VULNERABILITY FOR COCAINE DEPENDENCE

INVOLVEMENT OF μ-OPIOID RECEPTORS

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INVOLVEMENT OF μ-OPIOID RECEPTORS

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Rol van μ-Opioid Receptoren

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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"Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning."

Albert Einstein

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ANOVA	analysis of variance
CPP	conditioned place preference
CREB	cyclic AMP-response element-binding protein
DAT	dopamine transporter
EPSC	excitatory postsynaptic current
ERK	extracellular signal-regulated protein kinase
GABA	gamma-aminobutyric acid
i.c.v.	intracerebroventricular
ICSS	intracranial self-stimulation
IPSC	inhibitory postsynaptic current
IR	immunoreactivity
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
NET	noradrenalin transporter
NLX	naloxone
NTX	naltrexone
POMC	pro-opiomelanocortin
RT	room temperature
SERT	serotonin transporter
SNP	single nucleotide polymorphism
SNR	substantia nigra reticularis
TH	tyrosine hydroxylase
Δ^9-THC	Δ^9-tetrahydrocannabinol
TTX	tetrodotoxin
VTA	ventral tegmental area

THESIS OUTLINE

The sensitivity to the reinforcing effects of drugs of abuse constitutes a risk factor for drug dependence. The overall objective of this thesis was to investigate the neurobiological mechanisms of cocaine reinforcement and in particular the role of μ-opioid receptors in cocaine reinforcement.

General Introduction - In **Chapter 1** of this thesis, an introduction to drug addiction and preclinical addiction research is provided. Existing theories for mechanisms of addiction processes are discussed, with emphasis on the role of neurotransmitter systems, including endogenous dopamine and opioid systems, in drug reinforcement. In addition to pharmacological approaches, more recent studies have used gene knockout strategies to determine the role of specific genes in amongst others drug reinforcement. An overview of genetic approaches in addiction research, particularly preclinical, is provided in **Chapter 2**.

Endogenous opioid systems - The primary aim of the studies described in this thesis was to establish the role of the endogenous opioid system and, more specifically, of μ-opioid receptors in cocaine reinforcement. The studies in **Chapter 3** were designed to establish the involvement of μ-opioid receptors in cocaine reinforcement, which was determined with acquisition of cocaine self-administration by μ-opioid receptor knockout mice as a measure. It appears from previous studies with the opioid antagonist naltrexone (NTX), that opioid receptors in the ventral tegmental area (VTA) are of particular importance in endogenous opioid modulation of cocaine reinforcement (Ramsey et al., 1999). We therefore focused on the VTA and performed electrophysiology studies in horizontal slices of the VTA of μ-opioid receptor knockout and wild-type mice. Inhibitory post-synaptic current (IPSC) recordings were made from dopamine neurons in order to assess GABA mediated neurotransmission in the VTA in absence of μ-opioid receptors (**Chapter 3**). In addition, signal transduction pathways coupled to μ-opioid receptors in the VTA were explored in an *in vitro* study as described in **Chapter 4**. Slices of the VTA were treated with a specific μ-opioid receptor agonist, fentanyl, and were processed for immunohistochemistry with antibodies for different phospho-specific proteins for quantification of phospho-protein immunoreactivity.

Long-term exposure to drugs such as cocaine causes sensitization to that particular drug, which is manifested both by increased locomotor stimulant effects and augmented reinforcing properties of the drug and is considered to reflect neuroadaptations, which contribute to the actual development of drug dependence. In **Chapter 5**, the role of μ-opioid receptors in the acute locomotor response and in cocaine-induced behavioural sensitization was studied using μ-opioid receptor knockout mice, as a model for ectopic absence of μ-opioid receptors, and chronic NTX pre-treated mice, which is a model for transient opioid receptor over-expression (**Chapter 7**).

Besides endogenous opioid systems, dopamine is generally regarded to be a common factor in the effects of different drugs of abuse. Previous anatomical and electrophysiology studies

suggested interactions between opioid and dopamine systems in amongst others the mesolimbic system. Such interaction may be of relevance to μ-opioid receptor-induced modulation of drug reinforcement. In **Chapter 6** we therefore quantified levels of mRNA encoding the rate-limiting enzyme in dopamine synthesis, TH, and levels of dopamine receptor binding for mice with reinforcement relevant changes in μ-opioid receptor levels: μ-opioid receptor knockout mice and chronic NTX treated mice. In addition, locomotor activity in the open field and spontaneous climbing, behaviours which both involve dopamine were assessed for these mice.

In **Chapter 7**, the effects of chronic treatment with the opioid antagonist NTX upon opioid receptor levels were determined using quantitative autoradiography. Chronic NTX treatment has been shown to induce supersensitivity to morphine's analgesic effects and is known to increase opioid receptor numbers as assessed using whole brain homogenates. Interestingly, this same treatment is also effective in potentiating the reinforcing effects of both drugs of abuse, such as cocaine (Ramsey & Van Ree, 1990) and alcohol (Phillips et al., 1997). We performed a full quantitative mapping study for the three main opioid receptor subtypes: μ-, δ- and κ-opioid receptors. The chronic NTX model was used to investigate the role of (μ-)opioid receptors in cocaine-induced sensitization (**Chapter 5**) and in the study of opioid-dopamine interactions (**Chapter 6**).

Endogenous Cannabinoid systems – Recently, the endogenous cannabinoid system has been implicated in addiction processes. The studies described in **Chapter 8** were designed to further investigate the role of endogenous cannabinoids, through interactions with the CNS cannabinoid type 1 (CB1) receptor, in cocaine reinforcement and cocaine-induced behavioural sensitization. CB2 receptor expression is restricted to the periphery. For this purpose we used the selective CB1 receptor antagonist SR141716A, which was administered prior to cocaine self-administration or before the repeated intermittent cocaine injections for sensitization.

The results described in this thesis demonstrate an important and specific role of μ-opioid receptors in cocaine reinforcement, thus suggesting that variations in μ-opioid receptor levels might alter an individual's proneness to develop cocaine dependence. Further the neurobiological and behavioural findings are combined to a proposed mechanism through which μ-opioid receptors might modulate cocaine reinforcement in the General Discussion (**Chapter 9**).

CHAPTER 1

DRUG ADDICTION AND UNDERLYING NEUROBIOLOGICAL MECHANISMS; A PRECLINICAL PERSPECTIVE

Drug addiction is a major health issue worldwide and is characterised by its persistence and high rates of relapse. The mechanisms of this complex disease are only partly understood. In this chapter an overview will be provided of pre-clinical addiction research that has contributed to our present understanding of addiction processes. To this end, paradigms used in experimental addiction research and involvement of different neurotransmitter systems, *i.e.* dopamine, opioid, GABA, glutamate, serotonin and cannabinoid systems in experimental addiction will be discussed.

VULNERABILITY TO DRUG ADDICTION

Does drug use lead to drug abuse? Numbers from national drug surveys show that this is not necessarily the case: an individual who is exposed to an addictive drug does not in any case become an addict. For cocaine use, approximately 5-10% of the individuals who ever used cocaine in their life had recently used the drug, which is considered indicative of problematic cocaine use (Table 1A). The overall numbers for alcohol are higher, the percentage of the population that ever drank alcohol being approximately 80-85% as compared 2% (the Netherlands) and 12% (the United States) for cocaine. In the United States, approximately 7% of the population that ever drank alcohol in their lifetime was categorized as heavy drinkers (Table 1B). In the Netherlands 13% of the alcohol consuming population was categorized as heavy drinkers. These numbers suggest individual variation in the risk to develop drug dependence after being exposed to a drug. The current challenge is to determine which factors cause such individual variations in vulnerability for drug dependence. There is evidence for heritability in addiction proneness (see Chapter 2), although the manifestation of a genetic high risk to become an addict requires in any case the voluntary decision to use drugs in the first place and therefore depends upon environmental factors (Leshner, 2000).

It is likely that the sensitivity to the positive reinforcing effects, euphoric effects in humans, induced by a drug determine or at least contribute to repeated drug use, which may eventually lead to drug addiction (Haertzen et al., 1983) and hence is a possible risk factor for drug addiction. The aim of the studies described in this thesis was therefore to investigate the mechanisms underlying the reinforcing effects of in this case cocaine (see Box 1). As outlined

TABLE 1A Cocaine use in the Northern American and Dutch population: numbers from national drug surveys

Country	Year	% life time	% recent use	% recent of life time users
United States[1]	2000	11.2	0.5	4.9
United States[1]	2001	12.3	0.7	6.0
The Netherlands[2]	1997	2.1	0.2	9.5
The Netherlands[2]	2001	2.9	0.4	13.8

1 SAMHSA, National Household Survey on Drug Abuse

2. National Drug Monitor 2002, Trimbos Institute

TABLE 1B Alcohol consumption in the Northern American and the Dutch population: numbers from national drug surveys

Country	Year	% life time	% heavy drinkers	% heavy of life time drinkers
United States[1]	2000	81	5.6	6.9
United States[1]	2001	82	5.7	7.0
The Netherlands[2]	2000	86	13	15

1 SAMHSA, National Household Survey on Drug Abuse. Heavy drinking being defined as drinking five or more drinks on the same occasion on each of five or more days in the past 30 days

2 National Drug Monitor 2002, Trimbos Institute. Heavy drinking was defined as drinking more than 6 units of alcohol for at least one day every week

in the next section, animal models have been developed which allow the investigation of addiction processes, including drug reinforcement, which is a measure of facilitation by a drug of the acquisition of an instrumental response required to earn the drug. Reward, another widely used term in addiction research, expresses the positive subjective effects of a stimulus.

ANIMAL MODELS IN ADDICTION RESEARCH

Drug addiction is, as compared to other psychiatric diseases, relatively easy and reliably measurable in laboratory animals. Subjective measures such as reward or euphoria can not be measured as such in laboratory animals. However, operant conditioning tasks, amongst others based on Thordike´s law of effect "*behaviour that produces ´satisfying´ outcomes tends to be repeated"*, Skinner's analytic operant approach (Zimbardo et al., 1995), and conditioning paradigms based on, amongst others, classical conditioning introduced by the Russian physiologist Ivan Pavlov (1927, see Zimbardo et al., 1995), allow the investigation of addiction processes in laboratory animals (Van Ree, 1996).

In literature the effects of drugs of abuse are described in terms of reward and reinforcement. The distinction between reward and reinforcement is often vague and confusing. Reward expresses the positive subjective effects of a stimulus, which is not experimentally measurable. On the contrary, positive reinforcement can be assessed experimentally. For positive reinforcement is a measure of the facilitation by a stimulus, *e.g.* drugs of abuse, of the acquisition of an instrumental response required to earn the stimulus.

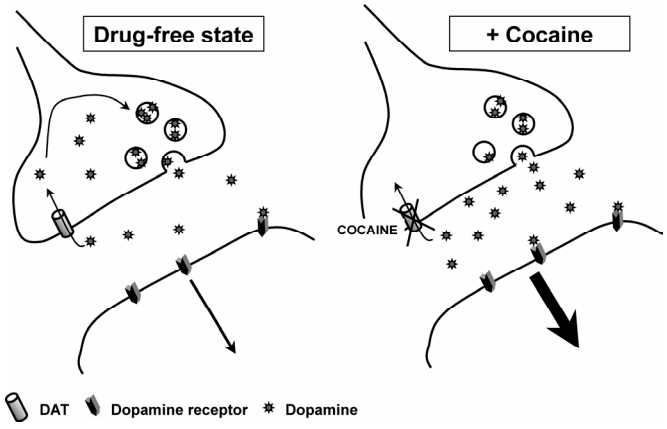
Drug addiction is a complex trait involving amongst others reinforcement, motivation and craving for the drug. The initiation of self-administration is mainly determined by the reinforcing effects of a drug (Van Ree et al., 1999) and is therefore most informative about drug reinforcement per se. In contrast, drug-maintained responding, drug-induced conditioned place preference (CPP) and relapse involve multiple factors such as motivation and craving, in addition to reinforcement.

BOX 1. COCAINE – A HISTORICAL PERSPECTIVE

Coca chewing is a Southern American habit/tradition, archaeological evidence for which dates back to at least 2500 BC when the first discoveries of the continent were made (Deng et al., 2002; Gardner & Vorel, 1998). South Americans chew coca, in combination with alkali, for medicinal reasons, but more importantly coca chewing was and still is essential to their daily life. Coca chewing makes people stronger and fitter, enabling them to work harder and longer, even without eating. In 1859, the German chemists Albert Niemann and Friedrich Wöhler isolated cocaine from Coca leaves, after which scientists and doctors started experimenting with cocaine. The effects of cocaine were magical: cocaine appeared a miraculous cure for different symptoms and pathologies. Ironically, heroin addiction was one of the diseases which people, including Sigmund Freud, believed they could now cure with cocaine. However as we realize today, cocaine was not a true medicine in this respect, it merely made the patients feel great for a while. Soon afterwards many of the patients, and their doctors, developed cocaine dependence: the first cocaine epidemy had emerged.

When dissolved in water cocaine can be snorted, *i.e.* nasally administered, or injected intravenously. Cocaine can not be smoked for it is unstable at high temperatures. South Americans did smoke cocaine, but this was a crude mixture, which included cocaine sulphate, which they called basé. North Americans though basé was the freebase of cocaine, *i.e.* lacking the hydrochloride group which is easily removed by adding strong alkali. The resultant product, which the North Americans started to smoke, was in fact pure cocaine base: the highly addictive crack, much different form the basé the South Americans smoked.

Cocaine is, like amphetamine, an indirect dopamine agonist. Upon interaction with monoamine transporters (Ritz et al., 1987), cocaine blocks dopamine re-uptake thereby increasing the available dopamine in the synaptic cleft thus causing more dopamine binding to post-synaptic dopamine receptors (see Figure). Recent studies, which used gene knockout mice for different monoamine transporters suggest that dopamine transporters (DAT) probably do not solely account for the effects of cocaine. Involvement of other monoamine transporters was suggested (Uhl et al., 2002).



In this thesis, the term reinforcement will be used in the context of acquisition of drug self-administration, while reward is chosen in the context of intracranial self-stimulation, maintenance (and relapse) of self-administration and in case of CPP.

Self-administration

The most widely used model in experimental addiction research is the self-administration paradigm. In this case, rats, monkeys and more recently also mice, earn an intravenous or intracerebral infusion of the drug of interest by execution of the instrumental response, *i.e.* lever pressing, nose pokes or head dipping. Researchers have shown, using this paradigm, that animals self-administer drugs which are known to have addictive potential in humans, *e.g.* morphine, heroin, fentanyl, cocaine, amphetamine, nicotine and alcohol whereas drugs with a low addictive potential are not readily self-administered by animals (Van Ree et al., 1978; Criswell, 1982; Grahame & Cunningham, 1995; Kuzmin et al., 1996; Kuzmin et al., 1997a).

Acquisition of drug self-administration is predominantly determined by the positive reinforcing efficacy of the drug of interest (Van Ree et al., 1999). Acquisition of self-administration is of particular interest with respect to individual variation in drug dependence for repeated exposure is required to become dependent upon a drug. Repeated drug exposure and hence drug dependence will not develop if the positive reinforcing efficacy is low to a given individual. Once the self-administration is acquired, animals will maintain stable responding. Secondary reinforcers such as environmental stimuli may contribute to maintenance of responding for self-administration.

Subsequently forced withdrawal of the drug can be induced by the experimenter, which typically leads to physical withdrawal signs that are best described for opioid dependence. The relevance of physical dependence, induced either by cessation of drug self-administration or of drug administration by an experimenter, is questionable. Dependence syndromes differ substantially across drugs of abuse. The dependence syndromes associated with psychostimulants, *e.g.* amphetamine and cocaine, are even the reverse of withdrawal syndromes characteristic for depressants, *e.g.* opioid drugs. Moreover, relief of withdrawal distress has not proven effective in the treatment of drug addiction (Wise & Bozarth, 1987).

After abstinence and extinction of responding on the previously drug-paired lever or hole, reinstatement of responding can be triggered in laboratory animals with a history of drug self-administration. Drug cues but also environmental cues associated with previous self-administration or stressors have been shown to induce reinstatement of lever pressing even if the drug is not available (Shalev et al., 2002). The reinstatement of responding on the previously drug-paired lever or hole is considered to reflect drug seeking and may reflect drug craving. As such, reinstatement may resemble human relapse (Epstein & Preston, 2003).

The self-administration paradigm has contributed substantially to our current knowledge of drug reinforcement. Moreover it has face and construct validity and has therefore been used

repeatedly to screen promising novel treatments. Drug self-administration is thus a powerful tool in experimental addiction research.

Conditioned place preference (CPP)

Another commonly used paradigm to evaluate rewarding effects of drugs of abuse is conditioned place preference (CPP). The place preference paradigm is based on the principal introduced by Pavlov, that neutral environmental cues can, by pairing them to affective stimuli, evoke similar approach behaviour as the affective (rewarding) stimuli. Typically a conditioned place preference apparatus consists of at least two compartments, one of which is paired to administration of a drug of interest. After the conditioning the animals will be allowed to choose between the two compartments in a drug-free state. If the animals spend more time in the previously drug-paired compartment the animal displays CPP. CPP has been described for morphine, heroin, cocaine, amphetamine, nicotine, alcohol and Δ^9-tetrahydrocannabinol (Δ^9-THC) (Stewart & Grupp, 1981; Mucha et al., 1982; Spyraki et al., 1982; Spyraki et al., 1983; Fudala et al., 1985; Lepore et al., 1995). Place preference probably reflects the desire/motivation to re-experience the rewarding effects of the drug, although the validity of CPP for the investigation of drug reward relevant to humans is speculative (Bardo & Bevins, 2000). For example, amphetamine-induced CPP was not predictive of subsequent intravenous self-administration of amphetamine (Bardo et al., 1999).

Intracranial self-stimulation (ICSS)

Intracranial self-stimulation has been widely used to explore the involvement of brain circuits in reward. In this paradigm, animals are trained to press a lever, nose poke or head-dip in order to obtain electrical stimulation in so-called ´pleasure centers´ *i.e.* the medial forebrain bundle, particularly the ventral tegmental area (VTA), and the lateral hypothalamus (Wise, 1998). Here the electrical stimuli serve as positive reinforcers. Different drugs of abuse, *i.e.* morphine, heroin, amphetamine, cocaine, phencyclidine and Δ^9-THC, all reduce the threshold current for ICSS. This facilitation of ICSS appears to be a common effect of drugs of abuse, despite their different primary sites of action (see Van Ree et al., 1999).

THEORIES FOR COMMON MECHANISMS OF DRUG REWARD

Intriguingly, various drugs of abuse, which use different primary targets, can all lead to the same phenomenon: drug dependence. Opioid drugs, such as morphine and heroin, interact with opioid receptors in the brain (Snyder & Pasternak, 2003), whereas psychostimulants, *i.e.* cocaine and amphetamine, act as indirect dopamine agonists through blockade of the dopamine transporter and the vesicular monoamine transporter (VMAT), respectively (Ritz et al., 1987; Pifl et al., 1995). Alcohol is thought to act through interactions with ligand-gated ion channels, examples of which are gamma-aminobutyric acid (GABA), serotonin, nicotinic

acetylcholine (nACh) and glutamate receptors (Soderpalm et al., 2000). Further, nicotine probably exerts its actions through interaction with nicotinic acetylcholine (nACh) receptors (Corrigall et al., 1992; Picciotto et al., 1998) and the effects of Δ^9-THC, the active component of cannabis/marihuana, involve binding to cannabinoid receptors (Gardner & Vorel, 1998; Childers & Breivogel, 1998). Despite their diverging primary sites of action these drugs all reduce the threshold for intracranial self-stimulation, illustrative of their rewarding properties (for review see Koob et al., 1998). Furthermore these drugs are self-administered by rodents and monkeys in operant tasks, indicative of reinforcing properties of these drugs (see Van Ree et al., 2000). Δ^9-THC may be an exception to this, for this drug is not readily self-administered by laboratory animals (Maldonado, 2002), although self-administration of Δ^9-THC by rats and squirrel monkeys has been shown (Van Ree et al., 1978; Tanda et al., 2000). It is likely that the actions of different classes of drugs converge to the same system, a ´reward system´. Here examples of candidates for a common 'reward system' will be outlined, with particular focus on endogenous dopamine and opioid systems.

Dopamine systems in reward

Dopamine systems and their topographical organization have been well described. There are different groups of dopamine neurons in the central nervous system (see Figure 1). The A8 and A10 neurons in the retrorubral nucleus and ventral tegmental area (VTA), respectively, constitute the mesolimbic and mesocortical systems with projections to limbic areas and the ventral striatum, olfactory tubercle, nuclei of the stria terminalis and the neocortex. A9 neurons in the substantia nigra account for the major input of the mesostriatal system with projections to the caudate putamen, globus pallidus, subthalamic nucleus and neocortex. Further, dopaminergic nuclei are organized in A11, A12, A13 and A14 diencephalic dopaminergic cell groups, A15 hypothalamic cells, A16 neurons in the olfactory bulb and retinal A17 dopaminergic neurons (Role & Kelly, 1991).

The existence of dopamine receptors in the brain was first proposed in 1978. Two subtypes of dopamine receptors were suggested, one of which was positively coupled to adenylyl cyclase (AC) whereas the other was not coupled to AC (Spano et al., 1978). The AC coupled receptors were called dopamine D1 receptors; the others were called D2 subtypes (Kebabian & Calne, 1979). The classification in D1 and D2 subtypes is still valid today and has been extended with pharmacological, biochemical, physiological and anatomical findings in which these subtypes have been shown to differ substantially (Missale et al., 1998). The first dopamine receptor to be cloned was the D2 receptor in 1988 (Bunzow et al., 1988). Two years later the dopamine D1 receptor was cloned by three different groups (Dearry et al., 1990; Monsma, Jr. et al., 1990; Zhou et al., 1990). Subsequently D3-, D4- and D5-dopamine receptors and several splice variants were identified, which will not be discussed here. The dopamine receptors are classified to D1-like receptors, comprising D1 and D5 receptor subtypes, and D2-like receptors, which include dopamine D2, D3 and D4 receptors, based on sequence homology, function and anatomical distribution (Missale et al., 1998).

Dopamine appears to be a common mediator of effects of different classes of drugs of abuse. An overview of studies to suggest a role of dopamine in drug reward/reinforcement but also of studies arguing against involvement of dopamine in these processes will be provided here.

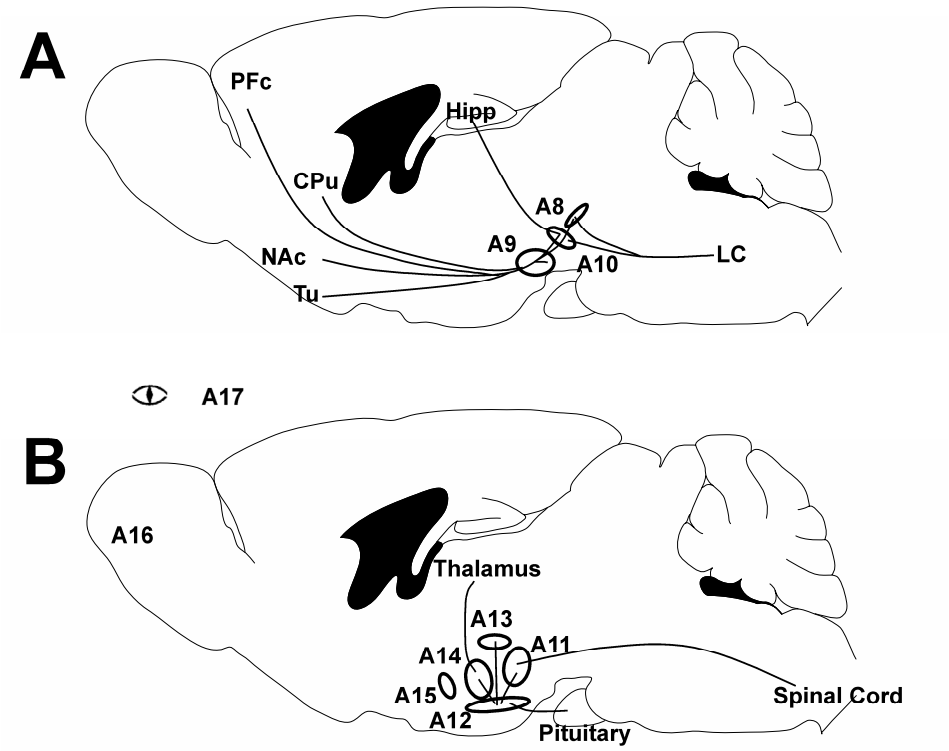


FIGURE 1
Dopamine systems in the brain.
A Dopamine cell groups in the midbrain, **A8** (retrorubral nucleus), **A9** (substantia nigra) and **A10** (ventral tegmental area), form the mesostriatal and mesolimbic systems.
B Other dopamine cell groups in the brain: **A11-A14** (diencephalic dopaminergic cell groups), **A15** (including preoptic areas and hypothalamus), **A16** (contains the olfactory bulb) and **A17** (retinal dopaminergic neurons).

Intracranial self-stimulation (ICSS)

The mesolimbic dopamine system, which originates in the VTA with projections to the nucleus accumbens and prefrontal cortex (the medial forebrain bundle), is generally considered the ´reward system´. In 1954 Olds and Milner first recognized the rewarding effects of electrical brain stimulation. They noted that rats acquired lever pressing in order to obtain electrical stimulation when the electrode was placed in certain brain regions. In their study the septum was particularly sensitive to the rewarding effects of electrical stimulation

(Olds & Milner, 1954). Later other groups described electrical self-stimulation for more brain regions. The medial forebrain bundle, the lateral hypothalamus and the VTA were most sensitive for ICSS (Wise, 1998). Although it is evident that the mesolimbic system supports ICSS, dopamine may not be critically involved in the rewarding aspects of ICSS as was demonstrated with haloperidol and 6-hydroxydopamine (6-OHDA) lesions (Fibiger et al., 1976).

Dopamine release

Further support of a role of dopamine in reward was provided by microdialysis studies. It was shown using this technique that drugs abused by humans, *e.g.* morphine, methadone, cocaine, amphetamine, alcohol and nicotine all increase dopamine release from the nucleus accumbens (Di Chiara & Imperato, 1988a; Pontieri et al., 1996; Tanda et al., 1997), particularly from the shell (Pontieri et al., 1995). Dopamine release from the nucleus accumbens shell is not only enhanced after experimenter-delivered drugs, but also after self-administration of cocaine and heroin (Gerrits et al., 2002). Related to dopamine release may be the psychomotor stimulant effects, which are common for different drugs of abuse. As proposed by Wise and Bozarth (1987), psychomotor activation induced by a drug is predictive of the reinforcing effect of the drug and thus its addiction liability.

