidence that scattered OT fibers projecting from magnocellular hypothalamic neurons lie in diverse brain regions including the Hip, cortex, Amy, substantia nigra (SN), VTA, dorsal raphe nucleus (DR), locus coeruleus, and densely throughout the brain stem and spinal cord (Sofroniew, 1983, Sofroniew, 1980).

Oxytocin peptide is known for its peripheral actions, reflecting its release into the bloodstream from the pituitary gland. Two of the classical roles assigned to OT is milk ejection from the mammary gland and uterine contraction to induce labor (see Gimpl and Fahrenholz, 2001). In addition to its peripheral actions, OT exerts several central effects by binding to OT receptors (OTR) expressed in the CNS; see Section 1.6.2. Indeed, the activation of OT receptors in the brain has been shown to be involved in a range of behaviours including autonomic function (Badoer, 2001, Mack et al., 2002, Petersson, 2002, Tang et al., 1998), social/sexual (Argiolas, 1999, Argiolas and Melis, 2004, Insel et al., 1997, Kendrick, 2000, Kendrick, 2004), anxiety-, depressive- and psychotic-like behaviours (Baumgartner et al., 2008, Dabrowska et al., 2011, Di Simplicio et al., 2009, Kirsch et al., 2005, Rosenfeld et al., 2011, Windle et al., 2004). For further elucidation of the functions of the oxytocinergic system, both oxytocin peptide knockout and oxytocin receptor knockout mice have been developed (see Sections 1.8.4 and 1.8.5, respectively).

1.6.2 Oxytocin receptors

The OTR gene sequence has been identified in several mammalian species including human (Kimura et al., 1992), rat (Rozen et al., 1995), sheep (Ivell and Richter, 1984a) and mouse (Kubota et al., 1996). Similarly to the human (Inoue et al., 1994) and the rat (Rozen et al., 1995), the mouse OT receptor gene contains 3 introns and 4 exons (Kubota et al., 1996); also see Figure 1.6. Exons 1-2 correspond to the 5'-prime non-
coding region. Exon 3 encodes a portion of the 5'-prime non-coding region, the start codon ATG, and the first 6 of the 7 transmembrane domains of the OTR. Exon 4 encodes the seventh transmembrane region, the stop codon TGA, the carboxyl terminal and the 3'-prime non-coding region (Kubota et al., 1996).

Figure 1.6: Organisation of the mouse oxytocin receptor gene.
The oxytocin receptor gene consists of 4 exons and 3 introns. The seven trans-membrane domains (I-VII) are indicated as well as the start (ATG) and the stop (TGA) codons of the gene. Modified from Gimpl and Fahrenholz, (2001).

The OTR belongs to the G protein-coupled receptor (GPCR) superfamily and it is functionally coupled to G_{q/11a} class GTP binding proteins, stimulating the activity of phospholipase C (PLC) (Kimura et al., 1992); also see Figure 1.7. This effect leads to the generation of inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 diffuses into the cytosol and, through its binding to calcium channels on the endoplasmic reticulum (ER), allows the release of calcium. In neurosecretory cells, rising calcium levels control neuronal excitability, modulate their firing patterns and lead to neurotransmitter release (see Berridge, 1998). DAG, in the presence of calcium, directly activates protein kinase C (PKC), which then phosphorylates several proteins.
Figure 1.7: Oxytocin receptor transduction mechanism and second messenger control of cellular effector systems.

Activation of the OTR by OT leads to the activation of phospholipase C, resulting in the generation of phosphatidylinositol bisphosphate and 1,2-diacylglycerol. Phosphatidylinositol bisphosphate is cleaved to activate inositol trisphosphate, which releases intracellular calcium from the endoplasmic reticulum. 1,2-diacylglycerol stimulates protein kinase C, which in turn is responsible for the phosphorylation of several other proteins in the cell. Abbreviations: Ca^{2+}, calcium; DAG, 1,2-diacylglycerol; GDP, guanine diphosphate; GTP, guanine triphosphate; IP_3, inositol trisphosphate; P, phosphate; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; OT, oxytocin; OTR, oxytocin receptor. Modified from Tom and Assinder, (2010).

Primary cell culture experiments indicate that OTRs are localised not only on hypothalamic neurons but also on astrocytes in rats (Di Scala-Guenot and Strosser, 1992). Concerning the regional distribution of OTR binding sites in the brain, critical species differences have been observed (see Table 1.4).
### Table 1.4: Distribution of oxytocin receptors in the brain of different species

<table>
<thead>
<tr>
<th>Brain Regions</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Olfactory system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior olfactory nucleus</td>
<td>N/R</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Olfactory tubercle</td>
<td>N/R</td>
<td>++</td>
<td>N/R</td>
</tr>
<tr>
<td>Islands of Calleja</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>N/R</td>
<td>N/R</td>
<td>+++</td>
</tr>
<tr>
<td>Entorhinal/perirhinal area</td>
<td>-</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td><strong>Cortical areas</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peduncular cortex</td>
<td>+/-</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>Insular cortex</td>
<td>N/R</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>N/R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>-</td>
<td>+/-</td>
<td>N/R</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>-</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>Taenia tecta</td>
<td>N/R</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Ventral limb of the Diagonal band of Broca</td>
<td>+</td>
<td>N/R</td>
<td>N/R</td>
</tr>
<tr>
<td>Basal nucleus of Meynert</td>
<td>+++</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td><strong>Basal ganglia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>-</td>
<td>++</td>
<td>N/R</td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td>++</td>
<td>+++</td>
<td>N/R</td>
</tr>
<tr>
<td>Glopus pallidus</td>
<td>++</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>-</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td><strong>Limbic system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral septum</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Bed nucleus of stria terminalis</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Amygdaloid-hippocampal area</td>
<td>-</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>CA1 region of Hippocampus</td>
<td>N/R</td>
<td>N/R</td>
<td>-</td>
</tr>
<tr>
<td>CA3 region of Hippocampus</td>
<td>N/R</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Central amygdaloid nucleus</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Medial amygdaloid nucleus</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basolateral amygdaloid nucleus</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Parasubiculum and presubiculum</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dorsal subiculum</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Ventral subiculum</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><strong>Thalamus and hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anteroventral thalamic nucleus</td>
<td>-</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td>Paraventricular thalamic nucleus</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ventromedial hypothalamic nucleus</td>
<td>-</td>
<td>++  +</td>
<td></td>
</tr>
<tr>
<td>Anterior medial preoptic area</td>
<td>++</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>-</td>
<td>+/-</td>
<td>N/R</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>-</td>
<td>+/-</td>
<td>N/R</td>
</tr>
<tr>
<td>Medial tuberal nucleus</td>
<td>+</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>Posterior hypothalamic area</td>
<td>++</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td>Supramammillary nucleus</td>
<td>-</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>Lateral mammillary nucleus</td>
<td>+</td>
<td>+</td>
<td>N/R</td>
</tr>
<tr>
<td>Medial mammillary nucleus</td>
<td>+</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td><strong>Brain stem</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>+++</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td>Ventral and dorsal tegmental area</td>
<td>-</td>
<td>-</td>
<td>N/R</td>
</tr>
<tr>
<td>Central gray</td>
<td>+</td>
<td>-</td>
<td>N/R</td>
</tr>
</tbody>
</table>
Dorsal raphe nucleus | + | - | N/R
Reticular nuclei | - | - | N/R
Medial vestibular nucleus | - | - | N/R
Hypoglossus nucleus | ++ | - | N/R
Nucleus of the solitary tract | +++ | +/- | N/R
Dorsal motor nucleus of the vagus nerve | + | + | N/R
Inferior olive nucleus | +/- | + | N/R
Substantia gelatinosa of trigeminal nucleus | +++ | + | N/R


+, low binding; ++, moderate binding; ++++, high binding; +/- at the detection limit; -, not detectable; N/R, not reported.

The OTR is widely distributed throughout the brain. Species differences exist in the OTR distribution and these differences are thought to elucidate several discrepancies in behaviour. In rats, OTR are especially prominent in the olfactory system, peduncular cortex, ventral pallidum, subiculum, BNST and amygdaloid nuclei (e.g. Jarrett et al., 2006, Ostrowski, 1998, Tribollet et al., 1992b). In mice, OTR binding is more abundant within the olfactory nucleus, LS, Pir, hippocampal formation (especially CA3 region) and PVN (e.g. Curley et al., 2009, Insel et al., 1991, Insel et al., 1993). In humans, high expression is found in the basal nucleus of Meynert, LS, VP, VDB, posterior hypothalamic area, SN, Sol and substantia gelatinosa of trigeminal nucleus (Loup et al., 1991, Loup et al., 1989). In parallel, OTR mRNA was detected in brain areas that mostly coincided with the occurrence of OTR binding sites (Yoshimura et al., 1993).
1.6.3 Oxytocin and the blood-brain barrier

Evidence suggests that central and peripheral OT can be independently regulated, meaning that plasma OT may provide a poor proxy for central oxytocinergic tone (Neumann et al., 1993a) due to the presence of the blood-brain barrier (BBB). This barrier’s limited permeability prevents numerous substances such as many drugs and endogenous chemicals from entering the brain. OT, being non-steroid and water soluble, is carried freely within the blood circulation; however, peptides have limited ability to cross the BBB; ~0.1% of systemic OT enters the brain (Jones and Robinson, 1982).

Nevertheless, there is some evidence suggesting that the OT peptide might cross the BBB. It has been shown that peripheral injections of OT can mirror some effects seen after central administration of the peptide. For example, in Mongolian gerbils, affiliation between monogamous females and their partners was significantly increased upon subcutaneous injections of OT (Razzoli et al., 2003). Having a short plasma half-life (3-5 minutes), OT’s prolonged effects remain unclear (Uvnas-Moberg, 1998, Engstrom et al., 1998). It might be possible that OT can cross the BBB via the circumventricular region which allows a less restricted transport of molecules into the brain (see Ermisch et al., 1985). Another possible mechanism by which systemically administered OT may affect the CNS is via the formation of active fragments that may have the capability to cross the BBB. In fact, fragments of OT have been found to cross the BBB and regulate several memory processes (Burbach et al., 1983). Once in the brain, OT may bind to OTRs on OT-containing neurons in the PVN and SON; thus, even small quantities may stimulate the release of significant quantities of endogenous OT to trigger a feed-forward response (Rossoni et al., 2008).
In addition, OT, when peripherally administered in rats, increased *c-Fos* expression in a number of brain regions including the SON, PVN, CeA, lateral parabrachial nucleus, and the locus coeruleus (Hicks et al., 2012). These are all OTR-dense brain regions which are implicated in a number of physiological processes including reproduction, anxiety, water balance, blood osmolality, and sleep. A double-labeling approach showed that peripherally administered OT increased *c-Fos* activation within OT-containing neurons of the PVN and SON, further supporting previous observations that OT is capable of stimulating its own release from central sites (Moos et al., 1984). Moreover, some effects of peripheral OT administration were abolished by central administration of OTR antagonists (e.g. Kovacs et al., 1998). Finally, intranasal administration of OT decreases Amy activation in response to fear in humans (Kirsch et al., 2005).

### 1.6.4 Oxytocin peptide knockout mice

Currently there are three different versions of the oxytocin deficient mouse (OT^−/−): (1) Gross et al., (1998) replaced all the three exons of the mouse OT peptide gene, thus eliminating the prepropeptide-neurophysin coding sequence. (2) Nishimori et al., (1996) deleted the first exon of the OT gene via homologous combination in embryonic stem cells. This deletion resulted in the elimination of the initiation ATG codon, the processing signal, the OT peptide, and the first few amino acids of neurophysin. (3) Young et al., (1996) replaced the second and third exons of the OT gene by using a neomycin resistance cassette, resulting in the deletion of the carrier polypeptide. Therefore, although OT is transcribed normally, it is not packaged or transported out of the cell. The deletion of the first exon has not been shown to affect arginine vasopressin mRNA, while in comparison, deletion of the second exon slightly decreased it. The distribution and the concentration of the OTRs were unaffected in these transgenic mice. Therefore, pre-synaptic OT is not necessary for the normal neuroanatomical
distribution of OTRs and subsequently does not affect the species differences in OTR
distribution in the brain. OT$^{−/−}$ mice are phenotypically healthy mice and both male and
female mice are fertile (see Winslow et al., 2000). However, these mice appear to have
differential behaviours compared to wild-types (see Table 2), i.e. deficits in social
recognition, reduced aggression but normal social interaction behaviours (Winslow et
al., 2000, Ferguson et al., 2000, Crawley et al., 2007).
Table 1.5: Oxytocin peptide knockout mice and behaviour

<table>
<thead>
<tr>
<th>Behavioural paradigm</th>
<th>Gender, age, background</th>
<th>Behavioural effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social recognition test</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model), 129SvEv x C57BL/6J, 8-9 weeks old</td>
<td>↓ social recognition memory</td>
<td>Ferguson et al., 2000</td>
</tr>
<tr>
<td>Isolation-induced aggression</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model), 129SvEv x C57BL/6J, 7 weeks old</td>
<td>↑ aggression</td>
<td>Winslow et al., 2000</td>
</tr>
<tr>
<td>Resident-intruder aggression</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model), 129SvEv x C57BL/6J, &gt;7 weeks old</td>
<td>↑ aggression</td>
<td>Winslow et al., 2000</td>
</tr>
<tr>
<td>Acoustic startle</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model), 129SvEv x C57BL/6J, &gt;7 weeks old</td>
<td>↓ acoustic startle response</td>
<td>Winslow et al., 2000</td>
</tr>
<tr>
<td>Elevated plus-maze</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model) 129SvEv x C57BL/6J, &gt;7 weeks old</td>
<td>↓ anxiety-related behaviour</td>
<td>Winslow et al., 2000</td>
</tr>
<tr>
<td>Elevated plus-maze</td>
<td>Female OT&lt;sup&gt;−/−&lt;/sup&gt; (Young’s model), C57BL/6J</td>
<td>↑ anxiety-related behaviour</td>
<td>Amico et al., 2004</td>
</tr>
<tr>
<td>Open-field test</td>
<td>Female and Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Young’s model), C57BL/6J, 11-15 weeks old</td>
<td>↔ locomotion</td>
<td>Crawley et al., 2007</td>
</tr>
<tr>
<td>Open-field test</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model), 129SvEv x C57BL/6J, 4-5 weeks old</td>
<td>↓ time in center</td>
<td>Crawley et al., 2007</td>
</tr>
<tr>
<td>Open-field test</td>
<td>Female OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model), 129SvEv x C57BL/6J, 4-5 weeks old</td>
<td>↔ time in center</td>
<td>Crawley et al., 2007</td>
</tr>
<tr>
<td>Accelerating rotarod test</td>
<td>Male and Female OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model) 129SvEv x C57BL/6J, 4-5 weeks old</td>
<td>↔ motor coordination</td>
<td>Crawley et al., 2007</td>
</tr>
<tr>
<td>Social interaction and preference for novelty tests (three-chambered box)</td>
<td>Male OT&lt;sup&gt;−/−&lt;/sup&gt; (Young’s model), C57BL/6J, 11-15 weeks old</td>
<td>↔ social interaction or preference for novelty</td>
<td>Crawley et al., 2007</td>
</tr>
<tr>
<td>Social interaction and preference for novelty tests (three-chambered box)</td>
<td>Male and Female OT&lt;sup&gt;−/−&lt;/sup&gt; (Nishimori’s model) 129SvEv x C57BL/6J, 4-5 weeks old</td>
<td>↔ social interaction or preference for novelty</td>
<td>Crawley et al., 2007</td>
</tr>
</tbody>
</table>

Mice with targeted mutation in the OT peptide gene showed deficits in social memory, decreased aggression, and normal sociability behaviours.
1.6.5 Oxytocin receptor knockout mice

In order to elucidate the central effects of the oxytocinergic system, two different types of OTR knockout (OTR"−") have been generated: (1) global OTR knockout mice (Takayanagi et al., 2005, Lee et al., 2008), and (2) mice lacking the OTR only in the forebrain – conditional OTR^{FB/FB} mice (Lee et al., 2008). Male OTR"−" mice pups generated by (Takayanagi et al., 2005) produced less ultrasonic vocalizations than wild-type littermates in response to social isolation (indicating decreased distress following social isolation). Adult mice of the latter strain displayed social amnesia and increased aggressiveness as measured by the social discrimination and resident-intruder aggression tests respectively (Takayanagi et al., 2005). The conditional knockout model has been shown to have reduced OTR binding on postnatal day 21 in forebrain regions, including the central and medial amygdala, the Hip, the piriform and perirhinal cortices (Pagani et al., 2011). Macbeth et al., (2009) investigated the performance of both strains of OTR knockout mice in a social discrimination test and showed that OTR"−" male mice were unable to discriminate individuals of the same strain but were capable to discriminate between females from different strains. This discrimination was not replicated in OTR^{FB/FB} mice. Possible reasons for this difference may rely on the spatial or temporal differences in diminished OTR expression between these two types of OTR knockout mice, as for instance postnatal delay and incomplete loss of forebrain binding in the OTR^{FB/FB} strain.
1.7 **Oxytocin and emotions**

Oxytocin, via its action on the OTRs in the brain, is involved in the regulation of a wide variety of CNS behaviours including sexual behaviour, maternal care and aggression, pair bonding, social behaviour, stress-related behaviour including anxiety and depression, learning and memory (see Neumann, 2008, Gimpl and Fahrenholz, 2001). This thesis concentrates on the role of OT in anxiety, depression and social behaviours, and the reader is directed to reviews on the central effects of OT by Gimpl and Fahrenholz, (2001), Wojciak et al., (2012) and Garrison et al., (2012) for further information.

1.7.1 **Anxiety**

Peripheral and central OT administration has been shown to reduce both hormonal stress-response of the HPA axis and anxiety levels (Neumann et al., 2000a). Numerous studies in mice and rats have illustrated the anxiolytic effects of OT. For instance, chronic infusion of synthetic OT into the lateral ventricles of female rats decreased the corticosterone response to psychogenic stress and reduced anxiety-related behaviour (Windle et al., 1997). Similarly, intracerebroventricular (i.c.v.) OT administration attenuated the high anxiety levels of rats selectively bred for high-anxiety behaviour (HAB), while infusion of an OT antagonist increased the anxiety levels of rats with low-anxiety behaviour (LAB) (Slattery and Neumann, 2010a), suggesting an anxiolytic effect of OT. Moreover, central administration of the OT analogue, deamino-1-monocarba-(2-O-methyltyrosine)-oxytocin (carbetocin, CBT), had profound anxiolytic effects in the rat (Mak et al., 2012). Interestingly, not only does central administration of OT exert anxiolytic effects, but peripheral administration of synthetic OT during adolescence also induces persistent reductions in the anxiety-like behaviour in rats as measured by the EPM (Bowen et al., 2011). Additionally, OT knockout mice showed
enhanced anxiety-like behaviours, increased corticosterone levels and higher markers of neuronal activity (c-Fos expression) in brain regions related with stress and anxiety (i.e., the Amy) after exposure to a stressor (Amico et al., 2004). These effects of OT in rodents have been shown to involve the OTR system in the CeA and the hypothalamic PVN in the brain since local microinjection of a selective OTR antagonist in these two areas was able to reverse anxiety-related behaviours (Neumann et al., 2000b, Neumann, 2002, Blume et al., 2008, Bale et al., 2001).

Similar to the animal studies, it has been suggested that OT may also decrease stress and HPA axis activity in humans. Studies examining OT’s effects on HPA axis activity and stress in humans were originally conducted in lactating women. During lactation, a time when circulating OT levels are high, women exhibited lower ACTH levels, indicating a decreased HPA axis activity, and lower cortisol levels after physical stress compared to non-lactating controls (Altemus et al., 1995, Bartz and Hollander, 2006). In another study, lactating women were randomly assigned to either hold or breast-feed their infant before undergoing psychosocial stress. While cortisol and ACTH were significantly increased in both groups in response to stress, women who breast-fed before stress had significantly lower post-stress plasma and salivary cortisol levels compared to those who only held their infant before stress (Heinrichs et al., 2001). These results suggest that the breast-feeding group had higher OT levels, which might have reduced cortisol. Similarly, Heinrichs and colleagues (2003) showed that men who received OT had lower salivary cortisol levels following a stressful event. Therefore, OT may be beneficial in buffering against the negative effects of stress. These results support the presence of HPA axis mediated mechanism for OT’s anxiolytic effects. However, many factors, including how an individual reacts to a stressful situation, can
impact the magnitude of the HPA axis activity and subsequent cortisol response (Lupien et al., 2007).

The exact mechanism through which OT affects the HPA axis and anxiety profile of both rodents and humans is not fully understood. One hypothesis suggests that OT influences the HPA axis by decreasing activity in the sympatho-adrenal system and increasing vagal nerve activity (an important part of the calming effect of the parasympathetic nervous system) (Higa et al., 2002, Uvnas-Moberg, 1997). Specifically, OT terminals in the brain’s solitary vagal complex may modulate reflex control of the heart to facilitate vagal activity and slow the heart (Higa et al., 2002). In animals, PVN OT neurons project to the vagal nuclei (Buijs et al., 1985), and plasma OT increases in response to vagal nerve stimulation (Stock and Uvnas-Moberg, 1988). Together, these data provide evidence that OT directly reduces HPA activity by promoting positive feedback of vagal reactions.

Another mechanism underpinning the anxiolytic properties of OT involves the reduction of fear and/or Amy activation, particularly in response to stress. In a functional magnetic resonance imaging (fMRI) study, it has been shown that intranasal OT administration decreases Amy activation in response to fearful and angry facial expressions (Kirsch et al., 2005). A more recent study suggests that OT reduces activation of the CeA, a structure influential in producing physiological responses to fear (Knobloch et al., 2012). CeA projects to the rostral ventrolateral medulla, a structure which may be essential in regulating cardiovascular responses (e.g., heart rate) to fear and anxiety triggered in the Amy. The medulla has also been shown to be deactivated in response to OT (Viviani and Stoop, 2008), possibly resulting in lower anxiety and increased calmness. However, as OT binds to receptors in many different
brain areas, this model represents only one possible pathway for OT’s anxiolytic effect. Overall, structures governing the fear response may also be responsible for OT’s anxiety-reducing properties.
1.7.2 Depression

Due to the high degree of comorbidity between anxiety and depressive disorders, common mediators are likely to underlie both conditions. Indeed, in addition to its anxiolytic effect, synthetic OT was shown to shift stress-coping in rodents towards a more active coping style, after either central or peripheral administration, indicating antidepressant-like effects (see Slattery and Neumann, 2010b).

The first report of an antidepressant-like effect of OT came from Arletti and Bertolini, (1987) who demonstrated that intra-peritoneal administration of OT, both acutely and chronically, decreased immobility time in the FST in mice. The FST results were replicated in rats following peripheral OT (Arletti et al., 1995) and also peripheral or central CBT administration (Chaviaras et al., 2010). In addition, subcutaneous OT was found to decrease the number of escape failures in the learned helpless test (Nowakowska et al., 2002), which is indicative of an antidepressant-like effect (Cryan et al., 2002). Recently, a novel non-peptidergic OT agonist, WAY-267464, while having an anxiolytic profile, did not show any antidepressant effects in the FST (Ring et al., 2010). Nevertheless, the extent to which this drug shows selectivity to OTRs has recently been contested (Hicks et al., 2012).

In depressed patients, evidence for an altered oxytocincergic system, as deduced from plasma and cerebrospinal fluid (CSF) levels, is limited and inconsistent. Zetzsche et al., (1996) reported lower levels of plasma OT in depressed patients compared with non-depressed controls. Similarly, low levels of plasma oxytocin were reported in depressed patients compared with non-depressed ones among fibromyalgia syndrome patients (Anderberg and Uvnas-Moberg, 2000). However, van Londen et al., (1997) found no significant difference in OT plasma levels between depressed patients and controls, and
a negative correlation was found between the scored symptoms of depression and plasma OT levels (also see Scantamburlo et al., 2007). Activation of the oxytocinergic system has also been reported among depressed patients. For example, in a post-mortem study, it has been shown that OT neurons are activated in the PVN in patients with depression, which may be associated with activation of the HPA axis activity (Purba et al., 1996). Similarly, increased OT mRNA in the PVN has been reported in patients with melancholic depression compared with controls (Meynen et al., 2007). The discrepancies between these studies may be the result of differences between the patient populations, between the results interpretation methods (i.e. not considering body mass index (BMI) or number of children) or between the kits used. For example, in the study by van Londen et al., (1997), OT levels were assessed without controlling possible confounding factors such as food consumption or medication intake (e.g. oral contraceptives).

It is therefore clear that oxytocinergic system is highly implicated in depressive-like responses. However, several questions remain; for example, whether such alterations in the oxytocinergic system represent causes or consequences of depression, and whether antidepressant treatment can normalise such changes.
1.7.3 Social deficits

Animal and human studies indicate a facilitatory role of the neuropeptide OT in a broad variety of social-enhancing responses. Following its central release, OT promotes important aspects of social behaviour, including social preference (Lukas et al., 2011), maternal care and aggression (Numan and Insel, 2003), sexual behaviour (Melis et al., 2007), pair-bonding in monogamous species (Donaldson and Young, 2008, Carter et al., 1995), and social cognition (Bielsky and Young, 2004, Gabor et al., 2012).

Intra-cerebral release of OT in specific brain regions was found to be increased in response to a variety of social stimuli, including mother–offspring interactions, maternal aggression, mating in males and females, and exposure to an adult con-specific during social defeat (see Neumann, 2009, Veenema and Neumann, 2008). In the context of social stimuli, increased OT release could be identified within the hypothalamic PVN (Neumann et al., 1993b, Waldherr and Neumann, 2007), the CeA (Bosch et al., 2005), the olfactory bulb (Levy et al., 1995), the mediolateral septum (Ebner et al., 2000), and the Acb (Ross et al., 2009). Also, OT has a role in social recognition in both male (Popik and van Ree, 1991) and female rats (Engelmann et al., 1998), which has been confirmed in OTR receptor knockout mice displaying impaired social memory (Ferguson et al., 2000, Choleris et al., 2003, Takayanagi et al., 2005). Furthermore, chronic central OT administration increases social interactions between male and female rats (Witt and Insel, 1991). Together, these results support the hypothesis that stimulation of the brain oxytocinergic system by social cues reinforces appropriate social interactions.

Central application of an OTR antagonist was able to attenuate social preference between male rats, and dose-dependently caused social avoidance between male mice (Lukas et al., 2011). These results provide direct evidence that OT can have a pro-social
effect on male-male interactions and not only on reproduction-related behaviours such as pair-bonding, maternal or sexual behaviours. Consequently, basal and/or social stimulus-induced release of OT within the brain and subsequent OTR interactions within discrete brain regions may underlie the motivation for social approach.

Human studies confirmed the pro-social effects of OT after intranasal administration in healthy subjects, as well as in patients with emotional or social dysfunctions (Meyer-Lindenberg et al., 2011, Campbell, 2010, Dai et al., 2012). Intranasal OT administration has been shown to affect many aspects of human sociability ranging from social perception, increased gazing toward the eye region (Gamer et al., 2010, Guastella et al., 2008), and improved recognition of emotional facial expressions (Domes et al., 2007b, Savaskan et al., 2008), to complex social behaviours like trust, social-risk taking, and empathy (Baumgartner et al., 2008, Hurlemann et al., 2010). Finally, OT has been shown to have a key role in the regulation of autism spectrum disorders, which are characterised by abnormalities in social behaviour and social cognition (see Stavropoulos and Carver, 2013). Specifically, children with autism showed to have decreased plasma OT levels (Modahl et al., 1998). Moreover, single nucleotide polymorphisms in the OTR gene (rs53576G) have been positively correlated with autism symptoms (see Brune, 2012). In addition, intravenous OT enhanced the performance of individuals with autism spectrum disorders on several social tasks such as recognition of affective speech (Hollander et al., 2007) and social cooperation in an online computer game (Andari et al., 2010).

Taken together, these findings support the general hypothesis for OT to be the “social” molecule as it enhances several social behaviours in humans and in animals. However, a
minority of human studies showed that OT actually produces anti-social effects under particular conditions (Bartz et al., 2011).
1.8 Oxytocin and drug addiction

Early evidence suggests that OT might play a role in drug addiction processes (Kovács, 1986, Kovacs and Telegdy, 1987). A growing body of evidence has shown that OT is implicated in addiction-related behaviours including reinforcement, reward, tolerance, withdrawal and reinstatement (see Tables 4-5). In support of this, recent findings showed that the pro-social properties of some drugs of abuse induce stimulation of the oxytocinergic system (Dumont et al., 2009), and there might be an overlap between the neural circuits underlying drug reward and social bonding (Liu et al., 2011, Young et al., 2011). The pro-social, anxiolytic and antidepressant effects of OT described in Section 1.7, along with the possible interactions between the oxytocinergic and DAergic systems in the brain (see Section 1.8.1), have led to the hypothesis that the oxytocinergic system might be a novel target for the treatment of addiction. As the focus of this thesis involves cocaine, opioid and MAP addiction, the role of OT in ethanol and nicotine addictions will not be discussed.

