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BRAIN OPIOID
MECHANISMS IN
AMPHETAMINE INDUCED
BEHAVIOURS

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To my family

ABSTRACT

Amphetamine abuse and dependence are global health problems, affecting large numbers of individuals. Despite extensive research efforts, there has been no evidence-based pharmacotherapy available for amphetamine dependence. However, recent clinical trials have suggested that the opioid receptor antagonist naltrexone reduces drug consumption and craving in amphetamine dependent patients. To further elucidate the interaction between amphetamine and the endogenous opioid system, the present thesis investigated the effect of naltrexone on amphetamine-induced behaviours in the rat. In addition, the effect of naltrexone on specific amphetamine-induced changes in brain neurochemistry, i.e. dopamine release in the striatum, was investigated using both *in vivo* microdialysis in animals as well as PET imaging in humans.

Firstly, the effect of naltrexone on drug-induced reinstatement was investigated in animals previously self-administering amphetamine. Naltrexone significantly attenuated drug-seeking behaviour induced by a low priming dose of amphetamine. The effect of naltrexone was not due to a general disruption of behaviour since the same doses of naltrexone had no effect on operant responding maintained by food.

Next, the effect of naltrexone on different phases of the conditioned place preference paradigm was investigated. Naltrexone had no effect on the acquisition, expression or on the reinstatement of amphetamine-induced place preference. Further, naltrexone by itself did not induce place preference or place aversion. In contrast, naltrexone significantly attenuated the locomotor response to a priming dose of amphetamine on reinstatement, without altering general locomotor behaviour.

A behavioural sensitization paradigm was used to further investigate the involvement of the endogenous opioid system in amphetamine cue- and drug-induced drug seeking behaviour. A single dose of naltrexone attenuated the amphetamine-induced locomotor activity in response to a drug challenge during the expression of sensitization. In addition, pre-treatment with naltrexone blocked the conditioned locomotor response induced by re-exposure to the context previously associated with amphetamine.

Finally, possible interactions between amphetamine and naltrexone were investigated using both preclinical and clinical experimental models, i.e. *in vivo* microdialysis in rats and PET imaging in humans. Amphetamine induced a dose-dependent increase in dopamine release in the rat striatum, and a reduction in [¹¹C]raclopride binding in the human that corresponded with a marked increase in self-reported rating of drug effects (high, arousal and liking). An acute dose of naltrexone attenuated the subjective effects of amphetamine, without altering the amphetamine-induced changes in [¹¹C]raclopride binding in the ventral striatum.

Taken together, the results from the present thesis show an involvement of the endogenous opioid system in some, but not all, amphetamine-induced behaviours. The results strengthen the hypothesis that the endogenous opioid system could be a potential target for the treatment of amphetamine dependence.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

- I. **J. Häggkvist**, S. Lindholm and J. Franck *The Opioid Receptor Antagonist Naltrexone Attenuates Reinstatement of Amphetamine Drug-Seeking in the Rat* Behavioural Brain Research, 2009, 197(1):219-224
- II. **J. Häggkvist**, S. Lindholm and J. Franck *The Effect of Naltrexone on Amphetamine-induced Conditioned Place Preference and Locomotor Behaviour in the Rat* Addiction Biology, 2009, 14: 260-269
- III. **J. Häggkvist**, C. Björkholm, P. Steensland, S. Lindholm, J. Franck, B. Schilström *Naltrexone Attenuates Amphetamine-Induced Locomotor Sensitization in the Rat* Submitted Manuscript
- IV. **J. Häggkvist** *, N. Jayaram-Lindström*, T Malmjöf, T. Saijo, C. Halldin, S. Lindholm, B. Schilström, A-L Nordstrom, J. Franck *Effects of Naltrexone on Amphetamine-Induced Changes in the Brain Dopamine System* Manuscript

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CONTENTS

1	Introduction	1
1.1	Amphetamine History	1
1.2	Substance Dependence	1
1.2.1	Transition from Drug-Use to Drug Dependence.....	3
1.3	Animal Models in Substance Dependence Research.....	4
1.3.1	Operant Self-Administration.....	5
1.3.2	Conditioned Place Preference.....	5
1.3.3	Behavioural Locomotor Sensitization.....	6
1.4	The Reward System	7
1.5	The Dopamine System	7
1.6	The Opioid System.....	9
1.7	Amphetamine	11
1.8	Naltrexone	12
1.9	Interaction Between the Endogenous Opioid System and Psychostimulants	13
1.9.1	Opioid and Dopamine Interaction.....	13
1.9.2	Endogenous Opioids and Psychostimulant Interaction.....	14
2	Aim of the Thesis.....	16
3	Materials and Methods	17
3.1	Animals	17
3.1.1	Ethical Approval.....	17
3.2	Drugs and Chemicals.....	17
3.3	Behavioural and <i>In Vivo</i> Neurochemical Studies	17
3.3.1	Intravenous Self-Administration.....	17
3.3.2	Conditioned Place Preference.....	18
3.3.3	Locomotor Sensitization.....	20
3.3.4	<i>In Vivo</i> Microdialysis.....	21
3.4	Human laboratory study.....	21
3.4.1	Positron Emission tomography (PET)	21
4	Results and Discussion.....	23
4.1	Naltrexone Reduces Amphetamine-Induced Drug-Seeking Behaviour in Animals Previously Self-Administering Amphetamine (Paper I) ...	23
4.2	The Effect of Naltrexone on Amphetamine-Induced Conditioned Place Preference (Paper II)	25
4.3	Naltrexone Reduces the Amphetamine-Induced Sensitized Locomotor Activity and Conditioned Locomotor Response (Paper III)	30
4.4	Effect of Naltrexone on Amphetamine-Induced Dopamine Release in the Nucleus Accumbens (Paper IV).....	32
5	Summary of Findings	35
6	General Conclusion	36
7	Populärvetenskaplig sammanfattning på svenska.....	39
8	Acknowledgements.....	41
9	References.....	45

LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ACTH	Adrenocorticotropin
Amph	Amphetamine
ANOVA	Analysis of Variance
BP	Binding Potential
COMT	Catechol-O-Methyltransferase
CPP	Conditioned Place Preference
δ	Delta
DSM-IV	Diagnostic and Statistical Manual of mental disorders - IV
DOPAC	Dihydroxyphenylacetic acid
DOPA	Dihydroxyphenylalanine
e.g.	Exempli gratia
FR	Fixed-Ratio
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
HVA	Homovanillic acid
i.e.	id est
i.p.	Intraperitoneal
i.v.	Intravenous
κ	Kappa
LSD	Least Statistical Difference
LST	Limbic Striatum
MRI	Magnetic Resonance Imaging
MSH	Melanocyte-Stimulating Hormone
MAO	Monoamine oxidase
μ	Mu
NTX	Naltrexone
ORL-1	Opioid receptor like-1
PET	Positron Emission Tomography
PR	Progressive-Ratio
POMC	Proopiomelanocortin
ROI	Region of Interest
SMST	Sensimotor striatum
S.D.	Standard Deviation
S.E.M	Standard Error of the Mean
s.c	Subcutaneous
VTA	Ventral Tegmental Area
VAS	Visual Analog Scale

1 INTRODUCTION

1.1 AMPHETAMINE HISTORY

Amphetamine belongs to the psychostimulants drugs, substances that increase wakefulness and activity levels, decreases fatigue as well as induces feelings of euphoria. The use of stimulant compounds dates centuries back in time. In China, the drug Ma-huang has been used for over 5000 years and in 1887 the Japanese scientist Nagai found that ephedrine was the active agent in Ma-huang. In the same year, the German chemist Edeleau synthesized amphetamine for the first time, which was found to be related to the natural psychostimulant ephedrine (see Lowinson et al., 2005). However, there was no formal indication for medical use of amphetamine at this time. It was not until in the late 1920s, in search for an artificial replacement of the naturally occurring ephedrine, that amphetamine was re-synthesised. In the 1930s, amphetamine was marketed as an inhaled pharmaceutical compound under the trade name Benzedrine, used for the treatment of nasal congestion. During World War II, amphetamine was commonly used to increase energy as well as to reduce fatigue and hunger in soldiers. When returning from the war, soldiers continued to use the drug, which gave rise to an epidemic of amphetamine misuse and dependence. After the war, Benzedrine inhalers were banned by the US Food and Drug Administration (FDA) in 1959 and amphetamine was limited to prescription use. Amphetamine became a schedule II drug under the Controlled Substances Act in 1971.

Globally, 35 million adults are reported to be using amphetamine-type stimulants (including ecstasy, an amphetamine derivative) and the only illicit drug that is used more often than amphetamines is cannabis. The use of amphetamines exceed the use of heroin and cocaine combined (UNODC 2008). In Sweden, it is estimated that around 30 000 individuals are heavy drug users, i.e. administer the drug intravenously (i.v.) or use the drug daily (irrespective of route of administration). Out of the 30 000 heavy drug users, about 50 % report having amphetamine as their primary drug of choice (Hakansson et al. 2007; CAN 2008). The cost of amphetamine abuse and dependence is significant to both society and the individual. However, despite extensive research efforts, no approved pharmacotherapy for amphetamine dependence is available (Srisurapanont et al. 2001; Elkashaf et al. 2008), although the field is developing rapidly (Jayaram-Lindström et al. 2008a).

1.2 SUBSTANCE DEPENDENCE

Substance dependence, or drug addiction, is a major health problem, affecting a large number of individuals. Substance *use* is defined as intake of the drug for non-medical purposes; however, the use of a drug does not automatically mean that a person will develop substance abuse or dependence. Substance *abuse*, on the other hand, is defined as continued substance use despite emergence of negative consequences, i.e. failure to fulfil work/family/school obligations, use of the drug in situation were it is physically hazardous, recurrent substance-related legal problems or interpersonal conflicts caused by substance intake. And lastly, substance *dependence* may be defined as a chronic relapsing disease

characterized by a compulsion to seek and take drugs despite severe adverse consequences, loss of control in limiting intake and emergence of negative emotional state (e.g. dysphoria, anxiety, irritability) when access to the drug is prevented (Koob and Moal 1997).

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), substance dependence is defined as a cluster of three or more of the symptoms listed in Table 1 (American Psychiatric Association 1994). The criteria are both of physiological and psychological character. Historically, substance dependence was characterized by physical withdrawal signs following cessation of drug taking or the drug-effect weans down. Today, there is greater emphasis on the psychological or motivational aspects of substance dependence as core symptoms rather than on the presence of physical symptoms of withdrawal and it is known that some substances, such as psychostimulants, do not induce measurable physical withdrawal symptoms. The psychological symptoms, such as drug craving and loss of control are known to remain long after cessation of drug use and the physical withdrawal signs have vanished.

Table 1. DSM-IV Criteria for Substance Dependence

A maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring any time in the same 12-month period:

1. Tolerance, as defined by either of the following:
 - (a) A need for markedly increased amounts of the substance to achieve intoxication or the desired effect
 - (b) Markedly diminished effect with continued use of the same amount of the substance
2. Withdrawal, as manifested by either of the following:
 - (a) The characteristic withdrawal syndrome for the substance
 - (b) The same (or closely related) substance is taken to relieve or avoid withdrawal symptoms
3. The substance is often taken in larger amounts or over a longer period than intended
4. There is a persistent desire or unsuccessful efforts to cut down or control substance use
5. A great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects
6. Important social, occupational, or recreational activities are given up or reduced because of substance use
7. The substance use is continued despite knowledge of having a persistent physical or psychological problem that is likely to have been caused or exacerbated by the substance

1.2.1 Transition from Drug-Use to Drug Dependence

The transition from drug use to dependence involves complex interactions between e.g. repeated exposure to the drug, biological (e.g. developmental and genetic) and environmental factors (e.g. social and economic variables, drug availability) (Volkow and Li 2005). In order to develop effective treatments of substance dependence, understanding the underlying nature of the disease is essential. Therefore, it is important, from not only a clinical perspective but also from a research perspective to differentiate between initial substance use, intermittent and compulsive drug use, withdrawal (acute and protracted abstinence), craving, as well as relapse to drug-taking behaviour (Fig. 1).

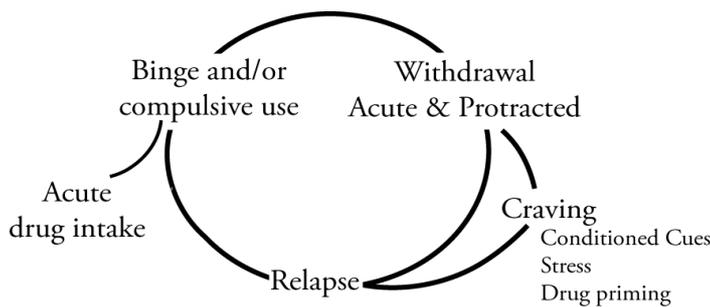


Figure 1. Proposed model for the different stages in substance dependence. Modified from Goldstein and Volkow (2002)

Initial drug taking depends, among other things, on the subjective effects induced by the drug (Wise 1980). Addictive drugs activate brain reward mechanisms, primarily the mesocorticolimbic dopamine system (see section 1.4). Initial drug intake can be driven by both positive and negative reinforcement, i.e. the drug is taken for its ability to increase positive subjective effects or to alleviate an existing aversive state (Koob and Moal 1997).

During continuous drug-intake, internal or external cues that are associated with drug-administration can acquire secondary positive reinforcing effects through conditioning and with repeated pairings, the cues can themselves serve as an incentive motivator for drug use (Robinson and Berridge 1993). In humans, such drug associated cues are known to induce craving that in turn may increase the propensity to relapse to continued drug-use (Childress et al. 1993). It has been hypothesised that with repeated drug use, the initial positive reinforcing effects of the drug decrease and there will be a transition from positive to negative reinforcement were drug-intake is driven by alleviation of withdrawal rather than by induction of euphoria.

One of the characteristics of substance dependence is the inability to stay drug free. The abstinence stage can be divided into two phases: the first phase i.e. acute withdrawal, appearing immediately after discontinuation of drug use, which is characterized by emergence of withdrawal symptoms. The withdrawal symptoms are specific for each class of drugs and the symptoms disappear by re-administration of the drug itself or by a closely related substance. The second phase, i.e. protracted abstinence, is present long after

discontinuation of drug-intake, and is characterized by more psychological and motivational features of substance dependence such as craving for the drug. According to the hedonic allostasis model proposed by Koob and Le Moal (1997), repeated drug-use leads to long-term neuroadaptations, which are characterized by a cycle of spiralling dysregulation in the reward system leading to a new reward set point. The changes in set point create an unpleasant state of withdrawal in a drug-free state, encouraging further drug-intake (Koob and Moal 1997).