Lesions

Different research groups have made use of 6-hydroxydopamine (6-OHDA) lesions of the mesolimbic dopamine system in order to determine the role of this system in drug reinforcement/reward. Cocaine-maintained self-administration was reduced in rats with 6-OHDA lesions in the nucleus accumbens or VTA (Roberts et al., 1977; Roberts & Koob, 1982; Pettit et al., 1984), although 6-OHDA lesions had only minor effects on the acquisition of cocaine self-administration (Gerrits & Van Ree, 1996). Acquisition and maintenance of amphetamine self-administration were reduced by 6-OHDA lesions in the nucleus accumbens (Lyness et al., 1979). In disagreement with the latter findings, facilitated acquisition of amphetamine self-administration was reported after radiofrequency or 6-OHDA lesions of the VTA (Le Moal et al., 1979; Deminiere et al., 1984). The discrepancies between these studies might be ascribed to differences in the extent of the lesion. Incomplete neuron loss may prompt the remaining neurons to compensate thereby facilitating rather than abolishing drug reinforcement. 6-OHDA lesions in the nucleus accumbens were however without effect on acquisition or maintenance of heroin self-administration (Pettit et al., 1984; Gerrits & Van Ree, 1996). In line with these findings, 6-OHDA lesions of the nucleus accumbens did not alter the dose-response curve for morphine-maintained self-administration (Dworkin et al., 1988) although the same group reported augmented morphine maintained self-administration after 6-OHDA lesions of the nucleus accumbens in a prior study (Smith et al., 1985). Finally, alcohol self-administration, both acquisition and maintenance, were not affected by 6-OHDA

lesions of the nucleus accumbens (Rassnick et al., 1993; Koistinen et al., 2001). To summarise, 6-OHDA lesion studies have shown a critical role of accumbens dopamine in cocaine and amphetamine reinforcement while dopamine in the nucleus accumbens is not required for the reinforcing effects of alcohol and opiates.

Dopamine receptors

If dopamine release in the nucleus accumbens contributes to the reinforcing/rewarding effects of drugs of abuse then dopamine antagonists should reduce these actions of drugs of abuse. Interestingly in this respect is the finding that dopamine D1 and D2 receptor antagonists increase the threshold for intracranial self-stimulation, indicative of reduced reward after dopamine receptor blockade (Baldo et al., 1999). Further evidence for a role of dopamine receptors in addiction processes from self-administration and CPP induced by different drugs will be outlined below.

Dopamine D1 receptor antagonists (SCH23390, A69045), D2 receptor antagonists (raclopride, eticlopride, YM-09151-2) or the mixed D2/5HT$_2$ antagonist risperidone, administered either systemically or directly into the VTA, nucleus accumbens or the amygdala, were shown repeatedly to enhance the rate of cocaine-maintained self-administration (Koob et al., 1987; Britton et al., 1991; Corrigall & Coen, 1991; Hubner & Moreton, 1991; Maldonado et al., 1993; McGregor & Roberts, 1993; Caine et al., 1995; Ranaldi & Wise, 2001). Interestingly, these effects of D1 and D2 antagonists upon cocaine-maintained responding were strain-dependent (Haile & Kosten, 2001). In contrast to the above, minor or no effects of the dopamine antagonist flupentixol (Negus et al., 1996), the D1 receptor antagonist SCH23390 and the D2 receptor antagonist pimozide (Woolverton & Virus, 1989) or the D2 antagonist spiperone (Koob et al., 1987) were also reported. The increase in cocaine-maintained responding induced by dopamine receptor blockade was interpreted as a compensation for the reduced reinforcing effects of cocaine. Studies which used a progressive ratio schedule of reinforcement, in which increasingly more instrumental responses are required in order to obtain a cocaine infusion, provided further evidence for this interpretation. In fact, progressive ratio studies revealed that dopamine D1 and D2 antagonists reduced the 'break-point', *i.e.* the maximum number of responses for a single reinforcer (Hubner & Moreton, 1991; McGregor & Roberts, 1993; Fletcher, 1998; Ranaldi & Wise, 2001). This reduction in the 'break-point' as observed for cocaine and amphetamine self-administration suggests that dopamine D1 and D2 antagonists indeed reduce the reinforcing effects of psychostimulant drugs.

Alcohol reward may also be modulated by dopamine. For example, the partial D1 agonist SKF38393 reduced both alcohol consumption and preference (Silvestre et al., 1996). Further, the dopamine antagonists fluphenazine and haloperidol reduced alcohol consumption and preference (Rassnick et al., 1992; Panocka et al., 1993). Similarly, alcohol but also sucrose-maintained self-administration was reduced by the dopamine D2 antagonist raclopride when injected in the VTA, although only the highest dose of raclopride was effective (Samson & Chappell, 1999). Hodge and colleagues studied the effects of agonists and antagonists for

dopamine D1 and D2 receptors upon alcohol-maintained responding. Reductions in alcohol self-administration were apparent after treatment with the D1 antagonist SCH23390, with high doses of the D2 receptor agonist quinpirole (which at low doses enhanced responding) and the D2 antagonist raclopride. The D1 agonist SKF38393 was without effect (Hodge et al., 1997). However, dopamine D1 and D2 receptor antagonists did not affect alcohol consumption in another study, at least not without a concomitant reduction in water intake (Silvestre et al., 1996). In agreement with these findings, Czachowski and co-workers reported reduced appetitive responding for alcohol after intra-accumbal raclopride administration, while alcohol consumption was not affected by this treatment (Czachowski et al., 2001). Further, clozapine, a dopamine D4 receptor antagonist, did not affect alcohol-induced CPP, although the locomotor activating effects of alcohol were reduced as was alcohol-induced conditioned taste aversion, although only for one dose (Thrasher et al., 1999).

With respect to opiates, acquisition of heroin self-administration was reduced but not abolished by systemic injections of haloperidol. In contrast, intracerebral injections of haloperidol in terminal areas of dopaminergic pathways, *i.e.* the striatum, nucleus accumbens, amygdala, prefrontal cortex and pyriform cortex, did not affect heroin self-administration in drug naive rats. The authors concluded that dopamine is not critically involved in opiate reward (Van Ree & Ramsey, 1987). No effects on acquisition of heroin self-administration were observed after intra-accumbens administration of the dopamine D1 antagonist SCH23390, although motor activity was effectively reduced by the same doses of SCH23390 demonstrating its effective blockade of D1 receptors at these doses. In the same study, systemic SCH23390 did reduce acquisition of heroin self-administration but responding on the non-reinforced lever was also reduced suggesting dopamine D1 receptors are not involved in heroin reinforcement (Gerrits et al., 1994).

At the time just prior to a scheduled self-administration session dopamine levels in the nucleus accumbens were reduced as compared to naive rats. This finding was interpreted such that dopamine may be involved in the desire for the drug or drug seeking (Gerrits et al., 2002). Reinstatement studies provided evidence for involvement of dopamine in drug seeking after a period of withdrawal. Cocaine-primed reinstatement of responding for cocaine was reduced by the D1 receptor antagonist SCH23390 but only when SCH23390 was injected in the accumbens shell and not the core or lateral septum (Anderson et al., 2002). A role of D1 receptors in cocaine reinstatement was however not supported by another study, which did describe reduced cocaine-induced reinstatement of responding for cocaine by the dopamine D2 antagonist eticlopride (Schenk & Gittings, 2002). Dopamine D1 and D2 antagonists reduced stimulus-induced reinstatement of alcohol seeking (Liu & Weiss, 2002). Finally, the D1 antagonist SCH23390, the D2 antagonist raclopride and mixed dopamine antagonists flupentixol and haloperidol reduced heroin-primed reinstatement of responding for heroin (Shaham & Stewart, 1996; Ettenberg et al., 1996).

The antipsychotic clozapine (D4 receptor antagonist) reduced cocaine-induced CPP and haloperidol reduced CPP induced by electrical prefrontal cortex stimulation (Duvauchelle & Ettenberg, 1991; Kosten & Nestler, 1994). D1 receptor antagonists consistently impaired

acquisition of CPP induced by cocaine, amphetamine, morphine, nicotine or diazepam (Acquas et al., 1989; Cervo & Samanin, 1996; Baker et al., 1998; Bardo et al., 1999; Rezayof et al., 2002; Zarrindast et al., 2003). Place aversion induced by naloxone or the κ-opioid receptor agonist U69593 was also reduced by the D1 antagonist SCH23390 (Shippenberg & Herz, 1988). It is not clear whether dopamine D1 receptors are involved in the expression of CPP for one study did and another did not observe effects of dopamine D1 receptor antagonists. Similarly, the role of dopamine D2 receptors in CPP is questionable for inconsistent findings were reported on this matter (Shippenberg & Herz, 1988; Cervo & Samanin, 1996; Baker et al., 1996; Bardo et al., 1999; Rezayof et al., 2002; Zarrindast et al., 2003). Taken together, these findings suggest a contribution of dopamine to motivational aspects of drugs of abuse.

Recently dopamine D3 receptors have received considerable attention in addiction research. Dopamine D3 receptors are potential targets in treatment of drug addiction (Le Foll et al., 2000). D3 receptor selective agonists have been shown to decrease cocaine self-administration in trained rats, indicating that dopamine D3 receptor agonists enhance cocaine's reinforcing effects (Caine & Koob, 1993). The partial D3 receptor agonist BP897 was found to reduce cue-controlled cocaine seeking without intrinsic reinforcing effects and without affecting cocaine self-administration on an FR1 schedule (Pilla et al., 1999). More recently, disruptions of amphetamine and nicotine cue-conditioned hyperactivity by BP897 were reported (Aujla et al., 2002; Le Foll et al., 2003). BP897 is currently in phase II clinical trials for it is regarded a potential therapeutic target in the treatment of cocaine abuse (Beardsley et al., 2002). Studies on a novel selective D3 receptor antagonist, SB-277011-A, showed reduced cue-controlled cocaine-seeking and reduced cocaine-enhanced reward in rats after treatment with SB-277011-A, although cocaine reinforcement was not affected (Di Ciano et al., 2003). In mice, reduced nicotine cue-conditioned hyperactivity has been shown after treatment with SB-277011-A (Vorel et al., 2002; Le Foll et al., 2002). Cocaine-induced place preference appeared not to be modulated by dopamine D3 receptor ligands (Gyertyan & Gal, 2003), whereas morphine-induced CPP was enhanced in dopamine D3 receptor knockout mice (Narita et al., 2003). A recent study reported that BP897 attenuated both the development and the expression of cocaine-induced CPP while not affecting morphine or food-induced CPP (Duarte et al., 2003). In summary, the notion has emerged that dopamine D3 receptors can modulate drug reinforcement, although they appear to have a more prominent role in drug seeking behaviour.

To summarise, dopamine systems are involved in reward as is evident from the support of intracranial self-stimulation by the mesolimbic system and the modulation of self-administration of different drugs after 6-OHDA lesion or dopamine antagonist treatment. However, it is important to note that acquisition and maintenance of heroin, morphine and alcohol were insensitive to 6-OHDA lesions and dopamine antagonists did not affect acquisition of heroin self-administration. These findings suggest that dopamine is not generally involved in reinforcement across drugs of abuse.

Endogenous opioid systems in reward

In the early 1970s, opioid binding sites were demonstrated in brain tissue (Pert & Snyder, 1973; Terenius, 1973; Simon et al., 1973). Shortly thereafter endogenous opioid peptides were identified: first Met-enkephalin and Leu-enkephalin (Hughes et al., 1975) and later β-endorphin (Bradbury et al., 1976) and dynorphin (Goldstein et al., 1979). Enkephalin, β-endorphin and dynorphin are derived from different precursor molecules, *i.e.* proenkephalin, pro-opiomelanocortin (POMC) and prodynorphin, respectively. Around the same time the existence of multiple opioid receptors was suggested based on pharmacological findings. Martin et al. described different syndromes in dogs that were chronically exposed to morphine (μ), ketocyclazocine (κ) or SKF-10,047 (σ) (Martin et al., 1976). Since σ-mediated effects were shown insensitive to naloxone, σ-sites are considered non-opiate sites. Another example for different opiate sites was the finding that, in contrast to guinea pig ileum, mouse vas deferens exhibited a higher affinity for enkephalins than for morphine, which suggested enkephalin-preferring sites (Lord et al., 1977). Besides the main opioid receptor classes, which are the μ-, δ- and κ-opioid receptors, other subtypes such as ε-, ι-, λ- and ζ-opioid receptors have been proposed (for review Akil et al., 1998). In the early 1990s the main opioid receptor subtypes were cloned and further characterized. The first to be identified was the δ-opioid receptor in 1992 (Evans et al., 1992; Kieffer et al., 1992). In the following year the μ- and κ-opioid receptors were also cloned (Thompson et al., 1993; Meng et al., 1993; Yasuda et al., 1993; Chen et al., 1993; Wang et al., 1993). Opioid receptors belong to the 7-transmembrane G-protein coupled receptor family; they are negatively coupled to G_i and induce a reduction in cyclic AMP. As reviewed elsewhere, μ-, δ- and κ-opioid receptors have a widespread expression pattern in the brain yet distinct from each other (Mansour et al., 1988; Mansour et al., 1995; Lesscher et al., 2003a). Amongst the areas where opioid receptor binding has been demonstrated are regions of the mesolimbic system. Interestingly, the endogenous opioid peptides have some selectivity for the μ-, δ- and κ-opioid receptors. β-Endorphin is relatively selective for μ-opioid receptors while enkephalin is more selective to δ-opioid receptors and dynorphin binds selectively to κ-opioid receptors (see Akil et al., 1998 and Gutstein & Akil, 2001 for review).

Endogenous opioids, particularly β-endorphin, have reinforcing properties. It has, for example, been demonstrated that β-endorphin is self-administered by rats (Van Ree et al., 1979) and that β-endorphin can induce conditioned place preference (Amalric et al., 1987). The reinforcing effects of endogenous opioid peptides suggested a potential role of these neuropeptides in reinforcement/reward induced by drugs of abuse. The impressive body of evidence for involvement of endogenous opioid systems in the effects of drugs of abuse, particularly alcohol, cocaine and heroin, has been reviewed elsewhere (see amongst others Ulm et al., 1995; Van Ree, 1996; Herz, 1997; Herz, 1998; Van Ree et al., 1999; Van Ree et al., 2000). Here, an overview will be given of pharmacological studies, which dealt with endogenous opioid systems in experimental addiction. This overview is not exhaustive and

further evidence for endogenous opioids in drug addiction from genetic studies will be discussed separately in Chapter 2 of this thesis.

Intracranial self-stimulation (ICSS) studies provided support for a role of endogenous opioid systems in brain reward. The non-selective antagonist naloxone (NLX) reversed cocaine-, amphetamine- and morphine-induced facilitation of ICSS (Bain & Kornetsky, 1987; Schaefer & Michael, 1990), although NLX was ineffective in reversing cocaine-induced facilitation of ICSS in another study (Van Wolfswinkel et al., 1988). The reduction of cocaine-induced brain reward by naltrindole may suggest involvement of δ-opioid receptors in drug reward (Reid et al., 1993). When administered alone, the opioid antagonists NLX and diprenorphine reduce ICSS responding, however only using a fixed-ratio schedule (Schaefer & Michael, 1988). Furthermore the μ- and δ-opioid receptor agonists DAMGO and DPDPE, administered in the nucleus accumbens, both reduced the threshold for ICSS (Duvauchelle et al., 1996; Duvauchelle et al., 1997).

Further, drugs of abuse induce changes in endogenous opioids in the brain. For example, β-endorphin immunoreactivity was reduced in limbic structures 18 hours after heroin and cocaine self-administration (Sweep et al., 1988). In a subsequent study Sweep and co-workers reported enhanced plasma β-endorphin levels in addition to the previously described reductions of β-endorphin in limbic regions. In contrast, immediately after the last self-administration session minor changes in β-endorphin immunoreactivity were noted (Sweep et al., 1989). More recently, Gerrits et al. studied opioid release using the indirect approach of *in vivo* autoradiography with the non-selective opioid antagonist [^{3}H]diprenorphine. Marked increases in opioid release were noted in restricted areas of the mesocorticolimbic system just before the scheduled self-administration session for both alcohol and cocaine. These findings suggested a role of endogenous opioids in drug craving (Gerrits et al., 1999). With microdialysis, enhanced β-endorphin levels in the nucleus accumbens were observed after acute administration of cocaine, alcohol and amphetamine treatment, while nicotine was without effect (Olive et al., 2001). Similar findings for cocaine were reported by Roth-Deri and co-workers who further reported enhanced brain activity in the nucleus accumbens and the arcuate nucleus, where the cell bodies of β-endorphin producing POMC neurons are localised (Roth-Deri et al., 2003). Marinelli and co-workers determined dopamine and β-endorphin concurrently after alcohol administration. An interesting observation was that the increase in dopamine release appeared to occur prior to the rise in β-endorphin levels in the nucleus accumbens, suggesting independent or at least different mechanisms of dopamine versus β-endorphin release (Marinelli et al., 2003).

Endogenous opioids and cocaine

Endogenous opioid systems are involved in cocaine reinforcement. This is evident from the impaired acquisition of operant responding for intravenous cocaine by NLX or naltrexone (NTX) treatment. Acute treatment with NLX and NTX just prior to self-administration caused rightward shifts in the dose-response curves for cocaine self-administration in naive rats and

mice, which is indicative of reduced reinforcing properties of cocaine (De Vry et al., 1989; Ramsey & Van Ree, 1991; Kuzmin et al., 1997a; Kiyatkin & Brown, 2003). Ramsey et al. have shown by local injections of NTX in different regions that the effects of NTX upon cocaine reinforcement are mediated by the ventral tegmental area (VTA) while NTX was ineffective when injected in the amygdala, caudate putamen, nucleus accumbens or prefrontal cortex (Ramsey et al., 1999). In contrast to the acute effects of opioid antagonists, chronic exposure to NTX facilitated the acquisition of cocaine self-administration (Ramsey & Van Ree, 1990). Several studies have addressed possible involvement of κ-opioid receptors in initiation of cocaine intake, which provided some conflicting findings. The κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI) impaired acquisition of cocaine self-administration in rats (Kuzmin et al., 1998) while the same group reported enhance reinforcing effects of cocaine after treatment with the κ-opioid receptor agonist U50,488H in drug naïve rats (Kuzmin et al., 1997b). Another study reported impaired acquisition of cocaine self-administration in rats treated with the κ-opioid receptor agonist U69593 (Schenk et al., 2001). Interestingly, acquisition of cocaine self-administration was also facilitated in adult rats that were prenatally exposed to the opioid agonist morphine (Ramsey et al., 1993). A recent study demonstrated altered μ-opioid receptor binding in amongst others the nucleus accumbens and the amygdala after prenatal morphine treatment in rats, although unfortunately the VTA was not included in their analysis (Vathy et al., 2003).

The involvement of opioid systems in cocaine-maintained self-administration is less clear as is apparent from the conflicting literature on this matter. In rats that were trained to self-administer cocaine, NTX was either ineffective (Ettenberg et al., 1982; Stromberg et al., 2002) or NTX enhanced cocaine-maintained responding (Carroll et al., 1986). In contrast, the μ-opioid receptor agonist DAMGO consistently reduced cocaine self-administration when administered in the VTA or the pontine nucleus (Corrigall et al., 1999a; Corrigall et al., 1999b; Corrigall et al., 2000). The shift in the dose-response curve for cocaine self-administration by intra-VTA DAMGO infusions was interpreted by Corrigall et al. to reflect increased reinforcing value of cocaine. However the same group failed to show convincing effects of the μ-opioid receptor antagonist CTOP upon cocaine responding (Corrigall et al., 1999a; Corrigall et al., 1999b). The irreversible μ-opioid receptor antagonist β-funaltrexamine (β-FNA) did not affect cocaine self-administration on a Fixed Ratio (FR) 1 schedule while responding for cocaine on a progressive ratio schedule of reinforcement was attenuated by β-FNA injected in the VTA or the nucleus accumbens (Ward et al., 2003). Conflicting results were obtained with the δ-opioid antagonist naltrindole, which reduced cocaine-maintained self-administration in one study (Reid et al., 1995) while naltrindole was ineffective in modulating cocaine responding in another study (De Vries et al., 1995). κ-Opioid receptors are probably not involved in cocaine dependence for only minor or no effects upon cocaine-maintained self-administration were noted after treatment with the κ-opioid receptor agonists U50,488H or U69593 and the κ-opioid receptor antagonist nor-BNI (Corrigall et al., 1999b; Schenk et al., 2001).

With respect to reinstatement, κ- but not μ-opioid receptors modulate reinstatement to cocaine seeking. For example, Comer et al. reported no effect of NTX or the μ-opioid receptor agonist etonitazene upon cocaine-primed reinstatement of cocaine seeking while the mixed μ-/κ-opioid receptor agonist/antagonist buprenorphine reduced cocaine seeking reinstated by cocaine (Comer et al., 1993). Schenk et al. later observed reduced cocaine-induced reinstatement after treatment with the κ-opioid receptor agonist U69593. This effect, which was reversed by the κ-opioid receptor antagonist nor-BNI, was also observed for reinstatement of cocaine seeking induced by the cocaine analogue RTI-55 but not for reinstatement induced by the dopamine transporter inhibitors GBR12909 and WIN35,428 (Schenk et al., 2000).

Also motivational aspects of cocaine involve endogenous opioid activity. NLX and NTX reduced the development of cocaine-induced CPP in rats and mice (Bilsky et al., 1992; Gerrits et al., 1995; Kuzmin et al., 1997a), although in one study effects of NTX were only noted on the third test day for expression of CPP (Houdi et al., 1989). Further the μ1-opioid receptor selective antagonist naloxonazine and the mixed μ-/κ-opioid receptor agonist/antagonist buprenorphine also reduced cocaine-induced place preference (Kosten et al., 1991; Rademacher & Steinpreis, 2002). Also δ-opioid receptors may contribute to the motivation for cocaine reward. Antisense oligonucleotides for the δ-opioid receptor reduced cocaine-induced CPP in mice and the δ-opioid receptor antagonist naltrindole reduced place preference cocaine (Suzuki et al., 1994), although these findings were not supported by another study (De Vries et al., 1995). More specifically, δ2- rather than δ1-opioid receptor subtypes have been implicated in cocaine-induced CPP (Suzuki et al., 1994).

Taken together, endogenous opioid systems are important in cocaine reinforcement and motivational aspects of cocaine, although their contribution to cocaine-maintained self-administration is less prominent.

Endogenous opioids and alcohol

Endogenous opioid systems have also been implicated in alcohol reinforcement. For example, NTX impaired the acquisition of alcohol self-administration and alcohol preference in mice and rats (Phillips et al., 1997; Davidson & Amit, 1997). In contrast, chronic exposure to NTX facilitated the initiation of alcohol consumption and enhanced alcohol preference (Phillips et al., 1997).

The effect of opioid antagonists upon alcohol-maintained self-administration has been widely investigated. The relatively non-selective opioid antagonists NLX and NTX were repeatedly shown to reduce alcohol maintained self-administration and alcohol preference in mice, rats and monkeys (Froehlich et al., 1990; Kornet et al., 1991; Hyytia, 1993; Phillips et al., 1997; Stromberg et al., 1998; June et al., 1999; Middaugh et al., 1999; Parkes & Sinclair, 2000; Koistinen et al., 2001; Goodwin et al., 2001; Hyytia & Kiianmaa, 2001; Shoemaker et al., 2002; Stromberg et al., 2002). Similar reductions in alcohol intake were noted after intracerebroventricular (i.c.v.) injection or local administration of NLX in the nucleus accumbens and the amygdala (Heyser et al., 1999). In contrast, Bienkowski and co-workers

did not observe effects of acute NTX upon alcohol consumption while small reductions in alcohol intake were noted after subchronic NTX treatment (Bienkowski et al., 1999).

Similar findings have been reported for μ-opioid receptor antagonists, *e.g.* the irreversibly μ-opioid receptor alkylating β-funaltrexamine (β-FNA) and the μ-opioid receptor antagonist CTOP reduced alcohol maintained responding (Hyytia, 1993; Stromberg et al., 1998; Hyytia & Kiianmaa, 2001). In contrast, the μ-opioid receptor agonist DAMGO enhanced alcohol intake when injected in the nucleus accumbens (Zhang & Kelley, 2002). The literature dealing with the involvement of δ-opioid receptors in alcohol maintained self-administration is ambiguous. Some groups found no modulation of alcohol intake by δ-opioid receptor antagonists naltrindole or ICI 174,864 (Hyytia, 1993; Stromberg et al., 1998; Middaugh et al., 2000) while another study reported reduced alcohol self-administration after naltrindole treatment (Hyytia & Kiianmaa, 2001). Further, June and co-workers described a similar reduction in alcohol responding induced by the δ_2-opioid receptor antagonist naltriben (June et al., 1999). There is no clear evidence for involvement of κ-opioid receptors in maintenance of alcohol self-administration, although the κ-opioid receptor agonist CI-977 may modulate alcohol drinking in a dose-dependent way (Holter et al., 2000).

With respect to relapse to alcohol consumption, the non-selective opioid receptor antagonist NTX, the δ-opioid receptor selective antagonist naltrindole and the μ_1-opioid receptor selective antagonist naloxonazine all reduced alcohol seeking in rats (Ciccocioppo et al., 2002). Moreover, renewed alcohol consumption by monkeys after a short period of abstinence was reduced by NTX (Kornet et al., 1991).

It is clear from these studies that endogenous opioid systems can modulate alcohol reinforcement and that opioid receptor activity may contribute to relapse to alcohol intake. In addition, the contribution of endogenous opioid systems to the motivational aspects of alcohol has been evaluated in alcohol-induced place conditioning paradigms. NTX did not affect the development and expression of conditioned place aversion induced by alcohol in rats (Bormann & Cunningham, 1997). Kuzmin et al. also failed to show effects of NLX upon the acquisition of alcohol-induced CPP although expression of CPP was reduced by NLX (Kuzmin et al., 2003). The latter study further demonstrated that NLX inhibited alcohol-primed expression of CPP after extinction of preference.

Endogenous opioids and opiates

Endogenous opioid involvement in heroin self-administration has also been demonstrated. For example, Martin et al. found a rightward shift in the dose-response curve for heroin self-administration after NTX treatment in drug naive rats. This suggests reduced reinforcing effects of heroin in presence of the opioid antagonist NTX (Martin et al., 1996). In drug-naive mice, the κ-opioid receptor agonist U50,488H facilitated morphine self-administration (Kuzmin et al., 1997b). Van Ree investigated the effects of β-endorphin and related peptides upon initiation of heroin self-administration in rats. He did not find heroin intake affected by β-endorphin itself although non-opioid fragments of β-endorphin either reduced (γ-type

endorphins βE_{2-17} and βE_{6-17}) or facilitated (β-endorphin$_{2-9}$) acquisition of heroin self-administration (Van Ree, 1983).

Opioid receptor antagonists also affected heroin-maintained self-administration. NLX and NTX enhanced heroin maintained responding when administered systemically, but also in the VTA, nucleus accumbens and lateral hypothalamus whereas local administration of NLX in the prefrontal cortex did not affect heroin self-administration (Koob et al., 1984; Vaccarino et al., 1985; Corrigall, 1987). In contrast, another study reported reduced heroin self-administration in trained rats (Oei, 1980) and Walker et al. found reduced heroin intake after NLX was administered in the bed nucleus of the stria terminalis (BNST) or the nucleus accumbens, but only in morphine dependent rats (Walker et al., 2000). Martin and co-workers made use of the irreversible μ-opioid antagonist β-FNA, which alkylates the μ-opioid receptor. I.c.v. β-FNA reduced heroin maintained self-administration (Martin et al., 1995), which was repeated in a later study by the same group (Martin et al., 1998). In yet another study β-FNA was injected in the caudal part of the nucleus accumbens and was found to cause a rightward and downward shift in the dose-response curve for heroin-maintained self-administration (Martin et al., 2002). The dose-effect curves for heroin responding in relation to β-FNA doses in time and the concurrently determined [^{3}H]DAMGO binding are complex. Nonetheless, it is clear from these studies that μ-opioid receptors are involved in heroin-maintained responding in rats. Finally, the δ-opioid receptor antagonist naltrindole did not affect morphine maintained responding (Reid et al., 1995) whereas the δ_2-opioid receptor antagonist naltrindole-5′-isothiocyanate attenuated heroin-maintained self-administration (Martin et al., 2000b).