1.8.1 Oxytocin - Dopamine interactions

Classically, most research in the drug addiction field has focussed on DAergic systems because both natural rewards, and the ‘reward’ obtained by taking drugs of abuse, acutely increases DA release in the Acb (Hernandez and Hoebel, 1988, Carboni et al., 1989, Mifsud et al., 1989, Rada et al., 1991). The interest for the involvement of the oxytocinergic system in drug addiction came from the initial findings that the brain systems involved in drug reward interact with systems involved in natural rewards such as sexual behaviour and social bonding. Emerging evidence implicates both the oxytocinergic and the DAergic systems in the regulation of certain social and affiliative behaviours, including pair bonding and sexual behaviour. It has been recently demonstrated that common DA- and OT- related brain areas are involved in pair
bonding and sexual behaviours including the Acb, VTA, SON, PVN, Amy and MPOA, regions also known to modulate several reward and addiction processes (Skuse and Gallagher, 2009). Early findings from Carmichael et al., (1987) outlined the ability of sexual self-stimulation to increase plasma OT levels in male and female humans. In rats, the DA agonist apomorphine has been shown to induce penile erection, which was reversed by the administration of an OTR antagonist (Argiolas et al., 1987), and OT-induced penile erection was inhibited by a DA receptor antagonist (Martino et al., 2005). Therefore, there is a possibility of a synergistic activation of both oxytocinergic and DAergic systems in the mediation of penile erection. In contrast, a recent study showed that OT microinjection within the subthalamic nucleus (STh) was able to prevent intra-STh DA-induced conditioned-place preference, an effect that was prevented by the co-administration of an OTR antagonist in this region (Baracz and Cornish, 2013). Taken together, these data suggest that there is a region-specific regulation of natural rewards (i.e. sexual behaviour) by the DA and oxytocinergic systems in the brain.

Sexual intercourse in prairie voles, a monogamous species, induces lifelong pair bonds with evidence for a facilitatory role of OT acting within the DAergic circuitry of the Acb (Lim and Young, 2006). The enduring partner preference that is formed by sexual intercourse in this species is abolished by both DA antagonists and OT antagonists injected into the Acb (Aragona et al., 2006, Lim and Young, 2006). These findings might suggest that social attachment and sexual intercourse is a different type of an addictive disorder, a hypothesis supported by Insel (2003), and that the same OT circuitry that is important in the regulation of “addiction to love” in prairie voles, may also regulate some drug addiction processes (Edwards and Self, 2006).
There is compelling evidence for the existence of direct OT-DA interactions within the brain. Early studies showed that intraventricular microinjections of OT into the brain were able to increase DA content in the mesencephalon of rats (Schwarzberg et al., 1981). In addition, Pfister and Muir, (1989) showed significantly increased synaptic DA levels in the Hyp following intravenous administration of OT in female rats. These findings were extended by Kovacs et al., (1990), who showed that both central and peripheral administration of OT acutely increased DA utilisation (i.e. decreased intracellular DA levels) within the Acb in mice. However, chronic subcutaneous administration of OT (0.2 mg/kg for 8 days) decreased DA utilisation (i.e. increased intracellular DA levels) within the basal forebrain (nucleus olfactorius posterior, Acb, septum) of mice (Kovacs et al., 1986). These discrepancies may indicate a neuroadaptive mechanism within the striatum, which could extend to the receptor level. In support of this, blockade of OTRs in the Acb prevented partner preferences induced by D2 receptor agonists in prairie voles (Liu and Wang, 2003). Moreover, OTRs undergo rapid (5-6 min in humans) desensitisation, clathrin-dependent internalisation and subsequently down-regulation following persistent agonist stimulation (e.g. chronic administration of OT) (Evans et al., 1997). Therefore, chronic administration of OT may have induced a desensitisation of OTRs in the Acb and subsequently caused a compensatory increase in the DA release.

In parallel, i.c.v. or peripheral administration of DA or the non-selective agonist apomorphine were found to increase hypothalamic and neurohypophysial OT release (Melis et al., 1989, Galfi et al., 2001). These data indicate a direct stimulatory effect of the DAergic system on the oxytocinergic system at both the hypothalamic and posterior pituitary level. Additionally, intra-VTA microinjection of OT induced increases in the
extracellular DA levels within the Acb and the PVN as measured by microdialysis in male rats (Melis et al., 2007)

In support of this, DA D₂ receptors are expressed in hypothalamic SON and PVN OT neurons (Baskerville et al., 2009), which are innervated by DAergic fibers originating from the zona incerta (Buijs et al., 1984, Decavel et al., 1987), suggesting a possible regulation of OT by the DAergic system. Interestingly, it has been recently shown that OTR are co-localised and functionally interact with DA D₂ in the Acb (Romero-Femandez et al., 2012) further supporting a bi-directional regulation between the two systems. DA D₂ receptors have been found to be expressed in cell bodies and dendrites of OT neurons within the MPOA, SON and PVN of male rats (Baskerville et al., 2009). This finding suggests that DA may bind to and activate OT cells via a D₂ receptor-mediated mechanism. Moreover, administration of the D₂/D₃ agonist quinpirone, but not a D₁ agonist, increased the exitability of OT neuronal populations within the hypothalamic SON, which was antagonised by a D₂ receptor antagonist (Yang et al., 1991). These common DAergic and oxytocinergic pathways point toward a possible beneficial role of the oxytocinergic system in the treatment of several DA-associated disorders including sexual dysfunction, autism, and addiction (see Baskerville and Douglas, 2010).

1.8.2 Opioid addiction

Early studies reported a role of the oxytocinergic system in the acute reinforcing effects of the opioid, morphine. Acute morphine administration decreased hypothalamic OT release in animals (Clarke et al., 1979) and also decreased OT release during suckling (Haldar and Sawyer, 1978). However, Kovacs et al. 1987 observed increased OT immunoreactivity in extra-hypothalamic regions including Hip, Amy and basal forebrain
suggesting differential effects of acute opioid administration in different areas of the brain.

Chronic morphine administration significantly decreased OT immunoreactivity within the Hip, and OT mRNA levels within the SON, ME and arcuate nucleus of the Hyp (ARC) (Kovacs et al., 1987a, Laorden et al., 1998). This down-regulation of the oxytocinergic system following chronic opioid administration, in comparison with the acute stimulatory effects of opioid administration in different brain regions, may be a result of several neuroadaptive changes in the oxytocinergic system caused by chronic exposure to opioids.

OT has been also shown to play a role during opioid tolerance. In accordance, both peripheral and central OT administration dose-dependently attenuated the development of rapid and chronic analgesic morphine tolerance in rodents (Kovacs and Telegdy, 1987, Kovacs et al. 1985; 1998). In support of this, tolerance to the antinociceptive effect of β-endorphin was attenuated by OT in a dose-dependent manner (Kovacs and Telegdy, 1987). These results might indicate the existence of an interaction between OT and endogenous opioid neuronal systems in the development of opioid tolerance. It is remarkable that the effects of OT in opioid tolerance could also be observed with non-analgesic effects of morphine. Accordingly, OT treatment inhibited the development of tolerance to locomotor hyperactivity following the administration of high doses of morphine in mice (Kovacs and Telegdy, 1987). Moreover, OT administration reduced heroin self-administration in heroin-tolerant rats, while it did not exert any effect in non-tolerant rats (Kovacs et al., 1998). The inhibitory effects of OT on the development of rapid-morphine tolerance (Sarnyai et al., 1988), or heroin self-administration (Ibragimov et al., 1987) were effectively antagonised by local microinjections of an OTR analogue, N-α-acetyl-[2-O-methyltyrosine]-OT into the limbic brain areas. It is therefore likely
that endogenous OT, via acting on OTRs in limbic areas of the brain, is able to modulate several adaptive mechanisms underlying opioid tolerance.

OT has also been found to play a role during opioid withdrawal. Peripheral OT administration decreased naloxone-precipitated morphine withdrawal symptoms: it decreased hypothermia and loss in body weight and increased the latency of the onset of stereotyped jumping (Kovacs et al., 1985). In parallel, several studies investigated the effects of opioid withdrawal on the endogenous oxytocinergic system. Acute precipitated morphine withdrawal induced by naloxone has been shown to significantly increase plasma OT levels and the firing rate of SON OT neurons in chronically morphine-treated lactating rats (Bicknell et al., 1988). Additionally, naloxone has also produced a large increase in OT levels within the CSF of opioid dependent rats (Coombes et al., 1991). Finally, naloxone-precipitated morphine withdrawal increased Fos protein expression within the SON (Murphy et al., 1997, Johnstone et al., 2000) and OT mRNA levels within the ME and PVN (Laorden et al., 1998), which may illustrate an increase of the biosynthesis of OT within the PVN that travels along the ME to be released into the plasma from the posterior pituitary gland.

Several mechanisms have been proposed to explain the hyper-activation of the oxytocinergic system following precipitated withdrawal from opioid administration. Briefly, the increase in plasma OT levels has been thought to be due to a physiological endogenous opioid peptide inhibition of the OT neurons. It is possible that naloxone, by blocking opioid receptors (e.g. Oishi et al., 1983, Kitano and Takemori, 1979), was able to disinhibit OT neuronal activity. In support of this, opioid peptide neuronal fibers and terminals are present in close proximity to OT neurons within the Hyp (Bicknell et al., 1988). Moreover, MOPr are present in the Hyp, and particularly within the SON and
PVN nuclei (Atweh and Kuhar, 1983). These findings indicate possible interactions between the opioid and oxytocinergic systems. However, there are no studies on co-localisation of opioid receptors with OTRs, thus further studies are needed to demonstrate if there is a functional interaction between these two systems.

1.8.3 Psychostimulant addiction

1.8.3.1 Cocaine addiction

The effects of OT on cocaine-induced behavioural and neurochemical alterations have been extensively studied in rodent models. Kovacs et al. (1990) showed that systemic administration of OT was able to block cocaine-induced DA utilisation in the Acb but not the CPu and to decrease cocaine-induced hyperactivity in mice (Kovacs et al., 1990). In addition, Sarnyai et al. (1993) demonstrated that systemic administration, i.c.v. or microinjections of synthetic OT into the Acb and Tu, but not the CPu or the CeA were able to abolish cocaine-induced sniffing. It has been suggested that OT is able to block these cocaine-induced behavioural effects due to its inhibitory effects on DA utilisation and on postsynaptic DA receptors across a network of limbic and basal forebrain areas of the brain (Kovacs et al., 1998). It is also possible that these effects of OT are due to the co-localisation and functional interaction of OTRs with DA D2 receptors in the Acb (Romero-Fernandez et al., 2012). It is important to mention that the inhibition of cocaine-induced sniffing behaviour by peripherally administered OT was reversed by the i.c.v. administration of an OTR antagonist (Kovacs et al., 1998). This finding suggests that at least a small amount of peripherally injected OT is able to pass the BBB and exert its effects by binding to the wide network of OTRs in the brain.

A large amount of evidence has also indicated profound effects of cocaine on the endogenous oxytocinergic system. In fact, OT immunoreactivity levels were decreased
within the basal forebrain following acute administration of cocaine whilst OT peptide levels were increased within the Hyp and Hip (Sarnyai et al., 1992c). In contrast, plasma OT levels as well as OT immunoreactivity levels within the Amy remained unaltered, suggesting region-specific effects of cocaine on the oxytocinergic system.

Chronic cocaine administration can lead to either tolerance or sensitisation, depending on the dose and route of administration, treatment schedule or environmental effects (Hammer et al., 1997, Johanson and Fischman, 1989). The effect of OT on the development of both tolerance and sensitisation to cocaine has also been studied in rodents. Repeated administration of cocaine produced a behavioural tolerance to the sniffing-inducing effects of cocaine (Sarnyai et al., 1992a). The development of tolerance was inhibited by the repeated peripheral daily administration of OT prior to cocaine injections. However, sub-chronic administration of cocaine-induced behavioural locomotor sensitisation (Sarnyai et al., 1992b), which was facilitated by the peripheral daily injections of OT prior to cocaine administration, suggesting that OT has also a role in the modulation of the development of tolerance or sensitisation to cocaine.

Pre-clinical evidence shows that chronic administration of cocaine induces a hypo-activity of the oxytocinergic system. For example, Sarnyai et al., (1992a) reported decreased plasma OT levels and decreased OT immunoreactivity within the Hyp and Hip following prolonged administration of cocaine in rats. Accordingly, chronic gestational and post-gestational cocaine treatment in rat dams decreased OT levels in the MPOA, VTA, and Hip, and OTR binding in the VMH and BNST (Johns et al., 1997, Johns et al., 1998, Johns et al., 2004, Jarrett et al., 2006). The detrimental effects of gestational cocaine treatment appeared to be long-lasting since young adult female offspring of cocaine-treated dams exhibited higher levels of aggression associated with lower levels
of OT in the Amy (McMurray et al., 2008). It is highly likely that these effects can be attributed to both gestational cocaine exposure *in utero* and maladaptive rearing conditions.

These pre-clinical findings have also been extended to humans. Light et al., (2004) provided evidence that mothers who used cocaine during pregnancy showed lower plasma OT levels, greater hostility and depressed mood, and less social support and adaptive coping strategies for stressful life events compared to cocaine-naïve mothers. Moreover, cocaine exposed mothers maintained higher blood pressure and urinary NA levels, while urinary cortisol and adrenaline levels were blunted, suggesting a dysregulation of the HPA axis stress system. Together these findings suggest profound alterations in the oxytocinergic system following acute and chronic cocaine use which is likely to affect behaviour.

**1.8.3.2 Amphetamine addiction**

Early research into the role of the adrenergic system in regulating neurohypophysial hormones showed that acute administration of amphetamine to rats significantly decreased hypothalamic and neurohypophysial OT levels (Guzek et al., 1978). Given that this study did not concentrate on the effects of amphetamine on OT, it is possible that other neurotransmitters were also involved in the observed down-regulation of the oxytocinergic system after amphetamine administration. Consequently, this oxytocinergic hypo-activity following acute amphetamine administration needs further investigation.

The effects of oxytocin on the behavioural effects of amphetamine were also examined. Systemic OT administration prior to amphetamine failed to alter the amphetamine-induced hyperlocomotion in mice (Kovacs et al., 1985) and to inhibit amphetamine-
induced stereotyped behaviour in rats (Samyai, 1993). In contrast, similar doses of OT showed inhibitory actions on the behavioural effects of cocaine (see Section 1.8.3.1). Differences in the pharmacological action of amphetamine and cocaine might partly explain the discrepancies between OT’s effect on the behavioural effects of these drugs. Cocaine, having a relatively short half-life, acts almost selectively to increase synaptic DA levels by blocking the action of DA transporters (DAT) for DA reuptake from the synaptic cleft (Thomsen et al., 2009). However, amphetamine reverses the DAT pump, markedly increasing DA release from the presynaptic terminals (Pifl et al., 1995). Furthermore, amphetamine inhibits the metabolism of DA by blocking the action of the enzyme monoamine oxidase (see Fowler and Benedetti, 1983). As a result, amphetamine has a relatively long half-life, depending on dose (see Cruickshank and Dyer, 2009). Therefore, given the prolonged action of amphetamine, it is likely that much higher doses of OT and a more proximal time-course of administration might be required before an effect can be observed.

The extended action of amphetamine on neuronal DA activity has been linked to a wide range of psychotic symptoms associated with schizophrenia in both animal and human models (see Hermens et al., 2009). Accumulating evidence suggests that dysregulation of the OTergic system may also be associated with the development of symptoms of schizophrenia (see MacDonald and Feifel, 2012). In order to investigate a possible role for OT as an antipsychotic agent, OT was systemically administered prior to amphetamine injection in rats that were tested for deficits in sensorimotor gating by using the prepulse inhibition (PPI) task. Briefly, PPI refers to the decrease in the magnitude of the startle reflex occurring when a weak sensory stimulus (the prepulse) is presented immediately before a startling stimulus (for an extended review on PPI see Larrauri and Schmajuk, 2006). Typically, patients with schizophrenia show deficits in
PPI and this can be modeled in laboratory animals by administration of psychostimulants, such as amphetamine (Mansbach et al., 1988). OT has been shown to exert beneficial effects on schizophrenic symptoms with high doses of systemically administered OT (1 mg/kg) being able to reverse the amphetamine-induced impaired PPI in rats (Feifel and Reza, 1999). These results suggest that OT is capable of reversing some of the “psychotomimetic” effects of amphetamine, pointing towards its use as an antipsychotic agent. Further investigation may determine how OT achieves this effect as PPI is considered to rely on a number of neuronal processes including DA and glutamate neurotransmission (see Braff, 2010). However, it should be noted that large doses of OT (i.e. 1 mg/kg) delivered systemically have been shown to cause sedation and thus attention should be taken in the elucidation of findings such as these (Uvnas-Moberg et al., 1994). Moreover, OT has also been found to facilitate the effects of sedating anxiolytics such as diazepam (Viviani et al., 2010). Therefore, it is possible that systemically administered OT may result in non-specific sedative effects as opposed to specifically blocking stimulant-induced behaviours.

Central administration of the OT agonist WAY-267464, has recently been shown to reverse the disruption of PPI in the acoustic startle reflex produced by amphetamines (Ring et al., 2010). However, as also mentioned in Section 1.7.2, the selectivity of this drug for OTRs remains unclear (Hicks et al., 2012).

1.8.3.3 Methamphetamine addiction

Several studies have examined the effects of OT in specific brain regions and investigated how OT affects several neurotransmitter systems involved in the formation of MAP-induced locomotor hyperactivity in mice. In a recent study, central OT administration dose-dependently reduced MAP-induced hyperlocomotion (Qi et al.,
This effect was accompanied by reduced ratios of the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) to DA in the striatum, indicating decreased DA metabolism. Generally, decreased extracellular levels of DA are associated with decreased motor activity in rodents (Alexander and Crutcher, 1990). Therefore, OT, possibly via causing a hypo-DAergic state in the brain, was able to reduce MAP-induced hyperlocomotion. Interestingly, central administration of the OTR antagonist, atosiban, attenuated these effects, suggesting that OT exerted its inhibitory effects on MAP-related behaviours via interaction with OTRs in the brain.

Following the demonstration of the effects of OT on the acute locomotor responses of MAP, Qi et al., (2009) examined the effects of OT administration on MAP-induced conditioned place preference (CPP). It was shown that centrally administered OT significantly inhibited the acquisition of a place preference for MAP, but had no effect on the expression of an already acquired CPP for MAP. Moreover, OT was able to facilitate the extinction of MAP-induced CPP and to abolish stress-induced but not MAP-primed reinstatement of CPP (Qi et al., 2009). The effect of OT in blocking stress-induced reinstatement to MAP-seeking has been attributed to the ability of OT to prevent increased glutamate levels in the medial prefrontal cortex (mPFC) following restraint stress- but not MAP-induced reinstatement of CPP. Evidence suggests that the ability of psychostimulants to trigger drug-induced reinstatement following abstinence involves their direct ability to activate the mesolimbic DA system, which in turn enhances glutamate release in the PFC. In contrast, stress can activate VTA DAergic neurons via the activation of an excitatory glutamatergic projection from the PFC innervating the VTA (Moghaddam, 1993). The stress-induced increase in glutamate levels is thought to be a mechanism underlying reinstatement following exposure to stress in rodents. Therefore, OT might differentially regulate glutamate release in the
mPFC, thus having a prominent role in stress-induced, but not MAP-primed, induced reinstatement.

Although CPP is considered to be a robust model of drug reward-related behaviour, it is widely accepted that drug self-administration is a more ethologically valid animal model of addiction. Systemic OT administration dose-dependently reduced MAP self-administration, MAP-induced hyperlocomotion and reinstatement of extinguished MAP-seeking behaviour caused by MAP challenge (Carson et al., 2010a). Taken together, these findings provide the first evidence that systemically applied OT could significantly inhibit MAP-induced addictive-like behaviours. The use of a peripheral route of OT administration is an important aspect as it is much more clinically relevant than central administration, a delivery that is highly unlikely to find a place in clinical practice.

The discrepancies between the findings of the studies carried out by Qi et al. (2009) and Carson et al. (2010a) on the blockade of reinstatement of MAP-seeking following a MAP challenge (priming-induced reinstatement) may be due to the different protocol of drug administration (i.e. contingent vs non-contingent). In support of this, it has been shown that contingent and non-contingent drug administration, including that of nicotine (Metaxas et al., 2010) and cocaine (Markou et al., 1999), exerted differential neurochemical as well as behavioural responses.

Moreover, peripherally administered OT significantly reduced MAP-induced c-Fos expression in the STh and Acb core, two regions highly implicated in impulsivity and drug addiction (Eagle and Baunez, 2010). Additionally, peripheral administration or microinjections of OT into the STh and Acb core attenuated the formation of MAP-induced CPP (Baracz et al., 2012). These findings provide further support for a key role
of the STh and Acb core in the inhibitory effects of OT on the rewarding properties of MAP. Indeed, a single-dose of DA injected directly in the STh of rats resulted in a significant acquisition of CPP, while the DA receptor antagonist, fluphenazine, or OT administered in to the STh blocked this effect (Baracz and Cornish, 2013). These data support the existence of local OTRs in the STh that exert an inhibitory action on DAergic reward processes associated with the effects of MAP.

1.8.3.4 MDMA (3,4-methylenedioxy-N-methylamphetamine) addiction

MDMA has repeatedly been shown to induce positive feelings and pro-social effects in individuals (Vollenweider et al., 2002), which might be a key factor contributing to the escalation of its use. Since OT has been shown to exert similar pro-social effects in humans (Lee et al., 2009), it has been postulated that the central oxytocinergic system might mediate several MDMA-related effects. A role for OT in MDMA addictive processes is further supported by the inhibitory effect of the OTR antagonist atosiban on the discrimination between MDMA and placebo as examined by the "drug discrimination paradigm" in rats (Broadbear et al., 2011), suggesting that the OTR system has a prominent role in MDMA's effects. In addition, acute administration of MDMA significantly increases plasma OT levels in humans which are positively correlated with OT levels rather than MDMA concentration in the blood (Dumont et al., 2009). These findings have been replicated in MDMA-positive clubbers who showed elevated plasma OT levels, a finding that was associated with changes in plasma osmolality and plasma sodium levels (Wolff et al., 2006).

Initial in vitro work by Forsling et al., (2002) showed that MDMA, when applied to isolated rat Hyp, resulted in substantial increases in OT release. Similarly, in an attempt to uncover the action of MDMA on the brain and behaviour at the neuropeptide level, Thompson et al., (2007) showed that acute administration of MDMA in rats induced a
substantial increase of *c-Fos* expression in OT-containing neurons in the Hyp as well as an increase in plasma OT levels. These results reflect the effects of MDMA in increasing social interaction via an oxytocinergic pathway (Clemens et al., 2004). The effects of MDMA on the endogenous oxytocinergic system were abolished by pre-treatment with the 5-HT₁a receptor agonist, WAY 100,635, and the OTR antagonist, tocinoic acid (Thompson et al., 2007). These results suggest that MDMA might exert its pro-social effects via an oxytocinergic mechanism involving possible interactions with the 5-HT system. In support of this, it has been recently shown that WAY 100,635 was able to inhibit MDMA stimulated *c-Fos* expression in the Hyp but not the striatum, Th or CeA (Hunt et al., 2011), further suggesting that MDMA exerts its acute effects on the oxytocinergic system via interactions with the serotonergic system.

In order to assess the potential long-term effects of MDMA in rats, van Nieuwenhuijzen et al., (2010) showed that a 4-week extinction period following 10 days of chronic MDMA administration increased OT mRNA levels in the Hyp, which might act as a compensatory mechanism to counteract the negative emotional effects associated with prolonged MDMA withdrawal. In humans, long-term abstinence from MDMA administration induces anxiety and depression (see Morgan, 2000). However, during protracted withdrawal, OTR mRNA levels remained unaltered in the Hyp, suggesting that the effects of prolonged withdrawal from MDMA might not be at the receptor level. Further research is essential to determine whether the effects of MDMA on the endogenous oxytocinergic system during withdrawal reflect counteractive mechanisms of the brain to reduce negative emotional withdrawal symptoms.
Table 1.6: Effects of oxytocin on opioid- and psychostimulant-induced behaviours