One of the major clinical problems in treating substance dependence is the high rates of relapse to drug use even after long periods of abstinence. Relapse is hypothesized to be partly due to an intensification of drug craving with repeated drug use. Craving can be induced by drug priming (Jaffe et al. 1989) drug associated cues (Childress et al. 1993) or by stress (Sinha 2001), all increasing the risk of relapse to former drug use. Relapse is characterized by a loss of control, where the substance is usually taken in larger amounts or over a longer period than intended as well as unsuccessful efforts to cut down or control substance use. According to the incentive-sensitization model proposed by Robinson and Berridge, drug priming and drug-associated stimuli can trigger incentive motivation for the drug, leading to compulsive drug seeking, drug taking and relapse (Robinson and Berridge 2000). Two major drug effects are believed to be sensitized, the psychomotor activating effect and the incentive motivational effect. The neural systems responsible for the sensitized incentive motivational effects can be dissociated from the brain systems that are involved in the liking of the drug. According to Robinson and Berridge, the “wanting” of the drug becomes sensitized without affecting “liking”. The sensitized motivational effect, i.e. the “wanting”, can lead to compulsive drug seeking, drug taking and relapse.

Taken together, it is hypothesized that the development of substance dependence involve a transition from initial positive reinforcement to a stage of negative reinforcement, where the drug taking is driven by an alleviation of negative state and strong wanting of the drug. Relapse to former drug use can be triggered by acute physical withdrawal symptoms or by motivational and physiological aspects of substance dependence such as drug craving, leading to the compulsive drug use that characterizes dependence.

1.3 ANIMAL MODELS IN SUBSTANCE DEPENDENCE RESEARCH

The use of laboratory animal models has contributed to the understanding of the neurobiological and behavioural mechanisms underlying substance dependence. While no animal model of substance dependence can fully imitate the human condition, animal models have proven to be very important tools in the medical development for substance dependence. By using behavioural models such as operant self-administration, conditioned place preference and behavioural locomotor sensitisation, major elements of the dependence process can be modelled, such as the reinforcing effects, conditioning effect, compulsive drug seeking and relapse behaviour (Sanchis-Segura and Spanagel 2006). In addition, other animal models, such as intra-cranial self-stimulation and drug-discrimination are commonly used in substance dependence research but are not included in this thesis and therefore not described. The experiments conducted in the present thesis are based on the following behavioural models.

1.3.1 Operant Self-Administration

Three distinct phases of drug-taking behaviour can be studied using the self-administration model, i.e. the initiation, maintenance and reinstatement phase. Animals voluntarily self-administer substances that have a high abuse potential in humans. When given free access to a drug, intake patterns gradually develop into similar behavioural patterns seen in humans and the self-administration model is considered to provide a valid measure of a drug's abuse liability.

During initiation, animals are trained to perform an operant behaviour, press a lever or nose poke, in order to receive the drug usually by i.v. infusion or by oral intake. Substances that have positive reinforcing and rewarding effects will be self-administered, and a stable pattern of drug intake develops with time (i.e. maintenance). In a simple schedule of reinforcement, each lever press, or nose poke, will result in one delivery of the drug (Fixed-ratio 1 – FR1). After a stable intake, the schedule can be shifted to a progressive-ratio (PR) schedule where the number of lever presses required to receive the drug is gradually increased. The most common index of PR performance is the so-called “break point”, i.e. the highest response rate accomplished in order to obtain a single reinforcer, and this is considered a measure of the motivational properties of the drug (for a review see Sanchis-Segura and Spanagel, 2006).

To mimic human relapse, the reinstatement model of operant self-administration has been widely used in experimental settings. In this model, animals are first trained to self-administer a drug until a stable intake is shown over consecutive days. The drug is then replaced by vehicle and as a result the animals will stop responding on the lever previous associated with drug delivery. Drug seeking behaviour (i.e. lever pressing or nose poke) can thereafter be triggered by different stimuli such as exposure to a stressor (Shaham and Stewart 1995; Erb et al. 1996; Shalev et al. 2000), presentation of stimuli previously paired with the drug (Meil and See 1996; Weiss et al. 2000) or acute exposure to the self-administered drug (de Wit and Stewart 1981). The face validity, including the translational value of the reinstatement model in substance dependence, is considered to be significant (Epstein et al. 2006).

1.3.2 Conditioned Place Preference

The conditioned place preference (CPP) paradigm is a non-operant procedure used for assessing the reinforcing efficacy of drugs using a classical or Pavlovian conditioning procedure. In the CPP paradigm, the laboratory animals learn to associate the effect of a reinforcing stimulus with a specific context by repeated pairings. Typically, a two compartment apparatus is used where each compartment has a distinct environment that includes contextual cues, such as colour or floor texture. The drug is conditioned with one compartment and vehicle with the other. After repeated pairings, the animal is given free access to both compartments, without administration of the drug. An increase in time spent in the drug-paired context is considered an indicator of the reinforcing value of the drug. If a substance instead induces aversive states, the animals tend to avoid the environment leading to conditioned place aversion.

The CPP paradigm can be divided into distinct phases: acquisition, expression, extinction and reinstatement. During acquisition, the animals learn to associate a context with the effect induced by the drug. In contrast to acquisition, the expression of CPP occurs in absence of the primary rewarding stimulus, e.g. amphetamine, and instead relies on the learned motivational properties of the environmental cues (Bardo and Bevins 2000; Tzschentke 1998). The third phase of the CPP is the extinction and reinstatement. After the CPP has developed, the learnt association between the context and the drug can be extinguished by repeated pairings with vehicle in the previous drug-paired context. The place preference can then be reinstated by exposure to stressors (Wang et al. 2000; Sanchez and Sorg 2001) or by priming dose of the drug (Mueller and Stewart 2000b; Wang et al. 2000). The CPP paradigm is one of the most commonly used experimental protocol for measuring drug reward in laboratory animals. If co-administration of a compound during acquisition alters the ability of a drug to induce CPP it is generally assumed that the treatment interfere with the motivational properties of the drug (Sanchis-Segura and Spanagel 2006). The CPP protocol is also commonly used for investigating potential target medications for the treatment of human drug craving. The expression of CPP is thought to model cue-induced craving whereas the reinstatement of CPP can be used as model both stress and drug-primed craving.

1.3.3 Behavioural Locomotor Sensitization

In the behavioural sensitization paradigm, changes in locomotor activity are used as a measure of the neuroadaptations induced by repeated drug administration. Repeated administration with the same dose of a drug, increase locomotor activity over time, a phenomenon known as sensitization. The neurobiological systems that mediate the psychomotor effects overlap with brain systems involved in reward and reinforcement and therefore the psychomotor sensitization is considered to model changes that may underlie aspects of substance dependence (Robinson and Berridge 2003).

The sensitized response, both on a behavioural and neurochemical level, is not only dependent on pharmacological effects of the drug but also on the environment surrounding drug administration. Animals that receive e.g. a psychostimulant in a “novel” test environment develop a greater behavioural locomotor sensitization than animals that receive the drug in a non-novel environment (i.e. home cage) (Badiani et al. 1995a; Badiani et al. 1995b). In addition, animals conditioned with repeated injections of a drug in a distinct environment show enhanced locomotor activity (conditioned locomotor response) when placed in the previously drug-paired environment (Tilson and Rech 1973; Beninger and Hahn 1983). This suggests that the drug-induced neuroadaptations, i.e. the sensitization, is a function of the interaction between the drug and the stimuli surrounding drug administration. Thus, the behavioural sensitization paradigm can be used to model drug craving and reinstatement of compulsive drug-seeking behaviour, triggered by drug priming as well as by drug-associated cues.

1.4 THE REWARD SYSTEM

Brain regions involved in reward and reinforcement were identified already in the 1950s by Olds and Milner (Olds and Milner 1954). An electrode was implanted into the brain of the rat and the animals were given the opportunity to voluntarily press a lever to give themselves intra-cranial electrical stimulation. When the electrode was placed in certain areas of the brain, the animals pressed the lever to the exclusion of other behaviours. The brain area stimulated was the median forebrain bundle (Olds and Olds 1969), a complex bundle of axons, including e.g. serotonergic projections from the raphe nucleus, noradrenaline projections from locus coeruleus, as well as dopaminergic projections from the ventral tegmental area (VTA).

The natural function of the reward system is to motivate learning about behaviours that are essential for the survival of the individual and or the specie, and natural stimuli such as intake of food and water as well as sexual behaviour activates the reward system (Hernandez and Hoebel 1988; Pfaus et al. 1990). In addition, drugs of abuse also activate and enhance neurotransmission in these brain areas, to an extent often more potent than natural rewards (Di Chiara and Imperato 1988; Hernandez and Hoebel 1988; Pfaus et al. 1990).

1.5 THE DOPAMINE SYSTEM

One of the essential neurotransmitters in the brain reward system is dopamine. In the 1950s, it was hypothesised that dopamine was only a precursor of other catecholamines such as noradrenaline and adrenaline. However, research by the Swedish scientist Arvid Carlsson and his co-workers, showed that dopamine was a neurotransmitter in its own right (Carlsson et al. 1958; Carlsson 1959).

With the development of the Falck-Hillarp fluorescence method, it became possible to visualize and study the neuronal distribution of the different neurotransmitters in the brain (Falck et al. 1962; Dahlström and Fuxe 1964). The dopamine neurons are organized into several major pathways that originate in the midbrain and project both to cortical and forebrain regions (Fig. 2). Neurons with cell bodies in the substantia nigra (A9) projects mainly to the dorsal striatum, i.e. the caudate putamen (Anden et al. 1964) forming the nigro-striatal pathway, particular important for the locomotor stimulating effects. Degeneration or destruction of the nigrostriatal pathway results in severe motor disturbances and Parkinson like symptoms (Ungerstedt and Arbuthnott 1970). In contrast, cell bodies originating in the VTA (A10) send their projections to both to cortical and subcortical areas, such as the prefrontal cortex, cingulate cortex, nucleus accumbens, amygdala, bed nucleus of the stria terminalis, hippocampus, olfactory tubercle, and septum (Dahlström and Fuxe 1964; Ungerstedt 1971), forming the mesocorticolimbic dopamine system, a pathway involved in emotions and reward.



Figure 2. Illustration of the dopamine pathways
Cell bodies originate in the midbrain, area A9 and A10, and send projections to both cortical and forebrain regions.
Illustration: Mattias Karlén

Synthesis and Metabolism

Dopamine is synthesised from the amino acid precursor tyrosine. In the dopaminergic neuron, L-tyrosine is converted into L-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme tyrosine hydroxylase. L-DOPA is in turn converted to dopamine by DOPA decarboxylase. When stimulated, the neurons release dopamine from the vesicles in a calcium-dependent manner. The clearance of dopamine from the synaptic cleft is regulated by both reuptake via the dopamine transporter, as well as via enzymatic breakdown of dopamine by the two main catecholamine-metabolizing enzymes, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). When dopamine is metabolized by the enzymes, two main products are produced, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), which are excreted in the urine and can provide an index of dopamine release (see Hardman et al., 1996).

Receptors

The dopamine receptors are divided into two classes, D1-like (including D1 and D5 receptors) and D2-like (including D2, D3 and D4) dopamine receptors, belonging to the G-protein-coupled seven-transmembrane receptor family. D1-like and D2-like receptors are expressed in distinct neuronal populations throughout the nervous system (Le Moine and Bloch 1995).

Both D1-like and D2-like receptors are located in the mesolimbic dopamine system but on different subset of neurons (Lu et al. 1998). In the nucleus accumbens, the D1-like receptors are expressed on GABAergic neurons, co-expressing substance P and dynorphin, projecting to the VTA and substantia nigra. D2-like receptors are located on neurons in the striatopallidal pathways on neurons that co-express enkephalin. Both D1-like and D2-like receptors are located post-synaptically, but the D2-like receptors are also expressed pre-synaptically on dopaminergic neurons functioning as auto receptors i.e. controlling phasic dopaminergic activity (Jaber et al. 1996; Le Foll et al. 2009)

Dopamine and Reward

Dopamine is involved in various functions, e.g. motor activity, motivation, emotion, cognition, endocrine secretion and reward. The mesocorticolimbic dopamine pathway has been hypothesised to be important for the reinforcing and rewarding effects of drugs of

abuse, since dopamine receptor-blocking by neuroleptic drugs causes devaluation of reward and reinforcement (Yokel and Wise 1975; De Wit and Wise 1977; Bozarth and Wise 1981). Further, using *in vivo* microdialysis in animals it has been shown that both natural rewards as well as addictive drugs increase the extracellular concentration of dopamine within the nucleus accumbens (Di Chiara and Imperato 1988; Hernandez and Hoebel 1988; Pfaus et al. 1990).

Recent advantages in technology and methodology in brain imaging has made it possible to study the effect of drugs of abuse in awake and conscious human subjects. Using positron emission tomography (PET) and the dopamine receptor radioligand [¹¹C]raclopride, changes in extracellular dopamine levels can be indirectly measured. Endogenous dopamine competes with the exogenous ligand [¹¹C]raclopride and an increased dopamine release will lead to a decrease in raclopride binding. Administration of drug of abuse decrease raclopride binding and the drug-induced dopamine release has been associated with positive mood states, such as euphoria and drug wanting (Schlaepfer et al. 1997; Drevets et al. 2001).

Despite the well-established effects of dopamine in response to acute drug administration, the neurobiology underlying substance dependence is complex and involves several neurotransmitter systems in addition to dopamine. One such system is the endogenous opioid system.

1.6 THE OPIOID SYSTEM

The use of opium, an extract from the poppy *Papaver somniferum*, dates back thousands of years and it has been used, for both social and medical purposes, due to its ability to induce euphoria, analgesia, sleep, as well modulate gastrointestinal function. In 1806, one of the main active alkaloid in opium was isolated by the pharmacist Sertürner and it was named morphine after Morpheus, the Greek god of dreams. The term *opioid* relates to any substance that induces morphine-like effects that are blocked by administration of opioid receptor antagonists such as naloxone or naltrexone. Endogenous and exogenous opioids are distinguished depending on whether the substances are normally present in the body or not (see Hardman et al., 1996).