With respect to reinstatement of extinguished responding for heroin, Stewart and Wise showed that morphine facilitated reinstatement of heroin self-administration. In contrast, NTX reduced the propensity to resume heroin consumption (Stewart & Wise, 1992).

NLX further impaired the development of place preference for heroin (Braida et al., 2001) and for β-endorphin (Amalric et al., 1987). Interestingly, chronic exposure to NTX enhanced cocaine-induced CPP (Bardo & Neisewander, 1987). This was however dose- and regimen-dependent: differences between chronic NTX and control rats were only observed in single trial morphine-induced CPP. There is no evidence for involvement of endogenous opioid systems in the expression of heroin-induced CPP (Hand et al., 1989).

Endogenous opioids and nicotine

Besides its involvement in alcohol, cocaine and opiate reward, endogenous opioid systems also modulate nicotine reward, although the literature dealing with opioids in nicotine addiction is relatively limited (Pomerleau, 1998). The role of endogenous opioids in the initiation of nicotine self-administration has not been investigated. However, reduced nicotine maintained self-administration in rats has for example been shown after administration of the μ-opioid receptor agonist DAMGO into the VTA (Corrigall et al., 2000).

Endogenous opioids and cannabinoids

The contribution of endogenous opioid systems to cannabinoid reward has been addressed using knockout studies, which will be discussed in chapter 2. However, there is also pharmacological evidence for opioid modulation of cannabinoid reward. For example, Braida et al. described reduced place preference induced by the cannabinoid agonist CP55,940 when given concurrently with NLX (Braida et al., 2001).

Mechanisms of opioid modulation of reward

As evident from the previous sections, endogenous opioid systems are involved in addiction processes of different classes of drugs of abuse. It appears that particularly opioid receptors in the VTA have a key role in opioid modulation of reinforcement. The μ-opioid receptor is most likely involved in this, for example because the VTA is relatively rich in μ-opioid receptors whereas the VTA contains little δ-opioid receptor binding sites (Lesscher et al., 2003a). Secondly, studies, which dealt with opioid modulation of initiation of drug self-administration, which involves predominantly the positive reinforcing effects of drugs, used either naloxone or naltrexone. These opioid antagonists have some selectivity for μ- over δ- and κ-opioid receptors, (Gutstein & Akil, 2001). Thus μ-opioid receptors in the VTA appear to have an important role in drug reinforcement. According to the current theory, μ-opioid receptors in the VTA increase dopamine neuron activity through disinhibition through a relief of GABAergic tone (Figure 2). In the VTA, two types of neurons have been characterised: principal dopamine-containing neurons and secondary non-dopaminergic neurons. The latter are sensitive to μ-opioid agonists and are presumably GABAergic interneurons (Gysling & Wang, 1983; Johnson & North, 1992b). Indeed, in the VTA μ-opioid receptors are expressed mainly by non-dopaminergic, presumably GABA containing neurons (Garzon & Pickel, 2001). Activation of μ-opioid receptors causes hyperpolarization of the secondary GABA containing neurons, thereby relieving inhibitory input to dopaminergic projection neurons (Johnson & North, 1992a). In line with this is the finding that intra-VTA administration of the $GABA_B$ receptor agonist baclofen impaired acquisition of cocaine self-administration and reduced responding for cocaine using a progressive ratio schedule of reinforcement (Brebner et al., 2000; Campbell et al., 2002). Baclofen locally injected in the VTA further reduced acquisition of heroin self-administration, enhanced the rate of heroin-maintained self-administration and reduced heroin-induced dopamine release (Xi & Stein, 1999). Similarly, increased mesolimbic GABA concentration which was achieved by local administration of the GABA transaminase inhibitor gamma-vinyl GABA (GVG) also impaired acquisition of heroin self-administration and reduced heroin reinforcement during maintenance of heroin self-administration (Xi & Stein, 2000). Corrigall and co-workers compared the effects of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen, respectively. Both GABA agonists reduced nicotine self-administration in trained rats but did not affect cocaine self-administration (Corrigall et al., 2000). It is further interesting to note in this context, that the

$GABA_A$ antagonist bicuculline is self-administered directly in the VTA by mice (David et al., 1997).

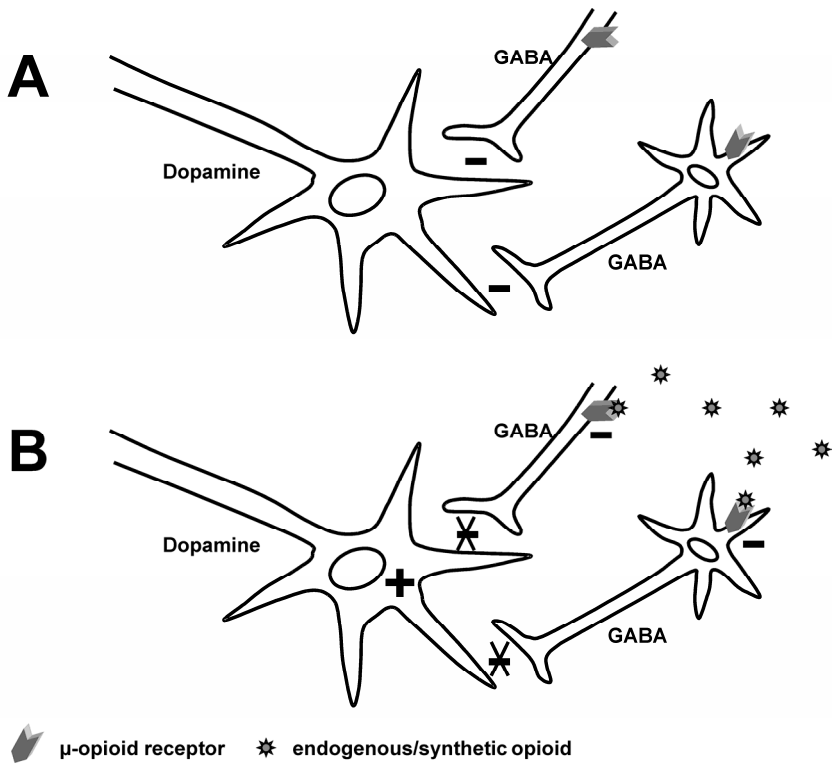


FIGURE 2
Schematic drawing of principal dopamine and secondary GABA neurons in the ventral tegmental area. **A** Dopamine neurons are tonically inhibited by GABA, which is released from the GABA (inter)neurons and interacts with GABA receptors, which are localized on these dopamine neurons.
B μ-Opioid receptors are localized on the GABA (inter)neurons in the VTA and when activated by opioids, either endogenous or exogenous, they cause the GABA neuron to hyperpolarize. Thereby, opioid ligands can relieve the inhibitory input onto dopamine neurons in the VTA thus causing increased activity of the dopamine neuron and hence of dopamine output to amongst others the nucleus accumbens.

Other neurotransmitter systems in reward

Besides dopamine and opioids, other neurotransmitters have also been implicated in modulating rewarding effects of drugs of abuse of which GABA was already mentioned in the previous section.

Glutamate and reward

Glutamate is among the examples of other neurotransmitters, which was studied by several groups for its possible contribution to drug addiction. A recent study, which used the mGluR5 antagonist MPEP, described reduced cocaine- and nicotine-maintained self-administration in mice after treatment with MPEP. In naive mice, MPEP reduced responding for nicotine although the intake of nicotine was not affected (Paterson et al., 2003). In line with these findings, NMDA antagonists dizolcipine, ketamine and AP-5 locally administered in the VTA reduced heroin reinforcement. AMPA antagonists only partly reduced heroin reinforcement (Xi & Stein, 2002) and did not affect cocaine-maintained responding when administered in the nucleus accumbens (Cornish et al., 1999). The latter study also explored the effects of AMPA agonists upon cocaine reinforcement and cocaine seeking. In fact, AMPA agonists effectively enhanced the reinforcing effects of cocaine as was evident from a reduction in the rate of cocaine-maintained self-administration. Moreover, AMPA agonists potentiated reinstatement of extinguished cocaine seeking. The NMDA antagonist MK-801 further impaired the acquisition of cocaine- and morphine-induced CPP (Cervo & Samanin, 1996; Kim et al., 1996), although amphetamine-induced CPP was not affected by MK-801 treatment (Hoffman, 1994). Interestingly, the AMPA antagonist DNQX impaired the expression but not the acquisition of cocaine-induced CPP (Cervo & Samanin, 1996). Of further interest in relation to glutamatergic involvement in addiction processes is the notion that synaptic plasticity (memory-like processes) occurs in reward-related regions such as the VTA. In 1999, Bonci and Malenka demonstrated long-term potentiation at synapses onto dopamine neurons in the VTA, which they induced with a pulse pairing protocol (Bonci & Malenka, 1999). Subsequently, a single exposure to cocaine was shown to induce long-term potentiation (LTP) of AMPA receptor-mediated currents at excitatory synapse onto dopaminergic neurons in the VTA (Ungless et al., 2001).

Serotonin and reward

Using agonists and antagonists selective for different subtypes of serotonin receptors, the contribution of serotonin systems to drug reward has also been investigated. The 5-HT_{1B} antagonist GR127935 did not affect cocaine self-administration (Castanon et al., 2000). Studies with 5-HT_{1A} and 5-HT_{1B} agonists yielded conflicting results. For example, the 5-HT_{1B} agonist CP93,129, but not the 5-HT_{1A} agonist 8-OH-DPAT, in the nucleus accumbens reduced amphetamine reinforcement as was concluded based on a reduction in the break-point for responding using a progressive ratio schedule of reinforcement (Fletcher et al., 2002). Another group reported enhanced cocaine reinforcement after treatment with the 5-HT_{1B} agonists RU24969, CP94,253 and CP93,129 (Parsons et al., 1998). Cocaine-induced CPP was not affected by the 5-HT_{1A} agonist buspirone (Ali & Kelly, 1997). Most studies agree on the lack of involvement of 5-HT2 receptors in drug reinforcement. Neither the 5-HT2 agonist DOI nor the 5-HT_2 antagonist ritanserin affected responding for amphetamine using a progressive ratio schedule (Fletcher, 1998; Fletcher et al., 2002). Moreover, the 5-HT_2 antagonist ritanserin did

not alter alcohol preference (Panocka et al., 1993) nor did another 5-HT_2 antagonist ketanserin affect cocaine-maintained self-administration (Ranaldi & Wise, 2001). In contrast, reduced alcohol consumption and cocaine reinforcement were observed after treatment with the 5-HT_{2C} antagonist amperozide (McMillen et al., 1993). Yet another study described reduced cocaine reinforcement after exposure to the 5-HT_{2C} agonist Ro 60-0175 although food-maintained responding and cocaine-induced hyperactivity were also reduced by this treatment (Grottick et al., 2000). Finally 5-HT_3 receptor antagonists did not affect cocaine-maintained responding (Peltier & Schenk, 1991) but reduced alcohol intake (Knapp & Pohorecky, 1992) and impaired the facilitation of intracranial self-stimulation by cocaine (Kelley & Hodge, 2003). Taken together serotonin systems can modulate drug reward. Knockout studies contribute further insight into the role serotonin systems have in reward processes, these will be discussed in Chapter 2 of this thesis.

Endogenous cannabinoids and reward

Endogenous cannabinoids have recently received substantial attention for their possible involvement in reward, as reviewed by Gardner and Vorel (Gardner & Vorel, 1998). Two cannabinoid receptors have been identified: the CB1 and the CB2 receptor (Childers & Breivogel, 1998). The CB1 receptor is expressed in brain while localisation of the CB2 receptor is restricted to the periphery. The central effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the active component of marijuana, are mediated by cannabinoid type 1 (CB1) receptors. Intracranial self-stimulation studies have confirmed the rewarding aspects of Δ^9-THC as was concluded from the reduction in threshold for ICSS caused by Δ^9-THC (Gardner et al., 1988) although the rewarding properties of Δ^9-THC seem to be strain-dependent for Δ^9-THC facilitated ICSS in Lewis but not Fisher rats (Lepore et al., 1996). In further support of involvement of cannabinoid systems in reward, the CB1 receptor antagonist SR141716A reduced responding for ICSS indicative of reduced rewarding effects of brain stimulation (Deroche-Gamonet et al., 2001). However, other studies suggest only a minor contribution of cannabinoid neurotransmission to reward. For example, the CB1 receptor agonist WIN55,212-2 did not affect ICSS itself, but impaired cocaine-induced facilitation of ICSS. This effect of WIN55,212-2 upon cocaine reward was blocked by the CB1 receptor antagonist SR141716A which itself was without effect on ICSS (Vlachou et al., 2003). Another study failed to show effects of another CB1 receptor agonist, CP55,940, upon ICSS while in this case SR141716A reduced ICSS responding however only at a very high dose at which SR141716A acts as an inverse agonist (Arnold et al., 2001).

Besides their possible intrinsic rewarding properties, endogenous cannabinoids have also been implicated in rewarding aspects of different classes of drugs of abuse. For example, the CB1 receptor antagonist SR141716A reduced heroin self-administration although the effects depended on the schedule of reinforcement. SR141716A effectively reduced heroin self-administration on a progressive ratio schedule of reinforcement while being modestly effective / ineffective on heroin-maintained behaviour (Solinas et al., 2003 but Navarro et al., 2001).

SR141716A further reduced acquisition of morphine self-administration, morphine-induced CPP in mice (Navarro et al., 2001) and heroin-induced CPP in rats (Braida et al., 2001). Further, pre-exposure to the CB1 agonist CP55,940 potentiated acquisition of morphine self-administration and morphine-induced CPP in rats (Norwood et al., 2003). Cocaine-maintained self-administration was not sensitive to effects of SR141716A but WIN55,212-2 reduced cocaine-maintained responding (Fattore et al., 1999). This may suggest either enhanced reinforcing effects of cocaine but may also reflect reinforcing effects of WIN55,212-2 itself.

Endogenous cannabinoid systems have been extensively studied for their role in alcohol reward. The cannabinoid agonist Win55,212-2 enhanced alcohol intake while water, food or sucrose intake were not affected (Colombo et al., 2002). Another CB1 agonist CP55,940 enhanced the breakpoint for self-administration of beer in a progressive ratio paradigm (Gallate et al., 1999). The CB1 receptor antagonist SR141716A reduced acquisition of alcohol drinking (Serra et al., 2001), it reduced alcohol and sucrose intake but not water or food intake in another study (Arnone et al., 1997), while yet another study did not reveal effects of SR141716A on alcohol intake (Colombo et al., 2002). An interesting study by Wang and co-workers demonstrated age-dependent effects of SR141716A on alcohol preference in mice. Young C57Bl/6 mice display greater alcohol preference as compared to adult mice from the same strain. While adult mice were not sensitive to SR141716A in relation to alcohol preference, SR141716A effectively reduced alcohol preference in young C57Bl/6 mice. This difference in sensitivity to cannabinoid receptor blockade may be related to reduced CB1 receptor G-protein coupling in old versus young C57Bl/6 mice (Wang et al., 2003). Further, SR141716A reduced the break-point for beer intake on a progressive ratio schedule of reinforcement, which was alcohol concentration dependent (Gallate & McGregor, 1999). This finding provided further evidence for cannabinoid involvement in alcohol reinforcement.

Cannabinoid involvement has further been suggested in MDMA-maintained self-administration, which was reduced when CP55,940 was co-administered with MDMA, and augmented with co-infusion of SR141716A (Braida & Sala, 2002). Further, nicotine-maintained self-administration was impaired by CB1 receptor blockade (Cohen et al., 2002)

Interestingly, the CB1 antagonist SR141716A effectively reduced reinstatement of cocaine seeking in rats primed by either cocaine, cues associated with cocaine or the CB1 agonist HU210 (De Vries et al., 2001). Footshock-induced reinstatement of cocaine seeking was not affected by SR141716A. Cocaine- or sucrose maintained self-administration was not affected by SR141716A in the latter study. Recently, the same group demonstrated that SR141716A also effectively reduced reinstatement of responding for heroin (De Vries et al., 2003). These findings suggest that CB1 receptors are promising targets for relapse prevention therapy.

CONCLUDING REMARKS

In this chapter an overview of the experimental addiction field is provided with particular focus on the role of different neuromodulators in reward and/or reinforcement. These appear to be more or less involved in different aspects of addiction processes.

A striking difference was noted in the involvement of dopamine in the reinforcing effects of classes of drugs: dopamine is required for the reinforcing effects of psychostimulants but not of alcohol and opiates. In contrast, the endogenous opioid system is involved in reinforcement across classes of drugs of abuse. Therefore, variations in the endogenous opioid system, which may be genetic or environmental in nature, might contribute to an individual's vulnerability to develop drug dependence in general.

The role of specific genes, including opioid genes, has been studied in experimental addiction using gene knockout studies, which will be discussed and related to genetic risk to develop drug dependence in humans in Chapter 2.

CHAPTER 2

GENETIC APPROACHES IN ADDICTION RESEARCH

Twin studies, with 1000-2500 twin pairs included, for alcohol, cocaine and opiates estimated that genetic factors account for 40-60% of the variation in liability to drug dependence (Prescott et al., 1999; Prescott & Kendler, 1999; Kendler et al., 2000). Such a high genetic contribution to vulnerability to drug addiction demands for knowledge of the role specific genes may play in addiction processes. Genetic technology advances of the past decade have allowed the investigation of specific genes, which may contribute to various diseases, including drug addiction. With the completion of the mapping and sequencing of the human genome (The International Human Genome Sequencing Consortium, 2001), the focus is now on the function of different genes and the contribution of mutations in specific genes to disease or vulnerability to disease, for example by single-nucleotide polymorphism (SNP) analysis. Efforts are currently made world-wide in order to establish a catalogue of common variants in the human population DNA, including SNP´s (Sachidanandam et al., 2001; Collins et al., 2003).

While appreciating the complexity of diseases such as psychiatric disorders, pre-clinical studies provide a unique tool for investigation of the function of specific genes in specific traits, including drug reinforcement for which excellent animal models have been developed over the years (Chapter 1). Here an overview will be provided of genetic pre-clinical studies, particularly using gene knockout mice, which have thus far contributed to our understanding of genes involved in addiction.

GENETIC STUDIES IN RODENTS

An impressive amount of literature is available dealing with rat strains, which were selectively bred for high or low alcohol preference. In 2002 Murphy and co-workers have reviewed the literature describing differences between the Indiana University Rat lines bred for high or low alcohol preference, *i.e.* the high/low alcohol drinking (HAD/LAD) rats and the alcohol-preferring/nonpreferring (P/NP) lines (Murphy et al., 2002). These lines obviously differ in their alcohol consumption and preference, but also in neurotransmitter systems including the serotonin, dopamine, GABA and opioid systems. Other lines selectively bred for their alcohol preference are the ALKO alcohol/nonalcohol lines (AA/ANA, the University of Chile A and B lines (UChA/UChB) and the Sardinian alcohol-preferring/nonpreferring lines (sP/sNP) (Murphy et al., 2002). Similarly, different strains of rats and mice have been compared for their reward profile. For example, C57Bl/6 mice appear more sensitive to cocaine reinforcement than BALB/c mice (Deroche et al., 1997) and DBA mice (Grahame & Cunningham, 1995), although DBA mice may acquire self-administration faster than C57Bl/6 mice (Rocha et al., 1998b). Further, alcohol consumption by C57Bl/6 mice is also higher as compared to DBA mice (Lessov et al., 2001). C57Bl/6 and DBA mice were also compared for morphine reinforcement. Drug naive DBA mice self-administered morphine while C57Bl/6 mice failed to do so. Interestingly, the same study demonstrated that emotional stress enhanced morphine self-administration in both strains: the dose-response curve for DBA mice was shifted to the left while C57Bl/6 mice responded for morphine with similar rates as DBA

mice after emotional stress (Kuzmin et al., 1996). Thus, both genetic and environmental factors determine the actual reinforcement phenotype of mice in this case. Studies, which used genetically distinct strains or selectively bred lines of rats or mice have pointed out that genetic influence exists in sensitivity to drugs of abuse. It is however difficult to identify specific genes, which may determine such differential sensitivity to drugs.

In order to pinpoint specific loci in the genome, which may determine for example an individual's sensitivity to alcohol reinforcement, Quantitative Trait Loci (QTL) studies have been exploited. These efforts in fact yielded several QTL's for murine responses to drugs of abuse (Crabbe et al., 1999). Knowledge of such gene locations enables us to pinpoint certain candidate genes, probably largely aided by pharmacology-based knowledge of systems involved in drug addiction.

The function of individual genes thus identified can and has been studied using gene knockout animals or using antisense oligodeoxynucleotides (ODN's). In the last decade knockout mice for genes encoding a wide variety of proteins have been generated (see Box 1 for gene knockout technology) and used to explore the role of specific opioid genes in reward/reinforcement.

In the next section, opioid gene knockout studies in addiction research are summarised. Subsequently, the contribution of gene knockout mice to the understanding of dopamine involvement in drug reward is discussed. Finally, an overview of the reward-related phenotypes described for miscellaneous other gene knockout mice, *e.g.* for serotonin, GABA, cannabinoid and other systems is provided.

Opioid gene knockout mice

As outlined in Chapter 1 of this thesis, endogenous opioid systems have been implicated in drug reinforcement. Endogenous opioids appear to be particularly involved in the initiation of drug self-administration. This has been shown both with opioid antagonists for different drugs of abuse, *i.e.* cocaine, alcohol and opiates. In the last decade knockout mice for genes encoding for opioid receptors and opioid precursors have been generated and used to explore the role of specific opioid genes in drug dependence. In this section, opioid gene knockout studies in drug dependence research are summarised.

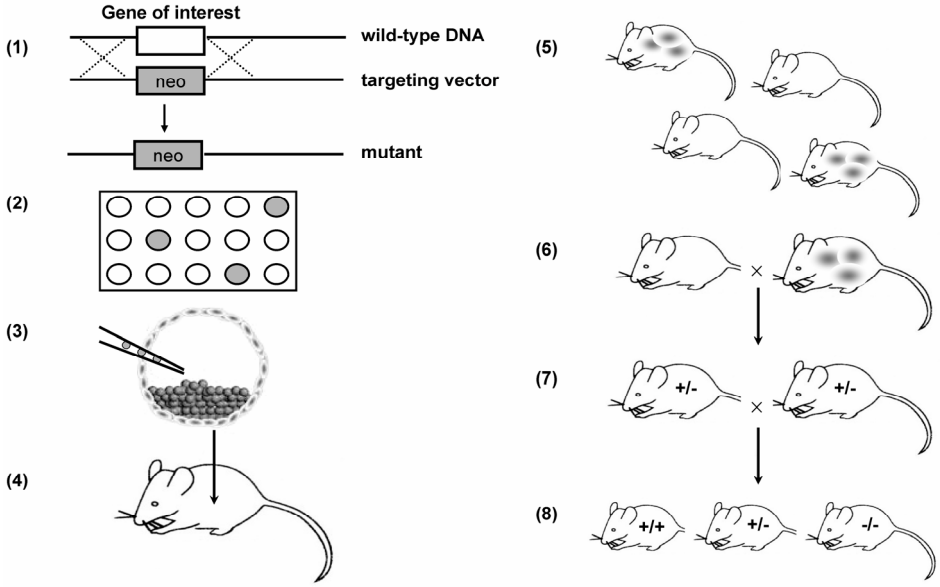
Opioid receptor knockout mice

Of the opioid receptor null mutants, the μ-opioid receptor knockout mouse was most extensively studied in drug reinforcement. Matthes and colleagues were the first to publish on μ-opioid receptor knockout mice (Matthes et al., 1996). These mice were generated by insertion of a Neo cassette in exon 2 of the μ-opioid receptor gene. In the years to follow other groups also developed μ-opioid receptor knockout mice, either by deletion of exon 1 (Sora et al., 1997; Tian et al., 1997; Schuller et al., 1999) or deletion of both exons 2 and 3 (Loh et al.,

BOX 1. GENE KNOCKOUT TECHNOLOGY

The most widely used approach to generate knockout mice lacking a specific gene is schematically represented in the figure below.

First mutant DNA should be prepared, which is usually done by replacement of the DNA containing the gene or part of the gene of interest, typically with marker-containing DNA such as the neomycine gene (1). Embryonic stem (ES) cells, usually derived from 129/Sv mice, will be transfected with the targeting vector. A proportion of the ES cells will incorporate the mutant DNA by homologous recombination. Subsequently the marker DNA, such as the neomycine gene encoding for neomycin-resistance enzymes, are used to select those ES cells in which the mutant DNA was successfully incorporated (2). The selected ES cells are injected into host blastocysts, typically taken from C57Bl/6 mice (3). These blastocysts containing the mutant-DNA containing ES cells are then applied to the uterus of a usually a C57Bl/6 pseudo-pregnant female (4). If the mutated stem cells developed into germ cells the offspring may contain chimeras, *i.e.* mice which have the mutation (5). The chimera males are then allowed to mate with wild-type, typically C57Bl/6, females (6). Successful germline transmission will yield mice heterozygous for the mutation in the offspring of this mating (7). Subsequent breeding of heterozygous females and males results in offspring containing knockout (-/-), wild-type (+/+) and heterozygous (+/-) mice in a ratio of 1:1:2 (8).



1998). Several groups have used these knockout mice as tools to investigate the role of the μ-opioid receptor in drug dependence processes. With regard to opiates μ-opioid receptor null mutants did not self-administer morphine (Becker et al., 2000). Moreover, morphine- and heroin-induced conditioned place preference (CPP) was respectively abolished (Matthes et al., 1996) or did not develop in these mice (Contarino et al., 2002). Alcohol reward in the μ-opioid receptor deficient mice was investigated in three laboratories. Roberts et al (2000) reported reduced alcohol intake in an operant self-administration paradigm, although responding for water was also reduced in μ-opioid receptor null mutants. μ-Opioid receptor knockout mice further displayed reduced preference for alcohol over water in a two-bottle choice task (Roberts et al., 2000). Data from another laboratory confirmed a role for μ-opioid receptors in alcohol consumption as evident from a two-bottle choice paradigm and CPP experiments, although the genotype effects were only observed in female mice (Hall et al., 2001). Finally, Becker et al. also observed reduced alcohol intake by μ-opioid receptor knockout mice as compared to wild-types, but alcohol-induced CPP was not different between genotypes in this study (Becker et al., 2002). With respect to psychostimulants, no difference was found between μ-opioid receptor knockout mice and wild-type controls in cocaine-induced CPP (10 mg/kg) (Contarino et al., 2002). Another group reported reduced CPP induced by 5 mg/kg cocaine in μ-opioid receptor knockout mice, whereas 10 mg/kg cocaine induced CPP in the knockout but not in the wild-type mice (Becker et al., 2002). Finally, place preference for Δ^9-tetrahydrocannabinol (Δ^9-THC) (Ghozland et al., 2002), nicotine (Berrendero et al., 2002) and deltorphin-II (Hutcheson et al., 2001) were also abolished in μ-opioid receptor null mutants.