<table>
<thead>
<tr>
<th>Addictive substance</th>
<th>Administration paradigm</th>
<th>Animal model</th>
<th>Oxytocin administration paradigm</th>
<th>Effect of OT</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>Morphine tolerance: 37.5 mg/kg morphine.HCl pellet, s.c./48 hours</td>
<td>Male CFLP mice (25±5g) housed in groups</td>
<td>50 µg and 100 µg/animal, s.c. (2 hours prior to morphine pellet implantation)</td>
<td>↓ development of tolerance</td>
<td>Kovacs et al., 1985</td>
</tr>
<tr>
<td>Morphine</td>
<td>Morphine tolerance: 30 mg/kg morphine.HCl, s.c. and 5 hours later 5 mg/kg morphine.HCl, s.c.</td>
<td>Male albino inbred mice (25±5g) housed in groups</td>
<td>1 µg i.c.v. or intra-CPu (1 hr prior the tolerance-inducing dose of morphine)</td>
<td>↔ development of tolerance</td>
<td>Sarnyai et al., 1988</td>
</tr>
<tr>
<td>Morphine</td>
<td>Morphine tolerance: 30 mg/kg morphine.HCl, s.c. and 5 hours later 5 mg/kg morphine.HCl, s.c.</td>
<td>Male albino inbred mice (25±5g) housed in groups</td>
<td>1 µg microinjection into: posterior olfactory nucleus, CeA, ventral hippocampus (1 hr prior the tolerance-inducing dose of morphine)</td>
<td>↓ development of tolerance</td>
<td>Sarnyai et al., 1988</td>
</tr>
<tr>
<td>Morphine</td>
<td>Morphine tolerance: 60 mg/kg morphine.HCl, s.c. and 5 hours later 1 mg/kg morphine.HCl, s.c.</td>
<td>Male CFLP mice (25±5g) housed in groups</td>
<td>0.002 mg/kg, s.c. (1 hr prior the tolerance-inducing dose of morphine)</td>
<td>↓ development of tolerance</td>
<td>Kovacs et al., 1987c</td>
</tr>
</tbody>
</table>
| **Morphine tolerance:**  
| 100 mg/kg morphine.HCl, s.c. and 5 hours later 1 mg/kg morphine. HCl, s.c. | Male CFLP mice (25±5 g) housed in groups | 0.002 mg/kg, s.c. (1 hr prior the tolerance-inducing dose of morphine) | ↓ development of tolerance | Kovacs et al., 1987c |
| **Morphine tolerance:**  
| 37.5 mg/kg morphine.HCl pellet, s.c./48 hours then, morphine.HCl (5 mg/kg, s.c.) | Male CFLP mice, housed in groups | 50 µg, s.c. | ↓ development of tolerance | Kovacs et al., 1984b |
| **Morphine tolerance:**  
| 37.5 mg/kg morphine.HCl pellet, s.c./48 hours then, morphine.HCl (5 mg/kg, s.c.) | Male CFLP mice, housed in groups | 0.005 or 0.5 µg/1µl, i.c.v. | ↓ development of tolerance | Kovacs et al., 1984b |
| **Morphine tolerance:**  
| 37.5 mg/kg morphine.HCl pellet, s.c./48 hours then, morphine.HCl (5 mg/kg, s.c.) | Male CFLP mice, housed in groups | 0.5 ng/1µl, into the dorsal hippocampus or the Acb | ↓ development of tolerance | Kovacs et al., 1984b |
| **Morphine tolerance:**  
| 37.5 mg/kg morphine.HCl pellet, s.c./48 hours then, morphine.HCl (5 mg/kg, s.c.) | Male CFLP mice, housed in groups | 0.5 ng/1µl, into the CPu, VTA or external cortical surface | ↔ development of tolerance | Kovacs et al., 1984b |
| **Naloxone-precipitated withdrawal:**  
<p>| 37.5 mg/kg morphine.HCl pellet, s.c./48 hours Naloxone: 1 mg/kg, i.p. | Male CFLP mice, housed in groups | 50 µg, s.c. | ↑ latency of naloxone-precipitated withdrawal | Kovacs et al., 1984b |</p>
<table>
<thead>
<tr>
<th><strong>Naloxone-precipitated withdrawal:</strong> 37.5 mg/kg morphine.HCl pellet, s.c./48 hours</th>
<th>Male CFLP mice, housed in groups</th>
<th>0.005 or 0.5 µg/1µl, i.c.v.</th>
<th>↑ latency of naloxone-precipitated withdrawal</th>
<th>Kovacs et al., 1984b</th>
</tr>
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<tbody>
<tr>
<td><strong>Naloxone-precipitated withdrawal:</strong> 37.5 mg/kg morphine.HCl pellet, s.c./48 hours</td>
<td>Male CFLP mice, housed in groups</td>
<td>0.5 ng/1µl, into the dorsal Hip or the mesolimbic Acb</td>
<td>↑ latency of naloxone-precipitated withdrawal</td>
<td>Kovacs et al., 1984b</td>
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<tr>
<td><strong>Naloxone-precipitated withdrawal:</strong> 37.5 mg/kg morphine.HCl pellet, s.c./48 hours</td>
<td>Male CFLP mice, housed in groups</td>
<td>0.5 ng/1µl, into the CPu, VTA or external cortical surface</td>
<td>↔ latency of naloxone-precipitated withdrawal</td>
<td>Kovacs et al., 1984b</td>
</tr>
<tr>
<td><strong>Naloxone-precipitated withdrawal:</strong> 37.5 mg/kg morphine.HCl pellet, s.c./72 hours</td>
<td>Male CFLP mice (25±5 g) housed in groups</td>
<td>50 µg and 100 µg/animal, s.c. (2 hours prior to morphine pellet implantation)</td>
<td>↓ withdrawal symptoms</td>
<td>Kovacs et al., 1985</td>
</tr>
<tr>
<td><strong>Heroin</strong></td>
<td>Male Wistar rats (200-220 g) housed individually</td>
<td>1.0 µg/animal, s.c. (1 hour prior to self-administration session on the day 7)</td>
<td>↓ self-administration</td>
<td>Kovacs and Van Ree, 1985b</td>
</tr>
<tr>
<td>Cocaine</td>
<td><strong>Acute Locomotion:</strong> 30 mg/kg, s.c.</td>
<td>Male CFLP mice (25-30 g) housed in groups</td>
<td>1.0 and 5.0 µg/animal, s.c. (30 min prior to cocaine)</td>
<td>↓ locomotor hyperactivity</td>
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<tr>
<td><strong>Acute stereotypic sniffing:</strong> 15 mg/kg, s.c.</td>
<td>Male Wistar rats (150-180 g) housed in groups</td>
<td>0.5 and 5.0 µg/animal, s.c. (60 min prior to cocaine)</td>
<td>↓ sniffing behaviour</td>
<td>Sarnyai et al. 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 and 50 ng/animal, i.c.v. (60 min prior to cocaine)</td>
<td>↓ sniffing behaviour</td>
<td>Sarnyai et al. 1991</td>
</tr>
<tr>
<td></td>
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<td>100 pg/animal; microinjection into the Acb and Tu</td>
<td>↓ sniffing behaviour</td>
<td>Sarnyai et al. 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 pg/animal; microinjection into the CeA, CPu</td>
<td>↔ sniffing behaviour</td>
<td>Sarnyai et al. 1991</td>
</tr>
<tr>
<td><strong>Tolerance:</strong> 7.5 mg/kg, s.c., 2/day/4 days</td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td>0.05 µg/animal, s.c. (4 days, 60 min prior to cocaine)</td>
<td>↓ development of tolerance</td>
<td>Sarnyai et al., 1992a</td>
</tr>
<tr>
<td></td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td>100 pg/animal, microinjection into the Hip</td>
<td>↓ development of tolerance ↔ sniffing behaviour</td>
<td>Sarnyai et al., 1992a</td>
</tr>
<tr>
<td><strong>Sensitisation:</strong> 7.5 mg/kg, s.c., 2/day/5 days</td>
<td>Male CFLP mice (25-30 g) housed in groups</td>
<td>0.5 µg/animal, s.c. (5 days, 60 min prior to cocaine)</td>
<td>↑ development of behavioural sensitisation</td>
<td>Sarnyai et al., 1992b</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Acute Locomotion: 1 mg/kg, s.c.</td>
<td>Male CFLP mice (25±5 g) housed in groups</td>
<td>Male Wistar rats (150-180 g) housed in groups</td>
<td>0.5, 5.0 and 50.0 μg/animal, s.c. (60 min prior to amphetamine) → locomotor hyperactivity</td>
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<tr>
<td>PPI: 2 mg/kg, s.c.</td>
<td>Male Sprague-Dawley rats (225-250 g) housed in groups</td>
<td>1 mg/kg, s.c.; (immediately prior to amphetamine)</td>
<td>Reversed amphetamine-induced impaired PPI</td>
<td>Feifel and Reza, 1999</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>Acute locomotion: 2 mg/kg, i.p.</td>
<td>Male Swiss mice (22-24 g housed in groups)</td>
<td>0.1, 0.5 and 2.5 μg/animal, i.c.v., (30 min prior to MAP) → dose-dependently ↓ locomotor hyperactivity</td>
<td>Qi et al., 2008a</td>
</tr>
<tr>
<td>CPP: 2 mg/kg, i.p.</td>
<td>Male Swiss mice (28-32 g) housed in groups</td>
<td>2.5 μg/animal, i.c.v., (30 min prior to MAP) ↓acquisition of CPP ↔ expression of CPP</td>
<td>↓extinction of CPP ↓restrain stress-induced but not priming (MAP 1mg/kg, i.p.)-induced reinstatement</td>
<td>Qi et al., 2009</td>
</tr>
<tr>
<td><strong>Self-administration:</strong>&lt;br&gt;7 days fixed ratio 1 schedule (0.1 mg/kg/infusion, i.v.), then 4 days progressive ratio schedule Challenge (1 mg/kg, i.p.) following 10 days extinction</td>
<td>Male Sprague-Dawley rats (307-420 g) housed in groups</td>
<td>Escalating doses (0.001 – 1 mg/kg, i.p.); 30 min prior to daily self-administration sessions</td>
<td>↓ self-administration sessions ↓ reinstatement of MAP- seeking</td>
<td>Carson et al., 2010a</td>
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<tr>
<td><strong>Acute locomotion:</strong>&lt;br&gt;1 mg/kg, i.p.</td>
<td>Male Sprague-Dawley rats (307-420 g) housed in groups</td>
<td>0.3 and 1 mg/kg, i.p.; 5 min prior to MAP</td>
<td>↓ locomotor hyperactivity</td>
<td>Carson et al., 2010a</td>
</tr>
<tr>
<td><strong>CPP:</strong>&lt;br&gt;1 mg/kg, i.p. (3 sessions)</td>
<td>Male Sprague-Dawley rats (200-250 g) housed in groups</td>
<td>0.6 mg.kg, i.p.; 10 min prior to MAP</td>
<td>↓ CPP</td>
<td>Baracz et al., 2012</td>
</tr>
<tr>
<td><strong>CPP:</strong>&lt;br&gt;1 mg/kg, i.p. (1 session)</td>
<td>Male Sprague-Dawley rats (200-250 g) housed in groups</td>
<td>Acb core bilateral microinjection: 0.6 ng/0.5 μl/animal/side STh bilateral microinjection: 0.6 ng/0.3 μl/animal/side</td>
<td>↓ CPP</td>
<td>Baracz et al., 2012</td>
</tr>
</tbody>
</table>

A detailed summary on the effects of oxytocin administration on the behavioural effects of opioid and psychostimulant administration in animals. Oxytocin has been shown to exert beneficial effects on several addiction processes including acute reinforcing effects, tolerance and negative withdrawal symptoms. ↑ increase; ↓ decrease; ↔ no effect; Abbreviations: Acb, nucleus accumbens; CeA, central amygdala; CPP, conditioned place preference; CPu, caudate-putamen; HCl, hydrogen chloride; Hip, hippocampus; s.c., subcutaneous; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.v., intravenous; MAP, methamphetamine; OT, oxytocin; STh, subthalamic nucleus; Tu, olfactory tubercle; VTA, ventral tegmental area.
Table 1.7: Effects of opioids and psychostimulants on the endogenous oxytocinergic system

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Addiction phase</th>
<th>Administration paradigm</th>
<th>Animal Model</th>
<th>Effect on Oxytocin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>Acute 4μg.</td>
<td>Lactating Wistar rats</td>
<td>↓ hypothalamic OT release</td>
<td>Clarke et al., 1979</td>
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<td></td>
<td>intraventricular</td>
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<td>10 mg/kg, s.c.</td>
<td>Swiss Webster mice between the 11th and 22nd</td>
<td>↓ OT release during suckling</td>
<td>Haldar and Sawyer, 1978a</td>
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<td></td>
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<td>day of lactation</td>
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<td></td>
<td>5 mg/kg, s.c.</td>
<td>Male CFLP mice (25±5 g) housed in groups</td>
<td>↑ OT immunoreactivity in Hip, Amy</td>
<td>Kovacs et al., 1987b</td>
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<td></td>
<td></td>
<td></td>
<td>and basal forebrain</td>
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<tr>
<td>Chronic</td>
<td>Morphine pellet</td>
<td>Male CFLP mice (25±5 g), housed in groups</td>
<td>↔ OT immunoreactivity in Amy and</td>
<td>Kovacs et al., 1987b</td>
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<tr>
<td></td>
<td>(37.5 mg</td>
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<td>basal forebrain</td>
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<td>morphine.HCl),</td>
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<td>s.c.</td>
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<td></td>
<td>Osmotic mini-</td>
<td>Male Sprague-Dawley rats (230-270 g) housed in</td>
<td>↓ OT immunoreactivity in the Hip,</td>
<td>Laorden et al., 1998, Laorden et al., 1997</td>
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<tr>
<td></td>
<td>pump (75 mg),</td>
<td>groups</td>
<td>SON, ME and ARC</td>
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<td></td>
<td>s.c., 1 on day</td>
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<td>↓ OT peptide levels in SON and ME</td>
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<td>0, 2 on day 2</td>
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<td>↔ OT peptide levels in PVN</td>
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<td>and 3 on day 4</td>
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<td>On day 8</td>
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<td>morphine.HCl</td>
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<td>(30 mg/kg, i.p.)</td>
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<td></td>
<td>Osmotic mini-</td>
<td>Lactating, primiparous Sprague-Dawley female</td>
<td>↔ plasma OT levels</td>
<td>Bicknell et al., 1988b</td>
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<tr>
<td></td>
<td>pump, s.c.,</td>
<td>rats (2-4 days post-partum)</td>
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<td>10μg/h/40hrs</td>
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<td>then 20μg/h/40hrs</td>
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<td>and then 50 μg/h/40hrs</td>
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<tr>
<td>Naloxone - Precipitated withdrawal</td>
<td>Morphine: osmotic mini-pump, s.c., 10μg/h/40hrs then 20μg/h/40hrs and then 50μg/h/40hrs Naloxone: 5 mg/kg, i.v.</td>
<td>Lactating, primiparous Sprague-Dawley female rats</td>
<td>↑ plasma OT levels; ↑ firing rate of OT neurons (SON)</td>
<td>Bicknell et al., 1988b</td>
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<td>Morphine sulphate (20-40 mg/kg) x 5 days, i.c.v (1 µl/hr) Naloxone: 5mg/kg, i.v.</td>
<td>Virgin Sprague-Dawley female rats (243-287 g) housed individually</td>
<td>↑ plasma OT levels; ↑ OT levels in CSF</td>
<td>Coombes et al., 1991a</td>
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<tr>
<td>Osmotic mini-pump (75 mg), s.c., 1 on day 0, 2 on day 2, 3 on day 4. Naloxone.HCl 1 mg/kg, s.c. (on day 7)</td>
<td>Male Sprague-Dawley rats (200-210 g) housed in groups</td>
<td>↑ OT mRNA levels in the ME and PVN</td>
<td>Laorden et al., 1998</td>
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<tr>
<td>Morphine: osmotic mini-pump, s.c., 10μg/h/40hrs, then 20μg/h/40hrs, then 50μg/h/40hrs Naloxone: 5 mg/kg, i.v.</td>
<td>Virgin Sprague-Dawley female rats (~250 g) housed individually</td>
<td>↑ plasma OT levels ↑ OT peptide levels in the mediolateral septum ↔ OT levels in the dorsal hippocampus ↔ OT levels in the nucleus of tractus solitarius</td>
<td>Russell et al., 1992</td>
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<tr>
<td>Cocaine</td>
<td>Acute</td>
<td>7.5 mg/kg, s.c.</td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td>↔ plasma OT levels ↓ OT levels in the basal forebrain ↑ OT levels in the Hip ↔ OT levels in the Hyp and Amy</td>
<td>Sarnyai et al., 1992c</td>
</tr>
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<tr>
<td></td>
<td>15 mg/kg, s.c.</td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td>↔ plasma OT levels ↓ OT levels in the basal forebrain ↑ OT levels in the Hip ↔ OT levels in the Hyp and Amy</td>
<td>Sarnyai et al., 1992c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 mg/kg, s.c.</td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td>↔ plasma OT levels ↑ OT levels in the Hyp ↔ OT levels in the Amy and Hip</td>
<td>Sarnyai et al., 1992c</td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>7.5 mg/kg, s.c., 2xdaily/4 days</td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td>↓ plasma OT levels ↔ OT levels in the basal forebrain and Amy ↓ OT levels in the Hyp and Hip</td>
<td>Sarnyai et al., 1992c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 mg/kg, s.c. 2xdaily throughout gestation (GD 1-20)</td>
<td>Gravid, nulliparous Sprague-Dawley rats (250-275 g)</td>
<td><strong>PPD1:</strong> ↓ OT levels in the MPOA ↔ OT levels in the Amy, VTA and Hip <strong>PPD2:</strong> ↓ OT levels in the VTA and Hip ↔ OT levels in the Amy and MPOA</td>
<td>Johns et al., 1997</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 mg/kg, s.c. 2xdaily throughout gestation (GD 1-20)</td>
<td>Virgin female Sprague–Dawley rats (200–250 g)</td>
<td><strong>PPD8 and PPD11:</strong> OT levels ↓ in the Amy</td>
<td>Johns et al., 1998</td>
<td></td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>Acute</td>
<td>2 mg/kg, i.p.</td>
<td>Male Sprague-Dawley rats (420-567 g) housed in groups</td>
<td>↔ firing rate of hypothalamic OT neurons</td>
<td>Carson et al., 2010b</td>
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<tr>
<td>Reinstatement</td>
<td></td>
<td>7 days fixed ratio 1 schedule (0.1 mg/kg/infusion, i.v.), then 4 days progressive ratio schedule Challenge (1 mg/kg, i.p.) following 10 days extinction</td>
<td>Male Sprague-Dawley rats (307-420 g) housed in groups</td>
<td>↔ plasma OT levels</td>
<td>Carson et al., 2010a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 mg/kg, s.c. 2xdaily throughout gestation (GD 1-20)</td>
<td>Virgin female Sprague-Dawley rats (200–250 g) housed in groups</td>
<td>PPD6: ↑ OTR binding and ↓ OTR affinity in the Amy; ↓ OTR binding and ↑ OTR affinity in the MPOA ↔ OTR binding or affinity in the Hip or VTA</td>
<td>Johns et al., 2004b</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg, s.c. 2xdaily throughout gestation (GD 1-20)</td>
<td>Virgin female Sprague–Dawley rats (200–250 g) housed in groups</td>
<td></td>
<td>↓ OTR binding in the VMH and BNST</td>
<td>Jarrett et al., 2006</td>
</tr>
<tr>
<td></td>
<td>7.5 mg/kg, s.c. 2xdaily/4 days</td>
<td>Male Wistar rats (180-220 g) housed in groups</td>
<td></td>
<td>↓ plasma OT levels ↓ OT immunoreactivity in the Hyp, Hip ↔ OT immunoreactivity in the Amy and basal forebrain</td>
<td>Sanyai et al., 1992a</td>
</tr>
<tr>
<td>Substance</td>
<td>Administration</td>
<td>Dose</td>
<td>Treatment</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
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<tr>
<td>Amphetamine</td>
<td>Acute</td>
<td>5mg/kg, i.p.</td>
<td>Male Wistar rats (320±28 g) housed in groups</td>
<td>↓ hypothalamic and neurohypophysial OT content</td>
<td>Guzek et al., 1978</td>
</tr>
<tr>
<td>MDMA</td>
<td>Acute</td>
<td>5mg/kg, i.p.</td>
<td>Male Australian Albino Wistar rats (344±10 g) housed in groups</td>
<td>↑ of activity of OT containing neurons in the Hyp (c-Fos expression) ↑ OT plasma levels</td>
<td>Thompson et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Chronic spontaneous withdrawal</td>
<td>5mg/kg, i.p. for 10 days following by four weeks extinction period</td>
<td>Male albino Wistar rats (220-300 g) housed in groups</td>
<td>↑ OT mRNA levels in the Hyp ↔ OTR mRNA levels in the Hyp</td>
<td>van Nieuwenhuijzen et al., 2010a</td>
</tr>
</tbody>
</table>

A detailed summary of the effects of opioids and psychostimulant drugs on the endogenous oxytocinergic system upon acute administration, chronic administration, as well as acute and chronic withdrawal. ↑ increase; ↓ decrease; ↔ no effect; Abbreviations: Amy, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; CSF, cerebrospinal fluid; HCl, hydrogen chloride; Hip, hippocampus; Hyp, hypothalamus; i.p., intraperitoneal; i.v., intravenous; ME, median eminence; MPOA, medial preoptic area; mRNA, messenger RNA; SON; supraoptic nucleus of the hypothalamus; OT, oxytocin; OTR, oxytocin receptors; PVN, paraventricular nucleus of the hypothalamus; s.c., subcutaneous; VMH, ventromedial hypothalamus; VTA, ventral tegmental area
1.8.4 The oxytocinergic system in drug addiction: a summary

The neurobiological mechanisms underlying comorbidity of addiction and other mental disorders remain unclear and thus this comorbidity is poorly treated. Emotional disturbances during abstinence from drugs of abuse are considered as a motivational trigger for relapse to drug-seeking; the hallmark for drug addiction treatment. Currently, there are no effective pharmacotherapies for the prevention of relapse following long-term drug cessation. Several neurotransmitter and neuromodulator systems have been previously implicated in drug addiction processes. During the last decade, the oxytocinergic system has been proposed as a potential mediator and modulator of several addiction processes (see Sanyai, 2011).

The key role of OT in drug addiction processes has become of great interest following several observations including the early evidence showing that OT was able to prevent tolerance to opioids and cocaine giving rise to the hypothesis for the involvement of OT in mesolimbic and forebrain areas. The oxytocinergic system is generally down-regulated as a result of repeated drug use and such adaptations might not only lead to tolerance development, but also to increased drug-seeking behaviour and a decreased interest in social rewards (McGregor et al., 2008). Recently, the importance of the oxytocinergic system has been extended at the level of reinstatement following MAP withdrawal. OT, having anxiolytic, antidepressant and social enhancing properties (see Section 1.7) may also be a promising candidate for the treatment of comorbidity disorders with drug addiction.

However, there are some critical characteristics of OT that might limit its clinical therapeutic prospects including its short half-life and its limited (if any) ability to cross
the BBB, pointing towards the need of using novel OTR agonists or antagonists for the treatment of drug addiction.
1.9 Hypothesis and aims

The role of the oxytocinergic system in the neurochemical and behavioural responses to drugs of abuse currently remains unclear. Drug addiction, and more specifically drug abstinence, is associated with negative emotional symptoms including social avoidance and mood disturbances that trigger relapse. A number of studies have shown that the OT peptide has antidepressant, anxiolytic and social enhancing effects, suggesting that it might also have beneficial effects in reducing impaired emotional response related to drug addiction.

The research in this thesis tests the hypothesis that the oxytocinergic system is involved in the modulation of several addiction processes and that administration of oxytocin receptor agonists will be beneficial in reducing the negative emotional symptoms associated with drug withdrawal.

Chapter 2: The oxytocin analogue carbetocin prevents social deficits and mood impairment in long-term abstinent mice.

Objectives:

- To investigate the effects of protracted morphine withdrawal on emotional-like behaviours of mice by using the three-chambered sociability test, the elevated plus-maze and the forced-swim test.
- To determine the effects of chronic morphine administration and withdrawal on OTR binding in the brain and OT levels in the plasma and brain of mice by using quantitative oxytocin receptor autoradiography and ELISA, respectively.
• To elucidate if administration of the oxytocin analogue carbetocin has any effect on the modulation of emotional-like behaviour of mice undergoing protracted withdrawal from chronic morphine administration.

Chapter 3: Chronic cocaine treatment and withdrawal induces a persistent oxytocin receptor up-regulation in the amygdala.

Objectives:

• To determine the effects of chronic cocaine administration and protracted withdrawal on stereotypy behaviour in mice.
• To investigate the effects of chronic cocaine administration and withdrawal on OTR binding by quantitative autoradiography of these receptors in mouse brains.

Chapter 4: Chronic methamphetamine treatment induces oxytocin receptor up-regulation in the amygdala and hypothalamus via an adenosine A2A receptor-independent mechanism.

Objectives:

• To investigate the effects of chronic MAP administration on OTR binding by quantitative autoradiography of these receptors in mouse brains.
• To assess the involvement of the adenosine A2A receptors in the modulation of MAP-induced neuroadaptations by using quantitative autoradiography of OTR in the brains of wild-type and A2A receptor knockout mice.
CHAPTER 2

The oxytocin analogue carbetocin prevents social deficits and mood impairment in long-term morphine abstinent mice
2.1 Introduction

Opioids are a class of drugs derived from the opium poppy, where the biologically active alkaloid, morphine, is abundantly found. Opioid drugs such as morphine, codeine or the semi-synthetic opiate heroin, act on three opioid receptor subtypes: the MOPr, the KOPr and the δ-opioid receptor (DOPr) (Martin et al., 1976, Lord et al., 1977).

Opioids can induce a variety of beneficial pharmacological effects including analgesia, primarily by acting at the $G_{i/o}$ MOPr in the spinal cord and in several areas of the brain such as the thalamus (Wang et al., 2009). Even though opioids have been proven to be effective in the treatment of pain and are widely used clinically in pain management (Corbett et al., 2006), their long-term prescription has been associated with the development of dependence (Fields, 2007). This effect of opioids has been at least partially attributed to their interactions with the MOPr in the VTA where opioids inhibit the γ-aminobutyric acid (GABA) release and disinhibit DAergic neurons projecting to the Acb (Di Chiara and Imperato, 1988). Increased DA release in the Acb can cause euphoric effects (see Sections 1.2.1.1), therefore, opioid drugs are misused for these rewarding effects.

Long-term use of opioids can lead to the development of dependence and the emergence of physical symptoms upon cessation of drug taking. Several acute physical symptoms of opioid withdrawal, which appear after 6-8 hours upon the cessation of use include tremor, restlessness, diarrhoea, vomiting and muscle weakness. Moreover, opioid addiction is also characterised by the emergence of negative emotional withdrawal symptoms (Jaffe, 1990, Martin and Jasinski, 1969, Nunes et al., 2004, Peles et al., 2007) which may serve as a motivational trigger to re-administer the drug and to relapse even after prolonged abstinence (Le Moal and Koob, 2007).
The main problem for recovering opioid addicts is the maintenance of the abstinence state. Despite the many physical symptoms of withdrawal typically decreasing after a short period of time, symptoms associated with emotional distress and dysphoria, such as anxiety, irritability, stress, depression, restlessness and anhedonia may linger for several months, or in certain instances even up to a year (protracted withdrawal) (Jaffe, 1990, Martin and Jasinski, 1969). The high prevalence (30%-50%) of major depression and anxiety in post-dependent heroin addicts has been well documented (Nunes et al., 2004, Peles et al., 2007). Moreover the negative impact of impaired social behaviour in recovering addicts has been recognised (Heinrichs and Gaab, 2007), especially in light of the significant benefits that psychosocial support have in maintaining addicts off the drug (Koerner, 2010). In animal studies, impaired emotional-like behaviour has been reported following short- or long-term withdrawal from opioids (Anraku et al., 2001, Grasing and Ghosh, 1998, Molina et al., 1994) and recently, Goeldner et al., (2011) reported an enhancement of despair-like behaviour and a reduction in sociability in mice withdrawn for 4 weeks from a chronic morphine administration paradigm.

Although several mechanisms have been suggested (e.g. Goeldner et al., 2011, Koob and Kreek, 2007, Koob, 2008), the molecular mechanisms underpinning the emotional impairment observed in opioid abstinent individuals remain unclear. Accumulating evidence showing a strong interaction between neuronal markers of social behaviour and those of stress and mood regulation (Dabrowska et al., 2011, Debiec, 2005, Di Simplicio et al., 2009, Heinrichs and Gaab, 2007, Windle et al., 2004) and of drug reward/withdrawal (Liu et al., 2011, Young et al., 2011), suggest that the “social” neuropeptide OT and its receptor may be implicated in the modulation of negative emotional aspects of protracted abstinence from opioids.
As discussed in Section 1.7, OT promotes social bonding and social memory (Keverne and Curley, 2004) and has a marked anxiolytic and antidepressant effect in humans when administered in the form of an intranasal spray (Baumgartner et al., 2008, Kirsch et al., 2005, Di Simplicio et al., 2009), or in animal models when administered centrally or peripherally (Dabrowska et al., 2011, Windle et al., 2004). This has triggered a growing interest in the involvement of OT in psychiatric conditions characterised by symptoms associated with increased anxiety and social withdrawal, such as autism and schizophrenia (Carter, 2007, Heinrichs and Gaab, 2007). Therefore, it is markedly possible for OT to have a key role in addiction processes (see Sanyai, 2011); also see Section 1.8.

There is emerging evidence supporting the involvement of OT in the effects of a number of drugs of abuse (see Broadbear et al., 2011, McGregor and Bowen, 2012, McGregor et al., 2008, Sanyai, 2011). With respect to opioids, peripherally administered OT dose-dependently attenuated morphine tolerance, blocked naloxone-precipitated morphine withdrawal, inhibited tolerance to the endogenous opioids β-endorphin and met-enkephalin (Kovacs et al., 1985, Kovacs et al., 1984) and also attenuated the maintenance of heroin self-administration (Kovacs and Van Ree, 1985) in animal models. In addition, marked alterations in the endogenous oxytocinergic system were observed after acute and chronic opioid administration as well as during naloxone-induced withdrawal (see Section 1.8.2). Briefly, OT peptide content and/or synthesis were altered in the forebrain, Hip, Amy and Hyp of rodents following acute and chronic opioid administration as well as acute precipitated withdrawal (Kovacs et al., 1987a, You et al., 2000). Although these findings are suggestive of major neuroadaptations of the oxytocinergic system by opioid exposure, it is unclear if these alterations persist during protracted opioid abstinence and whether they might
contribute to the emotional behavioural impairment observed in opioid abstinent addicts.

As a result, we hypothesised that withdrawal from chronic opioid administration causes profound dysregulation of the central oxytocinergic system and that stimulation of OTRs may attenuate the emotional impairment observed during protracted opioid abstinence. To test this hypothesis, we first established a mouse model for studying emotional consequences of protracted opioid abstinence and investigated the effect of protracted opioid withdrawal on different components of the endogenous oxytocinergic system in plasma and brain tissue of mice. We also examined the effect of the OT analogue CBT in preventing this emotional behavioural impairment. This analogue has been shown to be protected from aminopeptidase and disulfidase cleavage (Barth et al., 1974), hence it has much longer elimination half-life than OT; 85-100 min versus 3-4 min (Engstrom et al., 1998). It has been also suggested to be a potential candidate for the treatment of several psychiatric disorders (Quay, 2005).
2.2 Methods

2.2.1 Animals

Male C57BL/6J mice (7-week old, 20-25g, Charles River Laboratories, Kingston, UK), were housed individually in a temperature-controlled environment with a 12-hour light/dark cycle (lights on: 06:00 am). Food and water were available ad-libitum. Mice were left to acclimatise in their new environment for seven days prior to the experimental start and were handled daily by the experimenter. All experimental procedures were conducted in accordance with the U.K. Animal Scientific Procedures Act (1986).