Receptors

Since opium and morphine was known to activate the brain, it was long hypothesised that there should be endogenous binding sites for opioid-like substances. In 1973, the opioid receptors were discovered by three independent research groups (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). In 1976, Martin and co-workers proposed the existence of multiple opioid binding sites based on studies performed in dogs (Martin et al. 1976). They distinguished three different receptor types and named them based on their behavioural and neurophysiological effect, i.e. the μ -receptor (for morphine which induces e.g. analgesia, hypothermia, and meiosis), the κ -receptor (for Ketocyclazocine which induces e.g. depression of flexor reflexes and sedation) and the σ -type (for SKF10,047 or *N*-allylnormetazocine, which induces e.g. tachycardia, delirium, and increased respiration) (Martin et al. 1976). Later a fourth opioid receptor was identified and was named δ -

receptor, for vas deferens (Lord et al. 1977). Additional research showed that the σ -type receptor was a non-opioid receptor leaving three main types of opioid receptors, i.e. the μ , δ and κ -opioid receptors.

The opioid-receptors belong to the family of seven transmembrane G-protein-coupled receptors. Binding studies have revealed that each opioid receptor class has a distinct pharmacological profile with a specific distribution in the brain and spinal cord (Mansour et al. 1988; Mansour et al. 1995). In addition to the classical opioid receptors, a fourth receptor has been identified, the opioid receptor like-1 (ORL-1) receptor, which share significant sequence homology to the previously identified opioid receptors (Mollereau et al. 1994)

Opioid Peptides

Historically, the discovery of the opioid receptors preceded the isolation and characterization of the endogenous opioid peptides. Three distinct families of opioid peptides have been identified, which are synthesised by enzymatic cleavage of three precursor molecules, the proopiomelanocortin (POMC), proenkephalin and prodynorphin (see Lowinson et al., 2005). POMC generates the opioid binding peptide β -endorphin as well as several non-opioid peptides, e.g. adrenocorticotropin (ACTH) and, α -, β - and γ melanocyte-stimulating hormone (MSH). In contrast, splicing of proenkephalin leads to several active opioid peptides out of four is [Met]enkephalin and one [Leu]enkephalin and in addition there are also several extended forms of these peptides i.e. [Met]enkephalin-Arg⁶-Phe⁷ (MEAP) or [Met]enkephalin-Arg⁶-Phe⁷-Leu⁸. Lastly, splicing of the prodynorphin precursor molecule leads to generation of α - and β -neoendorphin, dynorphin A, and dynorphin B.

Each of the three opioid peptide precursor molecules have an unique anatomical distribution throughout the central nervous system (CNS) (Akil et al. 1984). POMC are mainly synthesized in the anterior and neurointermediate lobes of the pituitary gland, the arcuate nucleus of the hypothalamus as well as in the nucleus tractus solitarius and the cell bodies send widespread projections throughout the brain. Compared to POMC, proenkephalin and prodynorphin have a more scattered and widespread distribution through out the brain (for a review see Mansour et al., 1995).

Binding studies have demonstrated that the different endogenous opioid ligands show preference for the respective receptors, as shown in Table 2. β -endorphin have a high affinity for both μ - and δ -receptors, enkephalin has highest affinity for the δ - receptor but has also a high affinity for the μ -receptor. Dynorphins on the other hand, have high affinity for the κ -receptor with no or little binding to μ - and δ -receptors (see Hardman et al., 1996). None of the above mentioned opioid-peptides interacts with the ORL-1 receptor, however in 1995, two independent group identified and characterized an endogenous peptide that was named nociceptin or orphanin FQ (Meunier et al. 1995; Reinscheid et al. 1995). The precursor protein for nociceptin/orphanin FQ is encoded by a novel gene with significant sequence homology to the genes encoding prodynorphin, proenkephalin and POMC (Mollereau et al. 1996).

Table 2. Opioid precursor molecules, major opioid peptide and opioid receptor affinity

Precursor molecule	Major opioid peptide	Opioid receptor affinity
Proopiomelanocortin (POMC)	β -endorphin	$\mu \leq \delta$ -receptor
Proenkephalin (PENK)	[Met]enkephalin [Leu]enkephalin	$\delta < \mu$ -receptor
Prodynorphin (PDYN)	Dynorphin A Dynorphin B	κ -receptor
Prepronociceptin	Nociceptin/orphanin FQ	ORL-1

1.7 AMPHETAMINE

Amphetamine belongs to the group of psychostimulants, substances that act as indirect sympathomimetics, i.e. mimic the action of an activated sympathetic nervous system. Physical and psychological symptoms associated with amphetamine-intake are e.g. increased heart rate and blood pressure, increased physical activity and wakefulness, reduced fatigue and appetite, as well as increased mood and euphoria. The long-term effects of amphetamine use include e.g. confusion, paranoia and hallucinations, weight loss, tremors and convulsion and in severe cases cardiovascular collapse and death (Lowinson et al. 2005).

Amphetamines acts as indirect catecholamine agonists and administration of amphetamine causes a dose-dependent release of noradrenaline, dopamine and in higher doses serotonin (Azzaro and Rutledge 1973). Amphetamines increases and prolongs the concentration of the neurotransmitter in the synaptic cleft by stimulating release through reversing transport, inhibiting enzymatic breakdown as well as blocking reuptake of the neurotransmitter (Fig. 3).

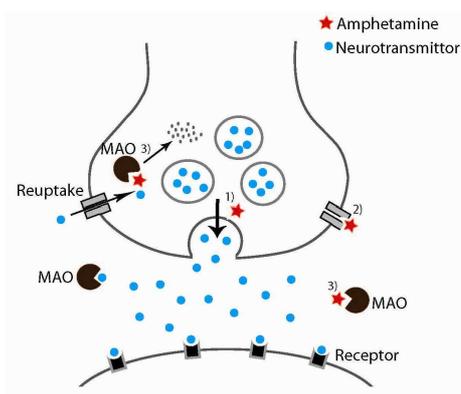


Figure 3. Mechanism of Action of Amphetamine

Amphetamine acts as an indirect catecholamine agonist by increasing the synaptic concentration of neurotransmitters through three different mechanisms; 1) reversing transporters of the neurotransmitter leading to pre-synaptic release, 2) blocking reuptake of the neurotransmitter and 3) inhibiting enzymatic breakdown of the neurotransmitter.

The importance of dopamine in amphetamine-induced neurochemical and behavioural effects has been repeatedly shown, in animals and human models of substance dependence. Similar to other drugs of abuse, amphetamine causes a dose-dependent increase in dopamine release in the striatum (Zetterstrom et al. 1986; Di Chiara and Imperato 1988) with a correlated effect on locomotor activity (Zetterstrom et al. 1986). In humans, using PET and [¹¹C]raclopride, it has been shown that amphetamine induces dopamine release, an increase correlated with the subjective-reported euphoria (Drevets et al. 2001). Collectively, the results from animal and humans studies suggest that dopamine release in the terminal region of the mesolimbic dopamine system is important for the reinforcing and rewarding effects of amphetamine.

The locomotor stimulating effect induced by amphetamine can be blocked by e.g. pre-treatment with dopamine receptor antagonist (Rolinski and Scheel-Kruger 1973), lesion of dopamine pathways (Fibiger et al. 1973) and blockage of dopamine synthesis (Weissman et al. 1966; Svensson 1970). The interaction between amphetamine and dopamine has been investigated using different animal models of reinforcement such as the CPP paradigm and the self-administration model. Dopamine receptor antagonists blocked both the acquisition and expression of amphetamine-induced CPP (Mackey and van der Kooy 1985; Hiroi and White 1991) and pre-treatment with either D1 or D2 receptor antagonists partially antagonized amphetamine self-administration (Phillips et al. 1994).

The direct role for dopamine in the subjective experience of reward/euphoria has been questioned in some recent human laboratory studies. For example, pre-treatment with dopamine receptor antagonists did not attenuate amphetamine-induced euphoria (Brauer and De Wit 1997; Wachtel et al. 2002) indicating that other neurotransmitters might also be involved in amphetamine-induced reward and reinforcement.

1.8 NALTREXONE

The opioid receptor antagonists naltrexone and naloxone have been investigated for their ability to modulate addictive behaviours for over 30 years. In 1994, the FDA approved naltrexone for the treatment of alcohol dependence based on two pivotal clinical trials showing that naltrexone reduces craving and relapse in alcohol dependent patients (O'Malley et al. 1992; Volpicelli et al. 1992). Findings from these and other clinical trials have been supported by pre-clinical studies showing that opioid receptor antagonism reduces alcohol intake in various species (Altshuler et al. 1980; Myers et al. 1986; Stromberg et al. 1998) as well as attenuates CPP induced by alcohol (Kuzmin et al. 2003). In addition to alcohol, opioid receptor antagonists also modulate neurochemical and behavioural effects induced by other drugs of abuse, such as nicotine, heroin and cocaine.

Naltrexone and naloxone are both full antagonists on all three typical opioid receptors, with a somewhat higher affinity for the μ - and κ -receptor compared to the δ -receptor (Uwai et al. 2004). Naltrexone has a longer half life than naloxone and may be administered orally (Blumberg and Dayton 1973), rendering naltrexone more efficacious in long-term treatment of addictive behaviours while naloxone is a better emergency antidote for opioid agonist overdose (Hardman et al. 1996). In a drug-naïve state, administration of opioid receptor antagonists has few effects, suggesting that the basal

activity of the endogenous opioid system is generally low. In humans, the most common side effects reported for naltrexone includes headache, nausea or vomiting. However, these symptoms generally diminish within a few days of treatment. In a large clinical study, the safety profile of naltrexone in the treatment of alcoholism was investigated and the authors concluded that naltrexone was safe to administer under a variety of conditions (Croop et al. 1997).

1.9 INTERACTION BETWEEN THE ENDOGENOUS OPIOID SYSTEM AND PSYCHOSTIMULANTS

1.9.1 Opioid and Dopamine Interaction

Interactions between the endogenous opioid system and the dopamine system are supported by anatomical, neurochemical and behavioural studies. Opioid receptors are found in areas with a high density of dopamine neurons and opioid peptide mRNA colocalize in postsynaptic neurons expressing dopamine receptors (Mansour et al. 1988; Sesack and Pickel 1992; Curran and Watson 1995; Mansour et al. 1995). In the nucleus accumbens, enkephalinergic neurons predominantly express D2-receptors whereas the dynorphinergic neurons express D1-receptors (Lu et al. 1998).

Several studies have shown that activation of opioid receptors can modulate dopamine release within the brain reward system. Administration of μ - and δ -opioid receptor agonists, either systemically or directly into either the VTA or substantia nigra, dose dependently increases the dopamine release in the mesolimbic dopamine system (Di Chiara and Imperato 1988; Spanagel et al. 1990; 1992; Devine et al. 1993). In the VTA, the opioid receptors are located on GABAergic neurons, and through GABAergic disinhibition, opioids can indirectly activate the dopamine neurons (Johnson and North 1992). Administration of κ -receptor agonists on the other hand, show opposite effects by decreasing the dopamine release. Subsequently, κ -receptor antagonists increase dopamine release in the nucleus accumbens (Spanagel et al. 1990; 1992; Devine et al. 1993). This implies an opioid-dopamine interaction that is upstream from the dopamine synapse in the nucleus accumbens.

In addition to the ability of opioid peptides to modulate dopaminergic activity, there is also evidence for a reverse interaction, where dopamine release modulates the opioid peptide expression, an effect downstream of the dopamine synapse in the nucleus accumbens. Local infusion of a selective D1-receptor agonist in the striatum increases dynorphin and GABA levels in the striatum (You et al. 1994). In addition, depletion of dopamine activity, induced by 6-hydroxydopamine (6-OHDA) decreases dynorphin expression, an effect reversed by administration of a D1-selective agonist (Gerfen et al. 1990). Conversely, dopamine denervation increased enkephalin expression and administration of D2-selective agonist reversed this effect an (Gerfen et al. 1990). These results imply that the dynorphin system is stimulated by dopamine via D1 receptors, whereas enkephalin is under inhibitory control by dopamine, through D2 receptors. In addition, local administration of dopamine in the nucleus accumbens increased β -endorphin release in the same area, as measured by *in vivo* microdialysis. The increased

release was blocked by pre-treatment with a dopamine receptor antagonist, suggesting that activation of dopamine receptors in the nucleus accumbens induces β -endorphin release (Roth-Deri et al. 2003).

In conclusion, the interactions described above indicate that the endogenous opioid system modulates dopamine release and activation of the dopamine system in turn regulates opioid peptide expression.

1.9.2 Endogenous Opioids and Psychostimulant Interaction

The evidence of a neuroanatomical and neurochemical interaction between the endogenous opioids and dopamine suggests that the opioid system could be involved in mediating the neurochemical and behavioural effects of amphetamine. Early studies showed that naloxone and naltrexone reduce amphetamine-induced locomotor activity in several species (Holtzman 1974; Dettmar et al. 1978; Adams et al. 1981; Winslow and Miczek 1988; Balcells-Olivero and Vezina 1997) as well as attenuate the threshold currents for self-stimulation by amphetamine in rats (Holtzman 1976; Schaefer and Michael 1990).

Pre-treatment with naloxone attenuates the increased dopamine release in the striatum induced by repeated and increasing doses of amphetamine (Hooks et al. 1992; Schad et al. 1995). The attenuating effect of naloxone seems to be dependent on activation of opioid receptors in the cell body region, since local administration of naloxone methiodide (a naloxone derivate) in the VTA or substantia nigra, but not in the striatum or nucleus accumbens, attenuated the amphetamine-induced dopamine release (Schad et al. 2002). Further administration of the enkephalinase inhibitor thiorphan, which slows degeneration of endogenous opioids, increased the dopamine response to amphetamine, indicating that endogenous opioids contribute to the neurochemical effects of amphetamine (Schad et al. 2002). These results are further supported by the fact that amphetamine-induced dopamine release in the nucleus accumbens activates postsynaptic dopamine receptors leading to a delayed and prolonged increase in opioid peptide gene expression in the striatal medium spiny neurons (Smith and McGinty 1994; Wang et al. 1995; Zhou et al. 2004). In addition, using *in vivo* microdialysis Olive et al. (2001) showed that amphetamine administration increases the release of endorphins in the nucleus accumbens, implying that the increase in endorphin may contribute to the motivational effects of amphetamine. Taken together, these amphetamine-induced neurochemical effects can be modulated by opioid peptides, and amphetamine can in turn modulate opioid peptide expression through dopamine receptor activation.