δ-Opioid receptor null mutants self-administered more alcohol than wild-type controls. The δ-opioid receptor null mutants further displayed a greater preference for alcohol over water as compared to wild-type mice, but only after operant self-administration experience (Roberts et al., 2001). Ghozland and colleagues reported no difference in the development of place preference for low doses of Δ^9-THC (1 mg/kg) and place aversion for a high dose of Δ^9-THC (5 mg/kg) between δ-opioid receptor knockout and wild-type mice (Ghozland et al., 2002).

κ-Opioid receptor knockout and wild-type mice were comparable in Δ^9-THC-induced place preference (low dose, 1 mg/kg), whereas place aversion induced by higher doses of Δ^9-THC (5 mg/kg) was impaired in κ-opioid receptor null mutants (Ghozland et al., 2002).

Opioid peptide precursor knockouts

Knockout mice for opioid peptide precursor genes have also been developed and used in research on the role of opioid peptides in drug dependence. β-Endorphin knockout mice were developed through truncation of the pro-opiomelanocortin (POMC) prohormone gene. These β-endorphin null mutants were used to investigate directly the role of β-endorphin in alcohol intake. Heterozygous mice (50% β-endorphin expression) consumed more alcohol than wild-type mice did in a two-bottle choice paradigm. The null mutants consumed more alcohol

compared to wild-type controls, but only for the 7% alcohol solution and not when 10% alcohol was presented (Grisel et al., 1999). In addition to this finding, β-endorphin deficient mice acquired operant responding for alcohol, although in this study wild-type mice did not (Grahame et al., 1998).

Preproenkephalin knockout mice were not different from wild-type mice with regard to alcohol consumption and preference in a two-bottle choice test nor were there differences between genotypes in alcohol-induced CPP (Koenig & Olive, 2002).

Prodynorphin null mutants developed morphine-induced CPP comparable to wild-type mice. In agreement with the κ-opioid receptor, place aversion induced by 5 mg/kg Δ^9-THC was impaired in mice lacking the prodynorphin gene (Zimmer et al., 2001). Place preference for a lower dose of Δ^9-THC, such as described by Ghozland et al. (2002), was not studied in prodynorphin null mice.

Dopamine gene knockout

The mesolimbic system is considered to play a key role in drug addiction. For example, intracranial self-stimulation is supported when electrodes are placed in the medial forebrain bundle (Koob et al., 1998, Chapter 1). In support of involvement of dopamine in reward is the finding that all drugs of abuse increase dopamine release from the nucleus accumbens, despite their different primary targets of action (Di Chiara & Imperato, 1988a; Pontieri et al., 1996; Tanda et al., 1997). Moreover, studies which used either 6-hydroxydopamine (6-OHDA) lesions or dopamine antagonists suggest a role of dopamine in reward (see Chapter 1), although dopamine may not be crucial to drug reinforcement (Pettit et al., 1984; Van Ree & Ramsey, 1987; Gerrits et al., 1994; Gerrits & Van Ree, 1996). To further evaluate the significance of dopamine genes to drug reward, knockout mice strategies for specific dopamine genes have been applied in experimental addiction research.

Dopamine receptors are classified to D1- or D2-like receptors. The D1-like receptors comprise the D1 and D5 receptor subtypes and D2-like receptors are subdivided in D2, D3 and D4 receptors (Missale et al., 1998). Of further interest is the primary target of cocaine, the dopamine transporter (DAT) and the vesicular monoamine transporter (VMAT) through which amphetamine is thought to act. Mice lacking either the dopamine D1, D2, D3, D4 or D5 receptor gene, DAT or VMAT have been developed, some of which were characterized with respect to their reinforcement phenotype as outlined below.

Dopamine receptor knockout mice

Mice deficient in dopamine D1 receptors consumed less alcohol and displayed less preference for alcohol over water as compared to wild-type mice (El Ghundi et al., 1998). In contrast, cocaine-induced place preference was not affected by dopamine D1 receptor knockout (Miner et al., 1995).

Studies with dopamine D2 receptor knockout mice have yielded conflicting results. Intracranial self-stimulation of the nucleus accumbens was retained in dopamine D2 receptor null mutants. Cocaine reinforcement was not affected by dopamine D2 receptor gene knockout: the D2 null mutants acquired cocaine self-administration as the wild-type mice (Caine et al., 2002). Morphine-induced CPP was acquired by wild-type and dopamine D2 receptor knockout mice when the mice were naive to morphine prior to conditioning. In contrast, dopamine D2 receptor knockout mice withdrawn from morphine did not develop morphine-induced place preference even though withdrawal signs were comparable between genotypes (Dockstader et al., 2001). Of the D2 receptor short and long forms have been identified. Genetic deletion of the D2 long gene abolished morphine-induced place preference as well as place aversion induced by naloxone-precipitated withdrawal (Smith et al., 2002). Finally, D2 receptor null mice, trained to lever press for water, did not maintain morphine-induced responding suggesting that morphine is not reinforcing in D2 receptor knockout mice (Elmer et al., 2002). Alcohol reinforcement probably also requires D2 receptor activity for D2 receptor knockout mice did not increase lever pressing for alcohol (Risinger et al., 2000) while consuming comparable amounts of water and food as wild-type mice. Alcohol consumption and preference were also reduced in dopamine D2 receptor null mutants while saccharin and quinine consumption was not affected by deletion of the D2 receptor gene (Phillips et al., 1998). Further, dopamine D2 receptor null mutants did not develop place preference for alcohol, although this finding may have been confounded by increased preference of dopamine D2 receptor knockout mice for the drug-paired grid floor when paired to saline (Cunningham et al., 2000).

There is little literature available on studies, which investigated the contribution of the other dopamine receptor subtypes, *i.e.* D3, D4 and D5 receptors, to addiction processes by means of gene knockout strategies. The role of dopamine D3 receptors in drug reinforcement has recently been studied by different groups with dopamine D3 receptor selective drugs (see Chapter 1). Dopamine D3 receptor knockout mice are also available, but thus far only one study reported on their reward-relevant phenotype: morphine-induced CPP was enhanced in dopamine D3 receptor knockout mice (Narita et al., 2003). Dopamine D4 receptor knockout mice were supersensitive to the locomotor activating effects of alcohol, cocaine and methamphetamine, however the reinforcing effects of these drugs are yet to be investigated in these D4 receptor deficient mice (Rubinstein et al., 1997).

Dopamine transporter knockout mice

Studies with dopamine transporter (DAT) mice have lead to unexpected findings. Most important in this respect was the finding that DAT null mutants acquired cocaine self-administration although slower than their wild-type littermates. Similarly, cocaine-induced CPP was retained in DAT knockout mice. This implicates that the DAT, which was considered the primary target for cocaine, is not required for the reinforcing effects of cocaine. Serotonin transporters (SERT) have been suggested to account for the retained rewarding

effects of cocaine in these mice (Sora et al., 1998; Rocha et al., 1998a). Interestingly, morphine-induced CPP was potentiated in absence of the DAT gene as was the increase in extracellular dopamine levels induced by morphine (Spielewoy et al., 2000). Further evidence for a role of the dopamine transporters in drug reward is derived from alcohol studies. Hall and co-workers described increased alcohol intake in heterozygous DAT knockout mice and mice deficient in the vesicular monoamine transporter 2 (VMAT2) gene (Hall et al., 2003b). However this increment in alcohol consumption was only observed for male mice. A trend towards reduced alcohol consumption and preference in DAT and VMAT2 knockout females, which is in agreement with another study (Savelieva et al., 2002), suggests a gender dependent role of DAT in drug reinforcement. Finally, mice heterozygous for the VMAT2 gene displayed impaired amphetamine-induced CPP, which underlines the critical role of VMAT2 in the actions of amphetamine (Takahashi et al., 1997).

Besides the dopamine transporter, cocaine can also act through other catecholamine re-uptake transporters, namely through norepinephrine and serotonin transporters (NET and SERT, respectively). Mice with a genetic deletion of the gene encoding the NET, display augmented CPP induced by cocaine (Xu et al., 2000). Similarly, cocaine-induced CPP is enhanced in SERT knockout mice (Sora et al., 1998; Sora et al., 2001). Combined knockout mice with genetic deletions of both the dopamine and the serotonin transporter gene caused a reduction in cocaine-induced CPP (Sora et al., 2001). These findings suggest that the NET and the SERT are, in addition to the DAT, involved in rewarding aspects of cocaine, which may explain the retained self-administration of cocaine by DAT knockout mice.

DARPP-32 knockout mice

An important regulator in dopaminoceptive neurons is dopamine and cyclic adenosine 3´5´-monophosphate-regulated phosphoprotein 32 kDa, abbreviated as DARPP-32. Upon activation of dopamine D1 receptors, DARPP-32 is phosphorylated while it is dephosphorylated upon activation of dopamine D2 receptors. When phosphorylated, DARPP-32 acts as an inhibitor of protein phosphates-1 (PP-1), which in a phosphorylated state regulates various downstream targets such as neurotransmitter receptors, ion channels and transcription factors (Greengard et al., 1999). Knockout mice for the protein phosphatase-1 (PP-1) inhibitors DARPP-32 and Inhibitor-1 (I-1) were impaired in cocaine-induced CPP while cocaine-induced locomotor activity and cocaine-induced dopamine release were not affected in these mice (Zachariou et al., 2002). Further, alcohol consumption and alcohol-induced CPP were also reduced in DARPP-32 null mutants (Risinger et al., 2001). These findings suggest a role of DARPP-32 and hence possibly dopaminoceptive neurons in rewarding aspects of alcohol and cocaine.

GABA-related gene knockout mice

Genetic deletion of the α1 subunit of the $GABA_A$ receptor did not affect CPP induced by either cocaine (Reynolds et al., 2003) or alcohol (Blednov et al., 2003). Similarly, alcohol-induced place preference was not different for $GABA_A$ α1 subunit knockout mice. However, α1 subunit null mutants displayed reduced alcohol and saccharin consumption, while their quinine consumption was comparable to that of wild-type mice (Blednov et al., 2003). In the same study the involvement of β2 subunits was also assessed using knockout mice. Reduced saccharin and quinine consumption were apparent in β2 null mutants while their alcohol consumption was comparable to wild-type mice. The authors concluded that neither α1 nor β2 subunits of the $GABA_A$ receptor are involved in alcohol reinforcement. Finally, knockout mice for the δ subunit of $GABA_A$ receptors consumed less alcohol and also displayed less severe withdrawal signs after discontinuation of chronic alcohol exposure (Mihalek et al., 2001).

Serotonin receptor knockout mice

A substantial amount of literature is available which deals with the role of $5HT_{1B}$ receptors in addiction processes, as studied using gene knockout strategies, which will be outlined here. Drug reinforcement is enhanced in $5HT_{1B}$ knockout mice as is evident from facilitated initiation of cocaine and alcohol self-administration (Rocha et al., 1997; Risinger et al., 1999) and enhanced cocaine self-administration using a progressive ratio schedule (Rocha et al., 1998c; Castanon et al., 2000). Once self-administration of cocaine or alcohol was acquired, the $5\text{-}HT_{1B}$ knockout mice were not different from wild-type control mice (Rocha et al., 1997; Risinger et al., 1999). Oral alcohol intake studies yielded contradicting results. Alcohol consumption was enhanced according to one study (Crabbe et al., 1996) while no difference between $5\text{-}HT_{1B}$ knockout mice and wild-type controls in alcohol consumption was apparent in another study where alcohol was continuously available in a two-bottle choice paradigm (Gorwood et al., 2002). Further, cocaine-induced locomotor activity was also potentiated in $5HT_{1B}$ null mutants (Rocha et al., 1998c; Castanon et al., 2000) indicative of a phenotype which was not limited to reinforcement. Castanon and colleagues included an analysis of the effects of a specific $5\text{-}HT_{1B}$ receptor antagonist in their study and found no knockout-like effects of acute $5\text{-}HT_{1B}$ receptor blockade upon cocaine self-administration (Castanon et al., 2000). The latter finding suggests that the reinforcement phenotype of $5\text{-}HT_{1B}$ null mutants probably reflects compensatory changes rather than merely the lack of $5\text{-}HT_{1B}$ receptors. In contrast to the studies above, suggesting enhanced rewarding effects of drugs in mice with a deletion of the $5\text{-}HT_{1B}$ receptor gene, alcohol-induced CPP was abolished in $5\text{-}HT_{1B}$ knockout mice (Risinger et al., 1996) while cocaine-induced place preference was retained (Belzung et al., 2000). Thus although the reinforcing effects of cocaine and alcohol appear to involve $5\text{-}HT_{1B}$ receptor activity this receptor may not play a key role in drug dependence.

Cannabinoid receptor 1 knockout mice

Mice deficient in the cannabinoid receptor subtype 1 (CB1) gene have been reported to fail to self-administer the cannabinoid agonist WIN55,212-2 and morphine while cocaine, nicotine and amphetamine self-administration is retained in these mice (Ledent et al., 1999; Cossu et al., 2001). Also reductions in alcohol consumption have been reported for CB1 receptor knockout mice, while food and fluid intake were not different from wild-type controls (Hungund et al., 2003). This study by Hungund and co-workers further revealed that alcohol did not enhance dopamine release from the nucleus accumbens in CB1 receptor null mutants. Place preference for morphine and nicotine was also abolished in CB1 receptor knockout mice (Martin et al., 2000a; Castane et al., 2002 but Rice et al., 2002). Further, conditioned place aversion induced by the κ-opioid receptor agonist U50,488 was absent in CB1 receptor knockout mice (Ledent et al., 1999). In contrast, cocaine-induced place preference was not different between CB1 receptor null mice and wild-type controls (Martin et al., 2000a). Interestingly, despite comparable alcohol preference of CB1 receptor knockout and wild-type mice, CB1 receptor null mutants failed to show a stress-induced increase in alcohol preference (Racz et al., 2003). This finding suggests an interaction between the CB1 receptor gene and an environmental factor, stress, which together determined the reinforcement phenotype in this case.

Miscellaneous other knockout mice

Further findings relevant to addiction processes obtained with knockout mice for various genes are summarised in Table 1. These include knockout mice lacking genes encoding the glutamate receptor mGluR5, nicotinic and muscarinic acetylcholine receptors, growth factors or protein kinase isoforms.

HUMAN GENETICS, SINGLE NUCLEOTIDE POLYMORPHISMS

With the completion of the human genome mapping, the focus in genetic research is now on functional mutations, such as single nucleotide polymorphisms (SNP's). Different groups have identified SNP's in various genes (Collins et al., 2003) and attempts have been made to find associations of SNP's with certain traits, including drug addiction. As an example of recent advances in human genetics in addiction research by SNP analysis, an interesting SNP in the μ-opioid receptor gene will be discussed here.

Amongst several SNPs identified in the μ-opioid receptor gene is the nucleotide substitution at position 118 (A118G), predicting an Asp40Asn amino acid change (Bergen et al., 1997; Bond et al., 1998). The A118G variant was associated with enhanced affinity for the endogenous opioid peptide β-endorphin, suggesting this is a functional variant of the human μ-opioid receptor gene (Bond et al., 1998, but Befort et al., 2001). Some studies suggested a significant association of the A118G variant with opiate dependence (Szeto et al., 2001; Tan et al., 2003)

TABLE 1
Overview of reward-related phenotypes of miscellaneous gene knockout mice.

Gene	Major findings		Reference
mGluR5	×	cocaine self-administration	(Chiamulera et al., 2001)
β2 subunit nACh receptor	=	cocaine self-administration	(Picciotto et al., 1998; Epping-Jordan et al., 1999)
	×	nicotine-maintained self-administration when substituted for cocaine	
nACh receptor	=	morphine and cocaine CPP	(Zachariou et al., 2001)
M5 Ach receptor	×	morphine CPP	(Basile et al., 2002)
dopamine β-hydroxylase	↓	alcohol consumption and preference	(Weinshenker et al., 2000)
α1b adrenergic receptor	↓	cocaine and morphine consumption (two-bottle choice)	(Drouin et al., 2002)
	×	morphine CPP	
Nociceptin	=	alcohol CPP	(Kuzmin et al., 2003)
CRH	↑	alcohol consumption	(Olive et al., 2003)
	↓	alcohol CPP	
CRH1 receptor	=	alcohol consumption, enhanced alcohol intake 3 weeks post-exposure to stress	(Sillaber et al., 2002)
Neuropeptide Y	↑/=	alcohol consumption and preference (background dependent; NPY overexpression ↓ alcohol intake & preference)	(Thiele et al., 1998; Thiele et al., 2000a)
NPY5 receptor	=	alcohol consumption	(Thiele et al., 2000a)
Alcohol dehydrogenase	↓	alcohol consumption and preference	(Isse et al., 2002)
RIIβ subunit of PKA	↑	alcohol consumption and preference	(Thiele et al., 2000b)
PKCγ	×	morphine CPP	(Narita et al., 2001)
PKCε	↓	alcohol consumption and preference (rescued by conditional expression of PKCε, Choi et al., 2002)	(Olive et al., 2000)
Nurr1	↓	alcohol consumption and reward	(Werme et al., 2003)
Neurokinin 1	×	morphine CPP and naloxone place aversion	(Murtra et al., 2000)
GDNF	↑	cocaine CPP (heterozygous mice)	(Messer et al., 2000)
BDNF	↓	cocaine CPP (heterozygous mice)	(Hall et al., 2003)
NOS	×	cocaine CPP	(Itzhak et al., 1998)
Ca channel $Cav2.3_{\alpha 1\varepsilon}$	=	cocaine induced CPP, but insensitive to D1 receptor antagonist SCH23390	(Han et al., 2002)
GIRK2 K^+ channel	=	alcohol preference	(Blednov et al., 2001)
	×	alcohol CPP	(Hill et al., 2003)
Kir3.2/3.3	↓	cocaine self-administration	(Morgan et al., 2003)
CD81	×	cocaine CPP	(Michna et al., 2001)
tPA	=	cocaine self-administration (acquisition and maintenance)	(Ripley et al., 1999)

mutant mice versus wild-type controls, = not different; × abolished; ↓ reduced and ↑ increased

or with alcohol dependence (Town et al., 1999; Schinka et al., 2002), the frequency of the A118G variant being lower in drug dependent subject groups. However, other studies failed to show a significant association of the A118G variant of the μ-opioid receptor gene with either opiate (Bond et al., 1998; Franke et al., 2001; Shi et al., 2002) or alcohol dependence (Bergen et al., 1997; Sander et al., 1998; Gelernter et al., 1999; Franke et al., 2001; Rommelspacher et al., 2001). Recent findings by Oslin and co-workers, although preliminary in nature, revealed an association of A118G with the effectiveness of naltrexone in relapse prevention. Relapse rates were lower and time to relapse was higher in naltrexone-treated subjects bearing the Asp40 variant as compared to subjects homozygous for the Asn40 allele (Oslin et al., 2003). Thus, A118G is an example of human gene variants that may contribute to individual variation in drug dependence and in addiction treatment efficacy.

CONCLUDING REMARKS

Genetic approaches, particularly gene knockout strategies, have been used extensively to explore the role of specific genes in addiction processes. Fascinating findings such as those regarding the role of the dopamine transporter in the rewarding effects of cocaine illustrate the value of such approaches. Particularly because cocaine is itself a catecholamine re-uptake inhibitor and selective blockers of catecholamine re-uptake transporter subtypes are not available. However, knockout technology is also limited in nature because of possible adaptation and compensation, which may have occurred in response to absence of the gene of interest from gestation. Knockout mice, conditional in time or place, may provide a much more reliable tool in that respect. These preclinical genetic studies guide the selection of candidate genes in human association studies. Human geneticists currently explore the association of specific gene mutations, such as the single nucleotide polymorphisms, with diseases or disease-related traits.

CHAPTER 3

REDUCED COCAINE REINFORCEMENT AND INCREASED GABAERGIC INHIBITION IN THE VTA OF μ-OPIOID RECEPTOR KNOCKOUT MICE

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IN PREPARATION

ABSTRACT

Endogenous opioid systems and particularly μ-opioid receptors have been implicated in modulating the reinforcing effects of drugs of abuse like cocaine and have been suggested to be involved in vulnerability to develop drug dependence.

We studied the role of the μ-opioid receptor in cocaine reinforcement using cocaine self-administration by drug-naive μ-opioid receptor knockout and wild-type mice. Compared to wild-type mice, cocaine self-administration by μ-opioid receptor knockout mice was impaired, thus demonstrating the critical role of μ-opioid receptors in cocaine reinforcement. In order to determine the regulation of μ-opioid receptor ligands by cocaine and the involvement of endogenous μ-opioid receptor ligands in cocaine reinforcement, the effects of actively self-administered or contingently administered cocaine on POMC mRNA expression in the arcuate nucleus was assessed in wild-type mice. Cocaine intake (mg/kg) was positively correlated with POMC mRNA levels after active cocaine self-administration but not in the case of contingently administered cocaine. In order to determine the mechanism underlying impaired cocaine reinforcement in μ-opioid receptor knockout mice electrophysiological recordings were made from neurons in the ventral tegmental area (VTA). The frequency of spontaneous inhibitory post-synaptic currents (sIPSC's), as recorded from dopamine neurons in the VTA, was increased in μ-opioid receptor knockout mice as compared to wild-type controls.

It is concluded, that μ-opioid receptors in the VTA are, through regulation of inhibitory input onto dopamine neurons in the VTA, important modulators of cocaine reinforcement.

INTRODUCTION

The positive reinforcing effects of drugs of abuse, *i.e.* the positive subjective effects which increase the probability of subsequent drug use, are important in the initiation of drug use, which may ultimately lead to drug dependence. Positive reinforcing effects of drugs of abuse can be readily assessed by intravenous self-administration in laboratory animals (Van Ree et al., 1999).

An impressive amount of preclinical research using this paradigm has pointed to a role of endogenous opioid systems in drug reinforcement (for review see Van Ree et al, 1999; Herz, 1997). Opioid antagonists reduce the acquisition and maintenance of cocaine and ethanol self-administration (*e.g.* De Vry et al., 1989; Van Ree et al., 1999;.Froehlich et al., 1990; Kornet et al., 1991; Phillips et al., 1997; Kuzmin et al., 1997a; Stromberg et al., 1998). Further, μ-opioid receptor knockout mice are impaired in self-administration or conditioned place preference for morphine, heroin, alcohol, Δ^9-tetrahydrocannabinol (THC), nicotine and deltorphin (Matthes et al., 1996; Becker et al., 2000; Roberts et al., 2000; Hall et al., 2001; Hutcheson et al., 2001; Ghozland et al., 2002; Contarino et al., 2002; Berrendero et al., 2002). Primarily opioid receptors in the ventral tegmental area (VTA) account for opioid modulation of drug self-administration as was demonstrated by local injection of the opioid receptor antagonist naltrexone in different areas of the mesolimbic system (Ramsey et al., 1999). Moreover, in support of involvement of μ-opioid receptors in cocaine reinforcement, intra-VTA administered DAMGO, a specific μ-opioid receptor agonist, enhanced cocaine-maintained self-administration in rats as was apparent from a left-ward shift in the dose-response curve for cocaine self-administration (Corrigall et al., 1999b). The μ-opioid receptor selective endogenous opioid peptide β-endorphin is likely involved in opioid modulation of drug reinforcement, particularly since it possesses reinforcing properties itself (Van Ree et al., 1979). Indeed, the expression of pro-opiomelanocortin (POMC, the precursor of amongst others β-endorphin) in the arcuate nucleus is regulated by cocaine (Zhou et al., 2002).

In the VTA μ-opioid receptors are present on secondary, GABAergic, neurons (Garzon & Pickel, 2001; Garzon & Pickel, 2002). Activation of the μ-opioid receptors hyperpolarizes these secondary GABAergic neurons, resulting in a disinhibition of the principal dopamine neurons (Johnson & North, 1992a). Indeed, GABAergic neurotransmission in the VTA has been implicated in drug reinforcement (David et al., 1997; Xi & Stein, 1999; Corrigall et al., 2000). As increased dopaminergic output, at least in part due to an increased firing frequency or switch to burst firing of the principal dopamine neurons (Miller et al., 1981; Overton & Clark, 1997), is a common effect of drugs of abuse (Di Chiara & Imperato, 1988a), increased inhibitory, GABAergic, input onto the principal dopaminergic neurons could provide an explanation for the reduced susceptibility of μ-opioid receptor knockout mice for drug reinforcement.

The primary aim of this study was to establish the role of μ-opioid receptors in cocaine reinforcement. In addition, the involvement of endogenous μ-opioid ligands and of

GABAergic neurotransmission in the VTA in cocaine reinforcement were assessed. We report that cocaine self-administration is impaired in μ-opioid receptor knockout mice and that POMC mRNA levels in the arcuate nucleus are correlated with cocaine self-administration. Further, GABAergic input onto dopaminergic neurons in the VTA is enhanced in μ-opioid receptor knockout mice.

MATERIALS AND METHODS

Animals

Male mice aged 2-3 months for behavioural studies, and male and female mice aged 10-17 days for electrophysiological studies, were group housed (2-4) in extended Macrolon© type I cages with water and food pellets available ad libitum. Environmental conditions were controlled (22°C and 50% humidity; lights on at 7:00 am and lights off at 7:00 pm, GDL Utrecht University). The experimental procedures were approved by the Ethical Committee for Animal Experiments of the University Medical Center Utrecht.

Wild-type C57Bl6/Jico mice were obtained from Charles River (l'Arbresle, France). The μ-opioid receptor knockout mice used in this study have been described previously and were on a mixed 129Sv/C57Bl6 background (Schuller et al., 1999). No detectable binding of [^{3}H]DAMGO or μ-opioid receptor transcript was present in μ-opioid receptor knockout mice (Schuller et al., 1999). There is no evidence for compensatory changes in other opioid receptor subtypes: binding to δ-opioid receptor subtypes was comparable between genotypes and δ- and κ- and ORL-1 receptor mRNA levels were also unchanged (Schuller et al., 1999). Wild-type (+/+) and homozygous knockout (-/-) mice were obtained from heterozygous breeding. The mice used in the present study were on a C57Bl6/Jico background after 6-7 back-crossings to C57Bl6/Jico mice (Charles River, l′Arbresle, France). The mice were genotyped by Polymerase Chain Reaction on genomic DNA isolated from tail tips. The mutant product was 700 bp, the wild-type product 525 bp; the three primers used were outside the mutation site (5′ GAC TTT CCT GGC TGA TGC AAA CAA CCT 3′), within the mutation site (5′ CAT GGT TCT GAA TGC TTG CTG CGG ACT 3′) and within the neomycin box (5′ CTA CCT GCC CAT TCG ACC ACC AA 3′).