2.2.2 Chronic “intermittent” escalating-dose morphine administration paradigm

Mice were randomly divided into chronic saline- and morphine-treated groups. Mice were injected (i.p.) with saline (4 ml/kg) or morphine given at the same volume as saline (4ml/kg) (Sigma-Aldrich, Poole, UK) with a chronic, “intermittent” escalating-dose administration paradigm (20 mg/kg on Day 1, 40 mg/kg on days 2 and 3, 80 mg/kg on days 4 and 5 and 100 mg/kg on day 6 and 7) twice per day at 9:00 and 17:00 in accordance with Goeldner et al., (2011), Muller and Unterwald, (2004) and Zhou et al., (2006), with minor modifications. Both groups of animals were left to spontaneously withdraw in their home cages for a period of either 1 and/or 4 weeks. Weight, food and water consumption were monitored every day at 7:00am.

2.2.3 Behavioural experiments

2.2.3.1 Locomotor activity

Locomotor activity of each mouse was measured daily (8 o’clock, 2 hours after the start of the light cycle) in locomotor chambers (40cm x 20cm x 20cm; Linton Instrumentation, Norfolk, U.K.). Each chamber had two lines of 16 photocells located at
right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 6 cm above the cage floor to measure horizontal and vertical activity (rears), respectively. Mice were treated with either chronic “intermittent” saline or escalating-dose morphine administration paradigm and then left to spontaneously withdraw for 7 days in their home cage as described in Section 2.2.2. Mice were habituated in locomotor chambers for 1 hour prior to saline/morphine injections in order to assess basal activity every day. Subsequently, mice received an i.p. injection of saline or morphine and returned immediately to the chamber where horizontal and vertical activity (rearing) was measured for 1 hour. Behavioural activity (pre- and post- injection) of each animal was monitored daily for the 7-day duration of the treatment (see Figure 2.1) and following 1 and 7 days withdrawal period after the last treatment injection (basal activity after 7-days withdrawal was monitored from 8:00 until 9:00). Horizontal and vertical activities were recorded as the number of sequential infrared beam breaks, every 5 mins. The average activity of the hourly 5 min bins were calculated daily. Faecal boli production was recorded daily after the 2 hours of locomotor testing. Mice were returned to their home cages each day following completion of the locomotor test.

Figure 2.1: Morphine injection timetable
Basal locomotor activity was measured daily from 8:00 until 9:00 during the chronic administration paradigm.

2.2.3.2 Physical symptoms of withdrawal

 Withdrawal jumps and number of faecal boli were recorded to assess the somatic signs of morphine withdrawal. Different cohort of male C57BL/6J mice were treated with an
identical saline or morphine administration paradigm as described above and were placed in locomotor chambers for 120 min following a 24 hr and 7-day withdrawal period from this paradigm. Number of jumps and number of faecal boli were recorded throughout the 120 min duration of the observation period. This procedure was carried out 2 hrs after the start of the light phase of the light/dark cycle (8:00 o’clock).

2.2.3.3 Emotional impairment testing

For the pilot emotional impairment testing, separate cohorts of C57BL/6J mice were treated with an identical chronic saline and morphine administration paradigm followed by a 6-8 days withdrawal period and then tested for social interaction preference/social novelty preference (6 days withdrawal), anxiety (7 days withdrawal) and depression-like behaviours (8 days withdrawal) using the 3-chambered social interaction, EPM and FST respectively. These three tests were performed 24 h apart in the same animals and repeated following a 4-week withdrawal period again in the same animals (28, 29, and 30 days withdrawal respectively). The order of testing was determined by the degree of anxiety inducing properties of each test with the least stressful conducted first and the most potentially distressing test last (Clemens et al., 2007).

Social interaction test: The three-chambered box (Figure 2.2) was used to assess social interaction behaviours in rodents and was conducted as previously described (Nadler et al., 2004). The “experimental” mouse was placed into the middle chamber of an interconnected three-chambered box (60cm length x 30cm wide x 30cm high). The test consisted of 3 phases. During the habituation phase, the test animal had free access to all chambers for 10 min. After this session, two identical small cages (15cm x 8cm x 10cm), comprised of 1 cm-spaced vertical bars, were placed in each end-chamber and secured with a weighted cup. An unfamiliar male C57BL/6J mouse (stranger 1) was
placed into one of the two smaller cages ("social cage") while the other cage remained empty. The "experimental" mouse was placed back into the centre chamber and was allowed to explore the apparatus for 10 mins (social interaction phase). At the end of the "social interaction phase" another 10-min session followed, the "preference for social novelty" phase, where another unfamiliar male C57BL/6J mouse (stranger 2) was placed in the previously empty cage, while the now familiar (stranger 1) mouse remained in its original cage. The "experimental" animal was permitted to explore the entire apparatus now consisting of a chamber containing a now familiar mouse and another chamber containing a novel unfamiliar mouse, for another 10 min. During all of the three phases of the test, time spent by the "experimental" animal in each of the three chambers was measured by an automated tracking system (EthoVision v.3.0, Noldus Information Technology, Wageningen, Netherlands) and validated by an observer blind to the treatment groups. Social preference was determined by calculating the time that the "experimental" mouse spent in the chamber containing the unfamiliar mouse (stranger 1) vs the empty chamber during the social interaction phase of the test. Preference for social novelty was determined by calculating the time that the "experimental" mouse spent in the chamber containing a novel unfamiliar mouse (stranger 2) vs the chamber containing a now familiar one (stranger 1) during the preference for social novelty phase of the test. The test was carried out in dim light conditions (10lux).
Figure 2.2: Three-chambered sociability apparatus.
The mouse has the choice between staying in the center chamber, spending time in the side chamber
with an unfamiliar mouse (stranger 1), or spending time in the side chamber with either a novel
object (empty cage) or a newly introduced mouse (stranger 2). The stranger mice are enclosed into
the small end-chamber cages.

*Elevated plus-maze:* The elevated plus-maze (Figure 2.3) was used to measure anxiety-like
behaviour. The apparatus (Linton Instruments, UK) consisted of four equal-sized
lanes (30cm long x 5cm wide) converging at a center square (5cm x 5cm) to form the
shape of a cross and elevated 40cm off the floor. Of the four arms, two were enclosed
by a 15cm high solid wall on the long sides of the arms (closed arms) and the other two
had no sides (open arms). Each mouse was allowed to habituate in the test room for 1
hour prior to test session. To assess anxiety-like behaviours, the “experimental” mouse
was positioned with its head and forelimbs in the centre square of the apparatus and was
allowed to explore the whole apparatus for 5 mins. The time spent in each arm was
measured for each mouse by an automated tracking system (EthoVision v.3.0, Noldus
Information Technology, Wageningen, Netherlands) and validated by an observer blind
to the treatment groups. Anxiety-like behaviour was determined by calculating the
amount of time spent and number of entries each mouse made in the open and closed
arms and reported as the total time and the percentage number of entries respectively.
The test was carried out in dim light conditions (10lux).
Elevated plus-maze apparatus.
The mouse has the choice between staying in the center of the apparatus, spending time in the open-arms, or spending time in the closed-arms. The apparatus is elevated from the floor.

Forced-swim test: The FST measures depressive-like behaviours as previously described by Porsolt et al., (1977). Mice were exposed to a 15 min pre-test swim followed by a 5 min test, 24 hours after the pre-test as described by Perrine et al., (2008) and Lucki, (1997). The pre-test has been shown to facilitate the development of immobility during the test session and to increase the sensitivity for detecting behavioural effects (Lucki, 1997). Briefly, for the pre-test, mice were placed individually into clear glass cylinders (25cm height x 17cm diameter) filled with 2.5 litres of water at room temperature (23±1°C) for 15 min. Animals were removed from the water, dried by the experimenter with a paper towel, placed into the recovery chambers for 10 minutes (30°C) and placed back in their home cages. Twenty-four hours following the pre-test, the animals were placed in the cylinders again for 5 min and the session was recorded using a videocamera (Sony Handycam CX-250, Sony, Japan). Immobility time, defined as passive floating with no additional activity other than that necessary to keep the mice head above water, was scored by two treatment-condition blinded observers during the 5-min test session. Latency to the first encounter
to immobility was also measured. Faecal boli of the animals were counted at the end of the FST. The test was carried out in dim light conditions (30lux).

2.2.4 Neurochemical analysis

Separate cohorts of C57BL/6J mice were treated with an identical chronic saline and morphine administration paradigm as described above, and were left to spontaneously withdraw in their home cage for 7 days following their last treatment injection. Mice were killed by decapitation 1 hour post-final injection for the chronic morphine/saline administration groups or 7 days post-final injection for the withdrawal groups.

2.2.4.1 Oxytocin receptor autoradiography

Cleaning and subbing of microscope slides: Microscope slides were left to soak overnight in Decon, a water-based detergent. Slides were then rinsed in hot running water for 15 minutes followed by a rinse in cold distilled water for a further 15 minutes. The slides were then soaked in a 10% hydrochloric acid-90% ethanol solution for 20 minutes followed by another 15 minute rinse in distilled water. Slides were coated by a 2-minute immersion in 1% gelatine/chrom-alum solution and left to dry before use.

Tissue preparation: Brains were removed and frozen in -25°C isopentane solution for 30 seconds and stored at -80°C until use. Prior to sectioning, brains were removed from -80°C and placed into a -21°C cryostat apparatus (Zeiss Microm 505E, Hertfordshire, U.K.) and coronally aligned by fixing the cerebellum onto a mounting stage using a plastic embedding liquid (O.C.T. compound, BDH chemicals, Dorset, U.K.). Adjacent 20μm coronal brain sections were cut at 300μm distances and thaw-mounted onto gelatine-coated ice-cold microscope slides to define receptor binding levels from fore-to hind-brain regions. Brain slides were stored at -4°C for 2 hours to prevent the
formation of ice crystals into the brain structures and then saved into -20°C storage for a minimum of 1 week prior to use.

**Autoradiographic binding of the oxytocin receptor:** OTR binding was carried out on sections from chronic saline- and morphine-treated and withdrawn mice in accordance with previously described methods (Jarrett et al., 2006), with minor modifications. Sections were rinsed twice for 10 min in a buffer solution (50mM Tris-HCl, pH 7.4, room temperature) to remove endogenous OT. Total binding was determined by incubating sections with 50pM \[^{125}\text{I}^-\]ornithine vasotocin (OVTA) in an incubation buffer medium (50mM Tris-HCl, 10mM MgCl\(_2\), 1mM ethylenediaminetetraacetic acid (EDTA), 0.1 % w/v bovine serum albumin, 0.05 % w/v bacitracin; Sigma-Aldrich, Poole, UK, pH 7.4 at room temperature). Adjacent sections were incubated with \[^{125}\text{I}^-\]OVTA (50 pM) in the presence of 50µM unlabelled (Thr\(^4\),Gly\(^7\))-oxytocin (Bachem, Germany), 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 MgCl\(_2\), 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 (Drierite-BDH Chemicals, Dorset, U.K.) for 2 days.

**Autoradiographic film apposition:** Slides were apposed to Kodak MR-1 films (Sigma-Aldrich, UK) in Hypercassettes with autoradiographic \[^{14}\text{C}^-\]microscales of known radioactive concentration (GE Healthcare Life Sciences, Amersham, U.K.) for 3 days. Sections for all treatment groups were processed in parallel and apposed to the same film at the same time.
Film development: Film development was carried out in the dark under red-filtered light. The films were developed in a 50% Kodak D19 developer solution (Sigma-Aldrich, Poole, UK) for 3 minutes. The films were then washed in distilled water containing glacial acetic acid for 30 seconds to stop the development reaction followed by 5 minutes fixation step in a Kodak rapid fix solution (Sigma-Aldrich, Poole, UK). Finally, films were rinsed in cold running water for 20 minutes and left to dry in a fume hood.

Image analysis (Quantitative autoradiography): Quantitative autoradiographic analysis of all structures were carried out by reference to the mouse brain atlas of Frankin and Paxinos (1997) and binding was analysed as previously described (Kitchen et al., 1997), using MCID image analyser (Image Research, Ontario, Canada). Briefly, optical density values, which were quantified from autoradiographic [\(^{14}\text{C}\)] microscales of known radioactive concentration (GE Healthcare, UK), were entered with their corresponding radioactivity values into a calibration table, and the relationship between radioactivity and optical density was subsequently determined using the MCID software. Specific binding was determined by subtracting the NSB from the total binding in the images of the brain sections.

2.2.4.2 Oxytocin peptide levels in the brain and plasma

The brains were removed following decapitation and were cut coronally from the olfactory bulbs up to the level of cerebellum. Hyp, Amy, septum and Hip were then micro-dissected by reference to the mouse brain atlas of Frankin and Paxinos (1997) and immediately preserved in dry ice and stored in -80°C until use. Peptides were extracted from the brain regions in accordance to previously described protocol by Christensson-Nylander et al., (1985), with minor modifications. Briefly, tissues were
mixed with 1M acetic acid, heated to 95°C for 10 min, homogenised and stored in -80°C overnight. The following day, the samples were thawed on ice and centrifuged at 9000 x g for 20 min (4°C) and the supernatant was collected and lyophilized.

For plasma oxytocin levels, trunk blood from the mice was collected in tubes containing EDTA and aprotinin (500 KIU/ml of blood). The samples were centrifuged at 1600 x g for 15 min (4°C) and supernatant stored in -80°C. Solid phase extraction of samples was performed using 200mg C18 Sep-Pak Columns (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany). The extraction of the peptide in plasma was followed according to the manufacturer's protocol (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany). The eluent from the extraction process was collected and lyophilised.

The oxytocin peptide levels were measured according to the manufacturer’s instructions (oxytocin EIA kit, Enzo Life Sciences, Ch). The residues reconstituted in 250μL (for the Hyp and septum) or 200μL (for the plasma, Amy and Hip) of EIA assay buffer (oxytocin EIA kit, Enzo Life Sciences, Ch). OT levels were determined in duplicate in a single assay.

2.2.4.3 Plasma corticosterone levels

Trunk blood from the mice was collected in EDTA-containing tubes and spun for 15 min at 2000 x g at 4°C. Blood was collected at 10:00 for all the groups of the animals. Plasma was collected and corticosterone levels were measured by using a rat/mouse corticosterone [125I] kit (MP Biomedicals, New York, NY, USA) according to the manufacturer’s instructions. All coricosterone levels were determined in duplicate in a single assay.
2.2.5 Carbetocin effects on morphine-induced behaviour

To assess the effect of the OT analogue CBT on protracted opioid withdrawal-associated anxiety, depression and social deficits, different cohorts of C57BL/6J mice treated with a chronic saline or "intermittent" escalating-dose morphine administration paradigm followed by 7 days withdrawal. Each test was performed on separate group of animals and only one test was carried out for each animal.

2.2.5.1 Pilot carbetocin dose-response experiment in prolonged morphine-withdrawn mice

The pilot experiment was carried out in order to determine the effective dose of CBT on anxiety-like and depressive-like behaviour as well as social interaction and social novelty preference deficits in mice undergoing protracted opioid withdrawal. Male C57BL/6J mice were treated with either saline or morphine with a 7-day "intermittent" morphine escalating-dose paradigm as described in Section 2.2.2 and left to spontaneously withdraw for a period of 7 days in their home cages. Seven days after their last treatment injection mice were challenged with either saline (4 ml/kg, i.p.) or CBT (2 or 6.4 or 20 mg/kg, i.p.) and 15 minutes later they were tested for depressive-like behaviour with the use of the FST for 5 min as described in Section 2.2.3.3. The mice were exposed to a 15-min pre-test forced-swim test session 24 hrs prior to the test session. Immobility time, latency to the first episode of immobility and number of faecal boli production were measured during the 5-min duration of the test.

2.2.5.2 Effect of carbetocin on locomotor activity of naïve mice

To test if the immobility reduction effect of CBT in the FST, at the chosen dose of 6.4 mg/kg, was due to a possible effect on locomotor activity rather than an antidepressant effect, the effect of CBT was tested in naïve C57BL/6J mice in the locomotor chambers. Naïve male C57BL/6J mice were placed in the aforementioned locomotor chambers.
equipped with horizontal and vertical photocells (Linton Instrumentation, Norfolk, U.K.) for 60 min before they were injected with either saline (4 ml/kg, i.p.) or CBT (6.4 mg/kg, i.p. – lowest effective dose in the pilot experiment). Following the injection, the mice were immediately placed in the locomotor chambers for a period of 40 min. Horizontal and vertical activities were recorded as the number of sequential infrared beam breaks every 5 min during the 40 min duration of the experiment. Faecal boli production was also measured.

2.2.5.3 Effects of carbetocin on the social impairment following prolonged morphine withdrawal

In order to test the effect of CBT on social deficits following protracted morphine withdrawal, male C57BL/6J mice were treated with chronic saline or “intermittent” escalating-dose morphine administration paradigm as described in Section 2.2.2 and after 7 days of withdrawal mice were administered with either saline (4 ml/kg, i.p.) or CBT (6.4 mg/kg, i.p.) 5 min prior to testing in the 3-chamber sociability test for social preference and preference for social novelty. The sociability test was conducted as described in Section 2.2.3.3.

2.2.5.4 Effects of carbetocin on the anxiety-like behaviour following prolonged morphine withdrawal

In order to test the effect of CBT on anxiety-like behaviours following protracted morphine withdrawal, male C57BL/6J mice were treated with chronic saline or escalating-dose morphine administration paradigm as described in Section 2.2.2 and after 7 days of withdrawal mice were administered with either saline (4 ml/kg, i.p.) or CBT (6.4 mg/kg, i.p.) 10 min prior to testing in the EPM. The test was conducted as described in Section 2.2.3.3
2.2.5.5 Effects of carbetocin on the depressive-like behaviour following prolonged morphine withdrawal

In order to test the effect of CBT on depressive-like behaviours following protracted morphine withdrawal, male C57BL/6J mice were treated with chronic saline or escalating-dose morphine administration paradigm as described in Section 2.2.2 and after 7 days of withdrawal mice were administered with either saline (4 ml/kg, i.p.) or CBT (6.4 mg/kg, i.p.) 10 min prior to testing in the FST. The test was conducted as described in Section 2.2.3.3

2.2.5.6 Effects of carbetocin on the hypolocomotion following prolonged morphine withdrawal

In order to investigate the effects of CBT on hypolocomotion following protracted morphine withdrawal male C57BL/6J mice were treated with a chronic saline or escalating-dose morphine paradigm (see Section 2.2.2) and left to spontaneously withdraw for 7 days. At this time point, mice were pre-injected with either saline (4 ml/kg, i.p.) or CBT (6.4 mg/kg, i.p.) and tested in the locomotor chambers for a period of 60 min.
2.2.6 Statistical Analysis

Body weight, food and water intake differences in morphine administered and withdrawn animals were analysed using two-way repeated measures ANOVA for factors ‘treatment’ and ‘day (repeated factor)’. Social interaction behavioural data were analysed using repeated measures two-way ANOVA for factors ‘treatment’ and ‘chamber (repeated factor)’. Basal locomotor activity data during the chronic saline/morphine administration paradigm were analysed using repeated measures two-way ANOVA for factors ‘treatment’ and ‘day (repeated factor)’. Locomotor data following the 7 day withdrawal period was analysed with an unpaired Student \( t \)-test. CBT effects on locomotor activity was analysed using repeated measures two-way ANOVA for factors ‘pre-treatment (saline/CBT)’ x ‘time (repeated factor)’. Faecal boli production in the FST was analysed with non-parametric Mann-Whitney \( U \)-test. For the analysis of the effect of morphine withdrawal on anxiety and depressive-like behaviour, an unpaired \( t \)-test was used. The effect of CBT on anxiety and depressive-like behaviour in opioid abstinent mice was analysed by two-way ANOVA for factors ‘treatment (saline/morphine)’ x ‘CBT effect’. The effect of CBT on social preference and social novelty preference data were analysed using repeated measures three-way ANOVA for factors ‘treatment (saline/morphine)’ x ‘chamber (repeated factor) x ‘CBT effect’. For analysis of the OT levels and OTR binding two-way ANOVA was performed in each individual brain region or plasma for factors ‘treatment (saline/morphine)’ and ‘withdrawal effect’. Corticosterone levels were analysed using two-way ANOVA for factors ‘treatment (saline/morphine)’ and ‘withdrawal effect’. All statistical analyses were performed using Statistica 8.0 analysis program (Statsoft Inc., France). Where ANOVA revealed a significant factor or interaction effect \( (p<0.05) \), a Bonferroni \( post-hoc \) test was carried out. All the values were expressed as mean ± SEM.
2.3 Results

2.3.1 Chronic morphine administration and withdrawal induced marked alterations in body weight, food and water intake

Morphine treatment reduced body weight ($F_{[1,34]}=12.20, p<0.001$), food consumption ($F_{[1,24]}=187.66, p<0.001$) and water consumption ($F_{[1,24]}=15.54, p<0.001$) over time ($F_{[7,238]}=52.76, p<0.001; F_{[7,168]}=38.01, p<0.001$ and $F_{[7,168]}=2.86, p<0.01$ respectively) (Figure 2.4). However, withdrawal from morphine significantly increased body weight, food and water consumption over time ($F_{[6,204]}=76.01, p<0.001; F_{[6,198]}=38.01, p<0.001$ and $F_{[6,204]}=9.31, p<0.001$ respectively). After 7 days of withdrawal (i.e. day 14), mice recovered body weight, food intake and water consumption similar to saline-treated animals ($p>0.05$, Figure 2.4).
Figure 2.4: Effects of chronic morphine administration and withdrawal on body weight, food and water consumption.
(A) Daily alterations in body weight, (B) food consumption, and (C) water intake during chronic morphine or saline administration and withdrawal period. Data are expressed as mean ± SEM (n=13-18 per group). *p<0.05, **p<0.01, ***p<0.001 vs saline control; "p<0.05, ""^p<0.001 vs day 7 (repeated measures two-way ANOVA followed by Bonferroni post-hoc test). Dashed lines indicate the start of withdrawal period.

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2.3.3 Chronic morphine administration induced marked increase in horizontal but not vertical locomotor activity

Morphine treatment increased horizontal activity ($F_{[1,34]}=59.46$, $p<0.001$) over time ($F_{[6,204]}=12.98$, $p<0.001$) during the chronic administration paradigm (Figure 2.5A). Following 5 days of morphine administration, tolerance to the hyperlocomotion was developed as indicated with a significant decrease in morphine-stimulated horizontal activity vs the first day of morphine administration (time effect: $F_{[6,204]}=19.71$, $p<0.001$)). Chronic morphine treatment did not induce any significant alterations in the vertical locomotor activity compared to saline controls ($F_{[1,34]}=0.36$, $p>0.05$) (Figure 2.5B). Saline animals showed decreased vertical locomotor activity over time ($F_{[6,204]}=3.01$, $p<0.01$) due to habituation with the locomotor chambers.
Figure 2.5: Chronic morphine administration increases horizontal but not vertical locomotor activity.
Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day). Stimulated (A) horizontal and (B) vertical activity (representing rearing) were measured daily in 5 min bins for 1 hour post-morphine or saline injection during the 7-day “intermittent” saline or escalating-dose morphine administration paradigm. The average activity of the hourly 5-min bins was calculated daily. Chronic morphine administration increased horizontal activity compared to saline. Tolerance to the locomotor hyperactivity has been developed following chronic administration of morphine. Data are expressed as mean ± SEM (n=18 per group). ***p<0.001 vs Saline; #p<0.001 vs Morphine Day 1; †††p<0.001 vs Saline Day 1 (repeated measures two-way ANOVA followed by Bonferroni post-hoc test).
2.3.4 Absence of physical withdrawal symptoms but reduction of basal locomotor activity following protracted morphine abstinence

Mice withdrawn from morphine for 1 day exhibited higher number of withdrawal jumping (40 ± 13 vs 0 ± 0; \( p<0.001 \)) and showed higher numbers of faecal boli (13 ± 1 vs 3 ± 1; \( p<0.001 \)) compared to saline-withdrawn animals. However, following a 7-day withdrawal from morphine, mice did not show any withdrawal jumping and faecal boli production was identical to saline-withdrawn mice (4 ± 1 vs 4 ± 1; \( p>0.05 \)) demonstrating an absence of physical withdrawal symptoms following a 7-day morphine abstinence period.

Morphine treatment reduced basal horizontal activity (\( F_{1,204}=16.84, \ p<0.001 \)) over time (\( F_{6,204}=6.15, \ p<0.001 \)) during the chronic administration paradigm (Figure 2.6A), which persisted following 7 days of withdrawal (\( p<0.001 \)). This reduction was also observed in vertical basal activity but only following 7 days of withdrawal (\( p<0.01 \)) (Figure 2.6B).
Figure 2.6: Chronic morphine administration reduces basal locomotor activity which persists after a 7-day withdrawal period.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 7 days (i.e. day 14). Basal (A) horizontal and (B) vertical activity (representing rearing) were measured daily in 5 min bins for 1 hour prior to morphine or saline injection (basal) during the 7-day “intermittent” saline or escalating-dose morphine administration paradigm. Basal activity was also measured for a period of 60 min, 7 days after the last treatment injection (day 14: withdrawal period). The average activity of the hourly 5-min bins were calculated daily. Chronic morphine administration decreased basal horizontal activity which persisted following 7-days of morphine withdrawal. Data are expressed as mean ± SEM (n=9-18 per group). *p<0.05, **p<0.01 vs Saline (repeated measures two-way ANOVA followed by Bonferroni post-hoc test); #p<0.01, ###p<0.001 vs Saline withdrawal (unpaired t-test).
2.3.5 Protracted abstinence from chronic morphine administration abolished social preference in mice

We assessed social preference and social novelty preference in chronically saline and morphine-withdrawn mice with the use of the 3-chambered sociability test following 1- and 4-weeks withdrawal.

1-week withdrawal (Figure 2.7): Repeated measures two-way ANOVA showed a significant main effect of ‘side’ ($F_{[1,10]}=42.63, p<0.001$) and significant ‘side’ x ‘treatment’ interaction ($F_{[1,10]}=28.79, p<0.001$) during the “social interaction” phase of the three-chambered sociability test (Figure 2.7A). Saline-withdrawn mice exhibited a significant preference for the chamber containing stranger 1 mouse vs the empty chamber (social preference) ($p<0.001$). However, morphine-withdrawn animals did not show any preference between the chamber containing stranger 1 mouse and the empty chamber ($p>0.05$), demonstrating a lack of social preference. Although there was a trend of increased amount of time that saline-withdrawn animals spent in the chamber containing the stranger 2 mouse compared to the amount of time spent in the chamber containing the stranger 1 mouse, repeated measures two-way ANOVA did not indicate any significant effects of chamber side ($F_{[1,10]}=2.72, p>0.05$) or significant chamber side x treatment interaction ($F_{[1,10]}=0.75, p>0.05$) during the social novelty phase of the three-chambered sociability test (Figure 2.7B). However, this is probably due to power issues since by increasing the n numbers to $n=10$ in the studies with CBT, saline control animals showed preference for social novelty (see Section 2.3.12 and Figure 2.17B). Morphine-withdrawn animals did not show any preference between the chamber containing stranger 2 mouse and that containing stranger 1 demonstrating a lack of social novelty preference.
4-weeks withdrawal (Figure 2.8): Repeated measures two-way ANOVA showed a significant main effect of ‘side’ ($F_{[1,10]}=5.30$, $p<0.05$) and significant ‘side’ × ‘treatment’ interaction (Figure 2.8A, $F_{[1,10]}=4.99$, $p<0.05$) during the “social interaction” phase of the three-chambered sociability test (Figure 2.8A). Saline-withdrawn mice exhibited a significant preference for the chamber containing stranger 1 mouse vs the empty chamber (social preference) ($p<0.05$). However, morphine-withdrawn animals did not show any preference between the chamber containing stranger 1 mouse and the empty chamber ($p>0.05$), demonstrating a lack of social preference. Although there was a trend of increased amount of time that saline-withdrawn animals spent in the chamber containing the stranger 2 mouse compared to the amount of time spent in the chamber containing the stranger 1 mouse, repeated measures two-way ANOVA did not indicate any significant effects of chamber side ($F_{[1,10]}=2.63$, $p>0.05$) or significant chamber side × treatment interaction ($F_{[1,10]}=2.26$, $p>0.05$) during the social novelty phase of the three-chambered sociability test (Figure 2.8B). Morphine-withdrawn animals did not show any preference between the chamber containing stranger 2 mouse and that containing stranger 1 demonstrating a lack of social novelty preference.
Figure 2.7: Abolition of social preference following 1-week withdrawal from chronic morphine administration.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 6 days. Mice were tested for (A) social preference, as defined by the time spent in the chamber containing the unfamiliar mouse (stranger 1) vs the empty chamber, during the 10 min “social interaction phase” of the three-chambered sociability test and (B) social novelty preference, as defined by the time spent in the chamber containing a novel unfamiliar mouse (stranger 2) vs the chamber containing a now familiar one (stranger 1) during the 10-min “preference for novelty” phase of the three-chambered sociability test. Mice chronically withdrawn from morphine exhibited a lack of social preference. Data are expressed as mean ± SEM (n=6 per group). ***p<0.001 vs Saline withdrawal (Stranger 1 Side), (repeated measures two-way ANOVA followed by Bonferroni post-hoc test).
Figure 2.8: Abolition of social preference following 4-week withdrawal from chronic morphine administration.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 28 days. Mice were tested for (A) social preference, as defined by the time spent in the chamber containing the unfamiliar mouse (stranger 1) vs the empty chamber, during the 10 min “social interaction phase” of the three-chambered sociability test and (B) social novelty preference, as defined by the time spent in the chamber containing a novel unfamiliar mouse (stranger 2) vs the chamber containing a now familiar one (stranger 1) during the 10-min “preference for novelty” phase of the three-chambered sociability test. Mice chronically withdrawn from morphine exhibited a lack of social preference. Data are expressed as mean ± SEM (n=6 per group). *p<0.05 vs Saline 4 weeks withdrawal (Stranger 1 Side), (repeated measures two-way ANOVA followed by Bonferroni post-hoc test).
2.3.6 Protracted abstinence from chronic morphine administration enhanced anxiety-like behaviour in mice

Anxiety-related behaviour in saline and morphine-withdrawn mice was assessed with the use of the EPM following 1- and 4-weeks withdrawal.