The interaction between the endogenous opioids and psychostimulants are also evident by animal studies modelling the different stages of substance dependence. Concerning the acute effects of psychostimulants, it has been suggested that the endogenous opioids might only have a modulatory effect on the reinforcing effects psychostimulant. Treatment with opioid receptor antagonists attenuates the initiation of cocaine self-administration, suggesting that opioid receptor blockage decreases but does not completely block the reinforcing effect of cocaine (De Vry et al. 1989; Kuzmin et al. 1997). Acquisition of psychostimulant CPP and locomotor sensitization was prevented by co-administration of opioid receptor antagonists (Trujillo et al. 1991; Gerrits et al. 1995; Sala et al. 1995;

Balcells-Olivero and Vezina 1997) indicating that opioids are involved in the conditioning effects of psychostimulants. In addition, the endogenous opioid system seems to have a pivotal role in psychostimulant reinstatement. By using different drug-seeking models, e.g. self-administration and expression of behavioural sensitization, it has been shown that opioid receptor antagonists decrease both cue-induced and drug-induced drug-seeking behaviour in animals with a history of psychostimulant administration (Magendzo and Bustos 2003; Anggadiredja et al. 2004; Gerrits et al. 2005; Burattini et al. 2008). Taken together, these results reflect a role of the endogenous opioid system in drug-induced reinforcement and drug-seeking behaviour with regard to psychostimulant drugs of abuse.

Interactions between the opioid system and psychostimulants have also been shown in humans. Chronic treatment with naltrexone reduces euphoria and the “crash” induced by i.v. cocaine (Kosten et al. 1992). Further support for the involvement of the endogenous opioid system in psychostimulant dependence stems from imaging studies using PET and the opioid receptor ligand [¹¹C] carfentanil. The μ -opioid receptor binding was increased in several brain regions in cocaine-dependent patients and the receptor binding was positively correlated with cocaine craving, suggesting an opioid mechanism in cocaine craving and reinforcement (Zubieta et al. 1996; Gorelick et al. 2005). Based on the potential interaction between psychostimulant and the endogenous opioid system, naltrexone was evaluated in recent clinical studies in amphetamine dependent patients. These studies demonstrated that acute administration of naltrexone attenuates the subjective effects induced by amphetamine (Jayaram-Lindström et al. 2008b). In addition, treatment with naltrexone reduced drug consumption as well as reduced craving for amphetamine (Jayaram-Lindström et al. 2005; Jayaram-Lindström et al. 2008a).

Taken together, the evidence from both animal and human studies indicates a relationship between the endogenous opioid system and certain effects of amphetamine.

2 AIM OF THE THESIS

The general aim of the present thesis was to investigate the interaction between amphetamine and the endogenous opioid system using rat behavioural models. In addition, neurochemical interactions between amphetamine and the endogenous opioid system were investigated, with a specific focus on the ventral striatum

Specific aims:

- To investigate the effect of the opioid receptor antagonist naltrexone on drug-seeking behaviour induced by a priming dose of amphetamine, using the intravenous self-administration model
- To examine the effect of naltrexone on amphetamine-induced associative-learning using the conditioned place preference paradigm
- To investigate the effect of opioid receptor antagonism on drug- and cue-induced locomotor sensitisation in amphetamine-conditioned animals
- To use *in vivo* microdialysis and PET to investigate the effect of naltrexone on amphetamine-induced changes in dopamine in the rat and human brain, respectively. In addition, in humans, to study changes in amphetamine-induced subjective effects induced by naltrexone

3 MATERIALS AND METHODS

Detailed description of the materials and methods employed in the present thesis is provided in paper I-IV. Below, a short description of each of the methods is presented.

3.1 ANIMALS

Male Wistar rats (200-300g) were obtained from Scanbur AB, Sollentuna, Sweden. Animals were housed in a temperature ($\pm 21^{\circ}\text{C}$) and humidity ($\pm 40\%$) controlled environment on a 12h light/dark cycle (lights on 7:00AM) with *ad libitum* access to food and water (except in paper I where animals were food restricted during food training). Before the start of the each experiment, animals were allowed one week to habituate to the new environment and during this time, daily handling was performed. All experiments were conducted during the light phase of the cycle. Animals were housed in groups of four animals per cage (paper II & III) or individually (paper I and in paper IV after surgery).

3.1.1 Ethical Approval

All animal experiments were performed in accordance with the guidelines of the Swedish National Board of Laboratory Animals under protocols approved by the Animal Ethics Review Board of Northern Stockholm, Sweden. The human laboratory study in paper IV was approved by the Stockholm Regional Ethics Review Board, the Safety and Radiation Committee, Karolinska Institutet and the Swedish Medical Products Agency and was conducted in accordance with Good Clinical Practice (ICH GCP, 1996) and with the Declaration of Helsinki.

3.2 DRUGS AND CHEMICALS

D-amphetamine sulphate (Apoteket AB, Sweden) and naltrexone hydrochloride (Sigma Sigma-Aldrich AB, Sweden) were dissolved in physiological saline (sodium chloride 0.9% (w/v), Apoteket AB, Sweden). In paper I, amphetamine was self-administered intravenously (i.v.) and naltrexone was administered subcutaneously (s.c.). In paper II-IV, animals were administered amphetamine and naltrexone intraperitoneally (i.p.). All systemic injections were given at a volume of 1 ml per kilogram of body weight. In study IV, the human subjects were given naltrexone or placebo orally in a capsule and dexamphetamine was given i.v. (both substances were prepared by Apoteket AB, Sweden).

3.3 BEHAVIOURAL AND *IN VIVO* NEUROCHEMICAL STUDIES

3.3.1 Intravenous Self-Administration

Food- and Self-Administration Training

To promote learning of the operant behaviour, animals were trained to press a lever for 45 mg food pellets (Bio-Serv® Bilaney Consultants, United Kingdom) under a fixed ratio-1 schedule (FR1) of reinforcement. The food training was conducted once per day. A session lasted for 60 minutes or until 100 pellets were delivered. The behaviour was considered stable when 100 food pellets were delivered in less than 20 minutes for three consecutive days. Following food training, an i.v. catheter (Brian Fromant, Cambridge, UK) was inserted into the right jugular vein. Animals were allowed one week of recovery after

surgery before the start of i.v. self-administration. Self-administration of amphetamine was performed using a FR1 schedule of reinforcement for 2h per day. Active lever response resulted in an i.v. infusion of amphetamine (0.1 mg/kg over 3s).

Extinction and Reinstatement

After a minimum of 20 days of i.v. amphetamine self-administration, animals went through extinction training where amphetamine was replaced with saline. When the behaviour was considered extinguished (less than 10 lever presses/day for three consecutive days), reinstatement testing was initiated. To examine the effect of naltrexone on amphetamine-induced reinstatement, naltrexone (0.3, 1.0 or 3.0 mg/kg), given in a counter balanced order using a Latin Square design, was administered subcutaneously 30 minutes before the amphetamine priming (0.5 mg/kg). Lever presses on both levers were recorded but had no programmed consequences. Animals were given 2 days with saline injection between the test sessions to ensure that they returned to baseline extinction levels between each testing day.

Operant Task Performance

Drug-naïve animals were trained to press a lever for food pellets under an FR1 schedule (100 pellets during 1h). When the behaviour was stable, animals were switched to the test schedule, i.e. 30 pellets within 15 minutes. Naltrexone (0.3, 1.0 or 10.0 mg/kg) was administered 30 minutes before the animals were placed in the self-administration box.

Statistical Analysis

Amphetamine reinstatement sessions were designed using a Latin square design and analysed by analysis of variance (ANOVA) for repeated measures, followed by Fisher's Least Significant Difference (LSD) post hoc analysis when appropriate. The effect of treatment with different doses of naltrexone on operant task performance (reinforced by food) was analysed similarly. A p-value <0.05 was considered statistically significant.

3.3.2 Conditioned Place Preference

Conditioned Procedure

The CPP experiments were performed in a two-compartment apparatus. The CPP procedure consisted of five different phases; pre-test, conditioning, expression, extinction and reinstatement (Fig. 4a). During conditioning, animals were injected with saline and placed on the preferred side for 30 minutes, and on alternate days injected with amphetamine (2mg/kg) and placed on the non-preferred side. This was repeated for six consecutive days. On expression, animals were given free access to both sides of the experimental box for 15 minutes. To study the effect of naltrexone on amphetamine place preference, naltrexone (0.3, 1.0 or 3.0 mg/kg) was given 30 minutes before amphetamine during conditioning (Fig. 4b) or as a single dose 30 minutes before the animals were given free access to both sides of the experimental box on the day of expression (Fig. 4c).

The learnt association between amphetamine and the drug-paired side was extinguished by injecting the animal with saline and placing it on the drug-paired side and the saline-paired side, respectively, on alternate days for 12 days. Reinstatement was induced by a priming dose of amphetamine (0.5mg/kg), preceded 30 minutes earlier by naltrexone pre-treatment.

(0.3 or 3.0 mg/kg). Place preference was measured by giving the animals free access to both side of the experimental box for 15 minutes (Fig. 4d)

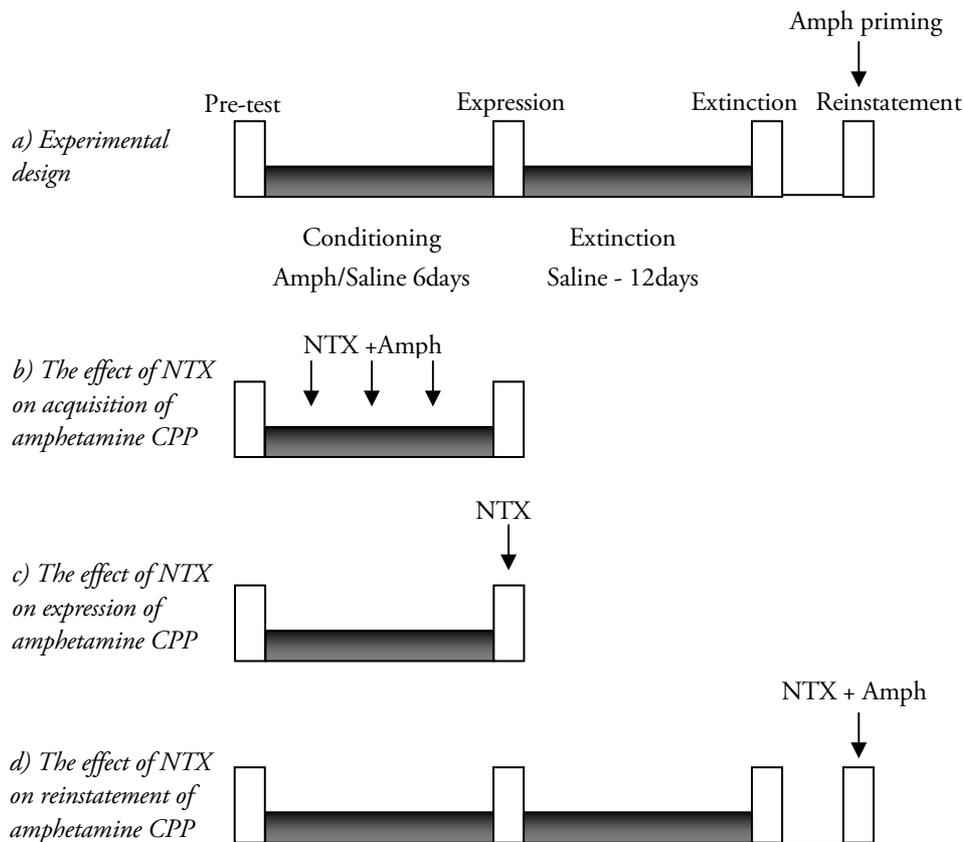


Figure 4. Conditioned Place Preference Design

a) Experimental design: animals were conditioned with saline and amphetamine on alternate days for six consecutive days. On the day of expression, animals were given free access to both sides of the experimental box. The CPP was extinguished by injecting the animal with saline and placing it on both the drug-paired side and on the saline paired side, respectively, on alternate days for 12 days. Reinstatement was induced by a priming dose of amphetamine. Each test session lasted 15 minutes. b) Naltrexone was administered 30 minutes before amphetamine during conditioning. c) On the day of expression, naltrexone was given 30 minutes before the animals were given free access to both side of the experimental box and in d) naltrexone was given 30 minutes before amphetamine priming at the reinstatement session. White box represents the test day. Abbreviations: NTX, Naltrexone; Amph, Amphetamine; CPP, Conditioned place preference

Statistical Analysis

The conditioning effect (CPP score) was analysed using two-way repeated measures ANOVA with one between factor (treatment) and one within group factor (session), followed by Bonferroni adjusted multiple comparisons when appropriate. All locomotor data were analysed by one-way ANOVA, followed by Newman–Keuls post hoc test when appropriate. A p-value <0.05 was considered statistically significant.

3.3.3 Locomotor Sensitization

Induction of Sensitization

Amphetamine or saline was administered once daily for 10 consecutive days. On day 1 and 10, the animals were first habituated to the box for 30 minutes. Animals were then given an amphetamine (2 mg/kg) or saline injection and were immediately returned to the locomotor box for another 60 minutes during which locomotor activity was measured. On day 2-9, the animals were given an i.p. injection of amphetamine or saline and were placed in the locomotor box for 30 minutes. After the 10th session, the animals were left undisturbed in their home cages for a 10-day drug free period. The animals were then assigned to either the expression of behavioural sensitisation experiment or the conditioned response experiment (see Fig. 5).

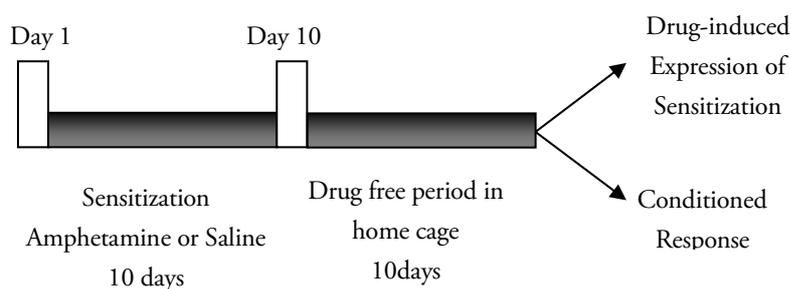


Figure 5. Experimental Design of the Behavioural Locomotor Sensitization Experiment

During sensitization (day 1-10), animals were treated with amphetamine or saline, once a day for 10 consecutive days. The locomotor behaviour was measured on day 1 and 10. After the last sensitization session, the animals were left undisturbed in their homecare for 10 days. The animals from each treatment group (amphetamine and saline) were assigned to either the drug-induced expression of sensitization experiment or the conditioned locomotor response experiment.