Intravenous cocaine self-administration

μ-opioid receptor knockout and wild-type mice were tested for cocaine self-administration as described previously (Kuzmin et al., 1997b). Briefly, the mice were tested in pairs, one active and one yoked control, in identical 8×8×8 cm test cages made from non-transparent material (RITEC, St. Petersburg, Russia). Each cage has a frontal nose-poking hole supported with infrared sensors interfaced to a computer. Mice were partially immobilized by fixing their

tails, which protruded through the vertical slot in the back wall, to the horizontal surface using tape. A ten minutes pre-test was performed on the test day. Based upon the pre-test results the mice were paired according to basal nose-poke responding. During the 30 minutes self-administration session, which commenced at least 2 hours after the pre-test, each nose-poke by the active mouse resulted in a contingent injection of 1.6 μl of either saline or cocaine solution in the lateral vein of both the active and yoked control mouse. The active groups were slightly bigger in number than the yoked control groups (1-2 more per group) for it was not always possible to form pairs due to failure in needle insertion. Nose-pokes by the yoked control mice were counted but had no programmed consequences.

Cocaine self-administration by μ-opioid receptor knockout mice and wild-type mice was compared. The doses used were 0.4, 0.8 or 1.6 μg per infusion. The data are expressed as the total number of nose poke responses or as total cocaine intake (mg/kg bodyweight) during the 30 minutes experiment. N = 6-8 per dose per type (active or yoked) per genotype.

POMC mRNA expression

In this experiment, C57Bl/6Jico (wild-type) mice were allowed to self-administer either 0.2, 0.4 or 0.8 μg per infusion with yoked controls as described for experiment 1. N = 5-6 mice per type (active or yoked) per dose. Because of failures in needle insertion, pairs could not always be formed, resulting in a slightly larger active group than the yoked control group (1-2 more mice per dose). The environmental conditions were different from those in the μ-opioid receptor knockout cocaine self-administration experiment, which might account for differences in the number of nose-poke responses between the experiments. One hour after the 30 minutes self-administration session was completed, the mice were sacrificed by cervical dislocation after which the brains were quickly dissected and frozen on crushed dry ice. The brains were stored at –80°C until further processing.

For *in situ* hybridisation, 16 μm coronal sections were cut at the level of the arcuate nucleus, nucleus accumbens and ventral tegmental area according to the mouse brain atlas (Paxinos & Franklin, 2001) using a cryostat (Leica, Rijswijk, NL) and thaw-mounted on Superfrost slides (Menzel, Germany). A 190 bp pro-opiomelanocortin cDNA fragment spanning exon 2 and the first 20 bp of exon 3 was subcloned into a PBS +/- vector (Promega, Leiden, NL). An antisense RNA probe was generated by *in vitro* transcription with 120 ng of linearised template DNA, 20 μCi [^{33}P]-UTP and 40 units SP6 RNA polymerase. The sections were post-fixed in a 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.4) for 10 minutes at room temperature (RT), washed twice in PBS for 5 minutes at RT and treated with 0.25% acetic anhydride in tri-ethanolamine (0.1 M, pH 8.0) for 10 minutes at RT. The sections were then rinsed in PBS for 5 minutes and in 0.83% NaCl for 5 minutes at RT. Subsequently the sections were dehydrated by immersing them in solutions with increasing concentrations of ethanol (50%, 70%, 80% and 100%) and dried to air. Hybridisation was performed in 50% deionised formamide, 10% dextran sulphate, 2×SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1x Denhardt's solution, 5 mM EDTA (pH 8.0), 10 mM phosphate buffer (pH 8.0) and

12.5 mg/ml tRNA, containing 0.8×106 cpm of the probe. The hybridisation mix was heated at 65°C for 5 minutes, transferred to ice and DTT was added to a final concentration of 2.5 M. Hybridisation was performed overnight at 55°C in a moist chamber, 100 µl hybridisation mix per slide. Coverslips were removed in 5×SSC at RT and the slides were briefly dipped in 2×SSC at RT, treated with RNAse A (2 mg/100 ml in 5 M NaCl, 1 M Tris, pH 8.0) for 15 minutes at 37°C and washed for 15 minutes at 37°C in 2×SSC. The slides were then washed twice in 2×SSC/50% formamide for 15 minutes at 60°C, twice in 2×SSC for 15 minutes at RT, dehydrated in graded ethanol concentrations and dried to air. Slides were apposed to Kodak Biomax MR films for 6 days.

Quantitative analysis of the POMC mRNA levels in the arcuate nucleus was performed by freehand drawing of the arcuate nucleus according to the mouse brain atlas (Paxinos & Franklin, 2001) using MCID image analyser (Interfocus, Suffolk, UK). There was no POMC mRNA expression in the nucleus accumbens or VTA. POMC mRNA levels are expressed in counts per minute (cpm) as calculated from a standard curve of diluted hybridisation mix, which was laid down with the film. For each animal 2-3 measurements were made for each hemisphere. Since no significant differences were found between hemispheres, the data were pooled. Each measure thus represents a mean of 4-6 measurements per animal.

Electrophysiology

Whole-cell patch-clamp recordings were made from VTA neurons in 200 µm thick horizontal slices. Animals were anaesthetised with isoflurane, decapitated and the brain was rapidly removed and kept in ice-cold high-magnesium artificial cerebral spinal fluid containing (in mM): NaCl 124, KCl 3.3, KH_2PO_4 1.2, $MgSO_4$ 2.6, $CaCl_2$ 2.5, $NaHCO_3$ 20, glucose 10, saturated with 95% O_2- 5% CO_2. Slices were cut in a horizontal plane using a vibratome (Leica, Rijswijk, NL). After preparation the slices were kept at 32°C for at least an hour in aCSF containing (in mM): NaCl 124, KCl 3.3, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 20, glucose 10, saturated with 95% O_2- 5% CO_2. Slices were then transferred to the recording chamber, where they were perfused at 2-3 ml/min with aCSF at RT.

Whole cell recordings from VTA neurons were made using borosilicate glass pipettes with a resistance of 4-6 MΩ. Individual neurons were identified using an upright differential interference contrast microscope (Olympus), with a differential interference contrast enhancement CCD camera. Presence of an I_h (> 100 pA) was used to distinguish between principal dopamine (DA) neurons and secondary GABAergic neurons (Mathon et al., 2003). In current clamp experiments, action potential characteristics were determined and used as an additional criterion to discriminate between principal DA and secondary GABA neurons. For current-clamp recordings the recording pipettes contained (in mM): K-gluconate 155, HEPES 10, Na^+-ATP 2, Na^+-GTP 0.4, EGTA 1, adjusted to pH 7.4 with KOH. For voltage-clamp recordings the recording pipettes contained (in mM): K-gluconate 78, KCl 77, HEPES 10,

Na^+-ATP 2, Na^+-GTP 0.4, EGTA 1, adjusted to pH 7.4 with KOH. For recording spontaneous and miniature inhibitory post-synaptic currents, cells were clamped at –70mV. To isolate inhibitory post-synaptic currents, 10 μM DNQX was added to the perfusion medium. When recording miniature post-synaptic currents additionally 10 μM TTX was present in the perfusing medium. Whole cell current- and voltage-clamp experiments were done using an EPC-9 patch-clamp amplifier (HEKA). Whole cell access resistance was typically between 10 and 35 MΩ. During recordings the access resistance was monitored and an increase of larger than 10% resulted in the experiment being terminated. Data was stored for analysis using Pulse software version 8.53 (HEKA) and on digital analogue tape. The voltage-clamp and current-clamp data was analysed using Signal (CED), WinWCP and WinEDR Strathclyde software.

Statistical analysis

SPSS10.1 was used for statistical analyses. The self-administration data were analysed by three-way ANOVAs. The independent factors were type (active or yoked), dose (μg/infusion) and genotype (+/+ or -/-). The intake data were analysed for the active mice only by two-way ANOVA with dose and genotype as factors.

The self-administration data for the POMC experiment were analysed by two-way ANOVA with dose and type (active or yoked) as factors and the number of nose-poke responses as the dependent variable. Since each mouse received a different amount of cocaine, depending on the number of nose-poke responses, bivariate correlation analysis was used to assess the effects of cocaine upon POMC mRNA expression in the arcuate nucleus. The POMC mRNA expression data were analysed separately for the active and yoked control mice.

sIPSCs, mIPSCs and firing frequencies were compared between μ-opioid receptor and wild-type mice by Student's *t* tests. The IPSC probability data were statistically analysed using the Kolmogorov-Smirnov test.

The data are expressed as mean ± SEM. Statistical significance was accepted at $P < 0.05$.

RESULTS

Intravenous cocaine self-administration by μ-opioid receptor knockout mice

In order to establish the role of μ-opioid receptors in cocaine reinforcement, cocaine self-administration by μ-opioid receptor knockout and wild-type control mice were compared.

Mice were placed in the self-administration boxes for 10 minutes at least 2 hours prior to the actual self-administration session to determine baseline levels of nose-poke responding. During this 10 minutes pre-test nose-poke responses did not result in a cocaine infusion. Baseline nose poke responding by μ-opioid receptor knockout mice was not different from wild-type littermates nor was there a difference between the dose groups (Table 1). During the 30 minutes cocaine self-administration test, the number of nose-poke responses by the active self-administering mice was significantly higher than the responding by yoked control mice (effect of type $F(1,75)=6.6$, $P < 0.05$, Figures 1A and 1B). This difference in nose-poke responding between active and yoked control mice is indicative of reliable cocaine self-administration in this experiment.

A significant effect of genotype on cocaine self-administration was revealed ($F(1,75)=5.8$, $P < 0.05$). Further, post-hoc analysis showed a significant genotype effect for the active responding mice ($F(1,36)=4.0$, $P = 0.05$) but not for the yoked control mice ($F(1,28)=2.7$, N.S.). These data demonstrate impaired cocaine self-administration by drug-naive μ-opioid receptor knockout mice as compared to wild-type littermates. Total intake is considered a more informative measure for the reinforcing efficacy of cocaine, and other drugs of abuse (Van Ree et al., 1999). Total cocaine intake, as calculated for the active mice of both genotypes, was reduced in μ-opioid receptor knockout mice, as is evident from a significant genotype effect (Figure 1C, genotype effect $F(1,41)=6.3$, $P < 0.05$), in addition to a significant effect of dose ($F(2,41)=12.3$, $P < 0.001$). These data further support a reduction in cocaine reinforcement in μ-opioid receptor knockout mice.

		Cocaine (μg/infusion)		
		0.4	**0.8**	**1.6**
+/+	Active	50.3 ± 8.2	62.9 ± 8.2	41.4 ± 8.2
	Yoked	53.6 ± 13.4	52.0 ± 13.0	38.9 ± 7.9
-/-	Active	39.2 ± 11.1	56.2 ± 5.8	46.8 ± 9.4
	Yoked	53.7 ± 6.9	55.1 ± 7.4	39.0 ± 7.0

TABLE 1

Nose poke responding during the 10 minutes pre-test for μ-opioid receptor knockout and wild-type mice. Data is expressed as mean ± SEM nose poke responses during 10 minutes per genotype per type (active or yoked) per dose. N = 6-8 per genotype per dose.

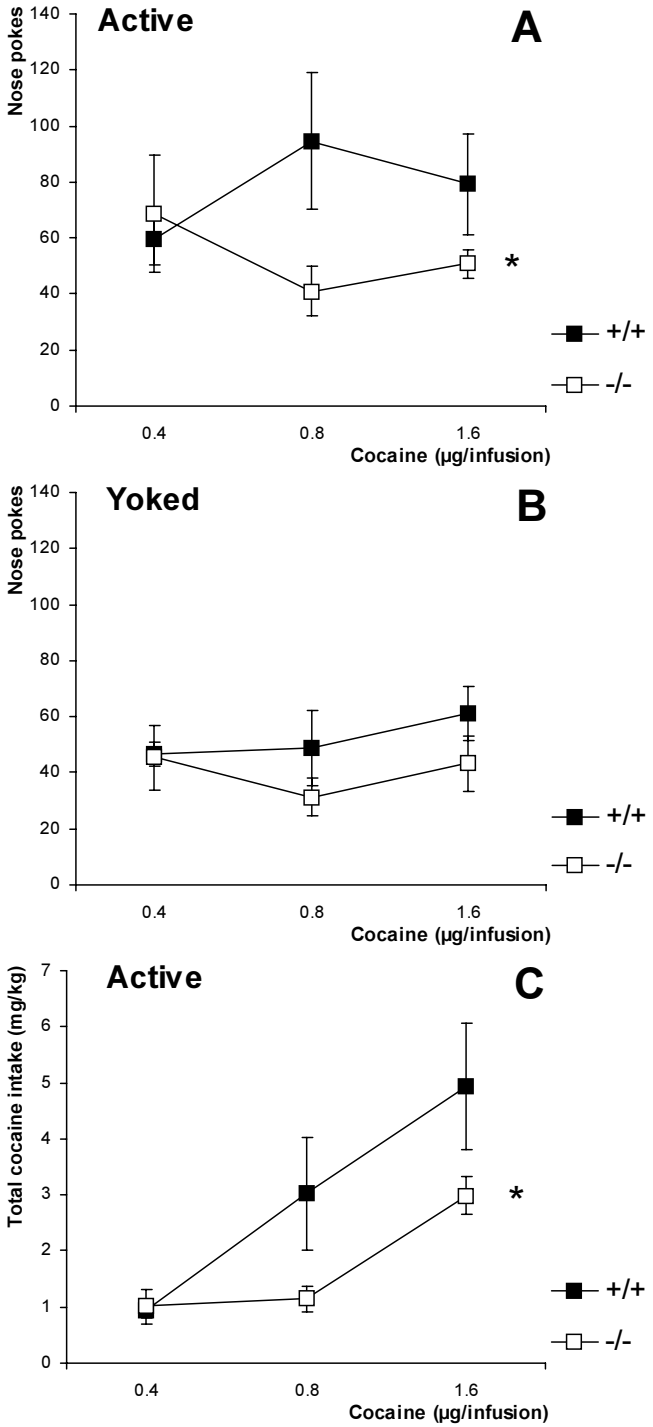


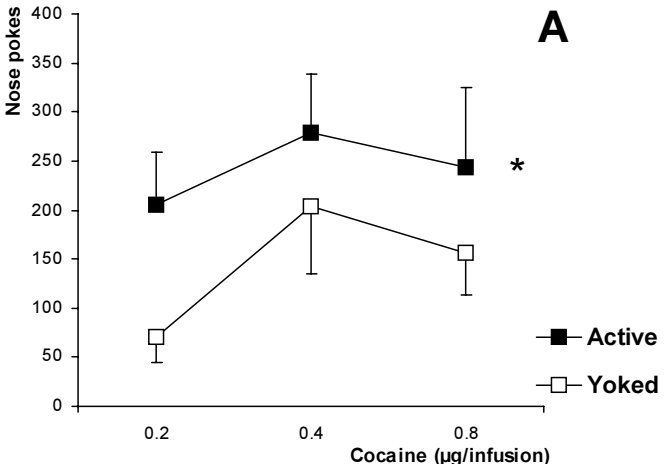
FIGURE 1

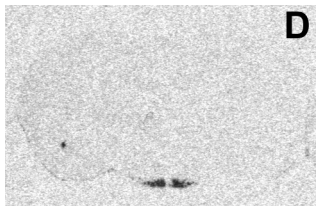
Cocaine self-administration by μ-opioid receptor knockout (-/-) and wild-type (+/+) mice. The number of nose-poke responses during the 30 minutes self-administration session by (A) the active mice for graded doses of cocaine by mice of both genotypes and (B) the yoked control mice of both genotypes across the different dose groups (0.4, 0.8 or 1.6 μg/infusion) are shown. The intake of cocaine by active μ-opioid receptor knockout and wild-type mice during the 30 minutes self-administration session is plotted in (C). Mean ± SEM; N=6-8 per genotype.

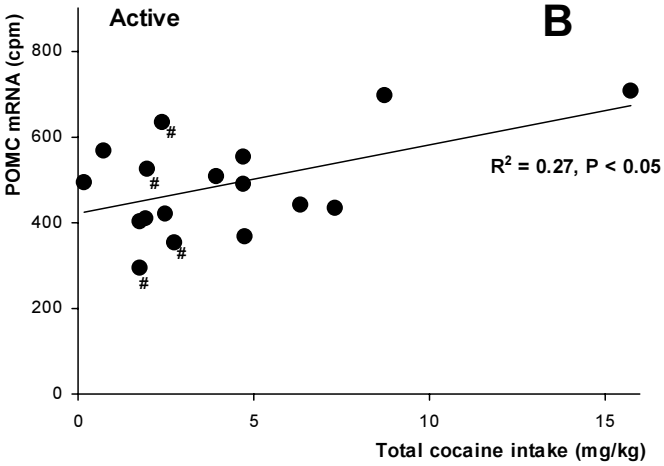
* $P \leq 0.05$, significant different from wild-type mice

Effects of active self- versus yoked-administered cocaine upon POMC mRNA expression in the arcuate nucleus

In this experiment the regulation of POMC, precursor of the μ-opioid receptor selective endogenous opioid peptide β-endorphin, by actively self-administered cocaine and by passively administered cocaine was determined and compared. C57Bl6/Jico mice were allowed to self-administer either 0.2, 0.4 or 0.8 μg cocaine/infusion. The yoked control mice received cocaine injections whenever the active mice responded. Overall analysis of the self-administration data revealed a significant effect of type (active or yoked; $F(1,29)=5.6$, $P < 0.05$), indicative of reliable cocaine self-administration in this experiment (Figure 2A). Inherent to the self-administration paradigm, each mouse received a different dose of cocaine depending on the number of nose poke responses exerted by each individual mouse. Therefore levels of POMC mRNA in the arcuate nucleus were plotted against the individual cocaine intake for each mouse (Figures 2B and 2C). Correlation analysis revealed a significant correlation between total cocaine intake and POMC mRNA levels in the arcuate nucleus of the active mice, which self-administered cocaine ($R^2 = 0.27$, $P < 0.05$). In contrast, total cocaine intake for the yoked control mice, which was controlled by the active mice, was not correlated with POMC mRNA levels in the arcuate nucleus ($R^2 = 0.02$, N.S.). Post-hoc analysis after correction for incomplete pairs (active N = 17, passive N = 13) confirmed significant positive correlation between the level of POMC expression and total cocaine intake for active self-administered cocaine ($N = 13$, $R^2 = 0.61$, $P < 0.05$, incomplete pairs are indicated by $^{\#}$ in Figure 2B). These findings demonstrate regulation of POMC expression by active cocaine self-administration, which is a measure for cocaine reinforcement. Therefore POMC, and its derivative β-endorphin, could be involved in the regulation of cocaine reinforcement.







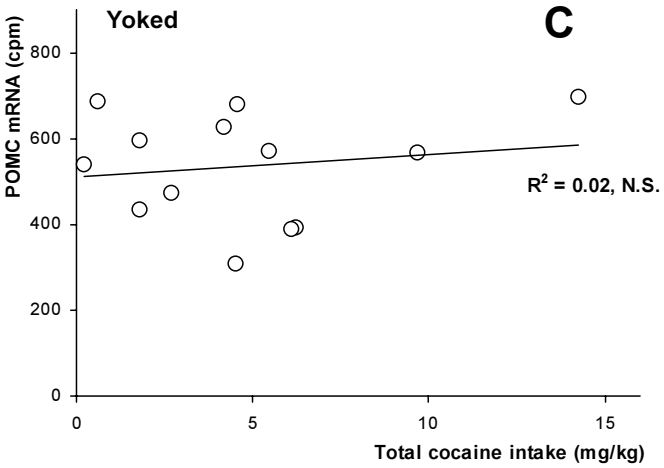


FIGURE 2

Effects of active and yoked administered cocaine upon POMC mRNA expression in the arcuate nucleus. The nose-poke responses (mean ± SEM) of active and yoked control C57Bl/6 mice is shown (A); N = 5-6 mice per dose per type (active or yoked). POMC mRNA plotted against individual cocaine intake is depicted for active (B) and yoked control (C) mice with N = 17 and N = 13 per group, respectively. R = Pearson-correlation coefficient. (D) Representative image of POMC mRNA expression in a coronal section of the arcuate nucleus, taken from an active mouse (0.8 μg cocaine/infusion).

* $P < 0.05$, overall significant different from yoked control mice.

Active mice from incomplete pairs, tested without a yoked control mouse.

GABAergic neurotransmission in the VTA

Here the consequence of the absence of μ-opioid receptors for GABAergic neurotransmission in the VTA was determined. Spontaneous IPSC's were recorded from dopaminergic neurons, firing frequencies of both GABAergic neurons and dopaminergic neurons were determined and miniature IPSC's were compared for wild-type and μ-opioid receptor knockout mice.

Principal neurons in the VTA were identified by the presence of a large hyperpolarization-activated depolarizing current, I_h. To evoke this current 2-second hyperpolarizing pulses (up to -120 mV) were given. To determine the GABAergic input onto principal dopaminergic neurons in the VTA, spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded from dopamine neurons (Figure 3). The frequency of sIPSCs was significantly higher in μ-opioid receptor knockout mice as compared to wild-type mice (3.38 ± 1.01 Hz versus 1.40 ± 0.17 Hz, $P < 0.05$, Student's *t*-test, Figure 3A), resulting in a rightward shift in the cumulative frequency distribution curve ($P < 0.001$, Kolmogorov-Smirnov, Figure 3B). The amplitude of sIPSCs was not different between μ-opioid receptor knockout mice and wild-type mice (34.24 ± 3.91 pA versus 28.77 ± 1.66 pA, N.S. Student's *t*-test, N.S. Kolmogorov-Smirnov, Figure 3C, 3D).

To determine the firing frequency of secondary GABA neurons, current-clamp recordings were made. Furthermore, current-clamp recordings were made from principal neurons to determine whether the increased inhibitory input resulted in a reduced activity of these neurons. In these experiments a number of action potential characteristics were also examined to add an extra criterion for establishing the identity of the neuron recorded from. Principal neurons had more depolarised action potential thresholds, longer duration action potentials and larger undershoots compared to secondary neurons (Table 2). Cells whose action potential characteristics did not correspond to the identification on basis of the presence of a large I_h were excluded from further analysis.

	Threshold	Amplitude	Undershoot	Width at ½ max
Principal	-21.17 ± 3.04	67.13 ± 1.92	-25.67 ± 3.43	4.27 ± 0.20
Secondary	-31.29 ± 1.70	74.61 ± 1.63	-20.74 ± 2.07	2.58 ± 0.15

TABLE 2

Action potential characteristics of principal dopamine and secondary GABA neurons in the VTA. The data represent mean ± SEM values for action potential threshold (mV), amplitude (mV), undershoot (mV) and the action potential width (ms at ½ max).

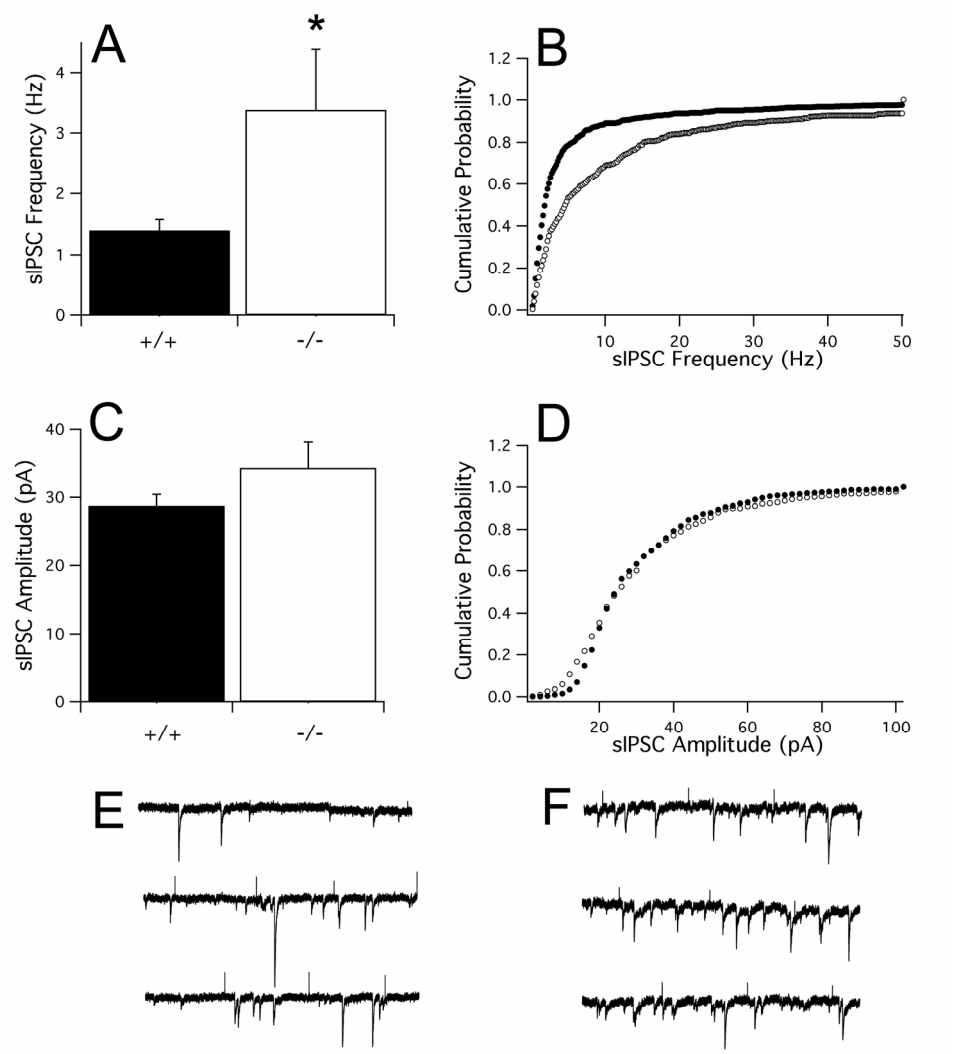


FIGURE 3

Enhanced IPSC frequency onto dopaminergic cells in VTA of μ-opioid receptor knockout mice. EPSC's were recorded from dopaminergic cells in the VTA from μ-opioid receptor knockout mice and wild-type controls.

(A) The average frequency of spontaneous IPSC's of -/- mice is enhanced as compared to +/+ mice (mean ± SEM). (B) Cumulative probability plots of the frequency distribution of spontaneous IPSC's of +/+ mice (●) and -/- mice (○) with significant genotype differences. (C) The average amplitudes of spontaneous IPSC's of +/+ and -/- mice (mean ± SEM). (D) Cumulative probability plots of the amplitude distribution of spontaneous IPSC's of +/+ and -/- mice. (E) and (F) Representative traces from wild-type (+/+) and μ-opioid receptor knockout (-/-) mice, respectively.