1-week withdrawal (Figure 2.9): Morphine-withdrawn animals spent significantly less time in the open arms compared to saline-withdrawn animals (Figure 2.9A, \( p<0.01 \)). In addition, morphine-withdrawn mice showed significantly less % of entries in the open arms compared to saline-withdrawn controls (Figure 2.9B, \( p<0.05 \)).

4-weeks withdrawal (Figure 2.10): Morphine-withdrawn animals spent significantly less time in the open arms compared to saline-withdrawn animals (Figure 2.10A, \( p<0.01 \)). In addition, morphine-withdrawn mice showed significantly less % of entries in the open arms compared to saline-withdrawn controls (Figure 2.10B, \( p<0.05 \)). These data clearly show that protracted withdrawal from morphine increases both measures of anxiety-like behaviour. The time spent by the 4-weeks withdrawn animals in the open arms showed a marked reduction compared with the time spent in the open arms of the EPM during the 1-week withdrawal. This reduction is due to the prior experience of the animals with the EPM that has been shown to cause a shift of the emotional state of the animals and to induce a phobic state instead of an unconditioned anxiety response (File et al., 1993).
Figure 2.9: Increase in anxiety-like behaviour following 1-week abstinence from chronic morphine administration.

Male C57BL/6J mice were treated with either saline or morphine with an "intermittent" 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 7 days. (A) Time in open-arm and (B) open-arm entries (%) were measured in saline and morphine-withdrawn animals during the 5 min duration of the elevated plus-maze test. Mice chronically withdrawn from morphine demonstrated increased anxiety-like behaviour. Data are expressed as mean ± SEM (n=6 per group). *p<0.05, **p<0.01 (unpaired t-test).
Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 29 days. (A) Time in open-arm and (B) open-arm entries (%) were measured in saline and morphine-withdrawn animals during the 5 min duration of the elevated plus-maze test. Mice chronically withdrawn from morphine demonstrated increased anxiety-like behaviour. Data are expressed as mean ± SEM (n=6 per group). *p<0.05, **p<0.01 (unpaired t-test).
2.3.7 Protracted abstinence from chronic morphine administration enhanced depressive-like behaviour in mice

Depression-like behaviour in saline and morphine-withdrawn mice was assessed with the use of the FST following 1- and 4-weeks withdrawal.

1-week withdrawal (Figure 2.11): Morphine-withdrawn animals showed significantly increased immobility time compared to saline-withdrawn animals (Figure 2.11A, $p<0.05$). Morphine-withdrawn mice also took significantly less time to reach immobility (latency to immobility) for the first time compared to saline-withdrawn mice ($p<0.01$) (Figure 2.11B). Number of faecal boli production of each mouse were also measured during the forced-swim session. Morphine-withdrawn mice produced significantly more faecal boli compared to saline-withdrawn mice (Figure 2.11C, $p<0.05$).

4-weeks withdrawal (Figure 2.12): Morphine-withdrawn animals showed significantly increased immobility time compared to saline-withdrawn animals (Figure 2.12A, $p<0.01$). Morphine-withdrawn mice produced significantly more faecal boli compared to saline-withdrawn mice (Figure 2.12B, $p<0.01$). These data clearly show that protracted withdrawal from morphine increases depressive-like behaviour.
Figure 2.11: Increase in depressive-like behaviour following 1-week abstinence from chronic morphine administration.

Male C57BL/6J mice were treated either with saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 8 days. (A) Immobility time, (B) latency to the first episode of immobility and (C) number of faecal boli production were measured in saline and morphine-withdrawn animals during the 5-min duration of the forced-swim test. Mice chronically withdrawn from morphine demonstrated increased depressive-like behaviour. Data are expressed as mean ± SEM (n=6 per group). *p<0.05, **p<0.01 (unpaired t-test); # p<0.05 (Mann-Whitney non-parametric t-test).
Figure 2.12: Increase in depressive-like behaviour following 4-weeks abstinence from chronic morphine administration.

Male C57BL/6J mice were treated either with saline or morphine with an "intermittent" 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 4 weeks. (A) Immobility time and (B) number of faecal boli production were measured in saline and morphine-withdrawn animals during the 5-min duration of the forced-swim test. Mice chronically withdrawn from morphine demonstrated increased depressive-like behaviour. Data are expressed as mean ± SEM (n=6 per group). **p<0.01 (unpaired t-test); ##p<0.01 (Mann-Whitney non-parametric U-test).
2.3.8 Carbetocin reversed depressive-like behaviour in 7-day morphine-withdrawn mice in a dose-dependent manner (pilot experiment)

This dose-response study was conducted to determine the optimal dose of CBT in reducing the emotional impairment during protracted morphine withdrawal in mice. Withdrawal from morphine significantly increased immobility time (Figure 2.13A, \(F_{[1,36]}=15.63, p<0.001\); Sal-Sal vs Mor-Sal, \(p<0.001\)), decreased latency to the first episode of immobility (Figure 2.13B, \(F_{[1,36]}=25.58, p<0.001\); Sal-Sal vs Mor-Sal, \(p<0.001\)) and increased faecal boli production (Figure 2.13C, \(F_{[1,36]}=9.38, p<0.001\); Sal-Sal vs Mor-Sal, \(p<0.01\)), indicating an enhancement of depression-like behaviour. Pretreatment with CBT dose-dependently decreased immobility time (\(F_{[1,36]}=4.88, p<0.01\); Mor-Sal vs Mor-CBT (6.4 or 20 mg/kg), \(p<0.01\)), increased latency to the first episode of immobility (\(F_{[1,36]}=3.86, p<0.05\); Mor-Sal vs Mor-CBT (20 mg/kg), \(p<0.05\)), and decreased faecal boli production (\(F_{[1,36]}=3.69, p<0.05\); Mor-Sal vs Mor-CBT (6.4 or 20 mg/kg), \(p<0.01\)) in 7-day morphine-withdrawn animals. Saline-withdrawn mice pretreated with CBT (2, 6.4 or 20 mg/kg) showed no significant change in immobility time, latency to immobility or faecal boli production (Figure 2.13, \(p>0.05\)). According to the results, the chosen dose for CBT was 6.4mg/kg since it is the lowest effective dose reducing depressive-like behaviour of morphine withdrawn animals in the FST.
Figure 2.13: Pilot dose-response carbetocin reversal of depressive-like behaviour in long-term morphine abstinent mice.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm and left to spontaneously withdraw for a period of 7 days. Seven days after their last treatment injection, mice were challenged with either saline or CBT (2 or 6.4 or 20 mg/kg, i.p) and 15 minutes later they were tested for depressive-like behaviour with the use of the forced-swim test. (A) Immobility time, (B) latency to the first episode of immobility and (C) number of faecal boli production were measured during the 5-min duration of the test. Withdrawal from chronic morphine administration induced depressive-like behaviour that was reversed by pre-treatment with CBT in a dose-response manner. Data are expressed as mean ± SEM (n=3-10 per group). **p<0.01, ***p<0.001 vs Sal - Sal; # p<0.05, ## p<0.01, ### p<0.001 vs Mor - Sal (two-way ANOVA for factors ‘treatment (saline/morphine)’ x ‘CBT effect’ followed by Bonferroni post-hoc test).
2.3.9 Carbetocin effects on locomotor activity in naive mice

Separate cohort of male C57BL/6J were randomly assigned to two treatment groups (saline or CBT 6.4mg/kg) and tested in the locomotor chambers to investigate the effects of the CBT on motor activity. There was no significant difference in basal horizontal (Figure 2.14A) or vertical activity (Figure 2.14B) of the animals (unpaired t-test, \( p>0.05 \)). Two-way repeated measures ANOVA revealed no significant differences in either horizontal (Figure 2.14C, \( F_{[1, 110]}=1.10, p>0.05 \)) or vertical (Figure 2.14D, \( F_{[1, 110]}=1.80, p>0.05 \)) locomotor activities following saline or CBT 6.4mg/kg injections.

**Figure 2.14:** Carbetocin administration did not alter locomotor activity in naive mice.

Naive male C57BL/6J mice were placed in locomotor chambers for 60 min before (pre-CBT injection) they were injected with either saline (4 ml/kg, i.p.) or CBT (6.4 mg/kg, i.p.) and average (A) horizontal and (B) vertical activities were measured for 1 hour. Following the injection (post-CBT injection), the mice were immediately placed in the locomotor chambers for a period of 60 min and (C) horizontal and (D) vertical activities were measured throughout the 60 min post-injection period. Data are expressed as mean ± SEM (n=6 per group).
2.3.10 Carbetocin reversed the enhancement of depressive-like behaviour in protracted morphine abstinent mice

Withdrawal from morphine significantly increased immobility time in the forced-swim test (Figure 2.15A, $F_{[1,28]}=24.67$, $p<0.001$; Sal-Sal vs Mor-Sal, $p<0.001$), decreased latency to the first episode of immobility (Figure 2.15B, $F_{[1,28]}=17.36$, $p<0.001$; Sal-Sal vs Mor-Sal, $p<0.001$) and increased faecal boli production (Figure 2.15C, $F_{[1,28]}=17.30$, $p<0.01$; Sal-Sal vs Mor-Sal, $p<0.01$), indicating an enhancement of depressive-like behaviours. Pre-treatment with CBT decreased immobility time (Figure 2.15A, $F_{[1,28]}=16.34$, $p<0.001$; Mor-Sal vs Mor-CBT $p<0.001$) and decreased faecal boli production (Figure 2.15C, $F_{[1,28]}=11.21$, $p<0.01$; Mor-Sal vs Mor-CBT $p<0.01$) only in morphine-withdrawn animals (morphine x CBT interaction: $F_{[1,28]}=19.11$, $p<0.001$). CBT pre-treatment showed a trend to increase the latency to the first episode of immobility only in the morphine-withdrawn animals (interaction: $F_{[1,28]}=5.37$, $p<0.05$), however, this effect did not reach statistical significance (Figure 2.15B, Mor-Sal vs Mor-CBT, $p=0.08$). Saline-withdrawn mice pre-treated with CBT showed no significant changes in immobility time, latency to immobility and faecal boli production (Figure 2.15, Sal-Sal vs Sal-CBT, $p>0.05$).
Figure 2.15: Carbetocin pre-treatment prevented the enhancement of depressive-like behaviour during protracted morphine abstinence.

Male C57BL/6J mice were treated with either saline or morphine with an "intermittent" 7-day escalating-dose paradigm. Seven days after their last treatment injection, mice were challenged with either saline or CBT (6.4 mg/kg i.p.), and 15 minutes later they were tested for depressive-like behaviour with the use of the forced-swim test. (A) Immobility time, (B) latency to the first episode of immobility and (C) number of faecal boli production were measured during the 5 min duration of the test. Pre-treatment with CBT reversed the enhancement of immobility time, the decrease in latency to the first episode of immobility and increased the number of faecal boli production in 7-days morphine-withdrawn animals. Data are expressed as mean ± SEM (n=6-10 per group). **p<0.01, ***p<0.001 vs Sal - Sal; ##p<0.01, ###p<0.001 vs Mor - Sal (two-way ANOVA followed by Bonferroni post-hoc test).
2.3.11 Carbetocin reversed the enhancement of anxiety-like behaviour in protracted morphine abstinent mice

Withdrawal from morphine significantly decreased time spent in the open-arms of the elevated plus-maze (Figure 2.16A, $F_{[1,29]}=6.80, p<0.05$; Sal-Sal vs Mor-Sal, $p<0.05$), which is an indication of increased anxiety-like behaviour in rodents. Pre-treatment with CBT (6.4 mg/kg, i.p.) increased the time spent in the open-arms only in morphine-withdrawn mice ($F_{[1,29]}=5.67, p<0.05$; Mor-Sal vs Mor-CBT, $p<0.05$). In addition, CBT pre-treatment significantly increased the % of open-arm entries in morphine-withdrawn animals (Figure 2.16B, $F_{[1,29]}=7.5455, p<0.05$; Mor-Sal vs Mor-CBT, $p<0.05$). Mice undergoing withdrawal from saline and pre-treated with CBT showed no significant changes in time spent in open-arms or % of open-arm entries (Figure 2.16, Sal-Sal vs Sal-CBT, $p>0.05$).
Figure 2.16: Carbetocin pre-treatment prevented the enhancement of anxiety-like behaviour during protracted morphine abstinence.

Carbetocin pre-treatment prevented the enhancement of anxiety-like behaviour during protracted morphine abstinence. Male C57BL/6J mice were treated with either saline or morphine with an "intermittent" 7-day escalating-dose paradigm. Seven days after their last treatment injection mice were challenged with either saline or CBT (6.4mg/kg, i.p.) and 15 minutes later they were tested for anxiety-like behaviour with the use of the elevated plus-maze. (A) Time spent in the open arms and (B) open-arm entries (%) were measured during the 5 min duration of the test. Pre-treatment with CBT reversed the decrease of open-arm time and open-arm entries following 7-days morphine withdrawal. Data are expressed as mean ± SEM (n=6-10 per group). *p<0.05 vs Sal - Sal; "p<0.05 vs Mor - Sal (two-way ANOVA followed by Bonferroni post-hoc test).
2.3.12 Carbetocin restored social preference in protracted morphine abstinent mice

In the social preference test, three-way repeated measures ANOVA for factors ‘treatment (morphine/saline)’ x ‘CBT effect’ x ‘side (stranger 1/empty cage)’ revealed a significant interaction between all three factors (Figure 2.17A, $\text{F}_{[1,25]}=11.16, p<0.01$). While saline-withdrawn animals showed significant preference to the chamber containing the stranger 1 mouse ($p<0.05$), morphine-withdrawn animals did not show any preference between these two chambers ($p>0.05$), indicating a lack of social preference. Pre-treatment with CBT significantly increased the time that morphine-withdrawn animals spent in the chamber containing the stranger 1 mouse (Figure 2.17A, Mor-Sal vs Mor-CBT, $p<0.001$; stranger 1 vs empty cage, $p<0.001$), indicating a restoration of social preference. CBT pre-treatment had no effect on social preference in mice undergoing withdrawal from saline (Figure 2.17A, Sal-Sal vs Sal-CBT stranger 1 side, $p>0.05$).

For social novelty, three-way repeated measures ANOVA showed a significant ‘side’ x “CBT effect” interaction ($\text{F}_{[1,25]}=46.66, p<0.001$) and a significant ‘side’ x ‘treatment’ interaction (Figure 2.17B, $\text{F}_{[1,25]}=12.14, p<0.001$). While saline-withdrawn animals showed significant preference for the chamber containing the novel stranger 2 mouse (i.e., unfamiliar animal) vs the chamber containing stranger 1 (i.e., familiar animal) ($p<0.05$), morphine-withdrawn mice did not show any preference between the two chambers ($p>0.05$), indicating a lack of social novelty preference. Pre-treatment with CBT increased the time that morphine-withdrawn mice spent in the chamber containing the unknown stranger 2 mouse compared to the chamber containing the familiar stranger 1 mouse, although this effect did not reach statistical significance ($p>0.05$).
CBT pre-treatment had no significant effect on preference to social novelty in mice undergoing withdrawal from saline (Figure 2.17B, \( p<0.05 \)).

![Graph A: Social Preference](image)

![Graph B: Preference for Novelty](image)

Figure 2.17: Carbetocin pre-treatment restored social preference during protracted morphine abstinence.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm. Seven days after their last treatment injection, mice were challenged with either saline or CBT (6.4 mg/kg, i.p.) and 25 minutes later they were tested for (A) social interaction as defined by the time spent in the chamber containing the unfamiliar mouse (stranger 1) vs the empty chamber during the 10-min “social interaction phase” of the three-chambered sociability test and (B) preference for social novelty as defined by the time spent in the chamber containing a novel unfamiliar mouse (stranger 2) vs the chamber containing a now familiar one (stranger 1) during the 10-min “preference for novelty” phase of the three-chambered sociability test. Withdrawal from chronic morphine administration abolished social preference which was restored by pre-treatment with CBT. Data are expressed as mean \( \pm \) SEM (\( n=6-10 \) per group). \( *p<0.05 \), \( **p<0.001 \) vs. stranger 1 side in the same treatment group; \( ***p<0.001 \) vs Mor - Sal stranger 1 side (repeated measures three-way ANOVA followed by Bonferroni post-hoc test).
2.3.13 Carbetocin increased hypolocomotion induced by morphine withdrawal in mice

*Horizontal locomotor activity:* Withdrawal from morphine significantly decreased horizontal locomotor activity (Figure 2.18A, $F_{[1,16]}=33.79, p<0.001$; Sal-Sal vs Mor-Sal, $p<0.001$). Pre-treatment with CBT (6.4 mg/kg, i.p.) showed a trend to increase horizontal activity only in morphine-withdrawn mice, however this effect did not reach statistical significance ($F_{[1,16]}=5.67, p<0.05$; Mor-Sal vs Mor-CBT, $p=0.07$). Mice undergoing withdrawal from saline and pre-treated with CBT showed no significant changes in horizontal activity (Figure 2.18A, Sal-Sal vs Sal-CBT, $p>0.05$).

*Vertical locomotor activity:* Withdrawal from morphine significantly decreased vertical locomotor activity (Figure 2.18B, $F_{[1,16]}=29.03, p<0.001$; Sal-Sal vs Mor-Sal, $p<0.001$). Pre-treatment with CBT did not show any significant effect in mice undergoing withdrawal from either saline or morphine (Figure 2.18B, Sal-Sal vs Sal-CBT, Mor-Sal vs Mor-CBT $p>0.05$).
Figure 2.18: Carbetocin pre-treatment increased hypolocomotion induced by protracted morphine abstinence.
Male C57BL/6J mice were treated with either saline or morphine with an "intermittent" 7-day escalating-dose paradigm. Seven days after their last treatment injection mice were challenged with either saline or CBT (6.4mg/kg, i.p.) and 15 minutes later they were tested for locomotion activity. (A) Horizontal and (B) vertical activities were monitored for 1 hour. Morphine withdrawal significantly decreased both horizontal and vertical activities. Data are expressed as mean ± SEM (n=6-10 per group). **p<0.01, ***p<0.001 vs Sal - Sal (two-way ANOVA followed by Bonferroni post-hoc test).
2.3.14 Protracted abstinence from morphine induced marked alterations in the oxytocin receptor binding in the brain of mice

A significant ‘treatment’ effect was found in the Pir (F_{1,16}=19.88, p<0.001), medial septum (MS) (F_{1,16}=10.06, p<0.01), ventral limb of the diagonal band (F_{1,16}=6.40, p<0.05), LS (F_{1,16}=13.72, p<0.01) and Amy (F_{1,16}=63.86, p<0.001). A significant withdrawal effect was observed in the Pir (F_{1,16}=17.89, p<0.001), the LS (F_{1,16}=9.60, p<0.01) and Amy (F_{1,16}=29.00, p<0.001) and a significant treatment x withdrawal interaction was identified in the Amy (F_{1,16}=4.75, p<0.05). Chronic morphine administration increased OTR binding in the Amy compared with saline control (p<0.001) which persisted following a 7-day withdrawal period (p<0.001) (Figure 2.19). Seven-day withdrawal from chronic morphine administration induced a significant increase in OTR binding in the Pir (p<0.05), MS (p<0.05) and LS (p<0.001) compared with saline-withdrawn animals (Figure 2.19).
Figure 2.19: Region-specific alterations of $[^{125}\text{I}]-\text{OVTA}$ binding following protracted withdrawal from morphine administration.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 7 days. **(A)** Computer-enhanced representative autoradiograms of adjacent coronal brain sections from chronic saline-, morphine-treated, saline-withdrawn and morphine-withdrawn mice at the level of the olfactory nuclei (Bregma 2.46mm, first row), the caudate putamen (Bregma 0.86mm, second row), the septum (Bregma 0.14mm, third row) and the thalamus (Bregma -2.06mm, fourth row). OTRs were labelled with $[^{125}\text{I}]-\text{OVTA}$ (50pM). The colour bar illustrates a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. Representative images for the non-specific binding (50pM $[^{125}\text{I}]-\text{OVTA}$ in the presence of 50pM unlabelled oxytocin) are shown for all the treatment groups. **(B)** Quantitative oxytocin receptor autoradiographic binding and in brain regions of mice treated with a chronic “intermittent” escalating-dose morphine administration paradigm and in mice-withdrawn for 7 days from this paradigm. Data are expressed as mean ± SEM $^\pm$ SEM (n=5) specific $[^{125}\text{I}]-\text{OVTA}$ binding (fmol/mg tissue equivalent). $^\text{***}p<0.001$ vs saline control; $^\text{*}p<0.05$; $^\text{**}p<0.01$ vs saline 7 days withdrawal (two-way ANOVA followed by Bonferroni post-hoc test). Abbreviations: Acb, nucleus accumbens; Amy, amygdala; AOM, anterior olfactory nucleus-lateral; AOV, anterior olfactory nucleus-medial; AOL, anterior olfactory nucleus-ventral; CgCx, cingulate cortex; CPu, caudate-putamen; Hip, hippocampus; Hyp, hypothalamus; LS, lateral septum; MS, medial septum; Pir, piriform cortex; PV Th, paraventricular nucleus of the thalamus; Th, thalamus; Tu, olfactory tubercle; VDB, vertical limb of the diagonal band of Broca.
2.3.15 Protracted abstinence from morphine induced alterations in the oxytocin peptide levels in the brain of mice

A significant 'treatment' effect ($F_{[1,22]}=15.81, p<0.001$) was observed on oxytocin levels in the Hyp. A 43% decrease in OT levels was observed in the Hyp of mice undergoing 7-day withdrawal from morphine vs 7-day saline-withdrawn animals (Figure 2.20, $p<0.05$). No 'treatment' or 'withdrawal' effects were observed in the septum ($p>0.05$), and OT content in the Hip and Amy were mostly below detection levels (<11.7 pg/ml, i.e. 1.4 pg/mg of tissue).

Figure 2.20: Brain region-specific alterations of oxytocin peptide levels following protracted withdrawal from morphine administration.

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 7 days. Data are expressed as mean ± SEM (n=5-7) OT peptide levels (pg/mg of tissue). *$p<0.05$; vs saline 7 days withdrawal (two-way ANOVA followed by Bonferroni post-hoc test). Abbreviations: Amy, amygdala; Hyp, hypothalamus.
2.3.16 Chronic administration and protracted withdrawal from morphine induced profound alterations in the plasma corticosterone and oxytocin levels

Plasma corticosterone levels: Two-way ANOVA showed a significant 'treatment' (F\([1,43]\)=10.81, \(p<0.01\)), 'withdrawal' (F\([1,43]\)=8.79, \(p<0.01\)) and interaction 'treatment' x 'withdrawal' effect (F\([1,43]\)=21.34, \(p<0.001\)). Chronic morphine treatment increased plasma corticosterone levels (Figure 2.21, \(p<0.001\)), but levels were comparable to the control group following a 7-day morphine withdrawal period (Saline withdrawal vs Morphine withdrawal, \(p>0.05\)).

![Figure 2.21: Normalisation of corticosterone levels following protracted withdrawal from morphine administration.](image)

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 7 days. Plasma corticosterone levels were measured in chronic saline-treated, morphine-treated, saline-withdrawn and morphine-withdrawn mice. Data are expressed as mean ± SEM (n=11-12 per group) ***\(p<0.001\) vs saline control; ####\(p<0.001\) vs morphine (two-way ANOVA followed by Bonferroni post-hoc test).
**Plasma oxytocin levels:** Two-way ANOVA showed a significant ‘treatment’ effect ($F_{[1,19]}=30.91$, $p<0.001$). A decrease in OT plasma levels was observed in chronic morphine-treated ($p<0.05$) and 7-day withdrawn ($p<0.001$) mice compared to their respective saline controls (Figure 2.22).

![Bar chart showing plasma OT levels](chart.png)

**Figure 2.22: Persistent decrease in plasma OT levels following chronic morphine administration and withdrawal.**

Male C57BL/6J mice were treated with either saline or morphine with an “intermittent” 7-day escalating-dose paradigm (2 x 20-100 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 7 days. Plasma OT levels were measured in chronic saline-treated, morphine-treated, saline-withdrawn and morphine-withdrawn mice. Data are expressed as mean ± SEM (n=5-6 per group) *$p<0.05$ vs saline control; ***$p<0.001$ vs saline 7-day withdrawal (two-way ANOVA followed by Bonferroni *post-hoc* test).
2.4 Discussion

The aim of this study was to investigate if protracted abstinence from chronic morphine causes dysregulation of the endogenous oxytocinergic system and if this is associated with the emergence of emotional impairment, characteristic for recovering abstinent opioid addicts. Moreover, we investigated the effects of CBT administration in reversing the emotional deficits induced following 1-week withdrawal from chronic morphine administration.

A mouse model of protracted opioid abstinence was established which mimicked the physical and emotional alterations taking place during long-term withdrawal in human opioid abstinent addicts. The significant reduction in food and water consumption as well as weight loss following chronic morphine administration, followed by the rebound over-eating, over-drinking and weight gain observed during acute abstinence from chronic morphine exposure in our model, is in line with the nutritional and weight changes observed in heroin addicts chronically exposed to the drug and during drug withdrawal periods (Mohs et al., 1990). These findings, along with the behavioural observations of this study, indicate the translational validity of the mouse model used in this study.

In addition, we demonstrated that acute (1 day) withdrawal from chronic morphine exposure triggered severe physical withdrawal symptoms including jumps and increased defecation which disappeared after 7 days of abstinence. This is in agreement with Goeldner et al., (2011) who observed a decrease in physical withdrawal symptoms following naloxone precipitation with increasing abstinence duration from morphine in the same strain of mice.
Despite the disappearance of the acute physical withdrawal symptoms and the normalization in food, water consumption and weight changes following 7 days of morphine abstinence, 7-day morphine-withdrawn mice exhibited higher levels of anxiety, depression and abolished social preference, which persisted following 4 weeks of withdrawal. These observations are consistent with the typical decrease of physical withdrawal symptoms following a short period of drug free detoxification in human heroin addicts (Nunes et al., 2004, Peles et al., 2007, Martin and Jasinski, 1969) and in line with the persistence of symptoms associated with emotional distress, dysphoria and social withdrawal lingering for months in recovering heroin addicts (Jaffe, 1990, Martin and Jasinski, 1969, McGregor et al., 2008). In support of this, the persistent reduction of basal horizontal activity during and following chronic morphine administration is likely to be a representation of anhedonia in these mice. Indeed, impaired locomotor activity has been associated with anhedonia and a loss of interest in exploration in rodents (Rygula et al., 2005), implying a deficit in motivation. The hypolocomotion observed during protracted morphine withdrawal might be associated with a decreased DAergic activity. Opioid withdrawal has been largely associated with a long-lasting reduction of mesolimbic DAergic neuronal activity (Diana et al., 1995) and a marked decrease in extracellular DA levels in the Acb (Pothos et al., 1991, Rossetti et al., 1992, Acquas et al., 1991), which have been associated with decreased locomotor activity in rats (Hooks et al., 1991).