Amphetamine-Induced Behavioural Sensitization

Animals were habituated to the experimental box for 30 minutes. To study the effect of naltrexone on the expression of amphetamine-induced behavioural sensitization, animals were pre-treated with either naltrexone (3 mg/kg) or saline following the 30-minute habituation period. After an additional 30 minutes, the rats were given a challenge of amphetamine (0.5 mg/kg) and were placed in the box for another 60 minutes (giving a total session length of 120 minutes).

Cue-Induced Conditioned Response

After the 10-day drug-free period, the animals were habituated in the locomotor box for 30 minutes. Animals were then pre-treated with naltrexone (3 mg/kg) or saline and after an additional 30 minutes given a challenge injection of saline. The locomotor activity was measured for 60 minutes after the last injection.

Statistical Analysis

The expression of amphetamine-induced sensitization in response to an amphetamine challenge as well as for the conditioned response experiment after a saline challenge was analyzed using a two-way ANOVA. When appropriate, the Fishers LSD post hoc test was performed. A p-value <0.05 was considered statistically significant.

3.3.4 *In Vivo* Microdialysis

Rats were implanted with a microdialysis probe in the nucleus accumbens, under Hypnorm[®] (Janssen-Cilag) and Dormicum[®] (Roche) anaesthesia. After surgery, animals were individually housed and were allowed 2 days of recovery before the microdialysis experiment. During the *in vivo* microdialysis experiment, dialysate was collected over 15 min intervals and automatically injected into a high performance liquid chromatography (HPLC) system. Baseline was defined as the average of the four samples immediately preceding treatment. After baseline, rats were pre-treated with naltrexone (3 mg/kg) 30 minutes before given an amphetamine (0.5 or 2 mg/kg i.p). Dialysate was collected for 180 minutes after the last drug administration.

Statistical Analysis

The mean percent changes from baseline (average of the four dialysate samples collected immediately before the pre-treatment injection) were then calculated for each 15 min sample. Data were analyzed by one- or two-way ANOVA followed by planned comparison. A p-value <0.05 was considered statistically significant.

3.4 HUMAN LABORATORY STUDY

3.4.1 Positron Emission tomography (PET)

Subjects

Seven healthy males between the ages of 20 and 45 years were recruited at Karolinska Institutet. Exclusion criteria included (1) DSM IV diagnosis of major Axis-1 psychiatric disorder including any history of substance abuse or dependence (and nicotine dependence) in self and in first degree relatives, (2) any use of psychoactive substance within the past 30 days, (3) serious medical condition such as history of cardiac or liver disease, (3) consumption of more than 60 grams of pure alcohol per week, (4) positive result on alcohol breath analyzer at each test session, (5) traces of opiates, cannabis, amphetamines or benzodiazepines in the urine at screening and during test days, (6) known allergy to naltrexone.

Experimental Procedure

This was a double-blind placebo controlled study. Prior to the test day, all subjects underwent a magnetic resonance imaging (MRI) scan, so that anatomically accurate region of interests (ROIs) could be drawn. In addition, subjects underwent a baseline PET scan with [¹¹C] raclopride. On the test days, subjects arrived to the laboratory at 8:00 am and filled out a questionnaire to rate their mood at that present time. In addition, physiological measures such as heart rate and pulse were monitored. The subjective and physiological measures were evaluated at designated time points through the entire experimental procedure. Subjects received either a capsule of naltrexone (50 mg) or placebo. One-hour post ingestion of study medication, subjects underwent the PET scan with [¹¹C]raclopride. The subjects received either an intravenous dose of amphetamine 0.3 mg/kg or placebo and this was immediately followed by a saline solution of [¹¹C]raclopride (223–268 MBq, specific radioactivity 193–1131 GBq/μmol) which was injected as a bolus during 2 seconds. The cannula was then immediately flushed with 10 ml saline and the scans were initiated thereafter. In total, each subject received three PET scans with [¹¹C]raclopride: a baseline scan, a scan after placebo + amphetamine administration, and a scan after NTX +

amphetamine administration (denoted as baseline, amphetamine, and amphetamine + NTX, respectively). Brain radioactivity was measured for 51 min after injection of [¹¹C]raclopride.

Regions of interest (ROI)

The striatum was divided into limbic (LST) and sensorimotor (SMST) sub-regions. A ROI for cerebellum was drawn below the appearance of the petrosal bone in five slices, corresponding to a thickness of 10 mm.

Subjective and Physiological Measures

To correlate the neurobiological changes with subjective reporting, by the human subjects a visual analog rating scale (VAS) was administered at designated time points, to describe current drug effects. The VAS comprised four scales: 'feel the drug', 'like the effect', 'feel aroused' and 'want more', providing a composite measure of the subjective effect. The subjects rated their experiences 60 minutes after ingestion of the first dose of NTX/placebo and then at designated time points during and after the PET scan. To measure physiological effects of amphetamine, heart rate and blood pressure were recorded manually. These recordings were made at 60 minutes after ingestion of the first dose of NTX/placebo and at designated time points during and after the PET scan.

Statistical Analysis

Statistical evaluation of the binding potential (BP) for each ROI was conducted using two-way repeated-measures ANOVA with Greenhouse-Geisser correction. Three comparisons of BP values were estimated by the ANOVA: (1) baseline vs. amphetamine, (2) baseline vs. amphetamine + naltrexone, (3) amphetamine vs. amphetamine + naltrexone. The secondary measure of the study, i.e. the effect of naltrexone on the subjective effects of amphetamine, was analyzed by repeated measures of ANOVA.

4 RESULTS AND DISCUSSION

4.1 Naltrexone Reduces Amphetamine-Induced Drug-Seeking Behaviour in Animals Previously Self-Administering Amphetamine (Paper I)

In paper I, the effect of naltrexone on reinstatement of self-administration in the rat was investigated. Animals were allowed to self-administer i.v. amphetamine for a minimum of 20 days after which the self-administering behaviour was extinguished by replacing amphetamine with saline. A priming dose of amphetamine readily reinstated drug-seeking behaviour, expressed as an increased number of responses on the lever previously associated with amphetamine (Fig. 6). Pre-treating the animals with naltrexone 30 minutes before the amphetamine priming significantly attenuated amphetamine-induced reinstatement. There were no statistical significant differences in lever presses on the inactive lever between extinction and reinstatement indicating that drug priming produced a specific drug-seeking behaviour and did not result in any unspecific increase in locomotor activity (Fig. 6).

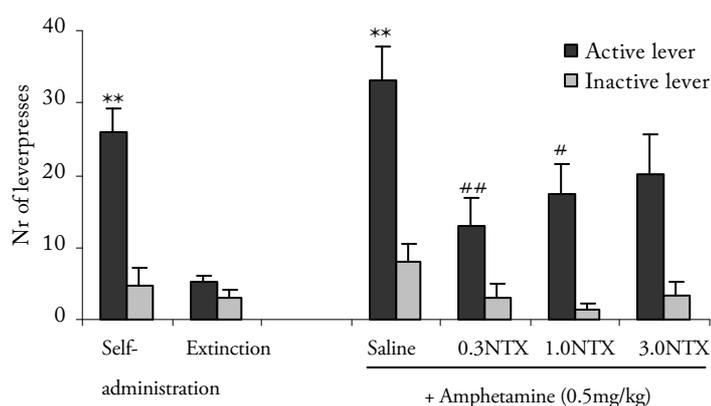


Figure 6. Effects of NTX on amphetamine-induced reinstatement

Data are presented as the mean \pm S.E.M of active and inactive lever presses during the last 3 days of self-administration, the last 3 days of extinction and on the reinstatement test session following amphetamine priming, respectively. During reinstatement, animals were pre-treated with NTX 30 minutes before the amphetamine priming, (n=12). **p<0.001 as compared to extinction, #p<0.05 ## p<0.01 as compared to vehicle + amphetamine. Abbreviations: NTX, Naltrexone

Earlier studies have shown that administration of an opioid antagonist can cause a suppression of spontaneous activity in laboratory animals, especially when given in relatively high doses (Amir et al. 1979; Rodgers and Deacon 1979; Walker et al. 1981; DeRossett and Holtzman 1982). To rule out any non-specific locomotor attenuating effect on operant behaviour by the doses of naltrexone used in the present study, animals were pre-treated with naltrexone before given the ability to press for food pellets. Pre-treatment with naltrexone did neither affect the number of pellets achieved in 15 minutes nor the time to reach the maximum number of pellets (i.e. 30 pellets) within the designated time (Fig. 7). These results suggest that naltrexone, at the doses used in the present study, does not impair the ability to perform operant responding, i.e. the capacity to press a lever.

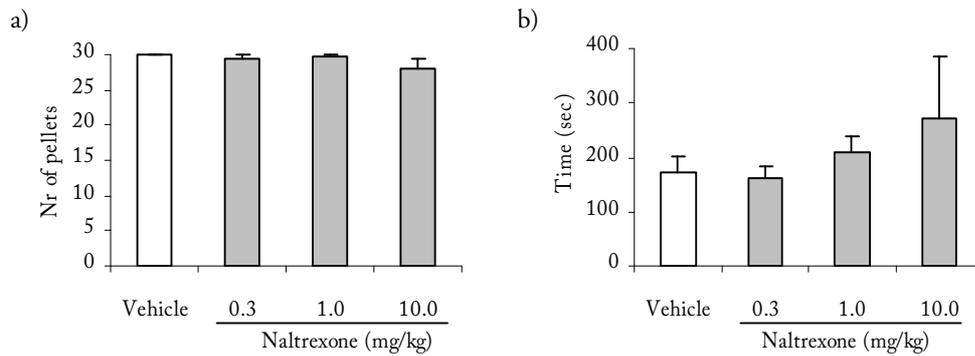


Figure 7. Effect of naltrexone on food-reinforced operant responding

Naltrexone did neither affect a) the number of pellets reached within 30 minutes (n=8), nor b) the time to reach 30 pellets (n=6). Data are presented as the mean \pm S.E.M (modified from paper I)

The results from the paper I are in agreement with earlier studies by Gerrits and co-workers (2005), showing that repeated administration of naltrexone attenuates reinstatement induced by cocaine (Gerrits et al. 2005). In addition, local infusion of the μ -opioid receptor antagonist Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) into the ventral pallidum blocked reinstatement induced by a systemic injection of cocaine (Tang et al. 2005). These results, together with the results from paper I, suggests an involvement of the endogenous opioid system on drug-induced reinstatement behaviour in animals previously self-administering psychostimulant drugs. However, in contrast to the above mentioned studies, Anggadiredja et al. (2004) showed that naltrexone attenuated cue- but not drug-induced reinstatement in animals previously self-administering methamphetamine (Anggadiredja et al. 2004). The discrepancy between the latter study and the data presented in paper I may be related either to pharmacodynamic differences between amphetamine and methamphetamine or to the significantly higher priming dose of methamphetamine compared to the dose of amphetamine used in the paper I.

The reinstatement model of self-administration is commonly used in substance dependence research as its face validity towards human relapse is considered to be significant (Epstein et al. 2006). In this paradigm, only drug seeking behaviour and not drug taking is examined, since pressing on the (previously active) lever during reinstatement does not result in infusion of the previously self-administered drug. In humans, a small dose of the drug can provoke drug craving, which could eventually lead to drug-seeking and relapse (Breiter et al. 1997). It has been shown, for example with alcohol, that substances that reduce reinstatement in animals may also reduce craving and relapse in patients with substance dependence (O'Malley et al. 1992; Volpicelli et al. 1992; Sass et al. 1996; Le et al. 1999). The results from paper I are in line with recent clinical trials showing that treatment with naltrexone reduces relapse and drug craving in amphetamine dependent patients (Jayaram-Lindström et al. 2005; Jayaram-Lindström et al. 2008a).

In summary, naltrexone attenuates amphetamine-induced drug-seeking behaviour, an effect specifically related to the ability of naltrexone to modulate the rewarding and reinforcing effect of amphetamine.

4.2 The Effect of Naltrexone on Amphetamine-Induced Conditioned Place Preference (Paper II)

The CPP paradigm is considered a valuable tool for measuring reward associated learning in laboratory animals. In paper II, the effect of naltrexone on conditioning, expression and reinstatement of amphetamine-induced CPP was investigated. In agreement with earlier studies (Spyraki et al. 1982; Trujillo et al. 1991; Shimosato and Ohkuma 2000), we showed that repeated administration of amphetamine produced a robust and reproducible place conditioning, highlighting the strong motivational and reinforcing properties of amphetamine.

Acquisition of Conditioned Place Preference

To study the effect of naltrexone on amphetamine-induced associative learning, animals were pre-treated with naltrexone 30 minutes before amphetamine during the conditioning phase. On the day of expression, the animals were given free access to the experimental box without any injections. Naltrexone, at all doses studied (0.3, 1.0 or 3.0 mg/kg), failed to modulate the amphetamine-induced CPP (Fig. 8a) indicating that naltrexone does not modulate the associative learning between the reinforcing properties of amphetamine and contextual cues, in this behavioural model. These findings are in line with an earlier study (Houdi et al. 1989) where pre-treatment with the opioid receptor antagonist naloxone during conditioning, failed to attenuate cocaine-induced CPP on the first day of expression. However, there are discrepancies in the literature as earlier studies have shown that opioid antagonists may attenuate CPP induced by both amphetamine (Trujillo et al. 1991) and cocaine (Menkens et al. 1992; Gerrits et al. 1995; Kuzmin et al. 1997). Compared to the study by Trujillo et al. (1991) the present study differed in several aspects, i.e. the dose of amphetamine, route of administration, time schedule and opioid antagonist (naltrexone vs. naloxone).