In both the wild-type and μ-opioid receptor knockout mice the majority of the secondary GABA neurons were silent. Only a small proportion of secondary neurons (WT: 3/12, KO: 3/10) fired sporadic action potentials (interval > 5 sec). The cumulative probability plots of the interval of the remaining neurons showed a significant leftward shift for the knockout animals compared to the wild-type animals showing an increase in firing activity ($P < 0.001$, Kolmogorov-Smirnov, Figure 4B). The resting membrane potential of the silent neurons was unaltered between the wild-type and μ-opioid receptor knockout mice (-35.7 mV ± 4.6 versus –39.7 mV ± 1.5 respectively, $N = 9$ versus 7, $P > 0.05$, Student's *t*-test). Principal neurons showed a much larger proportion of silent neurons: wild-type animals 16 of 22 neurons tested, knockout animals 21 of 27 neurons tested. The cumulative probability plot of the remaining neurons (Figure 4F) showed a small, but significant, rightward shift for knockout animals compared to wild-type animals indicating a decreased firing activity ($P < 0.001$, Kolmogorov-Smirnov). The resting membrane potential of the silent neurons was significantly more hyperpolarized in principal neurons from μ-opioid receptor knockout mice as compared to wild-type mice (-43.9 mV ± 1.6 versus -38.4 mV ± 1.7, $N = 21$ and $N = 16$, respectively, $P < 0.05$, Student's *t*-test). The increased sIPSC frequency observed in dopaminergic neurons of μ-opioid receptor knockout animals therefore appears to be at least partly due to an increase in firing frequency of local GABAergic interneurons. Furthermore, the increased GABAergic input results in reduced activity of the dopaminergic neurons.

There are indications that μ-opioid receptors are present on presynaptic GABAergic nerve terminals that synapse on dopaminergic neurons (Garzon & Pickel, 2001; Bergevin et al., 2002). The absence of μ-opioid receptors on presynaptic nerve terminals in knockout mice could increase the spontaneous release of GABA and thus contribute to the observed increased amount of sIPSCs observed in dopaminergic neurons. We therefore also examined action potential-independent release of GABA in wild-type and μ-opioid receptor knockout mice by recording mIPSCs in the presence of 1 μM TTX. The cumulative probability plot of the frequency was significantly shifted to the right for knock-out animals compared to wild-type animals (Figure 5B, $P < 0.01$, Kolmogorov-Smirnov), whereas the amplitude was unaltered (Figure 5D, $P > 0.05$, Kolmogorov-Smirnov). The increase in sIPSC frequency might therefore also be due to an increase in spontaneous GABA release in the μ-opioid receptor knockout mice.

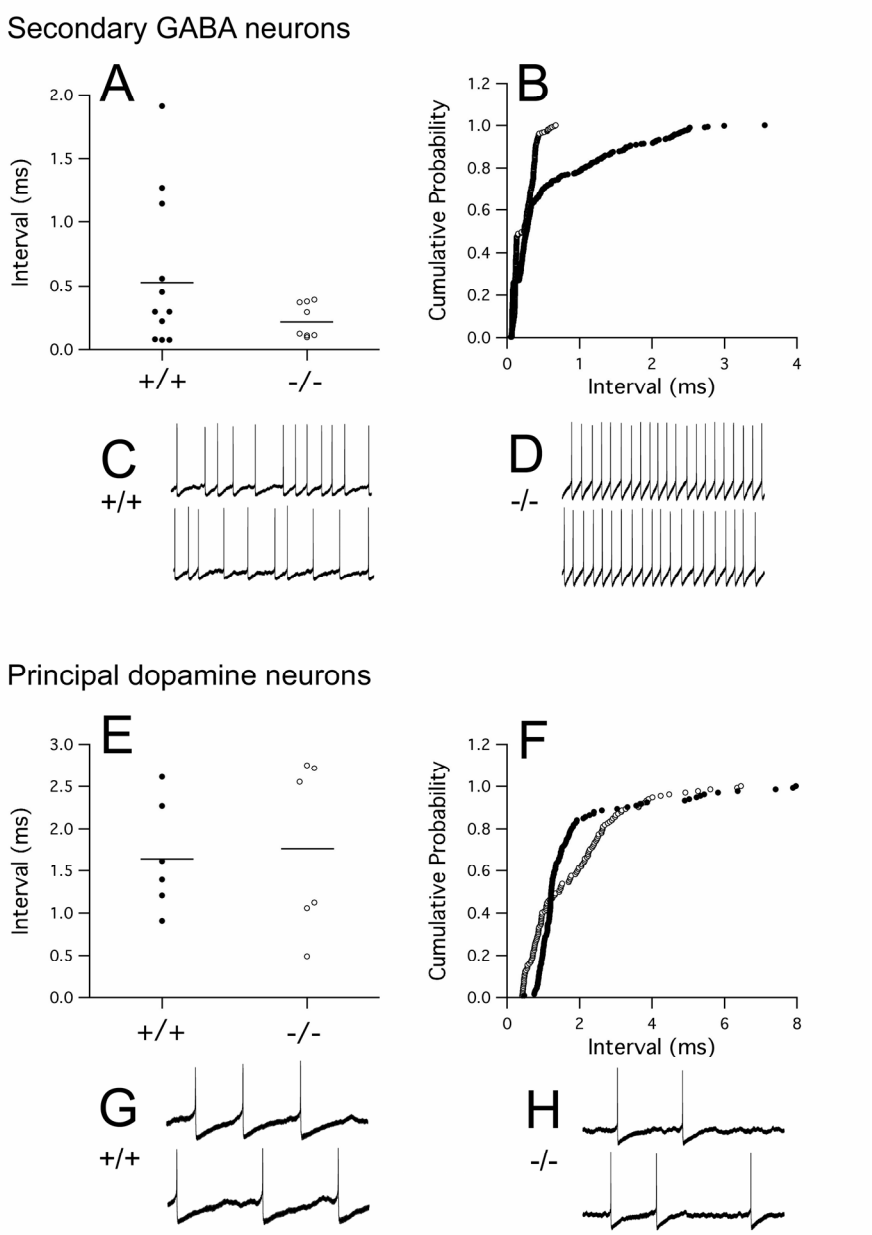


FIGURE 4

Firing frequency of secondary GABA neurons and principal dopamine (DA) neurons of wild-type (+/+) and μ-opioid receptor knockout (-/-) mice. Current-clamp recording were made from secondary GABA neurons and principal dopamine neurons in the VTA of +/+ and -/- mice.

(A) Between-event intervals for the firing GABA neurons. (B) Cumulative probability plot of the interval distribution of GABA neurons in the VTA of +/+ (●) and -/- (○) mice. (C) and (D) Example traces of secondary GABA neurons in the VTA of +/+ and -/- mice, respectively.

(E) Between-event intervals for the firing dopamine neurons. (F) Cumulative probability plot of the interval distribution of principal dopamine neurons in the VTA of +/+ (●) and -/- (○) mice. (G) and (H) Example traces of principal dopamine neurons in the VTA of +/+ and -/- mice, respectively.

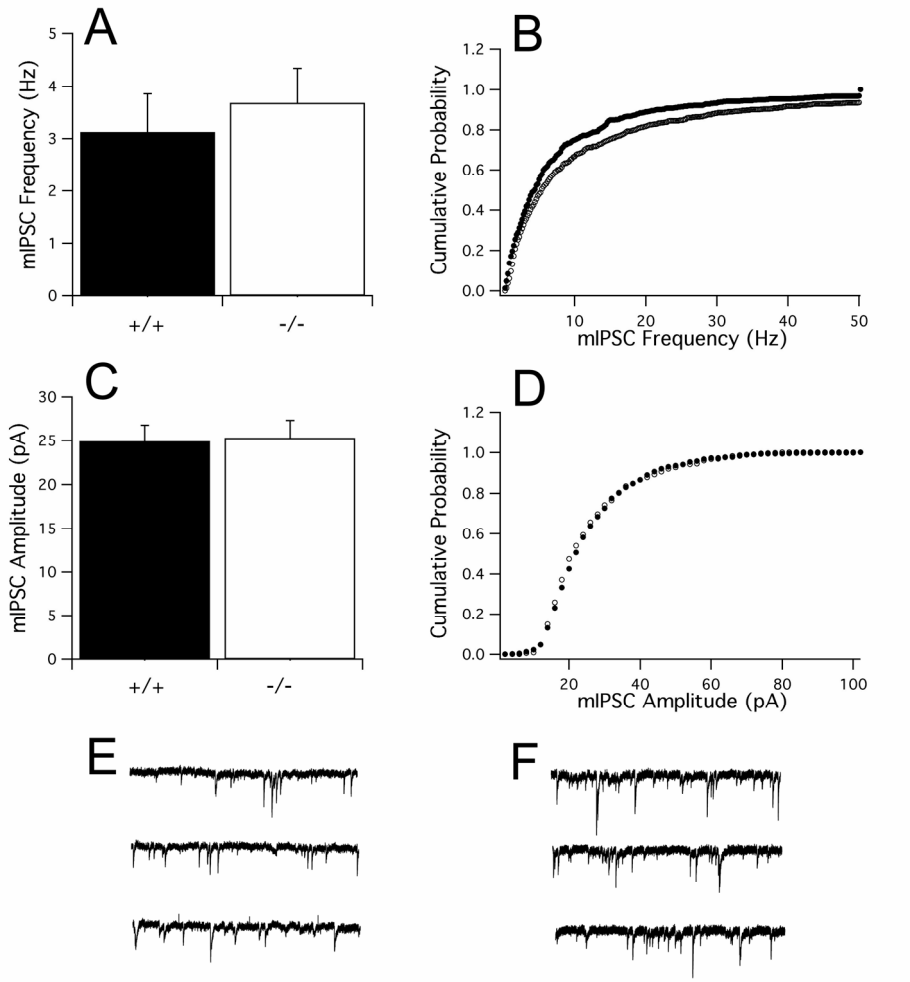


FIGURE 5

Miniature IPSC's recorded from principal dopamine neurons in the VTA of wild-type (+/+) and μ-opioid receptor knockout (-/-) mice in the presence of TTX. The average mIPSC frequencies and amplitudes are shown in (A) and (C) for +/+ (●) and -/- (○) mice, respectively (mean ± SEM). (B) and (D) represent cumulative probability plots of the mIPSC frequency and amplitute, respectively. Representative traces are shown for +/+ (E) and -/- (F) mice.

DISCUSSION

Endogenous opioid systems may modulate drug reinforcement through opioid receptors in the VTA (Herz, 1997; Van Ree et al., 1999). However, direct involvement of μ-opioid receptors in drug reinforcement as determined by acquisition of drug self-administration has not been established. We report that cocaine self-administration by drug naive μ-opioid receptor knockout mice is impaired as compared to cocaine self-administration by wild-type mice. This

shows an important role of μ-opioid receptors in cocaine reinforcement. The association of cocaine self-administration with POMC mRNA levels in the arcuate nucleus further suggests involvement of POMC, and possibly also of POMC-derived peptides such as β-endorphin, in the regulation of cocaine reinforcement. Finally, our data suggest that increased inhibitory GABAergic input onto principal dopaminergic neurons in the VTA of μ-opioid receptor knockout mice could provide a mechanism for the reduced reinforcing efficacy of cocaine in μ-opioid receptor knockout mice.

Involvement of μ-opioid receptors in cocaine self-administration

Cocaine self-administration by drug-naive μ-opioid receptor knockout mice is impaired as compared to wild-type control mice. This is the first report on cocaine self-administration in μ-opioid receptor knockout mice. Previous studies reported reduced self-administration or place preference for morphine, heroin, alcohol, nicotine, Δ^9-THC and deltorphin in μ-opioid receptor knockout mice (Matthes et al., 1996; Becker et al., 2000; Roberts et al., 2000; Hall et al., 2001; Hutcheson et al., 2001; Ghozland et al., 2002; Becker et al., 2002; Contarino et al., 2002; Berrendero et al., 2002). Merely the motivational properties of cocaine have been assessed in μ-opioid receptor knockout mice and were found not involve μ-opioid receptors (Contarino et al., 2002). The present findings demonstrate that μ-opioid receptors play a critical role in cocaine reinforcement.

The observed reduction in cocaine self-administration in μ-opioid receptor knockout mice can not be attributed to different activity levels as compared to wild-type mice, because there was no difference between genotypes in the number of nose poke responses during the 10 minutes pre-test. Furthermore, the nose poke responding of the yoked control mice was not different between genotypes although wild-type yoked control mice appeared to respond slightly more than yoked μ-opioid receptor knockout mice, which may in fact reflect the higher amount of cocaine to which yoked wild-type mice were exposed. The lack of a genotype effect upon non-reinforced nose pokes, both during the pre-test or by the yoked control mice, supports the specific involvement of μ-opioid receptors in cocaine reinforcement. Importantly, the locomotor response to cocaine in an open field is normal in μ-opioid receptor knockout mice (Lesscher et al., 2003c), showing that μ-opioid receptors are not required for the locomotor stimulant effects of cocaine. Thus, μ-opioid receptors may be specifically involved in cocaine reinforcement.

In wild-type mice, actively self-administered but not passively administered cocaine was positively correlated with POMC mRNA levels in the arcuate nucleus, that is POMC mRNA levels increased as total active cocaine intake increased. POMC is the precursor of, amongst others, the μ-opioid receptor selective endogenous opioid peptide β-endorphin. Indeed, β-endorphin levels increase in response to cocaine and also after administration of amphetamine and alcohol, at least in the nucleus accumbens of rats (Olive et al., 2001; Roth-Deri et al., 2003; Marinelli et al., 2003). Also, *in vivo* autoradiography revealed that opioid levels are increased after cocaine self-administration in rats (Gerrits et al., 1999). Active self-

administration is a measure for cocaine reinforcement, which requires μ-opioid receptor activation. The positive correlation of active cocaine self-administration with POMC mRNA, the precursor of β-endorphin, therefore suggests that β-endorphin, through interactions with μ-opioid receptors, may contribute to opioid modulation of cocaine reinforcement. However, other endogenous opioids such as the novel μ-opioid receptor selective endomorphins 1 and 2 (Zadina et al., 1997) or enkephalins, which also have affinity for μ-opioid receptors, may also be involved.

Electrophysiological changes in VTA of μ-opioid receptor knockout mice

The dopaminergic projections of the VTA to the nucleus accumbens are involved in mediating the reinforcing properties of cocaine (Koob & Nestler, 1997). The opioid antagonist naltrexone (NTX) reduced the initiation of cocaine self-administration and cocaine reinforcement only when naltrexone was administered directly in the VTA and not when NTX was applied to terminal regions of the mesolimbic system, *i.e.* striatum, prefrontal cortex, amygdala and nucleus accumbens (Ramsey et al., 1999). Based on these findings, it is likely that particularly the loss of μ-opioid receptors in the VTA contributes to the reinforcement phenotype of μ-opioid receptor knockout mice.

In the VTA μ-opioid receptors are located on secondary, GABAergic, neurons, of which at least a subset are thought to be local interneurons providing inhibitory input onto principal, dopaminergic, neurons (Johnson & North, 1992a; Johnson & North, 1992b; Garzon & Pickel, 2001; Garzon & Pickel, 2002). Activation of μ-opioid receptors on secondary neurons results in hyperpolarization and subsequent depression of spontaneous inhibitory potentials on principal neurons (Johnson & North, 1992a). Here, we show that the basal frequency of sIPSCs onto principal dopaminergic neurons is increased in mice lacking the μ-opioid receptor compared to wild-type mice, whilst the amplitude is unaltered. This shows an increased inhibitory input onto principal dopaminergic neurons in these mice.

The increased sIPSC frequency might be a reflection of increased secondary cell firing, as we find an increased firing frequency of spontaneously active secondary neurons in μ-opioid receptor knockout mice compared to wild-type mice. In addition, the increased inhibitory input onto principal neurons might also result from enhanced action potential-independent GABA release from GABAergic nerve terminals synapsing onto principal neurons. The increase in the frequency but unchanged amplitude of mIPSC's show that also action potential-independent release is increased. We therefore conclude that the increase in sIPSC frequency results from both increased secondary cell firing and increased action potential-independent GABA release.

The firing activity of active principal dopamine neurons was reduced in μ-opioid receptor knockout mice as compared to wild-type mice. Also, the resting membrane potential of principal neurons from μ-opioid receptor knockout mice was more hyperpolarized than that of principal neurons from wild-type mice, possibly as the result of increased inhibitory input to these neurons. GABAergic inhibition can indeed affect the firing activity of dopaminergic

cells. For instance, blockade of GABAergic projections to principal dopamine neurons in the substantia nigra pars compacta, caused burst firing *in vivo* (Tepper et al., 1995). Furthermore, activation of GABA $GABA_A$ and $GABA_B$ receptors reduces drug self-administration (Xi & Stein, 1999; Brebner et al., 2000; Corrigall et al., 2000; Xi & Stein, 2000; Campbell et al., 2002) and bicuculline, a $GABA_A$ receptor antagonist is self-administered locally in the VTA by mice (David et al., 1997). Addition of agonists for the μ-opioid receptor, which reduces firing of secondary neurons, does not result in increased firing of principal neurons *in vitro* (Johnson & North, 1992a; Korotkova et al., 2002). In addition, principal neurons do not show spontaneous burst firing behaviour *in vitro* (Johnson & North, 1992b; Seutin et al., 1993; Wang & French, 1993). This burst firing, which is observed *in vivo*, is an important mechanism through which principal dopamine neurons can increase their dopamine release in the nucleus accumbens (Suaud-Chagny et al., 1992). Principal neurons switch to burst firing when the animal is presented with certain salient stimuli that result in a behavioural response of the animal (Miller et al., 1981; Schultz et al., 1997; Schultz, 1998). Differences between μ-opioid receptor knockout mice and wild-type mice in the activity of principal neurons may only become apparent when bursting of principal neurons is required. In case of the μ-opioid receptor knockout mice the increase in inhibitory GABAergic input may result in a heightened threshold for the induction of burst firing and accompanying behavioural response.

In conclusion, the present finding of impaired self-administration by μ-opioid receptor knockout mice demonstrate an important role of μ-opioid receptors in cocaine reinforcement, probably through regulation of inhibitory neurotransmission in the VTA.

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CHAPTER 4

ERK1/2 ACTIVATION IN RAT VENTRAL TEGMENTAL AREA BY THE μ-OPIOID AGONIST FENTANYL: AN *IN VITRO* STUDY

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ABSTRACT

Opioid receptors in the ventral tegmental area, predominantly the μ-opioid receptors, have been suggested to modulate reinforcement sensitivity for both opiate and non-opiate drugs of abuse. The present study was conducted to study signal transduction proteins, which may mediate the functioning of μ-opioid receptors in the neurons of the ventral tegmental area. Therefore, brain slices of the ventral tegmental area were exposed *in vitro* to the specific μ-opioid receptor agonist fentanyl and immunohistochemically stained for four different activated proteins using phospho-specific antibodies. Fentanyl dose-dependently activated extracellular signal-regulated protein in brain slices of the ventral tegmental area. This activation was reversible with naloxone. Furthermore, naloxone itself also activated extracellular signal-regulated protein kinase. Under the present conditions fentanyl did not affect extracellular protein kinase kinase 1 and 2 (MEK1/2), Stat and cyclic AMP-response element-binding protein (CREB) activity. The direct activation of extracellular signal-regulated protein kinase in ventral tegmental area slices by the μ-opioid receptor agonist fentanyl may suggest a role of extracellular signal-regulated protein kinase in reward processes.

INTRODUCTION

Endogenous opioid systems have been implicated in reinforcement. Especially μ-opioid receptors in the ventral tegmental area (VTA) appear to be involved in both cocaine and ethanol reinforcement (Van Ree et al., 1999). For instance, self-administration studies in rats suggest that μ-opioid receptors in the VTA can modulate the initiation of cocaine self-administration. In both drug-naive rats and mice, treatment with opioid antagonists decreased cocaine intake (De Vry et al., 1989; Kuzmin et al., 1997a). In fact, naltrexone caused a rightward shift in the dose-response curve for cocaine, indicating that cocaine is less reinforcing after opioid blockade. Furthermore local injection of the opioid antagonist naltrexone into the VTA also reduced acquisition of cocaine self-administration, whereas injections of naltrexone in the caudate, amygdaloid or accumbens nuclei as well as in the prefrontal cortex did not affect cocaine self-administration (Ramsey et al., 1999). Injection of the specific μ-opioid receptor agonist DAMGO and the antagonist CTOP into the VTA also altered reinforcement processes for cocaine (Corrigall et al., 1999b). Taken together these studies suggest that μ-opioid receptors in the VTA are involved in sensitivity to drugs of abuse.

The cloning of the opioid receptors has facilitated the investigation of signalling pathways involved in opioid receptor mediated functioning. The elucidation of signal transduction pathways coupled to the μ-opioid receptor in neurons in the ventral tegmental area may add further to the understanding of mechanisms underlying differences in sensitivity to drugs of abuse. From studies using transfected cell lines it appears that opioid receptors are coupled to multiple signal transduction pathways. Due to the availability of antibodies against different phospho-proteins it is now possible to investigate signal transduction pathways *in situ* (Reijmers et al., 2000). *In vivo* studies suggest a role of cAMP-response-element binding protein (CREB), extracellular signal-related protein kinase (ERK) and ERK kinases 1 and 2 (MEK1/2) in opioid receptor mediated signalling (Guitart et al., 1992; Ortiz et al., 1995; Berhow et al., 1996; Widnell et al., 1996; Lane-Ladd et al., 1997; Schulz & Höllt, 1998). However these studies used nonselective opioid agonists which were injected systemically, either acute or chronically. Most of these studies did not concern the VTA: only Berhow et al. (1996) described activation of ERK in the VTA after chronic but not acute systemic morphine treatment. In the present study, signal transduction proteins coupled to the μ-opioid receptor in brain slices of the VTA were investigated. The *in vitro* approach was chosen to ensure detection of acute and local effects in the VTA. After stimulation with the specific μ-opioid receptor agonist fentanyl immunoreactivity was determined for phosphorylated CREB, ERK, MEK1/2 and Stat. These proteins were chosen based on known signal transduction pathways for μ-opioid receptors and availability of phospho-specific antibodies.

MATERIALS AND METHODS

Animals and tissue preparation

Male Wistar rats (200 gram; GDL Utrecht University) were housed in pairs in Macrolon© type III cages with water and food pellets ad libitum; environmental conditions were controlled (22°C and 50% humidity; lights on at 7:00 h and lights off at 19:00 h). The experimental procedures were approved by the Ethical Committee for Animal Experiments of the University Medical Center Utrecht.

For the *in vitro* procedures rats were killed by decapitation and brains were quickly dissected and transferred to ice-cold Krebs-Ringer solution (124 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 10 mM glucose, 20 mM $NaHCO_3$, 2.5 mM $CaCl_2$). Midbrain tissue blocks were cut from approximately -4.8 mm to -7.3 mm posterior to bregma, according to the Rat Brain atlas (Paxinos & Watson, 1998). The tissue was fixed on a specimen plate with cyanoacrylate glue and 2% agarose and subsequently 500 µm coronal vibratome (Vibratome® Series 1000) slices were cut in ice-cold Krebs-Ringer solution, oxygenated with 95%O_2 / 5%CO_2. As an example, one hemisphere of a slice is schematically depicted in figure 1A. Of each animal two slices cut at the level of the VTA were included in the experiment, which were assigned to one treatment group and were considered one sample.

In vitro procedures

The 500 µm thick VTA slices were allowed to rest for 1.5 h at room temperature in oxygenated Krebs-Ringer solution. Slices were then transferred to oxygenated 30°C Krebs-Ringer solution for another 30 minutes: 15 minutes acclimatisation followed by 15 minutes treatment.

Experiments

Experiment 1, screening

With phospho-specific antibodies the possible involvement of four intracellular signal transduction proteins in ventral tegmental µ-opioid receptor mediated signalling was studied. Herefore, rat VTA brain slices were stimulated with the µ-opioid receptor agonist fentanyl (Janssen Pharmaceutica B.V., Tilburg, The Netherlands) *in vitro* (N=4). The experimental procedures were validated using three control groups. *Fresh tissue* and *pre-incubated tissue*, *i.e.* tissue that was fixed after a total 2 h (1.5 h room temperature + 30 min 30°C) incubation in Krebs-Ringer without additives, were included to check for pre-incubation effects. Further, to check for tissue viability, tissue was stimulated with *50 mM KCl* (KCl was added to the medium at t = 105 min). For the experimental groups the sodium channel blocker *tetrodotoxin* (TTX, 1 µM, Tocris, UK) was used to prevent indirect effects due to depolarization of target

cells (TTX was added to the incubation medium at t = 90 min). The three experimental groups were TTX alone, TTX+ 0.1 µM fentanyl and TTX+ 0.5 µM fentanyl (fentanyl was added to the medium at t= 105 min). The 15 minutes incubation time with fentanyl was chosen since according to many studies the peak activity of most phospho-proteins lies within the range of 10-20 minutes. The fentanyl concentrations were chosen from a study on inositol (1,4,5)-triphosphate formation by fentanyl in SH-SY5Y neuroblastoma cells (Smart et al., 1994) and comparable studies on cell cultures which used either DAMGO or morphine (K_D in the range of that of fentanyl, (Johnson et al., 1994; Fukuda et al., 1996; Li & Chang, 1996; Gutstein et al., 1997; Selley et al., 1997; Polakiewicz et al., 1998; Ai et al., 1999; Schmidt et al., 2000). After a total 2 h of incubation the slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. The slices were stored in 0.1% sodium-azide in 0.1 M Tris-buffered saline (TBS, pH 7.4) until further processing.

Experiment 2, ERK activation repeat experiment

To confirm the observed dose-dependent activation of ERK by acute stimulation with fentanyl the experiment was repeated with the same doses of fentanyl: *TTX* alone, *TTX + 0.1 µM fentanyl* and *TTX + 0.5 µM fentanyl* (N=6). Fresh tissue was not included since ERK activity was not different in pre-incubated tissue compared to fresh VTA slices in experiment 1. As an internal control, additional measurements were made in the substantia nigra reticularis, in which area moderate levels of µ-opioid receptors are expressed (Ding et al., 1996).

Experiment 3, Specificity of ERK activation for the µ-opioid receptor

To check for specificity of the effects of fentanyl upon p-ERK immunoreactivity for the µ-opioid receptor the opioid antagonist naloxone was used. The concentration of naloxone was 5 µM; naloxone was added at t = 100 min. Previous studies used either 1 or 10 µM naloxone to demonstrate opioid receptor involvement in responses to DAMGO or morphine (Smart et al., 1994; Fukuda et al., 1996; Gutstein et al., 1997; Polakiewicz et al., 1998; Ai et al., 1999; Schmidt et al., 2000). Based on these studies the intermediate dose of 5 µM naloxone was chosen to examine opioid receptor involvement in the fentanyl-induced activation of ERK in this study. 1 µM TTX-treated slices and tissue slices exposed to the combination of 1 µM TTX and 0.5 µM fentanyl were included, which had been incubated in presence or absence of naloxone (N=8).

Immunohistochemistry

Polyclonal rabbit antibodies against phosphorylated kinases and transcription factors were chosen from a broad range of signal transduction pathways. We used antibodies against phosphorylated ERK (=p42/p44 ERK) from Promega (Madison, WI, USA), MEK1/2 (p-MEK1/2; Ser217/221) and p-Stat (Tyr705) from New England Biolabs (Beverly, MA, USA),

and p-CREB antibody from Upstate Biotechnology (Waltham, MA, USA). These antibodies were specific as checked on a western blot loaded with VTA homogenate.