This study demonstrated a clear causal link between opioid abstinence and the emergence of a negative emotional state and social deficits in mice. This is particularly important for the understanding the mechanisms underpinning the high comorbidity (30-50%) between heroin addiction and major depression and anxiety disorders (Nunes et al., 2004, Peles et al., 2007) as well as social withdrawal and self-isolation (Heinrichs...
and Gaab, 2007). There is a considerable body of literature suggesting that dyregulation of the HPA axis activity in opioid abstinent individuals might be involved in the mechanisms underlining at least some aspects of physical and emotional symptoms of withdrawal observed in opioid abstinent individuals (Koob and Kreek, 2007, Koob, 2008, Li et al., 2008, Zhang et al., 2008); also see Section 1.2.2.1. The results presented here indicate that although dysregulation of the HPA axis activity might be the case for the manifestation of acute physical withdrawal symptoms which is associated with a significant elevation of plasma corticosterone, this is unlikely to be the case for the manifestation of emotional behavioural impairment. This is supported by the normalisation of both physical withdrawal symptoms (jumping and increased defecation) as well as corticosterone levels following long-term morphine withdrawal in our animal model which is in accordance with Goeldner et al., (2011) and Zhou et al., (2008) who also reported normalisation of the HPA response following prolonged opioid abstinence in mice and rats respectively. All together, these data indicate the neuroadaptational alterations in other extra-hypothalamic stress circuitries and monoamine systems which might be also involved in the manifestation of the emotional deficits following chronic morphine withdrawal.

Additionally, this is the first study to demonstrate a dysregulation of the oxytocinergic system following protracted opioid withdrawal. Indeed, a marked decrease of OT levels in plasma of chronically morphine-treated mice that persisted following 7 days of withdrawal was observed. Lower levels of OT were also detected in the Hyp of morphine-withdrawn mice, clearly indicating a hypo-oxytocinergic state during this protracted opioid withdrawal period. This is coupled with an up-regulation of the OTR in the Amy, septum and Pir, where oxytocinergic neurons project (Gimpl and Fahrenholz, 2001), possibly as a compensatory consequence of a reduced oxytocinergic
activity. Generally, an increase in OT peptide or administration of an OTR agonist is concomitant with rapid OTR desensitisation, clathrin-dependend internalisation and subsequent down-regulation (Evans et al., 1997). Therefore, it is likely that the up-regulation of the OTR in the Amy, septum and Pir is the result of decreased OT levels in the brain. These findings are in agreement with deficits in plasma OT levels reported in mothers who were exposed to cocaine during pregnancy (Light et al., 2004) and they are also consistent with studies in rodents showing a decrease in OT content, synthesis or mRNA expression in several brain regions following chronic exposure to other drugs of abuse (see McGregor and Bowen, 2012), including morphine (Kovacs et al., 1987a, You et al., 2000). Considering the ability of the oxytocin analogue CBT to prevent the enhancement of anxiety- and depressive-like behaviour and to restore social preference in protracted opioid abstinent mice, this oxytocinergic dysfunction is likely to be at least partly responsible for the emotionally impaired behaviours observed in our mouse model. Interestingly, the up-regulation of OTR was observed in the Amy and septum, regions known to be responsible for the antidepressant, anxiolytic and/or pro-social effects of OT (Curley et al., 2012, Debiec, 2005, Domes et al., 2007a, Lukas et al., 2012). As there is considerable evidence demonstrating abnormalities of both these regions following chronic drug use (Cornish et al., 2012, Okvist et al., 2011), it is possible that the compensatory region-specific up-regulation of OTRs might represent a homeostatic mechanism to oppose the anxiogenic, depressive and anti-social consequences of chronic opioid use and withdrawal. In support of this, OTR binding has also been shown to be increased in the brain following chronic stress in rats (Liberzon and Young, 1997). Although the up-regulation of the OTR binding alone is unlikely to explain the effects of CBT in preventing the emotional impairment, the hypo-
oxytocinergic state observed in the Hyp supports the hypothesis that CBT may increase
the OT levels in the Hyp by acting on the central OTR.

In contrast with our results showing a decrease in OT peptide levels in the Hyp and a
concomitant up-regulation of the OTR in the brain, Curley et al., 2012, found up-
regulation of the OTR in the lateral septum, dorsal endopiriform nucleus and the dorsal
BNST under communal rearing in lactating female Balb/c mice with their pups, a period
that has been widely associated with elevated hypothalamic and plasma OT levels. The
discrepancy here may be due to sex-differences in mice (male mice in our study vs
female mice in Curley’s study), different strains (C57BL/6J vs Balb/c) and the fact that
during lactation and pup suckling hormonal differences might affect the oxytocinergic
system. Indeed, oxytocin release has been shown to increase during milk ejection and
suckling (see Gimpl and Fahrenholz, 2001).

At a molecular level, these findings may also suggest the presence of close interactions
between the opioid and oxytocinergic system in the brain. In fact, we showed that
manipulation of the opioid system by chronic administration of morphine or by
withdrawal induced profound alterations of OT levels in the brain of mice. This
interaction could also take place at the receptor level as persistent alteration of OTR
were observed following chronic opioid stimulation and following withdrawal. The
presence of a MOPr-OTR interaction is supported from data from our laboratory
showing significantly higher levels of OTR binding in the brains of MOPr knockout vs
WT mice (unpublished data). Interestingly, the up-regulation is localised in the Amy of
MOPr knockout mice which is supportive of a MOPr-OTR interaction in a region of
high MOPr (Kitchen et al., 1997) and OTR expression. The significance of this receptor
interaction in the modulation of mood, pain and addictive disorders remains to be
determined.
The results of this study provide strong evidence that the endogenous central oxytocinergic system might be a novel target for the effective prevention of the emotional deficits during opioid withdrawal. Indeed, the oxytocin analogue CBT completely abolished the anxiety- and despair-like behaviour observed following prolonged morphine abstinence highlighting the potential of oxytocinergic-based pharmacotherapy to assist with the prolongation of an abstinent state by attenuating the persistent emotional withdrawal symptoms. This is particularly important given the high prevalence of depression even in heroin addicts treated with methadone maintenance treatment (Peles et al., 2007) and the ambiguous efficacy of classic antidepressants in treating these symptoms in that population (Nunes et al., 2004). The effect of CBT on social withdrawal in morphine abstinent mice is of particular interest as it represents the first attempt to modulate the social consequence of opioid abstinence with a specific pharmacotherapy. Social enhancing effect of intra-amygdalar administration of OT was similarly observed in animals made socially withdrawn by repeated exposure to phencyclidine (PCP) (Lee et al., 2005). Considering the therapeutic success of social support programs (e.g. Alcoholics Anonymous; see Loder, (2009)) and the benefits of social rehabilitation and social reintegration (see McGregor and Bowen, 2012) in keeping addicts abstinent, the use of OT agents may prove an important tool to assist in relapse prevention by promoting pro-social behaviour.

Previous studies have shown that centrally but not peripherally administered CBT produces anxiolytic effects (Mak et al., 2012) and i.c.v., i.p. and i.v. administration of this compound exhibits antidepressant-like effect (Chaviaras et al., 2010) in naïve rats as measured in the EPM and FST respectively. However, in the present study CBT did not show a significant anxiolytic or antidepressant effect in control animals that were injected with saline for 7 days, at least at the dose of 6.4 mg/kg. This is likely to be due
to the already reduced baseline anxiety and aversive mood tone that the mice experience following repeated handling and injections over the 7-day injection period, which would make the detection of anxiolytic and antidepressant effect challenging. Nonetheless, our results indicate that the anxiolytic and antidepressant effect of CBT is specific for opioid withdrawal-induced symptoms, at least at the low dose of 6.4 mg/kg used. CBT, at this dose, did not alter the locomotor activity in naïve (Figure 2.14) and chronically saline-treated animals (Figure 2.18) suggesting that the decreased immobility measured in the FST in this study was not due to a stimulatory effect of CBT on motor activity but rather to an antidepressant effect, which is in complete accordance to similar observations in the rat (Chaviaras et al., 2010). The antidepressant-nature of the effects of CBT on the FST is further supported by its potent effect in reducing faecal boli production during the 5-min testing period. Indeed, antidepressant drugs were shown to significantly decrease defecation during the FST (Craft et al., 2010, Eissa Ahmed and Al-Rasheed, 2009).

Although activation of the oxytocinergic system is known to have a marked anxiolytic, anti-aggressive and antidepressant effect, primarily by inhibiting CRF release in the Hyp (Baumgartner et al., 2008, Kirsch et al., 2005, Di Simplicio et al., 2009, Dabrowska et al., 2011, Windle et al., 2004, Neumann et al., 2000b), this is unlikely to reflect the mechanism by which CBT exerts its antidepressant and anxiolytic effect during opioid abstinence, as basal HPA activity is normalised at that prolonged stage of abstinence in our animal model. However, it is likely that CBT may exert these effects as well as its pro-social effects, by modulating extra-hypothalamic activity (primarily in the Amy and septum). Indeed, the growing body of evidence demonstrating that the stress-regulating and pro-social behavioural enhancing properties of OT are partly due to its action in the Amy (Domes et al., 2007a) and the septum (Curley et al., 2012,
Lukas et al., 2012), strongly support our suggestion. The region-specific up-regulation of OTRs in the Amy and septum following long-term opioid withdrawal, which reflects an increase in sensitivity of the OTR system, may indeed assist with the high efficacy of CBT to reduce emotional impaired behaviour during abstinence, even at the relative low dose of 6.4 mg/kg.

The effects of CBT in our study are likely to be a consequence of OTR activation in the CNS. Indeed, the antidepressant effect of systemic CBT in the FST was shown to be blocked by i.c.v. administration of the selective OTR antagonist atosiban (Chaviaras et al., 2010) indicating a central OTR mediated effect of CBT. The normalisation of emotional impairment behaviour by CBT during protracted opioid abstinence suggests that oxytocinergic enhancing pharmacotherapy may be effective in assisting relapse prevention to opioid self-administration. This is indeed the case for MAP addiction, as OT facilitated extinction of MAP-induced CPP and prevented its stress-induced reinstatement in mice (see Section 1.8.3.3).

It is also highly likely that the pro-social enhancing effect of CBT in morphine abstinent mice may be due its effect on the mesolimbic DAergic system, given the strong interaction between the OT and DA system (see Section 1.8.1) which are common neuronal substrates for both social and addictive related behaviours (Baskerville and Douglas, 2010, McGregor and Bowen, 2012, McGregor et al., 2008). Nonetheless, it is unlikely that CBT itself is rewarding as intranasal OT is not euphorogenic in humans despite modulating social cognition (MacDonald et al., 2011, McGregor and Bowen, 2012) and only extremely high doses of systemically administered OT can induce CPP in rats (Liberzon et al., 1997, McGregor and Bowen, 2012). In accordance, it has been
recently shown that CBT 6.4mg/kg, i.p. is neither rewarding, nor aversive, as measured by the conditioned place preference paradigm (unpublished data).

In conclusion, we provide the first evidence that OTR analogues attenuate emotional impairment behaviour during protracted opioid abstinence in mice. We also demonstrate that long-term opioid abstinence induce profound neuroadaptations of the endogenous oxytocinergic system which are likely to modulate at least in part withdrawal-induced emotional impaired behaviour. These findings strongly suggest that the oxytocinergic system might emerge as a novel target for assisting long-term opioid detoxification and as a consequence might increase the chances of opioid addicts remaining in a long-term drug free abstinent state. OT itself is commercially available in an intranasal spray format, it is not euphorogenic itself (MacDonald et al., 2011) and is currently used as a treatment strategy for autistic spectrum disorder, social anxiety and obsessive compulsive disorders (OCD). Preliminary results from the first trial with intranasal OT treatment for acutely withdrawn alcoholics have revealed promising results in preventing withdrawal symptoms (Pedersen et al., 2013).
CHAPTER 3

Chronic cocaine treatment and withdrawal induces persistent oxytocin receptor up-regulation in the amygdala
3.1 Introduction

Cocaine is purified from the leaves of the Erythroxylum coca plant originally grown in South America, Mexico, Indonesia, and the West Indies (see Goldstein et al., 2009). Cocaine is a widely abused Class A psychostimulant drug acting on monoamine transporters in the brain to block the re-uptake of DA, NA and 5-HT, thus increasing their synaptic concentrations. The pre-synaptic blockade of the DA transporter in the DAergic neurons projecting from the VTA to the Acb causes increased DA levels in the synaptic cleft, which by binding to the DA receptors in the Acb cause euphoric effects (see Nestler, 2005b). Cocaine can be administered via several routes, including intravenous injection, intranasal administration of cocaine hydrochloride and smoking of cocaine base (“crack” cocaine) (Hatsukami and Fischman, 1996).

Administration of cocaine results in elevated motor responses and the emergence of stereotypic-like behaviours which are believed to be mediated by DAergic transmission in the mesolimbic system (see Kalivas and Stewart, 1991, Kalivas and Duffy, 1993, Robinson and Berridge, 1993). In animal models, psychostimulant-induced behaviour often manifests as escalated locomotor and/or stereotyped repetitive responses, such as rearing, grooming, biting or circling (see Creese and Iversen, 1972, Scheel-Krüger, 1972). Although the neural mechanisms underlying stereotypic behaviours are not fully elucidated, it is thought to be related with the psychostimulant-induced increased striatal DAergic transmission (Costall et al., 1977). Specifically, stereotypic responses to cocaine have been associated with increased DAergic transmission in the dorsal striatum (Staton and Solomon, 1984) and it has been shown that persistent vertical activity (rearing), a characteristic high-level stereotypic response to cocaine observed in C57BL/6J mice (Schlussman et al., 2003, Bailey et al., 2008), was correlated with high DAergic stimulation in the CPu (al-Khatib et al., 1995).
The major problem for cocaine addicts who are recovering from their addiction is as for opioids, the maintenance of a drug-free state since relapse is common. Cocaine withdrawal is not characterised by the substantial physical symptoms associated with opioid withdrawal (Gawin, 1991). Rather, acute cocaine withdrawal involves a spectrum of psychological side effects including dysphoria, anhedonia, anxiety, depression, sleep and appetite disturbances and craving (Gawin, 1991, Kampman et al., 2001, Malin et al., 2000, Montoya and Vocci, 2008, Mulvaney et al., 1999). These emotional effects associated with cocaine withdrawal are particularly crucial in terms of the treatment of cocaine addicts, as the expression of these symptoms has been shown to play a key role even after long-term abstinence, which often leads to relapse (Gawin and Kleber, 1986). Individuals crave the rewarding properties of cocaine and re-administer the drug to ameliorate the negative psychological effects associated with withdrawal (see Adinoff, 2004).

Several mechanisms have been proposed for the neurobiology underlying cocaine withdrawal negative emotional symptoms; however the exact mechanisms underpinning these affective behaviours remain unclear. Both pre-clinical and clinical studies demonstrated profound alterations in the HPA axis activity during acute as well as chronic cocaine administration and withdrawal. Both acute and chronic cocaine use induced hyper-activation of the HPA axis activity (see Section 1.2.2.1). Moreover, persistent elevations of plasma ACTH and corticosterone levels have also been observed following acute and prolonged spontaneous withdrawal from chronic escalating-dose cocaine administration in rodents (Mantsch et al., 2007, Zhou et al., 2011). In addition, Sarnyai and colleagues (1995) showed that during cocaine withdrawal, hypothalamic CRF-like immunoreactivity levels are decreased indicating increased CRF release. In contrast, POMC mRNA levels remain unchanged in the
pituitary (Zhou et al., 2003b). Cocaine abstinent individuals exhibit profound alterations in the HPA axis activity leading to stress-induced cravings of the drug as well as negative emotions (Fox et al., 2008, Sinha et al., 2003, Zhou et al., 2003a). Some human studies found that cocaine-addicted patients have higher basal plasma ACTH and cortisol levels persisting even up to three months of abstinence (e.g. Wilkins et al., 2005). Therefore, dysregulation of the HPA axis has been hypothesised to at least partially underlie the negative mood state during cocaine abstinence. Although CRF antagonists have been proved to be beneficial in reversing stress-induced reinstatement in drug-seeking after withdrawal and attenuating acute negative physical symptoms of drug withdrawal in rodents (Iredale et al., 2000, Lu et al., 2000), several clinical trials that investigated the effects of CRF antagonists on stress-related responses and anxiety in humans, yielded negative results in their treatment potential (Steckler and Dautzenberg, 2006, Binneman et al., 2008, Zorrilla and Koob, 2004, Holsboer and Ising, 2008). Given these negative results, prospects for the efficacy of CRF antagonists in the treatment of addiction and prevention of relapse after abstinence are not clear.

There is some evidence indicating a possible beneficial role of OT in the modulation of several negative emotional symptoms of prolonged withdrawal from cocaine. As it has been discussed in Section 1.7, OT has antidepressant and anxiolytic effects both in humans (Baumgartner et al., 2008, Kirsch et al., 2005, Di Simplicio et al., 2009) and in animals (Dabrowska et al., 2011, Windle et al., 2004). As a result, it is possible that some of the negative emotional effects associated with cocaine addiction and withdrawal may be at least partly modulated by the oxytocinergic system and that exogenous administration of OT might prove beneficial for the alleviation of these negative symptoms.
A number of pre-clinical as well as clinical studies have demonstrated profound dysregulation of the oxytocinergic system following chronic cocaine exposure and a possible role for exogenous administered OT in treating the behavioural and neurochemical consequences of chronic cocaine use (see Section 1.8.3.1). Briefly, both central and peripheral administration of OT have been found to decrease cocaine-induced hyperlocomotion (Kovacs et al., 1990), exploratory hyperactivity (Sanyai et al., 1990), stereotypic behaviour – e.g. sniffing (Sanyai et al., 1991), tolerance (Sanyai et al., 1992a) and intravenous self-administration (Sanyai and Kovacs, 1994).

Additionally, several studies reported profound effects of cocaine on the oxytocinergic system. For example, acute cocaine administration increased OT peptide levels within the Hyp and Hip (Sanyai et al., 1992c), while chronic administration of cocaine decreased both plasma levels as well as OT immunoreactivity within the Hyp and Hip (Sanyai et al., 1992a), and increased OTR binding in the Amy of rat dams (Johns et al., 2004). In accordance to these pre-clinical findings, mothers who used cocaine during pregnancy showed lower plasma OT levels (Light et al., 2004). The exact mechanisms underlying the interactions of the oxytocinergic system and cocaine remain unclear. The DAergic innervation of the hypothalamic OT-synthesising neurons might be a putative link between cocaine and oxytocinergic system since DAergic neurotransmission is activated following cocaine administration (see Section 1.2.1.1). In support of this, it has been observed that DA and DA agonists are able to increase OT hypothalamic secretion in vitro (Bridges et al., 1976). It has been also shown that OT administration was able to block cocaine-induced DA release in the Acb (Kovacs et al., 1990).

Although there is strong evidence for the involvement of the oxytocinergic system in cocaine addiction, it is currently not clear if chronic cocaine administration can dysregulate the OTR system and if this persists following prolonged periods of
abstinence. As a result, we hypothesised that chronic cocaine administration can cause alterations in the central OTR system, which persist following long-term withdrawal. To test this hypothesis, male mice were administered with a chronic escalating-dose "binge" cocaine paradigm to mimic escalating pattern of cocaine administration in humans (Mantsch et al., 2004, Picetti et al., 2012, Tsukada et al., 1996) and/or left to spontaneously withdraw for 14 days from this paradigm. Alterations of the OTR density in the brains of these animals were measured by means of quantitative $[^{125}\text{I}]$-OVTA autoradiography.
3.2 Methods

3.2.1 Animals

Male 7-week old C57BL/6J mice (20-25g, Charles River Laboratories, Kingston, UK), were single-housed in a temperature-controlled environment with a 12-hour light/dark cycle (lights on: 06:00 am). Whilst in home cages, mice had access to food and water ad-libitum. Mice were left to acclimatise in their new environment for 7 days prior to the start of experimental procedures and were handled daily by the experimenter. All procedures were conducted in accordance with the U.K. Animal Scientific Procedures Act (1986).

3.2.2 Chronic “binge” escalating-dose cocaine administration paradigm

Mice were randomly divided into 4 different treatment groups: (1) chronic saline, (2) chronic cocaine, (3) saline 14-days withdrawal, (4) cocaine 14-days withdrawal. Mice were injected (i.p.) with saline (4 ml/kg) or cocaine (Sigma-Aldrich, Poole, UK) in an escalating-dose “binge” paradigm as described by Bailey et al., (2005, 2007) to mimic a common pattern of self-administration in human cocaine abusers (Mantsch et al., 2004, Picetti et al., 2012, Tsukada et al., 1996). Three i.p. injections of saline or cocaine (1h apart) were administered daily, with the first injection given 4 hours after the start of the light cycle (i.e. 10:00am). The cocaine-treated animals received $3 \times 15$ mg/kg/day on days 1–4, $3 \times 20$ mg/kg/day on days 5–8, $3 \times 25$ mg/kg/day on days 9–12, and $3 \times 30$ mg/kg/day on days 13 and 14 (Figure 3.1). One hour (chronic treatment groups) or 14 days (protracted withdrawal groups) after their last treatment injection, animals were killed by decapitation after a 30-sec exposure to carbon dioxide ($CO_2$). Brains were rapidly removed, frozen in -25°C isopentane solution for 30 seconds, and stored at -80°C until use. Trunk blood was also collected in EDTA-containing tubes. Weight and food consumption of the animals were monitored during the whole drug-administration
period at 8:00am. Weight of the animals was also recorded during the withdrawal period.

Figure 3.1: Chronic cocaine administration and withdrawal protocol.
Male C57BL/6J mice were treated with chronic saline or “binge” escalating-dose (3 injections x 15-30 mg/kg, i.p./day) cocaine for 14 days and sacrificed (chronic administration groups). Another cohort of mice were treated with the same administration paradigm and left to spontaneously withdraw for 14 days (Day 28; 14-day withdrawal groups).

3.2.3 Behavioural experiments

3.2.3.1 Locomotor activity

Horizontal and vertical motor activities (basal and stimulated) were recorded everyday during the administration period, and 14 days following withdrawal (Day 28) as detailed in Section 2.2.3.1. The only difference being that motor activity was recorded for 60 minutes immediately following each of the three injections. Mice were returned to their home cages each day following completion of the locomotor activity measurement. Average activity from the 1h basal activity and average from the three daily injections were calculated.

3.2.3.2 Cocaine-induced stereotypy

All mice were scored for stereotypy behaviour 30 min after each injection for a period of 30 seconds. The rating was based on a slight modification of the scale used by Schlussman et al., (2003), Bailey et al., (2007), Schlussman et al., (2005) and Metaxas et al., (2012) and consists of a graded scale of behaviours, see Table 3.1. Stereotypy behaviour of each mouse was recorded during the saline/cocaine administration period (Days 1-14). Mice were also behaviourally assessed for stereotypy following 1, 8, 12
and 14 days of withdrawal (Days 15, 22, 26 and 28 respectively) at the same time points. The median stereotypy score of the three injections on each day was calculated for each treatment group.

Table 3.1: Cocaine stereotypy score

<table>
<thead>
<tr>
<th>Score</th>
<th>Predominant behaviour</th>
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<tbody>
<tr>
<td>1</td>
<td>Asleep, inactive</td>
</tr>
<tr>
<td>2</td>
<td>Alert, actively grooming</td>
</tr>
<tr>
<td>3</td>
<td>Increased sniffing (occasional light sniffing, often while exploring the cage)</td>
</tr>
<tr>
<td>4</td>
<td>Intermittent rearing and sniffing (two or three rears in a 20 sec period, with sniffing frequently at the apex of the rear)</td>
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<tr>
<td>5</td>
<td>Increased locomotion</td>
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<tr>
<td>6</td>
<td>Intense sniffing in one location (rapid sniffing, often with head down is the predominant behaviour displayed)</td>
</tr>
<tr>
<td>7</td>
<td>Continuous pivoting and sniffing with locomotion (no rearing)</td>
</tr>
<tr>
<td>8</td>
<td>Intermittent rearing and sniffing with locomotion (intermittent up and down rearing behaviour)</td>
</tr>
<tr>
<td>9</td>
<td>Maintained rearing and sniffing (animal remains up on hind legs throughout most of the observation period)</td>
</tr>
<tr>
<td>10</td>
<td>Splayed limbs</td>
</tr>
</tbody>
</table>

Mice were scored daily for saline- or cocaine- induced stereotypy behaviour for a period of 30 seconds, 30 min after each of the three administration injections and at specific days during withdrawal.

3.2.4 Neurochemical analysis

3.2.4.1 OTR autoradiography

Tissue preparation, quantitative autoradiographic binding of the OTR and subsequent autoradiographic procedures were carried out as detailed in Section 2.2.4.1.

3.2.4.2 Plasma corticosterone levels

Trunk blood from the mice was collected in EDTA-containing tubes and spun for 15 min at 2000 x g at 4°C. Plasma was collected and corticosterone levels were measured as detailed in Section 2.2.4.3.
3.2.5 Statistical Analysis

Body weight and food consumption changes were analysed using two-way repeated measures ANOVA for factors ‘treatment’ and ‘day (repeated factor)’. Basal and stimulated locomotor activity data during the chronic saline/cocaine administration paradigm were analysed using repeated measures two-way ANOVA for factors ‘treatment’ and ‘day (repeated factor)’. Basal locomotor data following the 14-day withdrawal period was analysed with an unpaired t-test. For the stereotypy behaviour repeated measures two-way ANOVA for factors ‘treatment’ and ‘day’ (repeated factor) was used. Because the expression of behavioural stereotypy was measured with a behavioural rating scale, non-parametric Mann Whitney U-test was used on individual test days to confirm ANOVA results. During withdrawal, stereotypy behaviour was analysed using Mann Whitney U-test.

For analysis of OTR binding and corticosterone levels, two-way ANOVA was performed in each individual brain region for factors ‘treatment (saline/cocaine)’ and ‘withdrawal effect’. All statistical analyses were performed using Statistica 8.0 analysis program (Statsoft Inc., France). Where ANOVA revealed a significant factor or interaction effect ($p<0.05$), a Bonferroni post-hoc test was carried out. All the values were expressed as mean ± SEM.
3.3 Results

3.3.1 Chronic cocaine administration and withdrawal induced marked alterations in body weight of mice

Cocaine administration reduced body weight (Figure 3.2, $F_{[1,10]}=14.477$, $p<0.01$) over time (Figure 3.2, $F_{[14,140]}=2.02$, $p<0.05$). This reduction in body weight persisted 1-day following withdrawal from chronic cocaine administration compared to saline controls ($p<0.001$). However, after 14 days withdrawal from cocaine (i.e. day 28) cocaine-withdrawn mice increased their body weight (Day 28 vs Day 14, $p<0.05$) and they did not show any significant differences compared to saline-treated animals (Figure 3.2, $p>0.05$).

![Weight changes](image)

**Figure 3.2: Effects of chronic cocaine administration and withdrawal on body weight of mice.**
Daily alterations in body weight during chronic cocaine or saline administration and withdrawal period. Cocaine administration significantly decreased body weight compared to saline controls which persisted 1-day following withdrawal (Day 15). No significant differences in body weight of cocaine withdrawn animals compared to controls were observed on Days 22, 26 and 28). Data are expressed as mean ± SEM (n=6 per group). *$p<0.05$ vs Saline control; †$p<0.01$ vs Saline 1-day withdrawal (Day 15); ‡*$p<0.05$ vs Cocaine Day 14 (repeated measures two-way ANOVA followed by Bonferroni post-hoc test). Dashed line indicates the start of withdrawal period.
3.3.2 Chronic cocaine administration significantly decreased food intake of mice

In order to investigate whether the decrease in body weight of animals following chronic cocaine administration was due to cocaine-induced hyperlocomotion or alterations in food intake, food consumption was recorded daily during the saline/cocaine administration paradigm. Cocaine administration reduced food intake (Figure 3.3, $F_{[1,10]}=121.91, p<0.001$) over time (Figure 3.3, $F_{[14,140]}=15.21, p<0.001$).

![Food Consumption](image.png)

**Figure 3.3: Effects of chronic cocaine administration on food intake of mice.**

Daily alterations in food intake during chronic cocaine or saline administration. Cocaine administration significantly decreased body weight compared to saline controls. Data are expressed as mean ± SEM (n=6 per group). **$p<0.01$, ***$p<0.001$ vs Saline control (repeated measures two-way ANOVA followed by Bonferroni post-hoc test).
3.3.3 Chronic cocaine administration increased locomotor activity of mice

Cocaine treatment increased horizontal activity ($F_{[1,9]}=88.74, p<0.001$) over time ($F_{[13,117]}=12.98, p<0.001$) during the chronic administration paradigm (Figure 3.3A). Cocaine-treated animals had increased locomotor activity compared to saline controls on days 2-14. Following 3 days of cocaine administration, there was a significant increase in cocaine-stimulated horizontal activity compared with that of Day 1 ($p<0.001$), indicating the acquisition of behavioural sensitisation to the locomotor-stimulating effect of cocaine. This increase in horizontal activity persisted following 14 days of withdrawal from this administration paradigm.