Expression of Conditioned Place Preference

Animals were conditioned with amphetamine and on the day of expression, they were given one single injection of naltrexone 30 minutes before given free access to both sides of the experimental box. Pre-treatment with naltrexone before placement in the box did not modulate the CPP induced by amphetamine (Fig. 8b). These results suggest that naltrexone failed to affect the learned association between amphetamine and the drug-paired compartment. Expression of CPP is believed to reflect drug-seeking behaviours induced by contextual cues, and the lack of effect of naltrexone suggests that it does not affect drug-seeking behaviour induced by contextual cues at least not as measured by using the rat CPP paradigm. In the present study, only one dose of amphetamine (2 mg/kg) was studied during conditioning. This could be a limitation of the study since earlier studies have shown that the effect of opiate antagonists on the expression of psychostimulant-induced CPP can be dose-dependent. In a study by Gerrits and co-workers (1995), naloxone only attenuated the expression of cocaine-induced CPP when the animals were conditioned with a lower dose but not a higher dose of cocaine (10 and 20 mg/kg, respectively) (Gerrits et al. 1995).

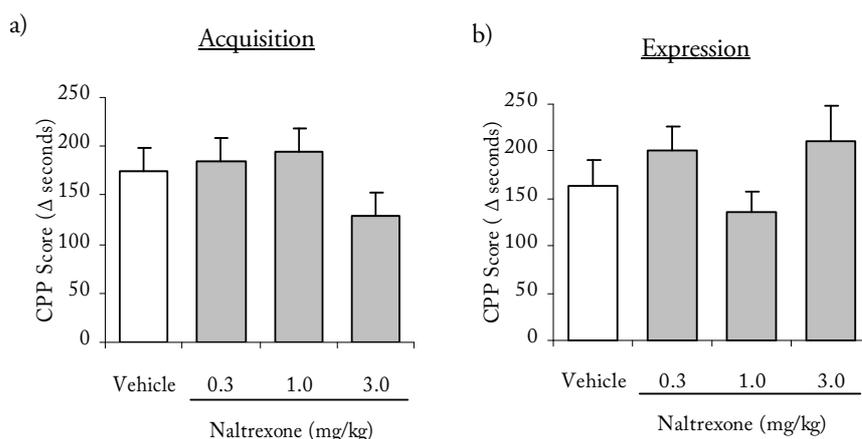


Figure 8. Effect of naltrexone on acquisition and expression of amphetamine-induced conditioned place preference.

In a) naltrexone (0, 0.3, 1.0 or 3.0 mg/kg) was administered 30 minutes before amphetamine (2 mg/kg) during conditioning. b) On the expression day, animals were given NTX (0, 0.3, 1.0 or 3.0 mg/kg) 30 minutes before free access to the experimental box. Data (CPP score) are presented as the difference in time spent on the drug-paired side between the expression day and initial preference day. Each value denotes the mean \pm S.E.M Abbreviations: CPP, Conditioned Place Preference

Reinstatement of Conditioned Place Preference

In addition to the acquisition and expression of conditioned place preference, the effect of naltrexone on reinstatement of amphetamine CPP was also studied. After conditioning, the behaviour was extinguished by repeated pairings of saline with the previously drug-associated compartment. Priming the animals with a small dose of amphetamine readily reinstated the preference for the drug-paired compartment in animals previously conditioned with amphetamine. Control animals, conditioned with saline and given their first amphetamine dose at reinstatement, did not change their time spent on either side of the experimental box. This indicates that the amphetamine priming specifically induces reinstatement and that the increase in time spent on the drug-paired side is not just a general increase in locomotor behaviour induced by amphetamine. Pre-treatment with naltrexone 30 minutes before amphetamine priming did not modulate the drug-induced reinstatement (Fig. 9).

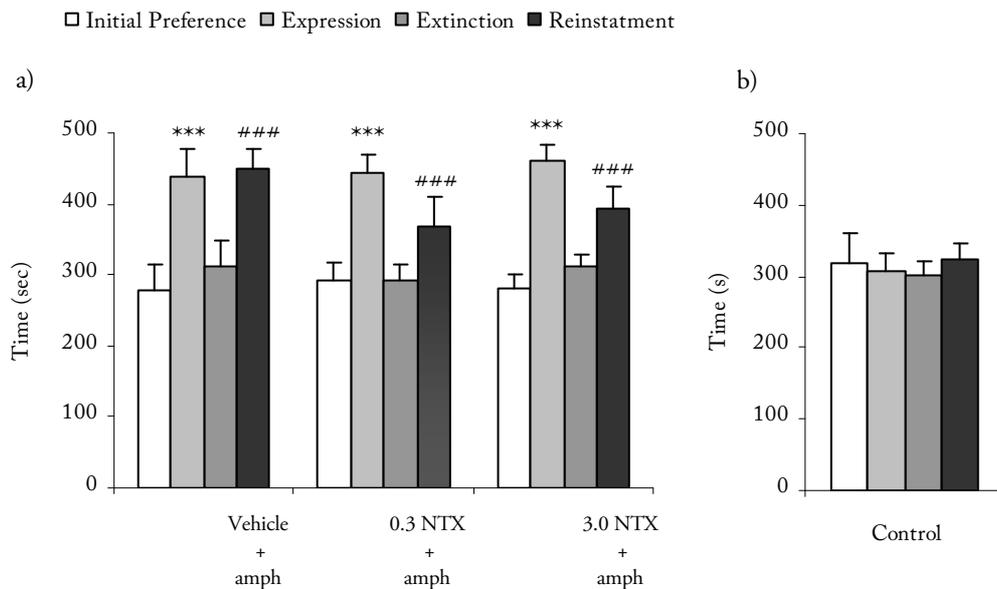


Figure 9. The effect of naltrexone on reinstatement of amphetamine-induced place preference
a) Animals were conditioned with amphetamine (2 mg/kg) for 6 days followed by saline for 12 days (extinction phase). During extinction, animals were placed on both the preferred and non-preferred side of the experimental box on alternate days. On the following day, the animals were given free access to both side of the experimental box for 15 minutes to evaluate extinction of the behaviour. On the reinstatement day, animals were given vehicle, 0.3NTX or 3.0NTX 30 minutes before a priming injection of amphetamine (0.5 mg/kg). b) Control animals received saline during conditioning and extinction. On reinstatement, they were pre-treated with saline and then given amphetamine priming. Each value denotes the mean \pm S.E.M *** $p < 0.001$ compared to initial preference, ### $p < 0.001$ compared to extinction Abbreviations: NTX, Naltrexone; Amph, Amphetamine

Naltrexone Place Conditioning

Earlier studies have reported that naltrexone and naloxone induce place aversion, especially when given at high doses (Trujillo et al. 1991; Gerrits et al. 1995) although there are conflicting results showing that conditioning with naltrexone neither produces place preference nor place aversion (Bossert and Franklin 2001; Braida et al. 2004). Since the effect of naltrexone seems to be dose dependent, with higher doses being more aversive, we investigated the effect of the highest dose of naltrexone (3 mg/kg) used in paper II in the place conditioning paradigm. Naltrexone was paired with either the preferred- or the non-preferred side of the experimental box. As compared to saline treated animals, animals conditioned with naltrexone did not change their preference when pairing was conducted with either side of the experimental box (Table 3). The results from the experiments suggest that the doses used in the present study do not cause negative associative learning or produce place aversion.

Table 3. Naltrexone place conditioning.

NTX preferred side: animals were administered with NTX and placed on the preferred side and on alternate days given saline and placed on the non-preferred side; NTX non-preferred side: animals were given NTX and placed on the non-preferred side and on alternate days administered with saline and placed on the preferred side. Saline treated animals received saline injections prior to placement on both sides. Each value denotes the mean \pm S.E.M. Abbreviations: NTX, Naltrexone

Treatment	Initial Preference		Expression	
	Black (s)	White (s)	Black (s)	White (s)
Saline (n=7)	632.7 \pm 26.0	265.6 \pm 25.4	587.4 \pm 25.0	310.5 \pm 25.1
NTX Preferred side (n=10)	633.0 \pm 20.1	266.9 \pm 20.1	597.7 \pm 27.7	292.8 \pm 25.5
NTX Non-preferred side (n=9)	645.6 \pm 19.6	248.1 \pm 17.6	592.3 \pm 37.2	307.6 \pm 37.2

The Effect of Naltrexone on Locomotor Activity during Conditioned Place Preference

Animals pre-treated with naltrexone during amphetamine conditioning showed no changes in any of the locomotor measures on the day of expression. Neither did a single dose of naltrexone given on the day of expression modulate the locomotor activity as measured by distance travelled, time moving or moves/counts. However, pre-treatment with the highest dose of naltrexone (3mg/kg) before the amphetamine challenge on the day of reinstatement significantly attenuated the amphetamine-induced locomotor activity (Fig. 10). There were no differences in general locomotor behaviour between the naltrexone pre-treated animals and the previously saline-conditioned animals indicating that naltrexone specifically blunts the amphetamine-induced locomotor response in animals previously conditioned with amphetamine.

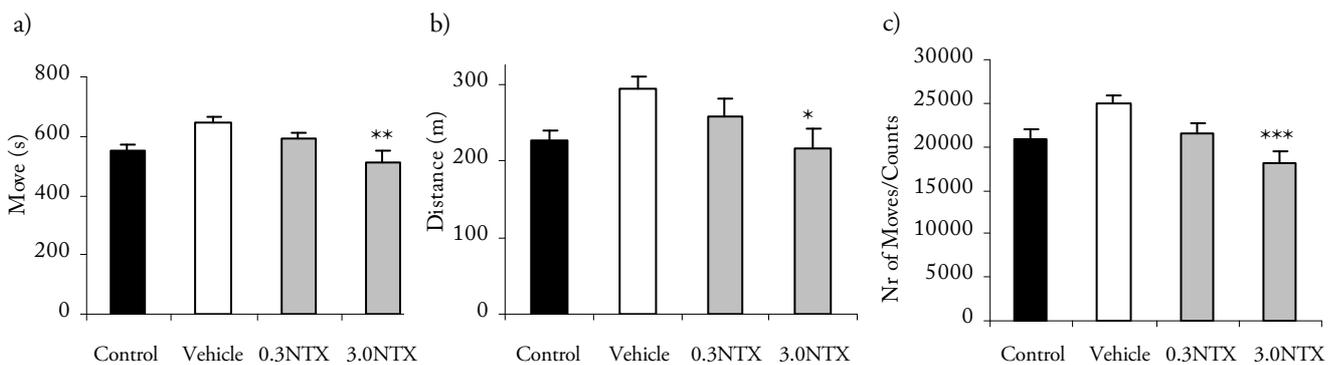


Figure 10. Effect of naltrexone on locomotor activity following a priming dose of amphetamine at reinstatement

Animals were previously conditioned with saline (control animals) or amphetamine (group a-c). Figure a) time moving and b) distance travelled c) moves/counts during the 15min test session. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to group (a) (animals conditioned with amphetamine and on the reinstatement day they were given amphetamine preceded by a saline injection). Each value denotes the mean \pm S.E.M. Abbreviations: NTX, Naltrexone

The CPP paradigm is one of the most commonly used experimental protocols for measuring drug reward in laboratory animals. However, interpretation of the results from CPP is not always easy to make. If an adjuvant treatment alters the ability of a drug to induce place preference (acquisition) it is generally assumed that the treatment interferes with the motivational properties of the drug (Sanchis-Segura and Spanagel 2006). However, a lack of CPP could also be due to e.g. that the adjuvant treatment interferes with the process of conditioning rather than diminishes the motivational and rewarding effects of the drug (Tzschentke 1998). In paper II, we found that naltrexone failed to modulate the acquisition of amphetamine-induced CPP. These findings indicate that naltrexone does not block the rewarding and reinforcing effects induced by amphetamine and that naltrexone does not impair associative learning. The results from paper II are supported by human laboratory studies where a single dose of naltrexone blunted, but did not completely block, the subjective effects produced by amphetamine both in patients with amphetamine dependence and in human subjects (Jayaram-Lindström et al. 2004; Jayaram-Lindström et al. 2008b). Considering that naltrexone may not completely block the subjective effects, it is possible that animals still experience the motivational and reinforcing effects induced by amphetamine and associate that with the context, leading to place preference.

In contrast to acquisition, the expression of CPP occurs in the absence of the conditioning stimulus, i.e. amphetamine, and instead relies on the learned motivational properties of the environmental cues (Tzschentke 1998; Bardo and Bevins 2000). Substances given on the day of expression and that reduce CPP have been considered as potential candidates in the treatment of human drug craving (Sanchis-Segura and Spanagel 2006). In addition to expression, the reinstatement model of CPP is also used to investigate potential anti-craving medications as this model also has high face validity towards relapse to drug use in humans (Epstein et al. 2006). The expression of CPP is dependent on cues associated with drug-administration while reinstatement of CPP can be induced by stressors (Wang et al. 2000; Sanchez and Sorg 2001) or by priming doses of the drug (Mueller and Stewart 2000a; Wang et al. 2000). The above mentioned stimuli also provoke drug craving and relapse in humans (Jaffe et al. 1989; Childress et al. 1993; Sinha 2001). In paper II, no effect of naltrexone was observed on the expression or the reinstatement of CPP. In contrast, recent human clinical trials have demonstrated an effect of naltrexone on amphetamine consumption and relapse (Jayaram-Lindström et al. 2005; Jayaram-Lindström et al. 2008a). The lack of effect of naltrexone on expression and reinstatement of CPP in the present study therefore suggests differences in the modulatory role of endogenous opioids between different stimulant-induced behaviours.

4.3 Naltrexone Reduces the Amphetamine-Induced Sensitized Locomotor Activity and Conditioned Locomotor Response (Paper III)

In paper III, the effect of naltrexone on both drug-induced locomotor sensitization as well as cue-induced locomotor activity was investigated. Animals were repeatedly treated with amphetamine for 10 consecutive days, which induced a robust sensitization. The sensitized response, induced by repeated treatment with amphetamine, is thought to reflect persistent neurophysiological adaptations that might underlie certain aspects of substance dependence (Robinson and Berridge 1993).

Naltrexone Attenuates Amphetamine-Induced Sensitization

After induction of sensitization, animals were left undisturbed in their home cage for 10 days. On the following day, animals (both amphetamine-conditioned and saline-conditioned) were challenged with amphetamine. Animals conditioned with amphetamine during induction of sensitization, had an increased horizontal locomotor activity as compared to control animals when challenged with amphetamine. Pre-treatment with naltrexone reduced this sensitized response (Fig. 11a). In contrast, naltrexone failed to attenuate the amphetamine-induced locomotor activity in saline-conditioned control animals ($p=0.07$). Concerning vertical activity, no statistically significant interaction effect was found (Fig. 11b). These results are in agreement with a study showing that naloxone attenuates the expression of amphetamine sensitization (Magendzo and Bustos 2003). In addition to the effect on expression of sensitization, co-administration of opioid receptor antagonists together with psychostimulants prevents the development of both cocaine and amphetamine-induced sensitization (Sala et al. 1995; Balcells-Olivero and Vezina 1997).