The fixed VTA slices were cut down to 40 µm thick vibratome slices and free-floating immunohistochemistry was performed. Slices (one in *experiment 1*, two in *experiment 2*) from two to three animals within one treatment group were processed within the same incubation chamber and a net-well system was used to ensure that incubation times were exactly the same for all groups. Slices were rinsed with TBS, preincubated with 5-10 mg/ml sodiumborohydride in TBS for 20 minutes, rinsed with TBS, preincubated with 3% H_2O_2 in TBS and rinsed again. The slices were incubated in supermix (TBS with 0.5% triton-X-100 and 0.25% gelatine) containing a phospho-specific antibody (anti-p-ERK (1:3200), anti-p-MEK1/2 (1:800), anti-p-Stat (1:800) or anti-p-CREB antibody (1:2500)) for 1 h at room temperature followed by 48 h at 4°C while shaking on a rocking table. Slices were again rinsed with TBS and incubated with biotinylated goat anti-rabbit IgG (1:500; Vector, Burlingame, USA) in supermix for 1 h at room temperature while shaking. After rinsing with TBS the slices were then incubated with avidin-biotin complex coupled to peroxidase (1:1000 Vectastain ABC; Vector) in supermix for 2 h at room temperature. Finally, the slices were rinsed with TBS and stained with 0.5 mg/ml diaminobenzidine (Sigma, Zwijndrecht, Netherlands) in TBS containing 0.2% nickelammoniumsulphate and 0.01% H_2O_2. The enzymatic reaction was stopped in TBS and the slices were mounted on gelatine-coated slides, dehydrated in graded ethanol, embedded and coverslipped.

Image Analysis

Slices were examined and images were taken using a MCID image analyser (Interfocus, Suffolk, UK) coupled to a microscope (Zeiss, NL). For quantitative analysis of the immunoreactivity (IR) for the different phospho-proteins, performed with the same MCID system, we used images of the VTA (20× objective) at -5.80 mm posterior to bregma, according to the Rat Brain atlas (Paxinos and Watson, 1998).

The staining pattern for p-CREB, p-MEK1/2 and p-Stat was punctate, hence proportional grain area was chosen for quantification of the IR for these proteins. The staining pattern for p-ERK was more diffuse and therefore optical density was used as the parameter for the quantification of p-ERK IR. Per animal average immunoreactivity was calculated from single measurements from both hemispheres of either one (experiment 1) or two slices (experiments 2 and 3).

Statistical Analysis

For statistical analysis of the data one- or two-way ANOVA (SPSS© 9.0 for Windows) was used, followed by post-hoc comparisons using Student's *t* tests. Overall analysis compared TTX, TTX + 0.1 µM fentanyl and TTX + 0.5 µM fentanyl groups for experiments 1 and 2. Similar analysis was performed after combination of the data of the experiments 1 and 2,

taking the factor experiment into account. For the naloxone experiment an overall analysis was performed for TTX, TTX + naloxone, TTX + fentanyl and TTX + fentanyl + naloxone. In addition, separate analyses examined the effects of fentanyl (TTX versus TTX + 0.5 μM fentanyl, in absence of naloxone) and effects of naloxone itself. Data are represented as mean ± SEM; significance was accepted at $P < 0.05$.

RESULTS

Validation of experimental procedures

The data for the control groups for the different experiments are summarised in Table 1. Compared to fresh tissue, p-MEK1/2, p-Stat and p-CREB immunoreactivity (IR) was reduced after pre-incubation (Table 1a). 50 mM KCl stimulation did not affect the immunoreactivity for p-MEK1/2, p-Stat and p-CREB. For p-ERK no difference between fresh and pre-incubated tissue was observed (Table 1b). In the repeat experiment only pre-incubated tissue was included. Exposure to 50 mM KCl increased p-ERK immunoreactivity. TTX did not significantly affect IR for p-MEK1/2, p-Stat, p-CREB and p-ERK as compared to pre-incubated tissue (compare Tables 1 and 2).

In vitro activation of signal transduction proteins by fentanyl in the ventral tegmental area

Out of four antibodies tested only phosphorylated ERK (p-ERK) showed a response to 15 minutes *in vitro* incubation with fentanyl, a specific μ-opioid receptor agonist. Microscopic examination of the VTA slices stained for p-ERK revealed differences in the density of the staining across groups (Figure 1): p-ERK IR was more dense for the fentanyl-treated slices than for the TTX-treated control slices.

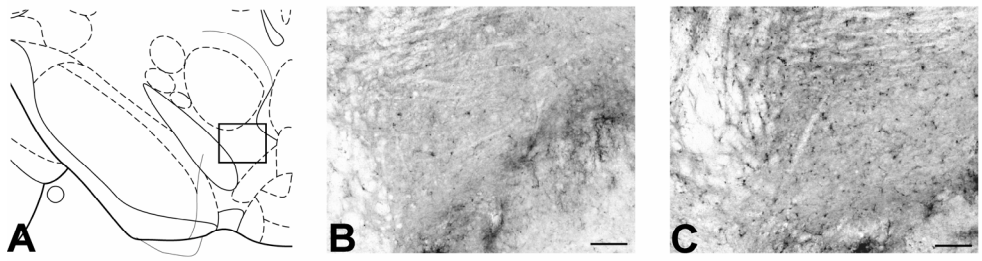

FIGURE 1
Phospho-ERK immunoreactivity in VTA brain slices treated *in vitro* with fentanyl. Images were taken at –5.80 mm to bregma according to the Rat Brain Atlas (Paxinos and Watson, 1998) as schematically drawn in (A). Panel (B) shows p-ERK immunoreactivity for TTX-treated tissue and in panel (C) a representative example is shown of TTX + 0.5 μM fentanyl-treated VTA slices. Calibration bar: 100 μM

Quantitative analysis of the slices, for which we used optical density as a parameter, confirmed the microscopic observations (Table 2). Fentanyl increased p-ERK immunoreactivity (IR) in *in vitro* treated VTA slices (overall effect: $F(2,11)=9.6$, $P < 0.01$). Post-hoc analysis revealed significant differences between TTX and TTX + 0.1 µM fentanyl ($P < 0.05$) and TTX + 0.5 µM fentanyl ($P < 0.01$), thus the ERK activation by fentanyl was dose-dependent. No effects of fentanyl upon p-MEK1/2, p-Stat or p-CREB IR (proportional grain area) were observed (see Table 2). Western blot proved specificity of the antibody for phosphorylated ERK (p42/p44 ERK) as shown in Figure 2.

A	p-MEK1/2	p-Stat	p-CREB
	Proportional grain area		
fresh tissue	0.046 ± 0.001	0.044 ± 0.009	0.038 ± 0.008
pre-incubated tissue	0.015 ± 0.002	0.015 ± 0.006	0.019 ± 0.008
50 mM KCl[a]	0.020 ± 0.005	0.015 ± 0.006	0.020 ± 0.021

B	p-ERK optical density	
	Experiments 1 + 2	Naloxone experiment (3)
fresh tissue	0.171 ± 0.037[b]	-
pre-incubated tissue	0.247 ± 0.039	0.237 ± 0.018
50 mM KCl[a]	0.338 ± 0.029	-

TABLE 1

Validation of experimental procedures. The data for the control groups for p-MEK1/2, p-Stat and p-CREB IR from experiment 1 (N=4) are shown in (A). The data for the control groups for p-ERK IR of experiments 1 and 2 together (N=10) and p-ERK IR of control tissue from the naloxone experiment (N=8) are summarised in (B). Data are represented as mean ± SEM.

[a] Pre-incubated tissue, [b] Data from experiment 1 only

	p-ERK	p-MEK1/2	p-Stat	p-CREB
	Optical density	Proportional grain area		
TTX	0.115 ± 0.021	0.013 ± 0.004	0.016 ± 0.004	0.005 ± 0.002
TTX + 0.1 µM fentanyl	0.219 ± 0.013[a]	0.022 ± 0.009	0.024 ± 0.003	0.010 ± 0.003
TTX + 0.5 µM fentanyl	0.246 ± 0.017[b]	0.020 ± 0.008	0.017 ± 0.003	0.008 ± 0.002

TABLE 2

Exposure of ventral tegmental brain area slices to fentanyl: effects upon signal transduction proteins (experiment 1).

TTX was added during pre-incubation. The data are shown for p-ERK (optical density) and for p-MEK1/2, p-Stat and p-CREB (proportional grain area). Data are represented as mean ± SEM.

Student's *t*-tests: [a] vs. TTX tissue, $P < 0.05$ and [b] vs. TTX tissue, $P < 0.01$ (N=4)

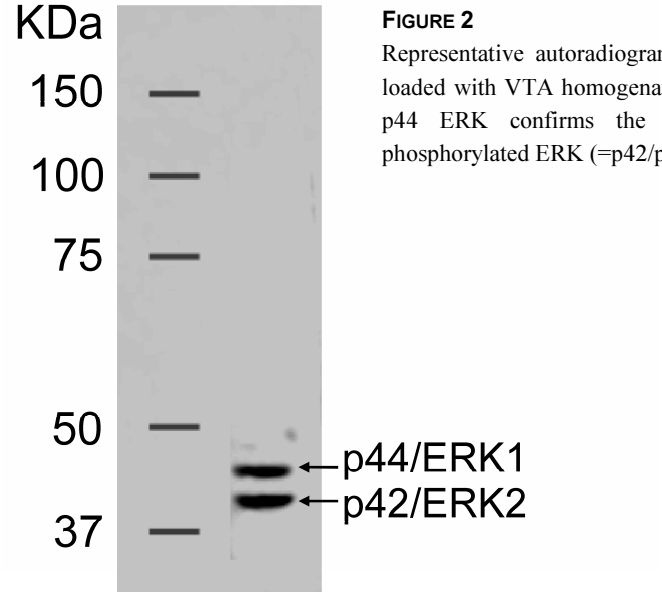

FIGURE 2
Representative autoradiogram of a phospho-ERK Western blot loaded with VTA homogenate. The detection of merely p42 and p44 ERK confirms the specificity of the antibody for phosphorylated ERK (=p42/p44 ERK).

ERK activation by fentanyl in VTA slices *in vitro*

To verify the finding that fentanyl activated ERK in the *in vitro* approach the experiment was repeated and similar results were found. Data for p-ERK IR from the two experiments revealed that there was an overall dose-dependent effect of fentanyl upon p-ERK IR: fentanyl increased p-ERK IR relative to TTX control slices ($F(2,30)=6.7$, $P < 0.01$) (Figure 3a). No interaction between treatment and experiment was present ($F(2,30)=0.37$, $P = 0.70$). Post-hoc analysis revealed significant differences in p-ERK IR between TTX and both 0.1 µM fentanyl and 0.5 µM fentanyl treated tissue ($P < 0.05$ and $P < 0.01$, respectively). Additional measurements from the substantia nigra reticularis (SNR), which were included as internal controls, not reveal activation of ERK by fentanyl. p-ERK IR in SNR: 0.124 ± 0.031 for TTX, 0.130 ± 0.015 for TTX + 0.1 µM fentanyl and 0.116 ± 0.028 for TTX + 0.5 µM fentanyl.

Involvement of µ-opioid receptors in fentanyl-induced activation of ERK

Overall analysis revealed a significant interaction between naloxone and fentanyl treatment (treatment×naloxone $F(1,19)=4.6$, $P < 0.05$, Figure 3b). Consistent with previous experiments we observed an increase in p-ERK IR in VTA slices after treatment with 0.5 µM fentanyl in presence of TTX ($F(1,15)=9.2$, $P < 0.01$). Naloxone itself increased p-ERK IR both in pre-incubated tissue without TTX treatment and in TTX treated tissue. A two-way ANOVA revealed an effect of naloxone ($F(1,28)=5.3$, $P < 0.05$).

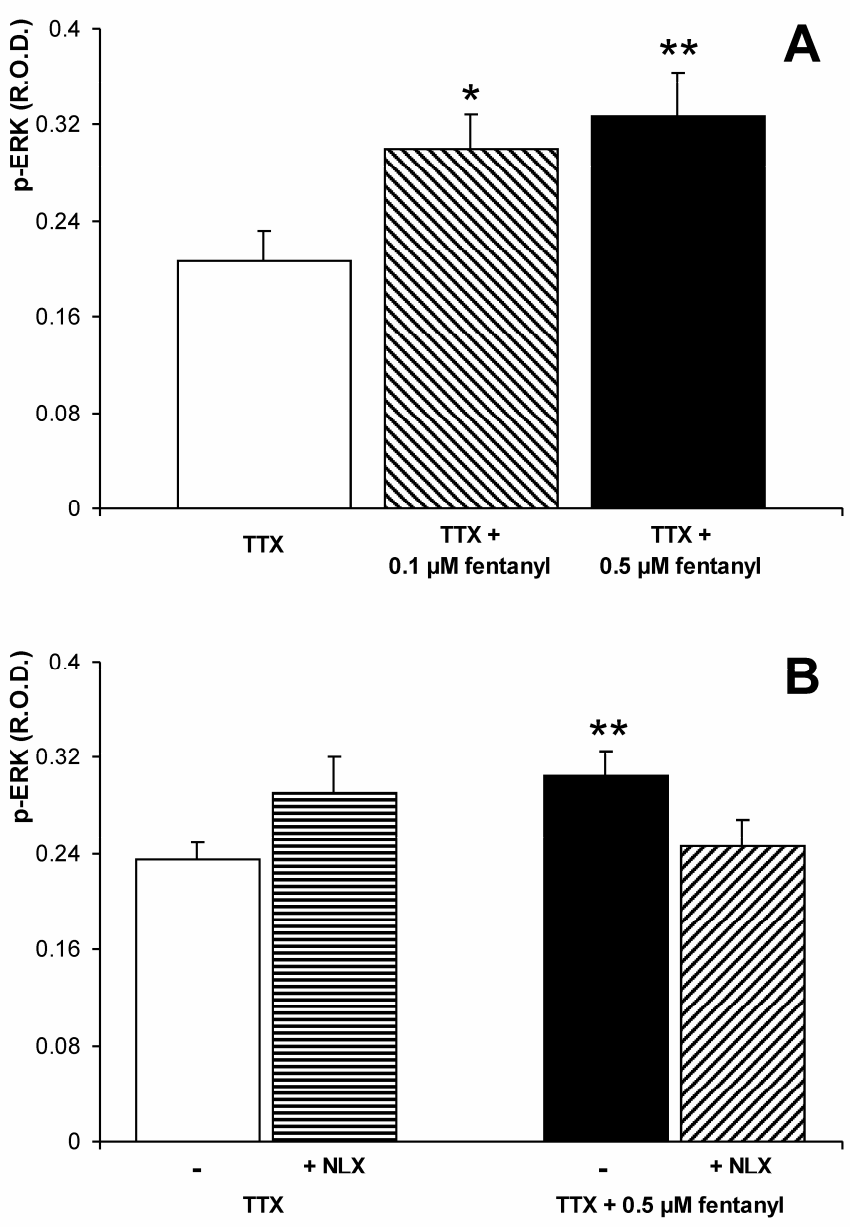

FIGURE 3

ERK activation by fentanyl in VTA brain slices *in vitro*. Fentanyl increased p-ERK IR dose-dependently in rat VTA slices (A) (N=10). The activation of ERK by fentanyl was reversed by the opioid antagonist naloxone (B) (N=8). Data are represented as mean ± SEM.

* $P < 0.05$, ** $P < 0.01$, significant from TTX treatment.

DISCUSSION

In the present study we show activation of ERK, a member of the mitogen-activated protein kinase (MAPK) family, by the specific μ-opioid receptor agonist fentanyl in rat VTA brain slices. Further, the opioid antagonist naloxone reversed the fentanyl-induced activation of ERK. Our data suggest that fentanyl activates ERK dose-dependently via an opioid-receptor mediated mechanism in VTA brain slices *in vitro*.

Out of four phospho-proteins only ERK showed a dose-dependent response to fentanyl treatment in VTA slices in an *in vitro* approach. Although opioid-mediated regulation of Stat has not been reported previously, effects upon ERK, MEK1/2 and CREB activity could be expected considering previous studies. For example, MEK1/2 activation by an opioid has been described previously, however only for the δ-opioid receptor agonist deltorphin (Hedin et al., 1999). Several studies reported CREB regulation by opioids. For example, Guitart and colleagues reported that acute morphine decreased the state of phosphorylation of CREB (Guitart et al., 1992). Acute precipitation of opiate withdrawal increased the levels of phosphorylated CREB. Further, chronic exposure to morphine increased levels of CREB in the locus coeruleus of the rat (Lane-Ladd et al., 1997). Widnell et al. (1996) showed decreased CREB immunoreactivity in the nucleus accumbens after chronic but not acute morphine. The lack of opioid-mediated regulation of MEK1/2 and CREB in the present study may be explained by the use of different opioid agonists. CREB and MEK1/2 activation has only been reported for the relatively non-specific opioid agonist morphine and for the δ-opioid receptor agonist deltorphin, respectively. Therefore opioid-induced activation of CREB and MEK may be mediated by δ- and κ- but not by μ-opioid receptors. Further different experimental conditions *e.g.* treatment time and the brain region studied may explain the lack of MEK1/2 or CREB activation in the present study. For example, MEK1/2 activation by deltorphin peaked at 5 min treatment time and reached basal levels after 10 min (Hedin et al., 1999), suggesting that after 15 min exposure as was done in the present study MEK1/2 phosphorylation levels may have returned to basal levels. Thus although no effects upon MEK1/2, CREB or Stat phosphorylation state were observed, the possibility of their involvement in opioid-mediated signalling can not be ruled out.

In transfected cell lines, ERK activation by opioid agonists has been shown previously (Fukuda et al., 1996; Li & Chang, 1996; Gutstein et al., 1997; Polakiewicz et al., 1998; Belcheva et al., 1998; Ai et al., 1999; Schmidt et al., 2000). *In vivo* studies on opioid-mediated ERK activation have mostly dealt with chronic effects of systemic morphine. Moreover, results of these studies were controversial: chronic exposure to morphine decreased ERK activity in one study (Schulz & Höllt, 1998), whereas the same treatment, but not acute morphine administration, increased ERK activity in other studies (Berhow et al., 1996; Ma et al., 2001). Berhow et al. found increased ERK activity after chronic morphine in the VTA and hence their results may agree with the present findings obtained in brain slices after *in vitro* exposure.

ERK activation induced by an acute stimulation with a specific μ-opioid agonist in a physiologically relevant system, as the VTA brain slices used for the present study has not been shown previously. Further, the *in vitro* approach, the use of the sodium channel blocker tetrodotoxin (TTX), preventing depolarisations, and naloxone blockade ensured the detection of merely direct cellular μ-opioid receptor mediated effects. Our findings indicate a possible role of ERK in the acute effects of μ-opioid agonists in the VTA. As such ERK may have a role in reward processes. In fact, a role of the mitogen-activated protein kinases (MAPK), of which ERK is a subtype, in cocaine responsiveness has been suggested previously. Treatment with a MAPK Kinase (MEK) inhibitor before cocaine reduced cocaine-induced hyperlocomotion (Valjent et al., 2000) and blocked sensitisation to the locomotor activating effects of cocaine (Pierce et al., 1999). Valjent et al. (2001) demonstrated ERK activation in the striatum and nucleus accumbens by Δ^9-tetrahydrocannabinol (Δ^9-THC). Furthermore inhibition of ERK by the MEK inhibitor SL327 impaired THC induced place preference. With regard to opioid reward, involvement of ERK has not been studied so far. It has been shown that MAPK is involved in μ-opioid receptor desensitisation: a feedback signal emanating from the MAPK pathway appears to be required for μ-opioid receptor desensitisation, although internalisation is not required for MAPK activation by opioids (Polakiewicz et al., 1998; Kramer & Simon, 2000).

Since ERK activation by fentanyl was dose-dependent, an *in vitro* approach as described here may further be applicable to monitor changes in sensitivity of the μ-opioid receptor system in, for example, animal models for altered sensitivity to reinforcing effects of drugs.

Interestingly, naloxone activated ERK in VTA slices for both preincubation and TTX conditions. Naloxone did block the effects of fentanyl upon ERK phosphorylation, thus acting as an antagonist in the presence of fentanyl as expected. Our data suggest that naloxone may act as a partial agonist in absence of fentanyl with antagonistic properties in presence of fentanyl. In support of non-classical behaviour of this opioid antagonist, partial agonist actions as well as inverse agonist properties have been suggested for naloxone previously in transfected cell lines (Fukuda et al., 1998; Wang et al., 1999). Further, Cruz and colleagues (1996) showed inverse agonist activity of naloxone in guinea-pig ilea preparations. However, studies which used μ-opioid receptor transfected cell lines do not support the present findings: naloxone blocked opioid-induced effects upon ERK activity, but when administered alone naloxone did not affect basal ERK activity in transfected CHO-K1 cells (Ai et al., 1999). Since there are no indications of partial agonist-like properties of naloxone in ERK-activation from studies using cell lines, we may speculate that cell-specific properties of receptor activation or participation of other receptor systems in the VTA may account for the naloxone-induced activation of ERK in the present study.

In conclusion we show that the specific μ-opioid receptor agonist fentanyl induced ERK activation in a dose-dependent manner in rat VTA brain slices. Assuming a significant role of VTA μ-opioid receptors in reward processes (Van Ree et al., 1999) the signal transduction pathways involving ERK may be involved in the cellular mediation of reward as supported by cocaine sensitization and Δ^9-THC place preference studies (Pierce et al., 1999). The direct and dose-dependent activation of ERK may further provide a tool to test opioid efficacy, possibly in animal models relevant for addiction proneness. Future research on the intracellular mechanisms coupled to μ-opioid receptor activation in the VTA may provide more insight in the mechanisms underlying individual proneness to addiction and hence may contribute to the prevention of drug dependence.

ACKNOWLEDGEMENTS

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CHAPTER 5

μ-OPIOID RECEPTORS ARE NOT INVOLVED IN ACUTE COCAINE-INDUCED LOCOMOTOR ACTIVITY NOR IN DEVELOPMENT OF COCAINE-INDUCED BEHAVIOURAL SENSITIZATION

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SUBMITTED

ABSTRACT

Although μ-opioid receptors have been extensively investigated for their role in drug reinforcement, little is known about the contribution of these receptors to the acute and sensitized locomotor response to cocaine. In this study μ-opioid receptor involvement in acute cocaine-induced locomotor activity and in the development of cocaine-induced behavioural sensitization was evaluated using μ-opioid receptor knockout mice and chronic naltrexone (NTX) pre-treatment as models. In addition, co-administration of the specific μ-opioid receptor antagonist CTOP with repeated saline or cocaine injections was used to establish the involvement of μ-opioid receptors in sensitization to the locomotor stimulant effects of cocaine.

The acute locomotor response to cocaine (3, 10, 20 or 30 mg/kg i.p.) of μ-opioid receptor knockout or chronic NTX pre-treated mice was not different from the cocaine response of their respective controls. With respect to cocaine-induced behavioural sensitization, induced by daily injections of 20 mg/kg cocaine for 11 subsequent days, μ-opioid receptor knockout mice developed behavioural sensitization to the locomotor stimulant effects of cocaine (challenge 10 mg/kg i.p.) comparable to wild-type littermates and the μ-opioid receptor antagonist CTOP did not affect cocaine-induced sensitization either. However, mice which were pre-treated with NTX exhibited augmented cocaine-induced behavioural sensitization relative to placebo pre-treated controls, which may be ascribed to increased δ-opioid receptor levels as has been described for chronic NTX pre-treated mice. The present findings suggest that μ-opioid receptors are not required for the acute locomotor response to cocaine nor are they essential for the development of cocaine-induced behavioural sensitization.

INTRODUCTION

Repeated intermittent exposure to cocaine enhances the locomotor stimulant effects of cocaine upon a subsequent exposure. This phenomenon, behavioural sensitization to the motor stimulant effects of drugs, is thought to reflect long-term adaptations to chronic drug exposure that may underlie certain aspects of drug addiction (Robinson & Berridge, 2000). A single exposure to cocaine can be sufficient to induce long-lasting sensitization to the locomotor stimulant effects of cocaine (Vanderschuren et al., 1999), possibly through long-term potentiation of dopamine neurons in the ventral tegmental area (VTA) (Ungless et al., 2001). Repeated intermittent pre-exposure to cocaine also facilitated the subsequent acquisition of cocaine self-administration (Horger et al., 1990; Piazza et al., 1990). Furthermore pre-exposure to *e.g.* amphetamine, cocaine and morphine enhanced the conditioned motivational effects of the respective drug (Lett, 1989; Shippenberg & Heidbreder, 1995a). Thus, repeated exposure to drugs of abuse induces long-term adaptations, which contribute to sensitization to the locomotor stimulant and motivational effects of these drugs.

Involvement of endogenous opioid systems in drug reinforcement has been demonstrated repeatedly in laboratory animals (Herz, 1997; Van Ree et al., 1999). Opioid antagonists reduce cocaine and ethanol self-administration (De Vry et al., 1989; Froehlich et al., 1990; Kornet et al., 1991; Kuzmin et al., 1997a; Stromberg et al., 1998), primarily through μ-opioid receptors in the VTA (Ramsey et al., 1999; Lesscher et al., 2003b). Endogenous opioid systems have also been implicated in behavioural sensitization induced by psychostimulants. For example, the non-selective opioid antagonist naltrexone (NTX) prevented the development of cocaine-induced behavioural sensitization (Sala et al., 1995). The selective δ-opioid receptor antagonist naltrindole blocked the development, but not the expression, of sensitization to the locomotor stimulant effects of cocaine (Heidbreder et al., 1993a; Heidbreder et al., 1996). Moreover, sensitization to the conditioned effects of cocaine was prevented when naltrindole was given together with repeated cocaine injections (Shippenberg & Heidbreder, 1995b). Effects of κ-selective opioid receptor agonists, both exogenous and endogenous, upon behavioural sensitization have been reviewed elsewhere (Shippenberg & Rea, 1997). In short, sensitization to the conditioned effects of cocaine or amphetamine, as apparent after pre-exposure to a psychostimulant, was abolished when κ-opioid receptor agonists were administered in combination with the psychostimulant (Shippenberg et al., 1996). In addition, κ-opioid receptor agonists reduced sensitization to the locomotor stimulant effects of cocaine (Heidbreder et al., 1993b; Heidbreder et al., 1995 but Vanderschuren et al., 2000). Taken together, these studies indicate that δ- and κ-opioid receptors are involved in the development of cocaine-induced behavioural sensitization. In contrast, little is known about the role of μ-opioid receptors in sensitization to the (locomotor) effects of cocaine. Interestingly, data from a recent study suggested that μ-opioid receptors may also contribute to cocaine-induced behavioural sensitization (Yoo et al., 2003).

The aim of this study was to establish the involvement of μ-opioid receptors in acute cocaine-induced locomotor activity and in cocaine-induced behavioural sensitization. The possible role

of μ-opioid receptors in the acute locomotor response to cocaine and in cocaine-induced behavioural sensitization was investigated using μ-opioid receptor knockout mice, co-administration of the μ-opioid receptor selective antagonist CTOP (Hawkins et al., 1989) and by means of chronic NTX pre-treated mice. Chronic NTX pre-treatment was included in this study because chronic exposure of mice to NTX results in an increase in μ-opioid receptor binding sites, although δ-opioid receptor binding is also increased by this pre-treatment albeit to a lower extent and up-regulation of κ-opioid receptors after chronic NTX exposure is restricted to cortical regions (Yoburn et al., 1988; Lesscher et al., 2003a).