Chronic cocaine treatment significantly increased vertical locomotor activity compared to saline controls on all treatment days (Figure 3.3B, $F_{[1,9]}=10.69, p<0.05$). No ‘treatment’ x ‘time (day)’ interaction effect was observed ($p>0.05$) indicating a lack of sensitisation for that behaviour.
Figure 3.4: Chronic cocaine administration increased horizontal and vertical locomotor activity.

Male C57BL/6J mice were treated with either saline or cocaine with a “binge” 14-day escalating-dose paradigm (3 x 15-30 mg/kg, i.p. per day). Stimulated (A) horizontal and (B) vertical motor activity (representing rearing) were measured daily in 5 min bins for 1 hour post- each saline or cocaine injection during the administration period. The average activity of the hourly 5-min bins (for all the three injections) was calculated daily. Chronic cocaine administration increased horizontal activity compared to saline. Data are expressed as mean ± SEM (n=6 per group). ***p<0.001, *p<0.05 vs Saline; ##p<0.01, vs Cocaine Day 1 (repeated measures two-way ANOVA followed by Bonferroni post-hoc test).
3.3.4 Increase of basal locomotor activity following chronic cocaine pre-treatment and following protracted abstinence

Cocaine pre-treatment increased basal horizontal activity ($F_{[1,10]}=8.00$, $p<0.05$) over time (Figure 3.5A, $F_{[13,130]}=3.80$, $p<0.001$). Cocaine pre-treatment also increased basal vertical (rearing) activity ($F_{[1,10]}=17.31$, $p<0.01$) during the chronic administration paradigm (Figure 3.5B). The increased basal horizontal ($p<0.05$) and vertical ($p<0.001$) activity persisted following 14-days withdrawal compared to saline-withdrawn animals (Day 28) (Figure 3.5).
Figure 3.5: Chronic cocaine pre-treatment increased basal horizontal and vertical locomotor activity which persisted following a 14-day withdrawal period.
Male C57BL/6J mice were treated with either saline or cocaine with a "binge" 14-day escalating-dose paradigm (3 x 15-30 mg/kg, i.p. per day) and left to spontaneously withdraw for a period of 14 days (i.e. day 28). Basal (A) horizontal and (B) vertical motor activity (representing rearing) were measured daily in 5-min bins for 1 hour prior to saline or cocaine injection during the 14 days of administration paradigm. Basal activity was also measured for a period of 60 min, 14 days after the last treatment injection (Day 28). The average activity of the hourly 5-min bins was calculated daily. Chronic cocaine administration increased basal horizontal and vertical activity which persisted following 14-days of cocaine withdrawal. Data are expressed as mean ± SEM (n=6 per group). *p<0.05, **p<0.001 vs Saline (repeated measures two-way ANOVA followed by Bonferroni post-hoc test); † p<0.05, ‡‡‡p<0.001 vs Saline withdrawal (unpaired t-test). Dashed line indicates the start of withdrawal period.
3.3.5 Chronic cocaine induced stereotypic behaviours which did not persist following withdrawal in mice

Cocaine administration significantly increased stereotypy behaviours in mice. Two-way repeated measures ANOVA for factors ‘treatment’ and ‘day (repeated factor)’ revealed that cocaine administration increased stereotypy behaviours (Figure 3.6) of mice ($F_{[1,10]}=1713.42, p<0.001$) over time ($F_{[13,130]}=7.40, p<0.001$). Following 3 days of cocaine administration, there was a significant increase in cocaine-induced stereotypy behaviour compared with Day 1 ($p<0.001$), indicating the presence of sensitisation to the stereotypy behaviour induced by cocaine. This increase did not persist following 1, 8, 12 and 14 days of withdrawal from the cocaine administration (i.e. Days 15, 22, 26 and 28 respectively), where stereotypic behaviour observations were similar to saline-withdrawn animals (Figure 3.6, $p>0.05$, non-parametric Mann-Whitney U-test).

**Stereotypy Scores**

![Graph showing stereotypy scores](image)

Figure 3.6: Effects of chronic cocaine administration and withdrawal on stereotypy behaviour of mice.

Cocaine treatment resulted in a significant increase in stereotypy scores and acute sensitisation. Cocaine-induced stereotypy behaviour was absent in 1-, 8-, 12- and 14-day withdrawn mice (i.e. Days 15, 22, 26 and 28 respectively). Data are expressed as mean ± SEM (n=6 per group). **$p<0.001$ vs Saline, *$p<0.001$ vs Cocaine Day 1** (repeated measures two-way ANOVA followed by Bonferroni post-hoc test). Dashed line indicates the start of withdrawal period.
3.3.6 Chronic administration and protracted withdrawal from cocaine induced profound alterations in the plasma corticosterone levels

Two-way ANOVA showed a significant 'treatment' effect ($F_{[1,35]}=15.88$, $p<0.001$) on the plasma corticosterone levels. Chronic cocaine treatment increased plasma corticosterone levels (Figure 3.6, $p<0.05$), which persisted following a 14-day withdrawal period (Saline withdrawal vs Cocaine withdrawal, $p<0.05$).

![Graph showing increased corticosterone levels following chronic administration and withdrawal from cocaine in mice.](image)

**Figure 3.7**: Increased corticosterone levels following chronic administration and withdrawal from cocaine in mice.

Male C57BL/6J mice were treated with either saline or cocaine with a “binge” 14-day escalating-dose paradigm (3 x 15-30 mg/kg, i.p. per day) or left to spontaneously withdraw for a period of 14 days. Plasma corticosterone levels were measured in chronic saline-treated, cocaine-treated, saline-withdrawn and cocaine-withdrawn mice. Data are expressed as mean ± SEM (n=6-12 per group) *$p<0.05$ vs Saline control; †$p<0.001$ vs Saline withdrawal (two-way ANOVA followed by Bonferroni post-hoc test).
3.3.7 Chronic administration and protracted abstinence from cocaine induced marked region-specific alterations in oxytocin receptor binding in the brain of mice

Quantitative autoradiographic binding of $[^{125}I]$-OVTA was performed in coronal brain sections of chronic saline-treated, cocaine-treated and protracted saline-withdrawn and cocaine-withdrawn mice. Regional 2-way ANOVA for factors ‘treatment’ x ‘withdrawal’ showed a significant ‘treatment’ effect on the OTR binding in the Pir ($F_{[1,18]}=11.86, p<0.01$), LS ($F_{[1,16]}=15.54, p<0.01$) and the Amy ($F_{[1,18]}=29.11, p<0.001$). A significant ‘withdrawal’ effect was observed in the MS ($F_{[1,18]}=11.26, p<0.01$) and the VDB ($F_{[1,18]}=6.38, p<0.05$) and a significant ‘treatment’ x ‘withdrawal’ interaction was identified in the MS ($F_{[1,18]}=7.07, p<0.05$) and the VDB ($F_{[1,18]}=16.13, p<0.001$). Chronic cocaine administration increased OTR binding in the Amy compared with saline control ($p<0.01$), which persisted following a 7-day withdrawal period ($p<0.05$) (Figure 3.7). Fourteen days withdrawal from chronic cocaine administration induced a significant increase in OTR binding in the MS ($p<0.05$), VDB ($p<0.01$) and the LS ($p<0.05$) compared with saline-withdrawn animals (Figure 3.7).
Male C57BL/6J mice were treated with either saline or cocaine with a “binge” 14-day escalating-dose paradigm (3 x 15-30 mg/kg, i.p. per day) or left to spontaneously withdraw for a period of 14 days. (A) Computer-enhanced representative autoradiograms of adjacent coronal brain sections from chronic saline-treated, cocaine-treated, saline-withdrawn and cocaine-withdrawn mice at the level of the olfactory nuclei (Bregma 2.46mm, first row), the caudate putamen (Bregma 0.86mm, second row), the septum (Bregma 0.14mm, third row) and the thalamus (Bregma -2.06mm, fourth row). OTRs were labelled with [125I]-OVTA (50pM). The colour bar illustrates a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. Representative images for the non-specific binding (50pM [125I]-OVTA in the presence of 50pM unlabelled oxytocin) are shown for all the treatment groups. (B) Quantitative OTR binding in brain regions of mice treated with a chronic “binge” escalating-dose cocaine administration paradigm and in mice-withdrawn for 14 days from this paradigm. Data are expressed as mean ± SEM (n=5-6) specific [125I]-OVTA binding (fmol/mg tissue). **p<0.01 vs Saline control; *p<0.05; ***p<0.01 vs Saline 14 days withdrawal (two-way ANOVA followed by Bonferroni post-hoc test). Abbreviations: Acb, nucleus accumbens; Amy, amygdala; AOL, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial; AOV, anterior olfactory nucleus-ventral; CgCx, cingulate cortex; CPu, caudate-putamen; Hip, hippocampus; LS, lateral septum; MS, medial septum; Pir, piriform cortex; PV Th, paraventricular nucleus of thalamus; Th, thalamus; Tu, olfactory tubercle; VDB, vertical limb of the diagonal band of Broca.

Figure 3.8: Region-specific up-regulation of [125I]-OVTA binding following protracted withdrawal from cocaine administration.
3.4 Discussion

The aim of this study was to determine the effects of chronic administration and protracted withdrawal from cocaine on the OTR system in the brain. This is the first study showing that prolonged abstinence from chronic cocaine administration induces marked up-regulation of the OTR binding in the Amy and septum of mice.

Food intake and weight changes that chronic cocaine addicts experience were mimicked in a mouse model (Brewer et al., 2008, Lynch et al., 2008, Williamson et al., 1997), with a significant decrease in food intake and body weight in mice chronically treated with cocaine and a rebound increase in weight gain following withdrawal. Indeed, cocaine abusers have repeatedly reported decreased appetite during cocaine use which returned to normal after cessation of drug taking (Lynch et al., 2008, Williamson et al., 1997). However, the nutritional effects of cocaine is beyond the scope of this thesis; thus, for further information the reader is directed to an extended review by Mohs et al., (1990).

Chronic “binge” escalating-dose cocaine administration increased both horizontal and vertical locomotor activities of mice. These findings are in line with previous findings showing increased locomotor activity following both steady-dose (e.g. Koff et al., 1994, Schlussman et al., 2003, Tolliver and Carney, 1994, Bailey et al., 2008, Bailey et al., 2007, Metaxas et al., 2012) and escalating-dose (Bailey et al., 2008, Schlussman et al., 2005) cocaine administration. Horizontal activity of cocaine-treated animals was significantly higher on Day 3 (15 mg/kg) compared to Day 1 (15 mg/kg) indicating the acquisition of rapid sensitisation to the locomotor activity which reached a plateau after the Day 4 of administration. Bailey et al., (2008) and Metaxas et al., (2012) observed a
similar pattern of cocaine-induced locomotor sensitisation by using a steady-dose “binge” cocaine administration paradigm in the same strain of mice.

Additionally, this is the first study to show increased basal horizontal and vertical locomotor activities following previous exposure to cocaine by using an escalating-dose “binge” administration paradigm, an effect which persisted following 14 days of spontaneous withdrawal. This finding shows a persistent cocaine-induced contextual sensitisation (i.e. the animals show behavioural sensitisation in an environment paired with the drug injection). The environment associated with drug administration has been shown to modulate both the development as well as the expression of behavioural sensitisation (Badiani and Robinson, 2004). Generally, in rodents, behavioural sensitisation is measured in terms of locomotor or stereotypic (including rearing) activity, although it is thought that locomotor sensitisation may only represent indirect evidence of hypersensitivity of the motivation circuit, which contributes to the addictive ‘wanting’ (see Robinson and Berridge, 2008). Locomotor sensitisation in the drug-paired environment has been linked with drug craving (see Robinson and Berridge, 2008), which might contribute to relapse following abstinence. Indeed, a low dose “challenge” with a previously paired drug during long-term abstinence from the administration paradigm induces the expression of behavioural sensitisation (see Pierce and Kalivas, 1997). The observed baseline sensitisation following cocaine pre-treatment is in contrast with the hypolocomotion induced by morphine in basal activity of the mice (see Chapter 2, Figure 2.6). This discrepancy is due to the anhedonic effects induced by chronic morphine administration and withdrawal and consequently the decrease in basal locomotor activity compared to the contextual sensitisation induced by cocaine administration. Opioids and psychostimulant drugs have been shown to exert
differential effects after chronic administration as well as during withdrawal, which affects their behavioural phenotype (Kreek et al., 2012).

We hypothesised that the oxytocinergic system may be involved in the observed expression of behavioural sensitisation in withdrawn mice since the oxytocinergic system interacts with the DA system (see Section 1.8.1) and DAergic neurotransmission in the striatum is involved in the modulation of cocaine-induced sensitisation (see Narendran and Martinez, 2008) and stereotypy behaviour (Staton and Solomon, 1984, Kuczenski et al., 1991, Segal and Kuczenski, 1992). Moreover, cocaine withdrawal has been found to induce a marked desensitisation of the DA D₉ autoreceptors (Jones et al., 1996, Pierce et al., 1995), an effect which is at least partly responsible for the expression of behavioural sensitisation (Antelman and Chiodo, 1981, Dwoskin and Zahniser, 1986). Since OTR is co-localised and facilitates allosteric functional interactions with D₂ receptors in the Acb (Romero-Fernandez et al., 2012), it is possible that OTR system is involved in the regulation of the basal hyperlocomotor activity observed during chronic protracted withdrawal from cocaine. Indeed, OT administration has been shown to facilitate cocaine-induced behavioural locomotor sensitisation in rodents possibly via interacting with the DAergic system within the striatum (Sarnyai et al., 1992b). Although the present study showed an up-regulation of OTR in the Amy and not the striatum following chronic administration of cocaine and withdrawal, it is important to highlight the possibility of OTR dysregulation taking place in the Amy, to influence DAergic neurotransmission in the Acb and thus modulating the observed basal hyperlocomotion and motivation to drug-taking. A clear connection between the Acb and the Amy has been proposed to control limbic function and motivation. For instance, it has been suggested that the CeA affects basal DAergic tone in the VTA,
thereby regulating DAergic tone in the Acb and modulating the incentive value of environmental stimuli (Phillips et al., 2003).

The dysregulation of the oxytocinergic system during cocaine withdrawal was localised in the septum and Amy. Considering the role of the Amy and septum in the antidepressant, anxiolytic and/or pro-social actions of OT (Curley et al., 2012, Debiec, 2005, Domes et al., 2007a, Lukas et al., 2012), the up-regulation of the OTR binding in these regions might be a neuroadaptive response of the oxytocinergic system to counteract the negative consequences of cocaine withdrawal. Indeed, comorbidity of cocaine addiction and depression has been reported (Schmitz et al., 2001) and early clinical observations demonstrated that cocaine use and abstinence are able to induce anxiety (Mackler and O'Brien, 1992, O'Brien, 1996); also see Section 1.5. These findings are supported by several pre-clinical evidence showing that withdrawal from cocaine can cause anxiety-and depressive- like behaviour in rodents (Paine et al., 2002, Blanchard and Blanchard, 1999, Basso et al., 1999, Ambrose-Lanci et al., 2010, Perrine et al., 2008). Therefore, the up-regulation of the OTR density following prolonged withdrawal may be a compensatory mechanism to counteract depressive- and anxiety-like behaviours that serve as motivational trigger to re-take the drug and relapse. Indeed, like the case of morphine withdrawal, OTR up-regulation in the Amy and septum was associated with emotional impairment (see Chapter 2).

The exact mechanism underlying the persistent central oxytocinergic system dysregulation following chronic administration of cocaine needs further investigation. However, it can be postulated that the OTR up-regulation may possibly be a compensatory consequence of a hypo-oxytocinergic state. In support of this, Sarnyai et al., (1992c) found decreased hypothalamic OT synthesis and reduced plasma OT levels in rats chronically treated with cocaine indicating a hypo-oxytocinergic state. Deficits in
plasma OT levels were also reported in mothers who were exposed to cocaine during pregnancy (Light et al., 2004). In fact, this is in accordance with the morphine data (see Chapter 2) where we observed hypo-oxytocinergic activity following chronic morphine administration and withdrawal. Generally, an increase in OT peptide or administration of an OTR agonist is concomitant with rapid OTR desensitisation, clathrin-independent internalisation and subsequent downregulation (Evans et al., 1997). Therefore, it is likely that the up-regulation of the OTR in the Amy and septum is the result of decreased OT levels in the brain, and this mechanism to be at least partly responsible for the emotional deficits induced by cocaine abstinence.

Another possible mechanism by which chronic cocaine administration and withdrawal induced up-regulation of the OTR binding in stress-related regions of the brain is via the hyper-activation of the HPA axis. Indeed, in the present study, we found increased corticosterone levels following 14 days of escalating-dose “binge” cocaine administration which persisted after 14 days of withdrawal in mice. This finding is consistent with Zhou et al., (1999a) who showed that 14-day “binge” cocaine administration increased plasma corticosterone and ACTH levels in rats, indicating an activation of the HPA axis. Dysregulation of the HPA axis has been associated with emotional impairment including depression (see Pariante and Lightman, 2008), anxiety (see Graeff, 2007) and social phobia (Condren et al., 2002), which are all consequences of cocaine addiction and withdrawal (see Sections 1.5). There is ample evidence showing that activation of the oxytocinergic system has a marked anxiolytic, anti-aggressive and antidepressant effect, partly by inhibiting CRF release in the Hyp (Baumgartner et al., 2008, Kirsch et al., 2005, Di Simplicio et al., 2009, Dabrowska et al., 2011, Windle et al., 2004, Neumann et al., 2000b). Therefore, the dysregulation of the OTR following cocaine administration and withdrawal may be a cause or a
consequence of a dysregulated HPA axis. However, this hypothesis needs further investigation.

In conclusion, this is the first study to demonstrate profound alterations in the oxytocinergic system following chronic cocaine administration and withdrawal in specific brain regions. It is also shown that this cocaine-induced up-regulation of OTR in the Amy and septum is accompanied by a dysregulation of the HPA axis. This may have implications in the understanding of the role of the oxytocinergic system in modulating a number of neurochemical and behavioural effects of cocaine including behavioural sensitisation, anxiety and depression. Understanding the role of OT may prove important for the development of drugs targeting the OTR as useful targets for treatment of cocaine addiction and the prevention of relapse.
CHAPTER 4

Chronic methamphetamine treatment induces oxytocin receptor up-regulation in the amygdala and hypothalamus via an adenosine $A_{2A}$ receptor-independent mechanism
4.1 Introduction

Methamphetamine, or methylamphetamine, is the N-methylated analogue of the psychostimulant amphetamine sharing common pharmacological properties (Melega et al., 1995). MAP is a widely abused drug and its use has become of public health concern in many parts of the world. The relatively easy clandestine manufacture and consequently low street cost of MAP has meant that its use has reached near-epidemic proportions in the United States (see Gonzales et al., 2010) and multiple cases of epidemic MAP use in Japan have been also reported (see Wada, 2011). MAP is a highly addictive psychostimulant drug (Woolverton et al., 1984) that acutely stimulates DA, NA and 5-HT release via either amine redistribution from synaptic vesicles to the cytoplasm, or by reversing the action of monoamine transporters (Krasnova and Cadet, 2009, see Sulzer et al., 2005). Actions of MAP on striatal DAergic nerve terminals are hypothesised to account for its acute rewarding effects (Vollm et al., 2004).

Chronic use of MAP leads to neurotoxic effects by either damaging the striatal DAergic neurons (Wagner et al., 1980), or by the formation of reactive oxygen species (O'Dell et al., 1991, Cubells et al., 1994, Yamamoto and Zhu, 1998, LaVoie and Hastings, 1999), or even via glutamate excitotoxicity as a consequence of MAP-induced glutamate release in the striatum (Nash and Yamamoto, 1992, Abekawa et al., 1994, Stephans and Yamamoto, 1994). Repeated administration of MAP has also been shown to induce emotional impairments including social avoidance and isolation, aggressiveness, depression and anxiety and can also induce psychotic behaviour in humans (Grant et al., 2012, McKetin et al., 2011). Currently, pharmacotherapy for addiction to psychostimulant drugs, including MAP, is limited (Ciccarone, 2011).
Although several mechanisms have been suggested, the molecular mechanism underpinning the addictive properties of chronic MAP use and its behavioural and emotional consequences remain unclear. More recently, centrally administered OT has been shown to reduce both MAP-induced hyperlocomotion and DA release in the Acb in mice (Qi et al., 2008), demonstrating a key role for OT in the modulation of behavioural effects of MAP, possibly via a DAergic-dependent mechanism. Moreover, i.c.v. OT treatment attenuated MAP-induced CPP and abolished stress-induced reinstatement to MAP-seeking in mice (Qi et al., 2009). In rats, peripheral treatment with OT reduced MAP self-administration and MAP-induced reinstatement (Carson et al., 2010a), providing further evidence for its “antireward/anticraving” and relapse prevention potential for MAP addiction. Interestingly, OT also reduced MAP-induced Fos neuronal activation within the STh and the Acb core of rats (Carson et al., 2010b), which are part of the basal ganglia. Given the prominent role of the basal ganglia in the rewarding, motivational, motor, compulsive and stereotypic behavioural effects of MAP (Bergman et al., 1990, Canales and Graybiel, 2000, Chiang et al., 2009, Klavir et al., 2009, Lardeux and Baunez, 2008, Smith-Roe and Kelley, 2000, Winter et al., 2008), these results provide further insight into the way in which OT might moderate rewarding and compulsive behavioural effects of MAP. Nonetheless, the mechanism by which the oxytocinergic system modulates these addictive processes remains unclear.

A mechanism underlying the beneficial effect of OT on modulating MAP’s behavioural effects may involve an interaction with adenosine A2A receptors. There is emerging evidence suggesting an involvement of central adenosine A2A receptors on endogenous oxytocinergic function in the brain. Indeed, the ubiquitous neuromodulator adenosine has been shown to play an integral role in the neuroendocrine function of SON neurons (vasopressin and oxytocin containing), via its action on A1 and A2A receptors. While A1
activation suppresses neurons of the SON (Ponzio and Hatton, 2005), activation of A2A receptors located on post-synaptic SON neurons and astrocytes causes depolarisation and excitation of those neurons (Ponzio et al., 2006). Although yet unknown, it is likely that A2A receptors may facilitate the excitation of other oxytocinergic neuronal sub-populations (e.g. PVN projections to the reward centers of the brain). Similarly to the effect of OT, activation of A2A receptors has a prominent role in modulating MAP behavioural effects including attenuation of MAP-induced hyperactivity (Shimazoe et al., 2000) and stimulus discrimination (Munzar et al., 2002), at least partly by inhibiting MAP-induced elevation in DA output in the striatum (Golembiowska and Zylewska, 1998, Yoshimatsu et al., 2001).

As a result, it is likely that OT might exert its modulatory effect on MAP-induced behaviours via an A2A receptor-dependent mechanism. At the receptor level, while structural interactions between OTR and A2A receptors have yet to be identified, A2A receptors are well known to be co-localised and form opposing functional interactions with DA D2 receptors in the striatum (Ferre et al., 2007, Ferre et al., 2008). DA D2 receptors are strongly associated with addictive processes and are consistently decreased in human drug addicts (Volkow et al., 2009) including MAP abusers (Volkow et al., 2001, Hume et al., 1996) and in animal models of MAP addiction (Kokoshka et al., 1998). As OTR and D2 receptors have recently been shown to co-localise and facilitate allosteric functional interactions in the Acb (Romero-Fernandez et al., 2012), it is tempting to speculate that OTR may also co-localise and/or functionally interact (i.e. form heterodimers) with A2A in the striatum and this interaction might be involved in the modulatory effect of OT on MAP-induced behavioural effects.
Based on the above, it has been hypothesised that chronic MAP use induces profound alterations in the oxytocinergic system, which may be involved in the modulation of various behavioural and neurochemical effects of the drug. To test this hypothesis, this study aimed firstly to investigate whether chronic MAP administration induces alterations in the central OTR system in the mouse brain with the use of quantitative receptor autoradiographic binding. Further, autoradiographic OTR binding in brain sections of wild-type (WT) and $A_{2A}$ receptor knockout ($A_{2A}R^{-/-}$) mice treated with a chronic steady-dose MAP administration paradigm was carried out to demonstrate whether possible MAP-induced alterations in the OTR system are mediated via an $A_{2A}$ receptor-dependent mechanism.
4.2 Methods

4.2.1 Maintenance and genotyping of the adenosine \( A_{2A} \) receptor knockout mouse colony

The CD-1 adenosine \( A_{2A} \)R\(^{+/-} \) mice were originally created by Ledent \textit{et al.} (1997) and generously supplied in order to maintain a breeding colony within the University of Surrey. The mice were bred from heterozygote breeding pairs which originated from the original crossing of a number of heterozygote males from the existing colony each with a WT female from an external supplier (Charles River, Margate, Kent, UK). The male and female heterozygotes from the litters of the newly created breeding pairs were paired and these formed the main breeding colonies which were maintained for approximately one year before new breeding pairs were created. The mice were housed in groups of approximately 4-5 mice per cage in a temperature-controlled environment on a 12 hour light-dark cycle (lights on: 06:00am) and were fed a standard diet with water available \textit{ad libitum}. All experimental procedures were conducted in accordance with the UK Animal Scientific Procedures Act (1986).

A polymerase chain reaction (PCR) based method was used to genotype the mice at weaning (three weeks old). Tail tip samples of no more than 3 mm were taken from each mouse and deoxyribonucleic acid (DNA) was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. In brief, all samples were incubated in a water bath at 55\(^\circ\)C with proteinase K (Sigma-Aldrich, Dorset, UK) overnight. The following day cells in the sample were lysed with ethanol and transferred to individual spin columns, composed of a silica-gel membrane allowing DNA to selectively bind. Two centrifugation steps with washes followed, removing any contaminants and binding the DNA to the column. The spin columns were transferred to collection tubes and the DNA extracted upon the addition of a buffer, releasing the
DNA from the membrane. The extracted DNA was then subjected to PCR amplification using puReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) and three custom primers (Sigma-Genosys, Suffolk, UK):

1) The common forward primer: AGTCATGGTTTCGGGAGATG
2) The wild-type reverse primer: ACCATGATGTACACCGAGGAG
3) Adenosine $A_{2A}$ receptor knockout primer: AAGGAAGGGTGAGAACAGAG

These were used to selectively amplify fragments of different sizes from the WT and adenosine $A_{2A}$ receptor knockout alleles. Primers 1 and 2 amplified a 229 base pair band from the WT allele, whereas primers 1 and 3 amplified a 572 base pair from the mutated allele. Heterozygous mice carrying a copy of both alleles resulted in 2 bands, enabling the identification of the 3 genotypes (Figure 4.1). A total volume of 25 μl per reaction was made up of 2 μl of each primer, 6 μl of DNA sample and 13 μl of double-autoclaved MilliQ water. The cycle parameters for the PCR were as follows:

1) **Pre-cycle** 2 minutes 94°C
2) **Denaturation** 30 seconds 94°C
3) **Annealing** 1 minute 15 seconds 72°C
4) **Elongation** 1 minute 72°C
5) **Post-cycle** 5 minutes 72°C

This process was repeated for 40 cycles.

The PCR products were separated by gel electrophoresis using 2% agarose double comb e-gels (Invitrogen, Carlsbad, CA, USA) allowing the identification of the genotypes by comparison to the HyperLadder I DNA ladder (Bioline, London, UK); see Figure 4.1.
Figure 4.1: Representative sample showing the separation of the PCR product of the genotyping reaction visualised by gel electrophoresis.
Lane 1: +/+ wild-type allele, 229 base-pairs (bp), Lane 2: +/- heterozygote, 229 and 572 bp, Lane 3: DNA Ladder, Lane 4: -/- A2A receptor knockout allele, 572 bp.
4.2.2 Animals

Male, 8-12 week old CD-1 wild-type and A2AR^{+/-} mice, derived from a heterozygous breeding programme were used in this study, as detailed in Section 4.2.1. Mice were singularly housed for a period of 7 days prior to the start of the study and throughout the whole study period. Mice were habituated to home cages and experimenter handling for one week before the start of experiment. The home cages were in a temperature-controlled environment and on a 12 hour light-dark cycle (lights on: 06:00am). Whilst in home cages, mice had access to food and water ad libitum. All experimental procedures were conducted in accordance with the UK Animal Scientific Procedures Act (1986).