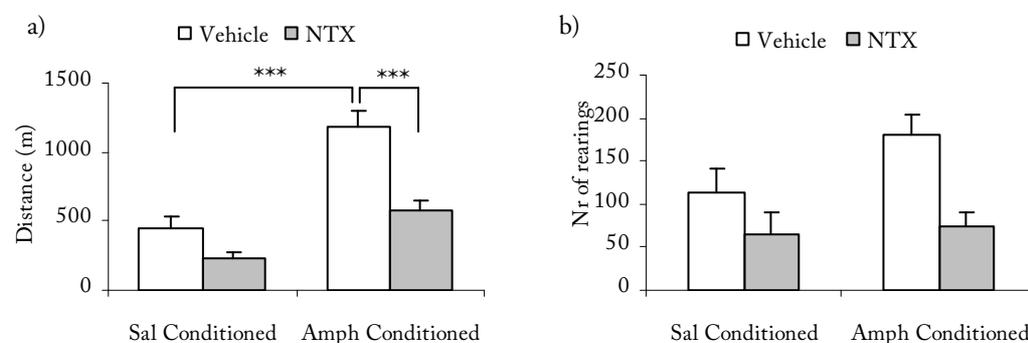


Figure 11. The Effect of Naltrexone on the Expression of Behavioral Sensitization

During the 10-day conditioning period, animals were given either saline or amphetamine (Sal conditioned; Amph conditioned). After a 10 day drug free period, the animals were challenged with amphetamine (0.5 mg/kg) preceded by NTX (3 mg/kg) or vehicle given 30 minutes before. a) Total distance during 60 minutes post injection is shown b) Total number of rearings during 60 minutes post injection *** $p<0.001$. The values represent the mean \pm S.E.M. ($n=7-8$ in each group) Abbreviations: NTX, Naltrexone; Amph, Amphetamine; Sal, Saline

Naltrexone Attenuates Conditioned Response in Animals Previously Sensitised with Amphetamine

To study the effect of naltrexone on cue-induced behaviours, animals were challenged with saline in a context previously associated with amphetamine, after the 10-day drug free period in the home cage. Animals given amphetamine during conditioning showed an increase in locomotor activity in response to a saline challenge, as compared to saline-conditioned animals, both on horizontal and vertical activity (Fig. 12). Naltrexone significantly attenuated the cue-induced increase in locomotor activity in amphetamine-conditioned animals. The inhibitory effect of naltrexone was specific to the amphetamine-induced conditioned locomotor response since naltrexone had no effect in control animals given saline during the 10-day conditioning phase (Fig. 12). The effect of naltrexone on cue-induced behavioural responses has previously been shown using other drugs of abuse, such as nicotine (Liu et al. 2009), ethanol (Bienkowski et al. 1999), methamphetamine (Anggadiredja et al. 2004), cocaine (Burattini et al. 2008) and opiates (Zhou et al. 2009).

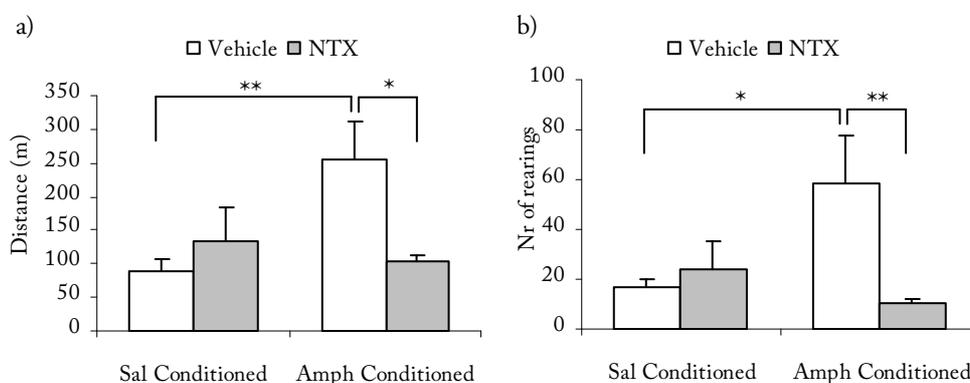


Figure 12. The Effect of Naltrexone on Conditioned Locomotor Activity

Animals were administered with either saline or amphetamine during the 10-day treatment period. After a 10-day drug-free period, animals were given a saline challenge preceded by an naltrexone or vehicle injection 30 minutes earlier. a) Distance travelled (m) and b) number of rearings are shown. * $p < 0.05$ and ** $p < 0.01$. The values represent the mean \pm S.E.M observed during 60 minutes after saline injection ($n = 7-8$ in each group). Abbreviations: NTX, Naltrexone; Amph, Amphetamine; Sal, Saline

Sensitization is defined as an increased response following repeated administration of a defined stimulus, e.g. amphetamine. With regards to substance dependence research, the locomotor activity is the most used measure of sensitization. The neurobiological systems that mediate the psychomotor effects overlap with brain systems involved in reward and reinforcement, and it is therefore considered that psychomotor sensitization is a good model for studying neuroadaptations induced by repeated administration of drugs of abuse. According to the incentive-sensitization model proposed by Robinson and Berridge (1993), not only psychomotor stimulation is increased with repeated drug treatment but also the incentive salience attributed to an initially neutral stimulus is progressively increased with repeated association between the stimulus and the drug.

The incentive sensitization theory has been proposed as a possible mechanism explaining the transition from controlled drug intake to compulsive drug-seeking and drug-taking

behaviour (Robinson and Berridge 1993). This sensitized neurochemical and behavioural response is evident both in response to a drug challenge, i.e. expression of behavioural sensitization (Robinson and Becker, 1986) as well as in a drug-free state when exposed to a context previously paired with drug administration, i.e. conditioned locomotor activity (Pickens and Crowder, 1967; Tilson and Rech, 1973). In paper III, naltrexone attenuated both the expression of behavioural sensitization as well as the conditioned locomotor activity in animals previously sensitized with amphetamine. These results suggest that the drug-seeking behaviour induced both by amphetamine priming as well as by amphetamine-associated cues, can be attenuated by an opioid receptor antagonist.

4.4 Effect of Naltrexone on Amphetamine-Induced Dopamine Release in the Nucleus Accumbens (Paper IV)

From a neurobiological perspective, it is well documented that release of dopamine within the nucleus accumbens plays an important role in the reinforcing and rewarding effects of drugs of abuse. Clinical studies have shown that naltrexone attenuates the subjective effects (high, arousal and liking) induced by amphetamine (Jayaram-Lindström et al. 2004; Jayaram-Lindström et al. 2008b). However, the neurobiological mechanism by which naltrexone mediates these pharmacological effects remains unclear. Based on the knowledge that amphetamine's reinforcing effects appear linked to alterations in brain dopamine levels, in paper IV, the effect of naltrexone on amphetamine-induced dopamine levels was investigated both in animal and human experimental models.

Naltrexone does not Modulate Amphetamine-Induced Changes in Extracellular Dopamine in the Nucleus Accumbens of the Rat

In agreement with earlier studies (Sharp et al. 1987), the present study showed that administration of systemic amphetamine to laboratory animals caused a dose-dependent increase in extracellular dopamine in the nucleus accumbens as measured by microdialysis, an effect that lasted up to 90-180 minutes depending on dose (Fig. 13). Systemic pre-treatment with naltrexone 30 minutes before amphetamine did not modulate the amphetamine-induced effects on dopamine. Naltrexone itself had no effect on extracellular dopamine levels in the nucleus accumbens (Fig. 13). Previous *in vivo* microdialysis studies investigating the effect of opioid receptor antagonists on amphetamine-induced dopamine release in the striatum have shown diverging results. Pre-treatment with naloxone (5 mg/kg) decreased dopamine release induced by repeated amphetamine-administration (Hooks et al. 1992; Schad et al. 1995) whereas a study from Feigenbaum and Howard (1997) demonstrated that 0.8 mg/kg naloxone produces an enhanced effect on central dopamine release (Feigenbaum and Howard 1997). The discordance between the results from paper IV and the above mentioned studies is not clear, but could possibly be explained by choice of opioid antagonist (naltrexone vs. naloxone), route of administration (i.p. vs. s.c.) or dosing of amphetamine (single dose of amphetamine or repeated administration).

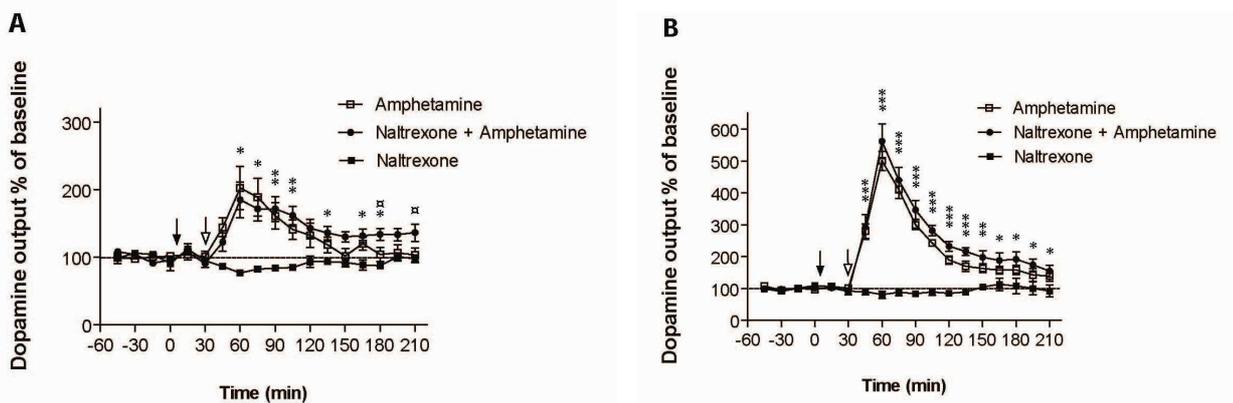


Figure 13. The effect of naltrexone on amphetamine induced dopamine release in the nucleus accumbens. Arrows indicate time of injection, black arrows represent naltrexone (3 mg/kg) or saline and white arrows represent amphetamine or saline. (a) Amphetamine 0.5 mg/kg (b) Amphetamine 2.0 mg/kg. Data are presented as the mean \pm S.E.M. Naltrexone + amphetamine compared to naltrexone (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Naltrexone + amphetamine compared to amphetamine (□ $p < 0.05$)

Naltrexone Does not Modulate Amphetamine-Induced Changes in Dopamine in the Striatum of Healthy Human Subjects

In the human experimental study, i.v. administration of amphetamine in healthy subjects increased dopamine release in the striatum as measured by a significant decrease in [^{11}C]raclopride binding (Fig. 14). In addition, there was a correlation between the decrease in [^{11}C]raclopride binding and an increase in the rating of subjective drug effects. The finding of a significant decrease in dopamine- D_2 receptor binding induced by amphetamine is in line with previous human PET studies (Drevets et al. 2001; Martinez et al. 2003). Pre-treatment with naltrexone one hour prior to amphetamine failed to modulate the drug-induced changes in [^{11}C]raclopride in the striatum (Fig. 14). In contrast, pre-treatment with naltrexone produced a significant reduction in the subjective effects of amphetamine (Fig. 15). The effect of naltrexone on subjective and rewarding effects induced by amphetamine is in agreement with previous studies in drug-naïve and amphetamine dependent individuals, respectively (Jayaram-Lindström et al. 2004; Jayaram-Lindström et al. 2008b).

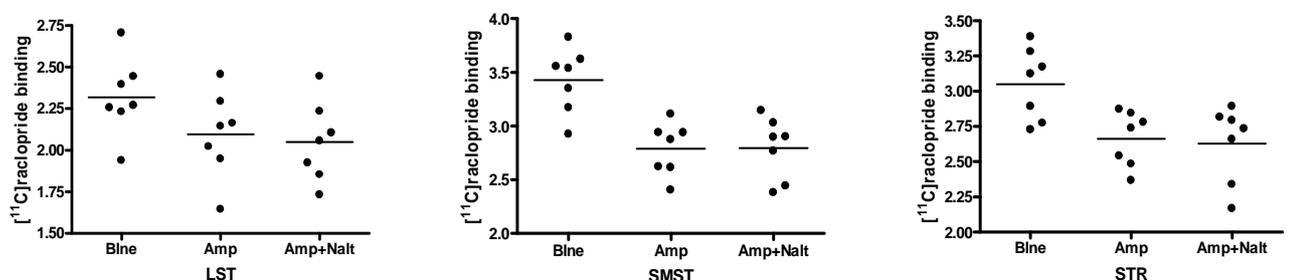


Figure 14. The effect of naltrexone on dopamine release in the striatum of healthy human subjects

The [^{11}C] raclopride binding potentials in the striatum of drug naïve healthy individuals ($n=7$) at baseline, after amphetamine administration, and after amphetamine and naltrexone administration in the striatum. Abbreviations: Amp, Amphetamine; Nalt, naltrexone; LST, limbic striatum; SMST, Sensorimotor striatum; STR, total striatum

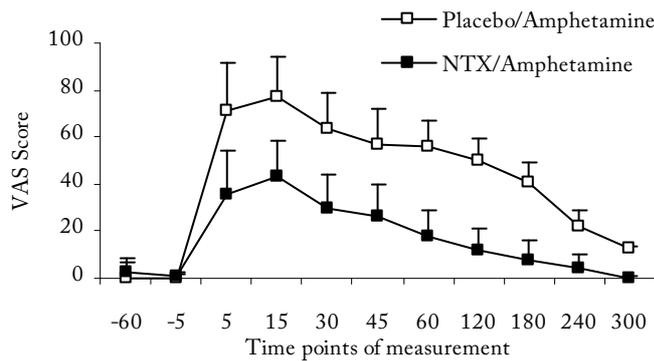


Figure 15. VAS scores of subjective effects in healthy human subjects Naltrexone (50 mg) significantly ($P < 0.001$) attenuated the subjective effects of intravenous amphetamine (0.3 mg/kg body weight) when compared to placebo. A fixed, single dose of naltrexone or placebo was administered at time point - 60 min and i.v. amphetamine at timepoint 0. $n=7$, Data is presented as Mean \pm S.D. Abbreviations: NTX, Naltrexone; VAS, Visual Analog Scale

The results from paper IV are in agreement with earlier studies showing an increase in dopamine release induced by amphetamine, an effect that was correlated with the subjective effects in the human laboratory part of the study. Pre-treatment with naltrexone failed to modulate amphetamine-induced dopamine release in the striatum, both in animals as well as in humans. However, the attenuating effect of naltrexone on the subjective effects induced by amphetamine, without alterations in dopamine release, suggests that this effect of naltrexone might be dopamine-independent, at least in the striatum.