4.2.3 Chronic steady-dose methamphetamine administration paradigm

Mice were randomly divided into four groups; chronic saline-treated WT and A2AR^{+/-} and chronic MAP-treated WT and A2AR^{+/-} treated animals. Chronic MAP-treated animals were injected i.p. for 10 days with MAP (1mg/kg) (Sigma-Aldrich, Poole, UK), once per day (11:00), in accordance with small modifications of studies carried out by Liang et al., (2006). The chronic saline-treated group were administered with saline (4 ml/kg, i.p.) for 10 days, once per day (11:00).

4.2.4 Autoradiographic binding of the adenosine A2A receptor

In order to confirm that the genotype of mice used had been correctly identified by PCR, autoradiographic binding of the adenosine A2A receptor was undertaken in all experimental mice used.

Sections were taken from the brains of all the experimental mice. Adjacent non-specific binding slides were not required to conduct qualitative confirmation of A2A receptor binding. Slides were defrosted thoroughly for 30 minutes and then pre-incubated at
room temperature for 30 minutes in a 170 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 2U/ml adenosine deaminase (Sigma-Aldrich, Dorset, UK). The A2A receptor agonist [³H]-CGS 21680 (Amersham International plc., Buckinghamshire, UK) was used to label A2A receptors at a 10 nM concentration, 3-4 times Kd of ligand as previously described in the literature (Johansson and Fredholm, 1995, Bailey et al., 2002b). Total binding was determined by incubating the sections with 10nM [³H]-CGS 21680 in an incubation buffer solution (50 mM Tris-HCl and 10 mM MgCl2, pH 7.4 for 2 hours at room temperature. The slides were then washed 3 times for 5 minutes in ice-cold rinse buffer (170 mM Tris-HCl, pH 7.4) followed by a 5 minute rinse in ice-cold distilled water. The slides were dried under a cold stream of air for 2 hours and stored in an airtight box with anhydrous calcium sulphate for 7 days at room temperature for further drying. Radioligand bound sections were apposed to film for a period of 3 weeks (at room temperature).

4.2.5 Autoradiographic binding of the oxytocin receptor

Tissue preparation, quantitative autoradiographic binding of the OTR and subsequent autoradiographic procedures were carried out as detailed in Section 2.2.4.1.

4.2.6 Statistical analysis

Three-way ANOVA for the factors treatment, genotype and brain region were used to compare receptor binding levels in saline- and MAP-treated, WT and adenosine A2A receptor−/− animals. Moreover, two-way ANOVA was performed in each individual brain region for factors ‘treatment’ and ‘genotype’. All statistical analyses were performed using Statistica 8.0 analysis program (Statsoft Inc., France). Where ANOVA yielded significant main effects (p<0.05), a Bonferroni post-hoc test was carried out. All the values are presented as mean ± SEM of 5-6 animals/group.
4.3 Results

4.3.1 Autoradiographic binding of the adenosine A$_{2A}$ receptor for genotype confirmation in wild-type and adenosine A$_{2A}$ receptor knockout mice used in methamphetamine experiments

Sections from the brains of each experimental animal were bound with the radiolabelled A$_{2A}$ receptor agonist $[^{3}H]$-CGS 21680 to confirm that the genotype determined by the PCR and DNA electrophoresis procedures were correct. Autoradiographic binding confirmed genotyping was correct in all animals. Representative images showing the presence (WT) and absence (A$_{2A}$R$^{-/-}$) of A$_{2A}$ receptors are shown in Figure 4.2.

![Figure 4.2](image)

Figure 4.2: Representative computer-enhanced pseudocolour autoradiograms of adjacent coronal brain sections labelled with $[^{3}H]$-CGS 21680 from wild-type and adenosine A$_{2A}$ receptor knockout mice. Brain sections were incubated for 120 min in $[^{3}H]$-CGS 21680 (10mM) to label A$_{2A}$ receptors. Sections presented are taken from the level of the striatum (Bregma 1.18mm and 0.62mm, first and second row respectively), at the level of the globus pallidus (Bregma -1.22 mm, third row) and at the level of the thalamus (Bregma -1.94 mm, fourth row).
4.3.2 Chronic administration of methamphetamine induced region-specific alterations of $[^{125}]$-OVT binding following chronic methamphetamine treatment in both wild-type and $A_{2A}$ receptor knockout mice

High levels of OTR binding (1.17-1.46 fmol/mg) were observed within the olfactory nuclei, moderate levels (0.33-0.85 fmol/mg) in the Pir, septum, Amy and Hyp, and low levels (0.03-0.24 fmol/mg) in the cingulate cortex, thalamus, Acb, olfactory tubercle, caudate-putamen in all treatment groups (Figure 4.3). Three way-ANOVA showed significant 'treatment' effect ($F_{[1,273]}=13.22, p<0.001$) and region effect ($F_{[4,273]}=4.27, p<0.001$). No effect of 'genotype' ($p>0.05$) or 'genotype' x 'treatment' interaction effects were observed ($p>0.05$). Two way-ANOVA for factors 'treatment' and 'genotype' in each individual brain region revealed a significant treatment effect in the Amy ($F_{[1,19]}=73.21, p<0.001$) and the Hyp ($F_{[1,19]}=28.08, p<0.001$) but no effect of genotype was observed in any of these brain regions ($p>0.05$). Chronic MAP treatment induced OTR up-regulation in both WT (78%) and $A_{2A}R^{−/−}$ mice (54%) in the Amy and 52% (WT) and 55% ($A_{2A}R^{−/−}$) in the Hyp (Figure 4.3). Chronic MAP administration significantly increased OTR binding in both the Amy ($p<0.001$) and the Hyp ($p<0.001$) of WT and $A_{2A}R^{−/−}$ mice compared with their saline controls (Bonferroni post-hoc analysis). No other treatment or genotype effect was observed in any other brain regions analysed ($p>0.05$).
Figure 4.3: Region-specific up-regulation of [$^{125}$I]-OVTA binding following chronic methamphetamine administration in wild-type and $A_{2A}$ receptor knockout mice.

Male WT and $A_{2A}^{R^-}$ mice were treated with either saline or MAP with a 10-day steady-dose paradigm (1 x 1 mg/kg, i.p. per day). (A) Computer-enhanced representative autoradiograms of adjacent coronal brain sections from chronic saline and MAP WT and $A_{2A}^{R^-}$ mice at the level of the olfactory nuclei (Bregma 2.46, first row), the caudate (Bregma 0.86mm, second row), the septum (Bregma 0.14, third row) and the thalamus (Bregma -2.06, fourth row). OTRs were labelled with [$^{125}$I]-OVTA (50pM). The colour bar illustrates a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. Representative images for the non-specific binding (NSB) (50pM [$^{125}$I]-OVTA in the presence of 50pM unlabelled oxytocin) are shown for all the treatment groups. (B) Quantitative oxytocin receptor autoradiographic binding from WT and $A_{2A}^{R^-}$ mice treated with a chronic MAP administration paradigm. Data are expressed as mean ± SEM (n=5-6 per group) specific binding of [$^{125}$I]-OVTA (fmol/mg tissue) in left and right brain hemispheres. **$p<0.01$, ***$p<0.001$ vs WT Saline; $*p<0.05$, ****$p<0.001$ vs $A_{2A}^{R^-}$ Saline (Two-way ANOVA followed by Bonferroni post-hoc test). Abbreviations: Acb, nucleus accumbens; Amy, amygdala; AOl, anterior olfactory nucleus-lateral; AOM, anterior olfactory nucleus-medial; AOV, anterior olfactory nucleus-ventral; CgCx, cingulate cortex; CPu, caudate-putamen; Hip, hippocampus; LS, lateral septum; MS, medial septum; Pir Cx, piriform cortex; Th, thalamus; Tu, olfactory tubercle; VDB, ventral limb of the diagonal band of Broca; VMH, ventromedial hypothalamus.
4.4 Discussion

This is the first study to demonstrate profound region-specific alterations of the OTR system in the brain following chronic MAP administration, which may be involved in the modulation of long-term behavioural and neurochemical adaptations taking place during and following chronic drug use. The present data show that in WT and A2AR \(^{+/−}\) mice, chronic MAP induces a 50-80% increase of OTR binding in the Hyp and Amy and also demonstrated that this dysregulation of the OTR system is not dependent on an A2A receptor-mediated mechanism.

Although dysregulation of the oxytocinergic system have been reported following acute or repeated alcohol (Kovacs et al., 1998), cocaine (Sarnyai, 1998, Sarnyai et al., 1992c), \(\gamma\)-hydroxybutyric acid (GHB) (van Nieuwenhuijzen et al., 2010), MDMA (Thompson et al., 2007) and morphine (Kovacs et al., 1987a) administration in different brain regions of rodents, this is the first study to report any oxytocinergic neuroadaptations following chronic MAP administration. The up-regulation of the OTR following chronic MAP treatment was localised in the Amy and the Hyp, two regions involved in stress regulation. This is in agreement with van Nieuwenhuijzen et al., (2009, 2010), who also showed an increase in hypothalamic OTR and OT mRNA levels following repeated administration of other drugs of abuse (GBH and MDMA respectively) in the rat; and an increase of OTR binding in the Amy following treatment with cocaine in rat dams (Johns et al., 2004). In contrast to the present results, Jarrett et al., (2009) observed a reduction in OTR binding density in the ventromedial hypothalamus and failed to demonstrate an increase of OTR binding in the central nucleus of the Amy of postpartum rat dams repeatedly treated with cocaine. Nonetheless, the MAP-induced up-regulation observed in our study was profound (50 %) and was replicated in genetically modified A2AR \(^{+/−}\) mice.
The significance of this MAP-induced up-regulation of OTRs is yet to be determined. However, given the ability of OT to modulate MAP-induced hyperactivity (Carson et al., 2010a, Qi et al., 2008) and to attenuate MAP-induced CPP (Qi et al., 2009) and stress- and priming-induced reinstatement of MAP self-administration (Carson et al., 2010a), or CPP (Qi et al., 2009), it is likely that this oxytocinergic alteration may underlie some behavioural consequences of chronic MAP use. Indeed, it has been suggested that based on the ability of OT to suppress c-Fos activity in brain regions associated with motivation and reward such as subthalamic nucleus (STh) and the Acb (Carson et al., 2010b), and the ability of OT to reduce MAP-induced DA activity in Acb (Qi et al., 2008), OT may be involved in the modulation of the incentive motivational properties of MAP. Based on these observations, alterations of OTR would have been expected in the STh and the Acb following chronic MAP treatment. The reason why these alterations were not observed in these regions may be due to the relatively low expression of OTR in these regions in the CD1 mouse used in the present study (Figure 4.3). This does not, however, preclude the possibility that changes in OT release occur in these brain regions following chronic MAP administration, which may indeed lead to long-lasting morphological, functional and behavioural changes.

It is also important to highlight the possibility of OTR dysregulation taking place in the Amy in the present study, to influence DAergic neurotransmission in the Acb and thus motivation of MAP-seeking behaviour. A clear connection between the Acb and the Amy has been proposed to control limbic function and motivation. For instance, it has been suggested that the CeA affects tonic DAergic tone in the VTA, thereby regulating DAergic tone in the Acb and modulating the incentive value of environmental stimuli (Phillips et al., 2003). As a result the profound up-regulation of OTR system following
chronic MAP administration in the Amy may affect DA levels in the Acb in order to modulate its addictive-related behavioural effects.

The Amy is a limbic structure which is involved in a number of facets of emotional analysis (Phelps and LeDoux, 2005) and is a key component of social cognitive circuitry in both animals and humans (Rosenfeld et al., 2011). There is considerable evidence demonstrating abnormalities in the Amy following chronic MAP use (Kim et al., 2011, London et al., 2004), which are likely to be at least partly responsible for the development of anxiety, stress, depression (Dawe et al., 2009, Goeldner et al., 2011, Morley et al., 2001, Thompson et al., 2004) and social cognitive deficits (Dawe et al., 2009, Volkow et al., 2011) following chronic drug use. As a result, it is possible that the up-regulation of amygdalar OTR following chronic MAP treatment might be involved in the modulation of the aforementioned behaviours. There is a growing body of evidence demonstrating the anxiolytic, antidepressant and stress relieving properties of OT partly due to its action in the Amy (see Debiec, 2005). In addition, the Amy is partly responsible for the pro-social behavioural enhancing properties of OT (Domes et al., 2007a). It is not clear if the up-regulation of OTR in the Amy is a rebound consequence of a possible reduction of OT release in the brain following chronic MAP administration or if there is an independent mechanism involved. Nonetheless, the results clearly demonstrate an increased sensitivity of the OTR system in the Amy which may represent a compensatory homostatic mechanism to oppose the anxiogenic, stress-inducing and/or social deficit consequences of chronic MAP use. In support of this compensatory mechanism, OT release and OTR binding has been shown to be increased in brain regions, including the Amy, following chronic stress in rats (Ebner et al., 2005, Landgraf and Neumann, 2004, Liberzon and Young, 1997) and OT$^{-/}$ mice exhibit increased anxiety (see Baskerville and Douglas 2010); also see Section 1.6.4.
Interestingly, the medial Amy is also strongly implicated in sexual behaviours and social bonding. These behaviours are strongly influenced by OT (see Baskerville and Douglas 2008). For instance, OT and MAP have been shown to act synergistically and there is some evidence that this synergistic interaction may enhance social and sexual responses (Holder et al., 2010). As a result the up-regulation of the OTR in the Amy may be involved in the sexual enhancing and pro-social properties of MAP. This is supported by Carson et al., (2010a) who suggested that exogenous OT treatment may be effective in decreasing MAP self-administration by enhancing the incentive salience value of social interactions relative to that provided by drug-related cues. In addition, Broadbear et al., (2011) recently demonstrated that the OT is involved in the pro-social effects of another psychostimulant drug, MDMA; however the pro-social effects of MDMA markedly depend on the activation of the oxytocinergic system (see McGregor et al., 2008), while this is not the case with MAP-induced pro-social effects that need further investigation.

Moreover, chronic MAP administration also induced an up-regulation of OTR in the Hyp, another brain region associated with stress regulation. Although the behavioural/functional significance of this up-regulation is unclear, it may represent a compensatory mechanism to oppose the anxiogenic, stress-inducing, consequences of chronic MAP use. OT release in the Hyp is known to regulate HPA axis activity and thus stress. OT has been shown to have a marked anxiolytic, anti-aggressive and antidepressant effect in humans (when administered as an intranasal spray) (Baumgartner et al., 2008, Kirsch et al., 2005, Di Simplicio et al., 2009) and in animal models (Dabrowska et al., 2011, Windle et al., 2004), primarily by inhibiting CRF release from the Hyp (see Neumann et al., 2000b). As a result, the MAP-induced activation of the OTR system may be counterbalancing the stress and anti-social effects
of MAP both by its action in the Hyp and in the Amy. This would imply that
dysfunction of this MAP-induced oxytocinergic system regulation may lead to the
release of the “brake”, thus triggering more severe anxiety, stress and social recognition
deficits; however, this hypothesis remains to be explored.

Based on the evidence suggesting an involvement of central adenosine $A_{2A}$ receptors on
the endogenous oxytocinergic function in the brain (Ponzio et al., 2006), we
hypothesised that the neuroadaptations of the OTR system following chronic MAP
administration may be mediated by an $A_{2A}$ receptor-dependent mechanism. However, as
in WT mice, the profound up-regulation of OTR following chronic MAP administration
was also observed in the Hyp and Amy of MAP-treated $A_{2A}R^{-/-}$ mice clearly
demonstrating a lack of $A_{2A}$ receptor involvement in this region-specific up-regulation.
The reason that we did not observe any modulation of this MAP-induced up-regulation
in $A_{2A}R^{-/-}$ mice might be due to the low expression of $A_{2A}$ receptors in these brain
regions (Figure 4.2). Although, there is some evidence of $A_{2A}$ receptor expression in the
Amy (Mak et al., 2012, Hume et al., 1996) and Hyp (Volkow et al., 2009, Kokoshka et
al., 1998), our autoradiographic binding revealed that $A_{2A}$ receptors are almost entirely
restricted to the striatum (Figure 4.2). As a result, the lack of $A_{2A}$ receptors in those low
$A_{2A}$ receptor expressing regions is unlikely to influence OTR binding. This of course
does not preclude the possibility of $A_{2A}$ receptors affecting oxytocinergic function by
modulating OT release. Indeed, $A_{2A}$ receptors have been shown to be localised post-
synaptically on SON neurons and activation of those receptors to cause depolarisation
and excitation of OT-containing neurons (Ponzio et al., 2006). Therefore, it would be
worth investigating if OT peptide release is affected by $A_{2A}$ receptor deletion in MAP-
treated mice and whether $A_{2A}$ receptors are involved in the modulatory properties of OT
on methamphetamine-induced behaviours, by modulating its release.

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It is important to point out here that, despite its significant contribution towards understanding the mechanisms underlying the effects of drugs of abuse, full developmental knockout mouse technology does not come without limitations (see Yoo et al., 2012). One of the problems arising from studies in knockout mice is the emergence of compensatory adaptations of various systems in the mouse triggered by the gene deletion. For instance, an up-regulation of opioid receptors has been observed in preproenkephalin and preprodynorphin knockout mice (Clarke et al., 2003). In the case of $A_2A R^{-/-}$ mice, we showed no compensatory changes in $D_2R$, $MOPr$, $DOPr$, $KOPr$, $\alpha 4\beta 2$ and $\alpha 7$ nAChR binding (Bailey et al., 2004, Bailey et al., 2002a, Campbell et al., 2009). No change in OTR was also observed in saline-treated $A_2A R^{-/-}$ vs WT in the present study. As a result, we can conclude that it is unlikely that the up-regulation of OTR in chronically MAP-treated $A_2A R^{-/-}$ mice are caused by compensatory neuroadaptations induced by $A_2A$ gene deletion, but rather to the effect of the psychostimulant itself.

This is the first study to show profound alterations in the oxytocinergic system following MAP administration in specific brain regions. We showed that this MAP-induced up-regulation of OTR in the Hyp and Amy does not depend on the presence of $A_2A$ receptors. This has implications for the delineation of the role of endogenous oxytocinergic system in modulating a number of MAP effects including social behaviour, anxiety and addiction. Understanding the role of the oxytocinergic system may prove important for the development of drugs targeting the OTR as useful pharmacotherapy for treatment of MAP dependence.
CHAPTER 5

General Discussion
5.1 General Discussion

This thesis investigated the role of the neuropeptide oxytocin in psychostimulant and opioid addiction. Previous research has raised questions as to whether the oxytocinergic system is involved during chronic administration and withdrawal from addictive substances and whether it is implicated in the emergence of emotional impairment following prolonged abstinence from drug administration. The studies presented in this thesis provide clear evidence for critical alterations that occur in the oxytocinergic system during protracted withdrawal from drug administration that is accompanied by negative emotional symptoms and social deficits. Moreover, we show that an acute administration of an oxytocin analogue prevents the emotional impairment associated with long-term abstinence from opioid administration.

5.1.1 Oxytocin analogues: Novel pharmacotherapy to assist with relapse prevention

The present pre-clinical work demonstrated for the first time that oxytocin may be an effective therapeutic intervention to prevent emotional deficits during protracted abstinence from drugs of abuse and, thus, to assist with relapse prevention. Indeed, the presence of mental comorbidities (e.g. depression, anxiety, social phobia) among addicts is associated with poor treatment outcomes (Deas, 2006), and serve as a motivational trigger to re-take the drug and relapse during abstinence (Le Moal and Koob, 2007). The effectiveness of classic antidepressants in treating these comorbidities among opioid-abstinent individuals is limited (Nunes et al., 2004), therefore, novel pharmacotherapies are needed to assist with relapse prevention. The present work, not only adds to the existing evidence for an important role of the oxytocinergic system in opioid addiction but it also points towards the need for randomised double-blind control
trials with opioid addicts undergoing detoxification to investigate the effects of intranasally administered OT in reducing comorbid emotional disorders and preventing relapse. In fact, we have recently shown that CBT is able to prevent stress-induced reinstatement to opioid-seeking following withdrawal, by using the conditioned-place preference paradigm in mice (unpublished data).

Furthermore, the present study is the first study to show that a single administration of an oxytocin analogue was able to restore social deficits associated with protracted opioid abstinence. This finding is of particular importance since prolonged use of drugs of abuse results in disintegration of the social lives of drug addicts and may lead to social isolation and poor decision making in their social domain at the expense of compulsive pre-occupation with the drug and its related cues (Dawe et al., 2009, Volkow et al., 2011). Impaired social behaviours have been linked with the propensity of addicts to relapse after long-term abstinence (see Section 1.5.3). Therefore, considering the therapeutic effects of social support programs (e.g. Alcoholics Anonymous) and the benefits of social rehabilitation and social reintegration in keeping addicts abstinent from the drug (Koerner, 2010, McGregor and Bowen, 2012), our findings may implicate the use of oxytocin agents as an adjunct to Cognitive Behavioural Therapy as a novel effective “psycho-biological therapy” for the prevention of relapse to drug-seeking. In support of this, there is clinical evidence for a possible beneficial role of OT in the treatment of other disorders characterised by social cognitive impairment including autistic spectrum disorders and schizophrenic patients (Carter, 2007, Heinrichs and Gaab, 2007).

Even though the present thesis clearly demonstrated the dysregulation of the oxytocinergic system at least at the receptor level following cocaine withdrawal it did
not extend on the behavioural consequences of cocaine abstinence and the possible
effects of the oxytocin analogues in preventing any emotional impairment. Therefore,
future work must be focusing to investigate the behavioural effects of other drugs of
abuse during abstinence and the effects of the activation of the oxytocinergic system at
this time point. Although the present study clearly showed that acute administration of
the oxytocin analogue carbetocin blocks emotional despair during protracted opioid
withdrawal, one concern for studies looking at effects of exogenously administered OT
is that OT has a very short plasma (3-5 min) and central (30 min) half-life (Ludwig and
Leng, 2006, Uvnas-Moberg, 1998, Engstrom et al., 1998). However, intranasal
administration of OT has been shown to induce more prolonged release of at least 80
min (Burri et al., 2008) and has extended biological (endocrine and sexual) activity,
even after a single dose in humans (Uvanas-Moberg et al., 2005). Moreover,
intranasally administered oxytocin has been shown to cross the BBB and to exert central
effects (Born et al., 2002, Chang et al., 2012, Pedersen et al., 2013). Nonetheless, the
development of smaller non-peptide OT agonists with high specificity for central OTRs
is undoubtedly desirable.

One important question that has yet to be addressed is whether long-term administration
of oxytocin can cause any side effects. In fact, high doses of i.v. OT have been
associated with severe cardiovascular side-effects including hypotension and
myocardial ischemia (Dyer et al., 2011). Concerns also include the safety of oxytocin
administration in females at different reproductive phases due to the peripheral effects
of OT (i.e. milk ejection, labour induction). Also, chronic OT administration has the
potential to cause electrolyte imbalances due to its structural similarity to arginine
vasopressin and its effects in the kidneys (Rasmussen et al., 2004). Furthermore, it must
be considered as to whether OT-based interventions are rewarding and/or have
dependence liability. However, CBT, at least for the dose tested in this thesis (6.4 mg/kg), was found to be neither rewarding, nor aversive, in mice.

5.1.2 Dysregulation of the oxytocinergic system: Common neurobiological mechanism underlying negative emotional symptoms of drug abstinence

The data presented in this thesis strongly suggest that the potential therapeutic effects of oxytocin analogues on the negative emotional symptoms induced by drug abstinence are mediated by alterations in the central oxytocinergic system and recognise the Amy as a potential site for this action. More specifically, we have demonstrated a hypo-oxytocinergic state as observed by decreased OT levels in Hyp and plasma, and a common up-regulation of OTR binding at the level of Amy following protracted withdrawal from opioids and an up-regulation of the OTR binding in the Amy following both cocaine and MAP administration, which in the case of cocaine it persistent after protracted withdrawal in mice.

Although the behavioural consequences of dysregulation of the oxytocinergic system during abstinence need further investigation, based on the findings of the present work it is highly likely to be involved in the modulation of withdrawal-associated anxiety, depression and social deficits. Given the anxiolytic, antidepressant and social enhancing effects of OT administration in humans when administered with an intranasal spray (Baumgartner et al., 2008, Kirsch et al., 2005, Di Simplicio et al., 2009), or in animal models when administered centrally or peripherally (Dabrowska et al., 2011, Windle et al., 2004), this dysregulation of the OTR system in the Amy may constitute a neurobiological mechanism to counteract the negative emotional state induced by chronic drug administration and abstinence. This hypothesis is further supported by the presence of a similar pattern of hypo-oxytocinergic state and OTR up-regulation in the Amy to that observed in other mental disorders characterised by emotional and social
impairments, including schizophrenia (see Kring and Moran, 2008). For example, chronic administration of the NMDA receptor antagonist phencyclidine that induces similar behaviours to schizophrenia including social withdrawal (Sams-Dodd, 1999, Qiao et al., 2001), decreased OT mRNA expression in the Hyp and increased OTR binding in the CeA (Lee et al., 2005). In addition, direct OT infusion into the CeA restored the social behaviour of these animals to a level similar to the controls (Lee et al., 2005).

Clearly, further work is required to investigate the underlying neurobiological mechanisms underpinning the dysregulation of the oxytocinergic system in the Amy and the association of these alterations with emotional impairment during drug abstinence. Firstly, it would be of great interest to investigate whether abstinence from other addictive substances such as nicotine and alcohol induce similar dysregulation of the oxytocinergic system within the Amy. Moreover, microdialysis studies are needed to measure the exact OT release in several brain nuclei, especially the Amy, since an important limitation of the ELISA assay that has been used in the present study is that it can only detect peptide content and not release. In addition, the development of a knockout mouse model lacking the OTR gene specifically in the Amy and its behavioural phenotypic characterisation under basal and drug withdrawal conditions would be of particular importance. This model would potentially give an insight into the behavioural alterations induced by dysregulation of the OTR in the Amy. With regards to translational studies, directions for future research would be to measure Amy activation after an intranasal OT administration during protracted drug withdrawal by means of a double-blind placebo-controlled fMRI scanning trial in humans. In fact, OT administration has been shown to attenuate Amy activation to fear in humans (Kirsch et al., 2005), which further implicates the amygdalar oxytocinergic system in the
modulation of emotional impairment. In addition, the development of PET ligands for the OTR may be important in the understanding of the changes taking place during drug administration and withdrawal in humans. However, the OTR levels in the brain are very low in humans (Loup et al., 1991) and the development of PET ligands remains a challenging area of research.

The current study also raises the question of whether people with oxytocin receptor gene polymorphisms have a differential propensity to develop emotional deficits and relapse during long-term withdrawal from drug-taking. There is recent evidence that the OTR polymorphism rs53576G rendered social support more effective in buffering against stress in humans (Chen et al., 2011). This is an important finding since psychosocial support has been shown to assist with the prolongation of a drug-free abstinent state in recovering addicts. Therefore, OTR polymorphisms in the Amy may predict the efficacy of psychosocial support in retaining abstinence from drug-taking.

5.1.3 Concluding remarks

Collectively, the results described in this thesis show that the central oxytocinergic system is involved in different phases of drug addiction and that the exogenous administration of an OT analogue is beneficial in preventing the negative psychological withdrawal symptoms of opioids during long-term abstinence (for a proposed mechanism see Figure 5.1). These results are supportive of the oxytocinergic system as a novel target for the treatment of addiction-mood disorders comorbidity, which could assist with relapse prevention. They also highlight the dysregulation of this system as an underpinning mechanism linking mood disorder comorbidity, at least during opioid abstinence. In light of the significant benefits that psychosocial support has in preventing relapse to drug-taking after abstinence, the combination of both social
support and oxytocin administration might be proved a novel beneficial and effective “psycho-biological therapy” for the treatment of addiction.

Figure 5.1: Proposed mechanism for the modulation of the negative emotional symptoms of protracted opioid withdrawal by oxytocin. Protracted abstinence from opioid administration brings about decreased oxytocin levels within the hypothalamus and negative emotional symptoms. A rebound up-regulation of oxytocin receptor binding within the amygdala may act as a compensatory mechanism to counteract the negative emotional state of opioid abstinence. Oxytocin analogues activate the oxytocinergic system, thereby blocking this negative “psychological” state and may be able to prevent relapse to opioid-seeking following abstinence. Dashed line indicates a hypothetical effect of oxytocin analogues in reducing relapse to drug-seeking following abstinence.


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