The role of dopamine in the liking and wanting effects of drugs of abuse has been widely debated during recent years. Reduction of dopamine function, either by destruction of ascending dopamine projections by 6-hydroxydopamine or by administration of neuroleptic drugs, does not impair “liking” reactions elicited by e.g. a sweet taste (Berridge et al. 1989; Pecina et al. 1997). Further, administration of dopamine receptor antagonists failed to antagonize the psychostimulant induced subjective effects in humans (Brauer and De Wit 1997; Wachtel et al. 2002). Taken together, this suggests that dopamine might not be essential for pleasurable effects of drugs.

There are now increasing evidence for a reward pathway that mediates the pleasure or reward signalling beyond the nucleus accumbens to the ventral pallidum, and it has been hypothesized that enkephalin may be its primary neurotransmitter (Gardner 2005). Microinjection of opioids in the nucleus accumbens and ventral pallidum amplifies liking of sweet taste rewards (Pecina and Berridge 2005; Smith and Berridge 2005). Intracranial self-stimulation of the mesolimbic pathway, e.g. in the VTA, induces endogenous opioid release in the ventral pallidum (Stein 1993). In addition, lesion of the nucleus accumbens–ventral pallidum circuit reduces cocaine self-administration as well as decreases the break point in a cocaine progressive ratio paradigm, indicating that lesion of this pathway causes an attenuation of the rewarding effects of cocaine (Hubner and Koob 1990). This lends support to the present finding of dissociation between subjective effects of amphetamine and striatal dopamine function.

5 SUMMARY OF FINDINGS

In the present thesis, the effect of the opioid receptor antagonist naltrexone on amphetamine-induced behaviours was investigated.

- Naltrexone attenuated amphetamine-induced drug-seeking behaviour in animals previously self-administering amphetamine. The effect of naltrexone was not due to a general disruption of behaviour, since the same doses of naltrexone had no effect on operant responding maintained by food
- Naltrexone had no effect on the acquisition, expression or the reinstatement of amphetamine-induced conditioned place preference. In contrast, naltrexone significantly attenuated the locomotor response to a priming dose of amphetamine on reinstatement without affecting general locomotor behaviour
- A single dose of naltrexone attenuated the amphetamine-induced locomotor activity in response to a drug challenge during the expression of sensitization. In addition, pre-treatment with naltrexone blocked the conditioned locomotor response induced by re-exposure to the context previously associated with amphetamine
- Amphetamine induced a dose-dependent increase in extracellular dopamine in the striatum of the rat. In addition, in humans amphetamine increased dopamine release in the striatum as measured by a significant decrease in [¹¹C]raclopride binding, corresponding with a marked increase in self-reported rating of drug effects (“high”, arousal and liking). Pre-treatment with an acute dose of naltrexone attenuated the subjective effects of amphetamine, without altering the amphetamine-induced changes in [¹¹C]raclopride binding in the ventral striatum

6 GENERAL CONCLUSION

Although it should be noted that no animal model could fully imitate the human condition, animal models have proven to be valuable tools for studying specific elements of the process underlying substance dependence. As described in the Introduction, substance dependence can be divided into different phases of drug taking and drug seeking behaviour. Here, the role of the endogenous opioid system in different phases of the cycle was investigated, i.e. the acute interaction between amphetamine and naltrexone, the effect of naltrexone on the conditioning effects and lastly, the effect of naltrexone on both cue- and drug-induced drug-seeking behaviour. In addition, the neurochemical interactions between amphetamine and the endogenous opioid system were investigated by measuring amphetamine-induced changes in extracellular dopamine in the striatum using both in vivo microdialysis in animals as well as PET imaging in humans.

Initial drug intake is associated with the induction of euphoria and feelings of well being. The positive reinforcing and rewarding effects of the drug may promote repeated drug-taking behaviour. In paper IV, healthy human subjects reported that amphetamine increased subjective effects, i.e. liking the drug, feeling the drug, and wanting more. Pre-treatment with naltrexone attenuated, but did not completely block, these amphetamine-induced subjective effects. Concerning the behavioural interaction between naltrexone and amphetamine in the rat, naltrexone failed to significantly attenuate locomotor activity in response to an acute amphetamine challenge (although there was a trend; $p=0.07$) (paper III). This might indicate that endogenous opioids have a modulatory effect on the acute reinforcing effects of this psychostimulant.

In the CPP study (paper II), pre-treatment with naltrexone during the acquisition phase did not modulate the amphetamine-induced place preference, indicating that naltrexone does not interfere with the associative learning process between the reinforcing effect of amphetamine and the stimuli associated with drug-intake. Since naltrexone attenuated, but did not completely block, the subjective effect of amphetamine in human subjects (paper IV), it can be hypothesised that the animals can still experience the motivational and reinforcing effects, enabling place preference to be formed.

In the present thesis, the effect of naltrexone on both cue-induced and drug-induced drug-seeking behaviour was investigated in amphetamine treated animals. With repeated intake of the drug, stimuli surrounding drug-administration can acquire secondary positive reinforcing effect through conditioning and the drug-associated stimuli, can in absence of the drug, induce craving for the drug. Cue-induced behaviour was investigated using both the CPP paradigm (paper II) and the locomotor sensitization paradigm (paper III), but the results from the two studies are diverging. Naltrexone had no effect on the expression of amphetamine-induced CPP. In contrast, the conditioned locomotor activity induced by placement of the animal in the previously amphetamine-paired context, was significantly attenuated by naltrexone pre-treatment. There are several methodological differences between the two behavioural models. In the CPP paradigm the animals were treated with amphetamine for 3 days, as compared to the sensitisation model where 10 consecutive amphetamine injections were given to the animals. In addition, the drug-free time (before testing) was different between the two studies, with a 10-day withdrawal period in the

sensitisation paradigm as compared to one day in the CPP-paradigm. It has been suggested that the involvement of the opioid system in the expression of behavioural sensitisation is dependent on the time of withdrawal, evident by a study where naloxone blocked sensitization after long-term but not short-term withdrawal periods (Magendzo and Bustos 2003). Another essential difference between the two models was the length of time of behavioural assessment, 15 minutes in the CPP-paradigm vs. 60 minutes in the conditioned locomotor activity experiment.

In humans, a small priming dose of a drug can trigger incentive motivation for the drug, leading to compulsive drug seeking, drug taking and relapse. In the present thesis, the effect of naltrexone on amphetamine priming was investigated using the self-administration model (paper I), the CPP model (paper II), as well as in the locomotor sensitization model (paper III). Naltrexone failed to modulate reinstatement of amphetamine-induced CPP. In contrast, naltrexone pre-treatment significantly attenuated reinstatement of self-administration as well as blocked the expression of behavioural sensitization in response to amphetamine priming. In the CPP reinstatement experiment, the behaviour was only measured for 15 minutes as compared to 60-120 minutes in the self-administration and sensitisation models, which could possibly explain the difference between the studies.

When evaluating a pharmacological treatment several issues are important to consider, one of them being unwanted and aversive side effects. In humans, naltrexone is considered to be a safe medication under a variety of conditions (Croop et al. 1997) and the most commonly reported side effects for naltrexone is nausea and vomiting. In the present thesis, no non-specific locomotor attenuating effects of naltrexone were found, neither as measured by operant behaviour (paper I) nor on spontaneous locomotor activity in drug-naïve animals (paper III). In addition, naltrexone alone did not induce place preference or place aversion (paper II). This indicates that naltrexone by itself does not cause any unspecific behavioural suppression, nor is it aversive, at least not at the doses studied.

Given the interaction between amphetamine and naltrexone, as shown by both animal and human studies, the mechanism underlying this effect was further investigated. From a neurobiological perspective, it is well documented that amphetamine, similarly to other drugs of abuse, increases dopamine levels in the nucleus accumbens, and that the increase in extracellular dopamine is linked to the rewarding and reinforcing effects of addictive drugs. Naltrexone did not modulate amphetamine-induced changes in extracellular dopamine, neither as measured by *in vivo* microdialysis in rats nor by PET in humans. Nevertheless, in agreement with earlier studies (Jayaram-Lindström et al. 2004; Jayaram-Lindström et al. 2008b), naltrexone significantly reduced the subjective effects induced by amphetamine in healthy human subjects. The results from paper IV therefore suggest a dissociation between amphetamine-induced dopamine release in the nucleus accumbens, and the effect of opioid receptor antagonism on ratings of subjective effects.

Taken together, the results of the present thesis show an involvement of the endogenous opioid system in some, but not all, amphetamine-induced behaviours. Based on these and earlier studies it is therefore hypothesized that repeated treatment with amphetamine induces changes in the opioid system, and that modulation of the opioid system may reduce amphetamine drug seeking behaviour in animals. The exact mechanism behind the

interaction between amphetamine and opioids needs to be further evaluated. However, the studies included in the present thesis are in agreement with recent clinical trials of naltrexone (Jayaram-Lindström et al. 2005; Jayaram-Lindström et al. 2008a) and strengthen the hypothesis that the endogenous opioid system may constitute a target for the treatment of amphetamine dependence.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Beroende av alkohol och narkotika utgör en av våra vanligaste och mest allvarliga folksjukdomar. I ett historiskt perspektiv har missbruk och beroende tidigare betraktats som ett slags karaktärsfel, men ökade kunskaper har medfört att beroende idag ses som ett multifaktoriellt sjukdomstillstånd. Utvecklingen av missbruk och beroende bygger på ett samspel mellan konstitutionella biologiska faktorer (exempelvis gener), miljöfaktorer (exempelvis attityder, socialt gruppptryck och tillgång till droger) samt den effekt som själva drogen har på hjärnan. Ett upprepat intag av beroendeframkallande droger förändrar hjärnas funktionssätt vilket kan leda att drogen "tar över" självkontrollen och individen hamnar i ett tvångsmässigt drogtagande. Längtan efter att få tag på och inta drogen kan bli så stark att den drabbade åsidosätter arbete, familj och fritidsaktiviteter.

Amfetamin tillhör gruppen psykostimulantia, vilka kännetecknas av att de ökar den psykiska energin och aktiviteten, samt minskar hunger, trötthet och sömnhet. Amfetamin är den näst vanligaste illegala drogen efter cannabis, med ca 34 miljoner regelbundna användare i världen. Trots intensiv forskning har det inte funnits någon evidensbaserad läkemedelsbehandling för amfetaminberoende. Kliniska prövningar från vår forskargrupp har nyligen visat att det kroppsegna opioida systemet förklarar vissa av de subjektiva effekter som orsakas av amfetamin. Naltrexon, ett ämne som blockerar opioidreceptorer, minskar den subjektiva upplevelsen av amfetamin samt minskar återfall i amfetaminmissbruk. Syftet med denna avhandling var att undersöka samspelet mellan naltrexon och amfetamin med hjälp av olika beteendeeexperiment på råttor, samt även hjärnavbildningsundersökning i människa.

Djurmodeller har visat sig värdefulla i sökandet efter nya möjliga läkemedel för beroende. Genom att använda olika beteendemodeller som självadministrering, beteende-sensitisering och konditionerad platspreferens (Pavloviansk inlärning) kan man studera specifika faser i utvecklingen av beroende. Belönande effekter, inlärning, tvångsmässigt drogintag samt drogsökande beteende och återfall är några aspekter som kan studeras i sådana djurmodeller.

Initialt drogintag är kopplat till en positiv upplevelse och eufori. Resultaten i denna avhandling, tyder på att det naltrexon kan påverka upplevelsen av amfetamin, men inte helt blockera de akuta effekterna av drogen (delarbete II-IV). Detta kan ha klinisk betydelse - om den upplevda effekten hämmas skulle merbegäret för amfetamin och därmed även konsumtionen av drogen kunna minska.

En av de största utmaningarna i behandling av missbruk och beroende är den höga risken för återfall, även efter lång drogfrihet. Återfall kan utlösas av akuta kroppsliga abstinenssymptom eller av mer psykologiska symptom som drogsug. Drogsug kan exempelvis framkallas av stress, specifika situationer eller känslor (stimuli) som man tidigare associerat med sitt drogtagande, eller av en liten dos av drogen. I denna avhandling studerades effekten av naltrexon på både droginducerat och stimuliinducerat återfall till drogsökande beteende i råttor. Resultaten visar att naltrexon minskar amfetamininducerat

drogsökande beteende i djur som tidigare självadministrerat amfetamin (delarbete I), samt i djur som sensitiserats (behandlats) med amfetamin i 10 dagar (delarbete III). I samma sensiteringsmodell minskade naltrexon även det drogsökande beteende som utlöstes av tidigare drogassocierade stimuli (delarbete III). Däremot sågs inga effekter av naltrexon på amfetamininducerad platspreferens (delarbete II). Skillnaderna i resultaten mellan de olika studierna kan bero på flera faktorer, exempelvis antalet doser av amfetamin eller observationstidens längd.

Gemensamt för beroendeframkallande medel är att de aktiverar hjärnans belöningssystem och frisätter signalsubstansen dopamin. I delarbete IV studerades samspelet mellan amfetamin och naltrexon på neurokemisk nivå, med hjälp av mikrodialys i råttor, och hjärnavbildningstekniken positron emission tomografi (PET) i människa. Amfetamin ökade dopaminfrisättningen i belöningssystemet, och hos friska försökspersoner korrelerade ökningen av dopamin med den subjektivt upplevda effekten av amfetamin. Naltrexon hade ingen effekt på dopaminfrisättningen, varken hos människa eller hos råttor, men däremot minskade naltrexon den subjektiva upplevelsen hos försökspersonerna.

Sammantaget visar resultaten från denna avhandling att det kroppsegna opioida systemet påverkar vissa, men inte alla, amfetamininducerade beteenden och stärker hypotesen att det kroppsegna opioida systemet kan utgöra ett mål för läkemedelsbehandling av amfetaminberoende.

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