MATERIALS AND METHODS

Animals

Male mice, either C57Bl/6Jico mice (Charles River, l'Arbresle, France) or μ-opioid receptor knockout and wild-type mice derived from heterozygous breeding (GDL, Utrecht), aged 2-3 months were group housed (2-4) in extended Macrolon© type I cages with water and food pellets available ad libitum. Environmental conditions were controlled (22°C and 50% humidity; lights on at 7:00 a.m. and lights off at 7:00 p.m., GDL Utrecht University). The experimental procedures were approved by the Ethical Committee for Animal Experiments of the University Medical Center Utrecht.

μ-Opioid receptor knockout mice

The μ-opioid receptor knockout and wild-type mice used for this experiment have been described previously and were on a mixed 129Sv/C57Bl6 background (Schuller et al., 1999). No detectable binding of [^{3}H]DAMGO or μ-opioid receptor transcript was present in μ-opioid receptor knockout mice and there is no evidence for compensatory changes in other opioid receptor subtypes: binding to δ-opioid receptor subtypes was comparable between genotypes and δ- and κ- and ORL-1 receptor mRNA levels were also unchanged (Schuller et al., 1999). Wild-type (+/+) and homozygous knockout (-/-) mice were obtained from heterozygous breeding. The mice used in the present study were on a C57Bl6/Jico background after 6-7 back-crossings to C57Bl6/Jico mice (Charles River, l´Arbresle, France). The mice were genotyped by PCR on genomic DNA isolated from tail tips. The mutant product was 700 bp, the wild-type product 525 bp; the three primers used were outside the mutation site (5′ GAC TTT CCT GGC TGA TGC AAA CAA CCT 3′), within the mutation site (5′ CAT GGT TCT GAA TGC TTG CTG CGG ACT 3′) and within the neomycin box (5′ CTA CCT GCC CAT TCG ACC ACC AA 3′).

Chronic naltrexone (NTX) treated mice

At least one week after transportation, C57Bl/6Jico mice from Charles River (L'Arbresle, France) received a pellet containing 15 mg NTX or a corresponding placebo pellet, which was implanted subcutaneously in the nape of the neck under isoflurane anaesthesia (2% / 53% N_2O / 45% O_2) (day 1). NTX and placebo treatments were randomly assigned and mice of both treatment groups were housed together (2 of both per cage). On day 8 the pellet was removed (2% isoflurane / 53% N_2O / 45% O_2). All experiments described here commenced 48 hrs after pellet removal, *i.e.* on day 10.

Acute cocaine-induced locomotor activity

Experiment 1. μ-Opioid receptor knockout mice; Experiment 2. Chronic NTX treated mice

Clear plexiglass cylinders of 20 cm in diameter and 30 cm in height were used as open fields. The mice were allowed to acclimatise to the experiment room for at least 1 hour prior to placement in the open field. The mice were then placed in the open field and motor activity was monitored for 1 hour. Thereafter saline was injected i.p. and the mice were monitored for another hour. Finally, cocaine was injected i.p. (3, 10, 20 or 30 mg/kg) after which the mice were returned to the open field and their locomotor activity was determined for another 30 minutes. During the total 2.5 hour of the experiment, the activity pattern of the mice was tracked and analysed for the total distance moved in the open field per 5 minutes intervals using Ethovision Color-Pro 2.3 software (Noldus Information Technology, Wageningen, NL). N = 6 for 3, 20 and 30 mg/kg treatment groups. N = 8 for the 10 mg/kg cocaine treatment group of the μ-opioid receptor knockout experiment. N = 10 for the 10 mg/kg cocaine treated chronic NTX or placebo pre-treated groups.

Cocaine-induced behavioural sensitization

Experiment 3. Cocaine-induced behavioural sensitization in wild-type mice

16 C57Bl/6Jico mice were randomly assigned to either cocaine or saline treatment groups. During 11 days the mice received a daily i.p. injection of either cocaine (20 mg/kg) or saline. 72 Hrs after the last cocaine or saline injection, the mice were transported and allowed to acclimatise to the experiment room for at least 1 hour. Thereafter, the mice were placed for the first time in the open field as described. During the first hour in the field the locomotor activity was measured to monitor the adaptation of the mice to the novel environment. Subsequently all mice received a saline injection (i.p.) after which they were returned to the open field and monitored for another hour. Thereafter all mice received a cocaine challenge (10 mg/kg i.p.) and their locomotor activity in the open field was determined during 30 minutes. The distance moved in the open field was measured as described above.

Experiment 4. μ-Opioid receptor knockout and wild-type mice

At 2-3 months of age the behavioural sensitization commenced as described for experiment 3. Mice of both genotypes (wild-type (+/+) and μ-opioid receptor knockout mice (-/-)) were randomly assigned to either saline or cocaine treatment groups. Group sizes were either 7 mice for the +/+ saline treatment group or 8 mice for +/+ cocaine, -/- saline and -/- cocaine treatment groups.

Experiment 5. CTOP co-administration

For this experiment 32 male C57Bl/6JIco mice from Charles River (L'Arblesle, France) were used. After transportation, the mice were allowed to acclimatise for at least one week before the behavioural sensitization commenced. The mice were randomly assigned to one of four treatment groups: placebo/saline, placebo/cocaine, CTOP/saline or CTOP/cocaine. CTOP (1 mg/kg) or placebo was administered i.p. 30 minutes prior to the daily saline or cocaine injections (co-administration) (Kim et al., 2000). Further procedures were similar to those described for experiment 3. N = 8 per treatment group.

Experiment 6. Effects of chronic NTX pre-treatment upon cocaine-induced behavioural sensitization

For this experiment mice were, prior to the behavioural sensitization protocol, pre-treated with naltrexone or placebo by subcutaneous implanted pellets as described above for experiment 2. The sensitization protocol (see Experiment 3) commenced 48 hrs after removal of the pellet, *i.e.* on day 10. N = 8 for placebo/saline, placebo/cocaine, NTX/saline and NTX/cocaine treatment groups.

Drugs

Cocaine (cocaine-HCl, OPG, Utrecht, The Netherlands) and CTOP (Tocris, Bristol, UK) were dissolved in saline, control mice received saline injections. Cocaine, CTOP and saline were injected i.p. in a volume of 5 ml/kg. NTX and corresponding placebo pellets (Research Triangle Institute, North Carolina, USA) were implanted subcutaneously in the nape of the neck as described above.

Statistical analysis

For statistical analyses SPSS10.1 was used. Open field activity is expressed as distance moved in 5 minutes intervals. Analyses of variance (ANOVAs) with repeated measurements were used to analyse the data with distance moved as the dependent variable. For the acute experiment only the locomotor activity data for the 30 minutes after cocaine injection was analysed with genotype (+/+ or -/-, experiment 1) or pre-treatment (placebo or NTX,

experiment 2) as factors. For the sensitization experiment, the data for the first hour, the hour after saline injection and the 30 minutes after cocaine challenge were analysed separately. The independent factors were treatment (saline or cocaine) and either genotype (+/+ or -/-, experiment 4), co-administration (placebo or CTOP, experiment 5) or pre-treatment (placebo or NTX, experiments 6). When appropriate, post-hoc analyses were performed using Student's *t*-tests. The data are expressed as mean ± SEM distance travelled in 5 minutes intervals in centimetres. Statistical significance was accepted at $P < 0.05$.

RESULTS

Acute cocaine-induced locomotor activity

Experiment 1. Acute locomotor response to cocaine in μ-opioid receptor knockout versus wild-type mice

After two hours adaptation to the open field (data not shown), the mice received 3, 10, 20 or 30 mg/kg cocaine i.p. The dose-response curve for cocaine-induced locomotor activity of μ-opioid receptor knockout mice and wild-type mice is shown in Figure 1. Cocaine increased the locomotor activity of both μ-opioid receptor knockout and wild-type mice in the open field in a dose-dependent way (dose effect $F(3,48)=31$, $P < 0.001$). The genotypes were comparable in their response to cocaine for there was no significant time × genotype × dose interaction, no significant effect of genotype, nor was there a genotype × dose effect.

Experiment 2. Effects of chronic NTX pre-treatment upon acute cocaine-induced locomotor activity

After two hours adaptation to the open field (data not shown), the mice received 3, 10, 20 or 30 mg/kg cocaine i.p.. The dose-response curve for cocaine-induced locomotor activity of chronic NTX and placebo pre-treated mice is depicted in Figure 2. Cocaine increased the locomotor activity of the mice in this experiment in a dose-dependent manner as is apparent from a significant dose effect ($F(3,47)=45$, $P < 0.001$) and a significant time × dose interaction ($F(15,235)=4.2$, $P < 0.001$). Further, a significant time × pre-treatment × dose interaction was observed ($F(15,235)=2.9$, $P < 0.001$), which was caused by a significant time × pre-treatment interaction within the 30 mg/kg cocaine dose group ($F(5,50)=3.0$, $P < 0.05$). It appears that the response to cocaine is somewhat altered for the NTX pre-treated mice as compared to the placebo controls (Figure 2).

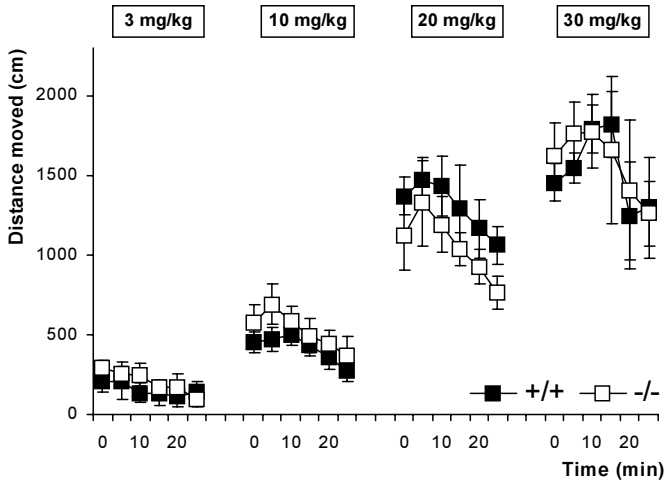

FIGURE 1

Acute cocaine-induced locomotor activity for μ-opioid receptor knockout (-/-) and wild-type (+/+) mice. The distance moved in cm during 30 minutes subsequent to 3, 10, 20 or 30 mg/kg cocaine administration i.p. is shown for both genotypes. N per genotype was 6 for 3, 20 or 30 mg/kg and N = 8 for 10 mg/kg cocaine. Mean ± SEM.

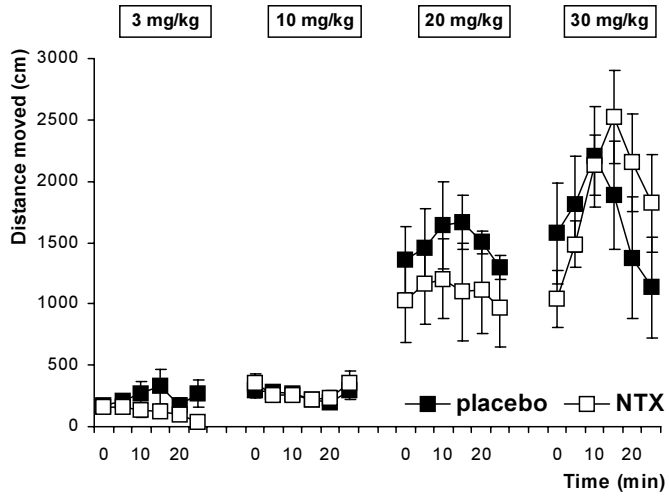

FIGURE 2

Effects of chronic NTX pre-treatment upon the acute response to cocaine. The distance moved in cm during 30 minutes subsequent to 3, 10, 20 or 30 mg/kg cocaine administration i.p. is shown for both pre-treatment groups. N per NTX or placebo pre-treatment group was 6 for 3, 20 or 30 mg/kg and N = 10 for 10 mg/kg cocaine. Mean ± SEM.

Cocaine-induced behavioural sensitization

Experiment 3. Cocaine-induced behavioural sensitization in wild-type mice

The locomotor activity in the open field and the response to a 10 mg/kg cocaine challenge of C57Bl/6Jico mice after repeated administration of 20 mg/kg cocaine or saline injections is shown in Figure 3.

During the first hour in the open field the activity of the mice declined (time $F(11,143)=7.5$, $P < 0.001$) and there was no effect of cocaine treatment upon the activity in the unfamiliar open field. Subsequent to saline administration, a further decline in the activity of the mice was apparent (time $F(11,143)=3.2$, $P < 0.01$) without cocaine treatment effects. After the 2 hours of adaptation to the open field, 10 mg/kg cocaine was administered and induced an increase in the locomotor activity of the mice (Figure 3). The cocaine and saline treated mice responded differentially to the 10 mg/kg cocaine challenge, as is evident from a significant time × treatment interaction ($F(5,65)=4.8$, $P < 0.01$) and an overall effect of treatment ($F(1,13)=4.9$, $P < 0.05$). Post-hoc analyses revealed significant differences between saline and cocaine treated mice for the first 15 minutes after the 10 mg/kg cocaine challenge was administered; $P < 0.05$, $P < 0.01$ and $P < 0.05$ for the locomotor activity during 0-5, 5-10 and 10-15 minutes after cocaine injection, respectively. This sensitization protocol was subsequently used for the experiments 4, 5 and 6.

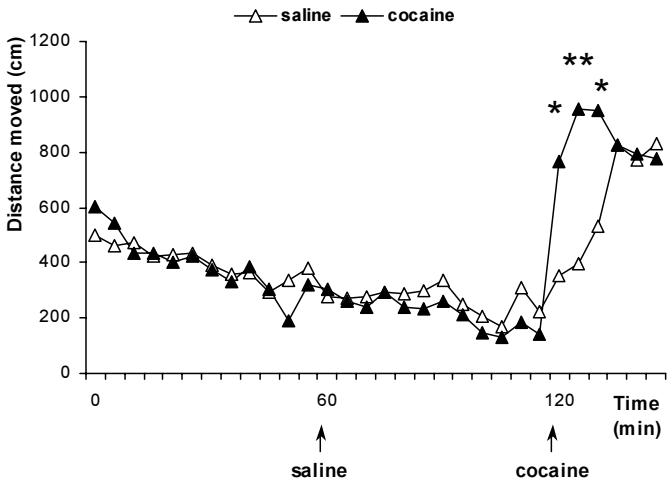

FIGURE 3

Development of behavioural sensitization in C57Bl6/Jico mice. Sensitization was induced by repeated intermittent treatment of C57Bl/6Jico mice with saline or cocaine (20 mg/kg) for 11 days. The time-course of the activity in the open field, 72 hrs after cessation of the sensitization protocol, is shown with 1 hour adaptation to the field, followed by 1 hour in the field after an i.p. saline injection and 30 minutes after an i.p. injection of 10 mg/kg cocaine. The activity in the open field is expressed in the total distance moved in centimetres during 5 minutes intervals. Mean ± SEM, N = 8 per treatment.
* $P < 0.05$, ** $P < 0.01$, significant difference between saline and cocaine treated mice.

Experiment 4. Cocaine-induced behavioural sensitization in μ-opioid receptor knockout and wild-type mice

Locomotor activity and the response to a cocaine challenge of μ-opioid receptor knockout mice (-/-) and wild-type controls (+/+) are shown in Figure 4.

The locomotor activity of μ-opioid receptor knockout and wild-type mice reduced during the first hour in the open field (effect of time: $F(11,297)=10$, $P < 0.001$). During the 60 minutes after saline injection the locomotor activity of the mice did not further decline and a significant time × genotype interaction was observed ($F(11,297)=2.0$, $P < 0.05$): the μ-opioid receptor knockout mice were less active as compared to wild-type mice 15-20, 35-40 and 40-45 minutes after saline injection ($P < 0.05$). There was no effect of treatment upon the activity of the mice in the open field and all mice were comparable in their locomotor activity just prior to cocaine injection.

The locomotor response to the 10 mg/kg cocaine challenge was augmented in cocaine treated mice as compared to saline treated mice, illustrating the occurrence of behavioural sensitization to cocaine (time × treatment $F(5,135)=2.3$, $P < 0.05$). Subsequent post-hoc tests revealed that cocaine treated mice responded with a higher increase in locomotor activity as compared to saline treated mice during 0-5, 5-10 and 15-20 minutes after the cocaine challenge was administered ($P < 0.05$). No genotype or genotype × treatment interaction was

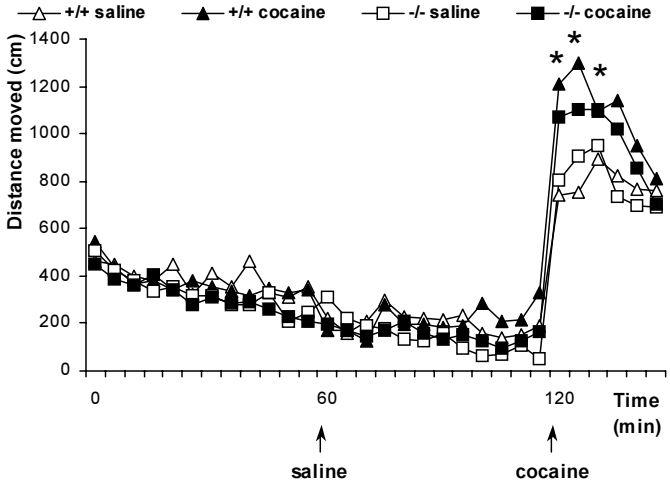

FIGURE 4

Behavioural sensitization in μ-opioid receptor knockout mice (-/-) and wild-type controls (+/+). Sensitization was induced by repeated intermittent treatment with saline or cocaine (20 mg/kg) for 11 days. The time-course of the activity in the open field, 72 hrs after cessation of the sensitization protocol, is shown with 1 hour adaptation to the field, followed by 1 hour in the field after an i.p. saline injection and 30 minutes after an i.p. injection of a 10 mg/kg cocaine challenge. The activity in the open field is expressed in the total distance moved in centimetres during 5 minutes intervals. Mean ± SEM, N = 7-8 per treatment per genotype.

* $P < 0.05$, significant difference between saline and cocaine treated subjects.

observed, suggesting that the development of cocaine-induced behavioural sensitization was comparable for μ-opioid receptor knockout and wild-type mice. There was no difference between saline treated wild-type and μ-opioid receptor knockout mice in their locomotor response to cocaine in the open field.

Experiment 5. Effects of CTOP co-administration upon cocaine-induced behavioural sensitization

In Figure 5 the locomotor activity and the locomotor response to a cocaine challenge is shown for mice, which received either CTOP or placebo co-administered with the repeated intermittent saline or cocaine injections.

During the first hour in the open field the mice reduced their activity (effect of time, $F(11,308)=28$, $P < 0.001$), indicative of adaptation to the open field. There was a significant co-administration × treatment effect upon the activity during the first hour in the open field ($F(1,28)=6.8$, $P < 0.05$), which was caused by higher locomotor activity of the CTOP/saline treated mice as compared to saline/saline and CTOP/cocaine treated mice. The locomotor activity declined further during the hour after saline injection (time effect $F(11,308)=4.9$, $P < 0.001$) and a significant time × co-administration × treatment effect was observed ($F(11,308)=2.1$, $P < 0.05$), which as post-hoc tests revealed was caused by minor differences between groups. That is, 0-5 minutes after saline administration the saline/cocaine treated mice were more active than CTOP/cocaine treated mice ($P < 0.05$). Further, 20-25 minutes after saline was administered, CTOP/cocaine treated mice were more active than mice from the CTOP/saline group ($P < 0.05$) and 35-40 minutes after saline injection, the mice which received repeated CTOP/saline treatment were more active in the open field as compared to saline/saline treated mice ($P < 0.05$). All groups were comparable in their locomotor activity during the last intervals prior to cocaine administration.

Overall analysis of the locomotor activity of all groups during the 30 minutes after administration of the 10 mg/kg cocaine challenge dose revealed a significant time × treatment (saline or cocaine) interaction ($F(5,140)=28$, $P < 0.001$). Cocaine treated mice responded with a higher increase in locomotor activity to the 10 mg/kg cocaine challenge during 0-5, 5-10 and 10-15 minutes after cocaine was injected ($P < 0.001$, $P < 0.001$ and $P < 0.01$). There was no effect of CTOP co-administration nor was there a significant time × co-administration × treatment interaction, suggesting that the μ-opioid receptor antagonist CTOP did not affect the development of cocaine-induced behavioural sensitization. CTOP co-administration did not affect the acute locomotor response to cocaine as CTOP/saline treated mice were not significantly different in their response to cocaine from placebo/saline treated mice.

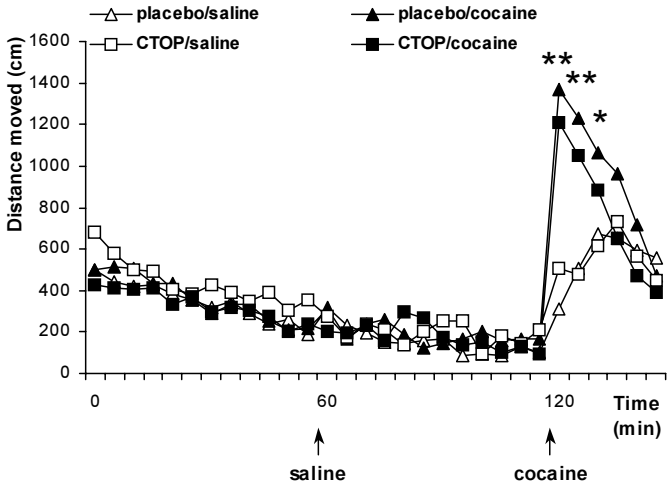

FIGURE 5

Effects of CTOP upon the development of cocaine-induced behavioural sensitization. CTOP or placebo were injected 30 minutes prior to repeated intermittent saline or cocaine (20 mg/kg) injections for 11 days (co-administration). The time-course of the activity in the open field, 72 hrs after cessation of the sensitization protocol, is shown with 1 hour adaptation to the field, followed by 1 hour in the field after an i.p. saline injection and 30 minutes after injection of a 10 mg/kg cocaine challenge. The activity in the open field is expressed in the total distance moved in centimetres during 5 minutes intervals. Mean ± SEM, N = 8 per treatment (saline or cocaine) per co-administration (placebo or CTOP). * $P < 0.01$, ** $P < 0.001$, significant difference between saline and cocaine treatment groups.

Experiment 6. Effects of chronic NTX pre-treatment upon cocaine-induced behavioural sensitization

The locomotor activity in the open field and the response to a 10 mg/kg cocaine challenge of chronic NTX and placebo pre-treated mice is depicted in Figure 6.

During the first hour in the open field the mice adapted to the open field, as reflected by a decrease in locomotor activity (effect of time $F(11,308)=14$, $P < 0.001$). There was no effect of chronic NTX pre-treatment upon the locomotor activity during the first hour in the open field. The locomotor activity during the hour subsequent to saline injection declined further (effect of time: $F(11,308)=3.7$, $P < 0.001$), without group differences.

The cocaine-induced locomotor response was augmented in cocaine treated mice (effect of treatment $F(1,28)=58$, $P < 0.001$). Chronic NTX pre-treatment enhanced the development of behavioural sensitization to cocaine, which was apparent from a significant pre-treatment × treatment interaction ($F(1,28)=7.2$, $P < 0.05$). Separate analyses of the groups confirmed the development of cocaine-induced behavioural sensitization in placebo pre-treated mice (time × treatment $F(5,70)=4.8$, $P < 0.01$ and treatment $F(1,14)=10$, $P < 0.01$) and chronic NTX pre-treated mice (treatment $F(1,14)=62$, $P < 0.001$). Post-hoc analyses revealed that placebo/cocaine treated mice were significantly more active than placebo/saline treated mice during the first 20 minutes after cocaine challenge injection ($P < 0.01$). Across the entire 30

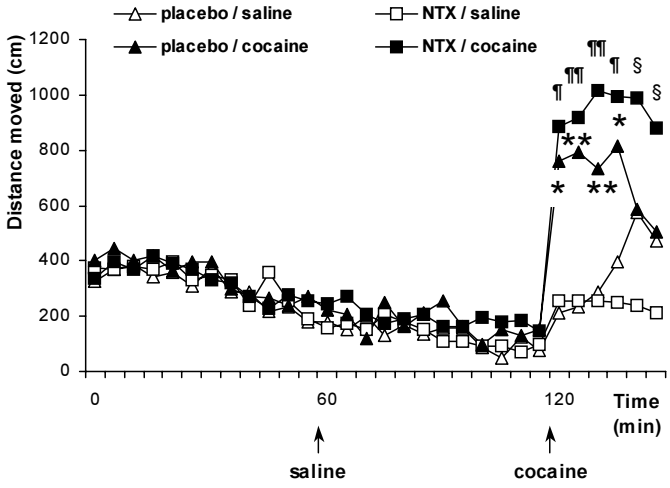

FIGURE 6

Effects of chronic placebo or NTX pre-treatment upon the development of behavioural sensitization. Starting 48 hrs after placebo or NTX pellet removal, repeated intermittent daily injections of saline or cocaine (20 mg/kg) were given for 11 days. The time-course of the activity in the open field, 72 hrs after cessation of the sensitization protocol, is shown with 1 hour adaptation to the field, followed by 1 hour in the field after an i.p. saline injection and 30 minutes after an i.p. injection of a 10 mg/kg cocaine challenge. The total distance moved in centimetres during 5 minutes intervals is shown. Mean ± SEM, N = 8 per treatment (saline or cocaine) per pre-treatment (NTX or placebo).

* $P < 0.05$, ** $P < 0.01$, significant difference between saline and cocaine treated mice within the placebo pre-treated group. § $P < 0.05$, ¶ $P < 0.01$, ¶¶ $P < 0.001$, significant difference between NTX pre-treated, saline controls and NTX pre-treated, cocaine treated mice.

minutes after cocaine injection, the NTX pre-treated mice that received repeated intermittent cocaine injections were significantly more active than NTX pre-treated mice that received repeated saline injections ($P < 0.01$). Placebo and chronic NTX pre-treated mice, which received repeated saline injections during the 11 days of sensitization protocol, were not different in their response to the 10 mg/kg cocaine challenge.

DISCUSSION

Here we investigated the role of μ-opioid receptors in the acute locomotor response to cocaine and in the development of cocaine-induced behavioural sensitization using μ-opioid receptor knockout mice, co-administration of the μ-opioid receptor antagonist CTOP and chronic NTX pre-treatment. Chronic NTX pre-treatment is known to induce increases in μ-opioid receptor binding, but also in δ- and κ-opioid receptor binding, although to a lesser extent (Lesscher et al., 2003a). Our findings indicate that μ-opioid receptors are not required for acute cocaine-induced locomotor activity nor are they essential for cocaine-induced behavioural sensitization to develop.

Acute cocaine-induced locomotor activity

The locomotor response to cocaine was not different between μ-opioid receptor knockout and wild-type mice suggesting that μ-opioid receptors are not required for cocaine-induced motor activity. Similarly, no differences were apparent between chronic NTX pre-treated mice and placebo pre-treated controls, except for the response to 30 mg/kg cocaine, which appeared somewhat altered for the chronic NTX pre-treated group. Cocaine-induced locomotor activity has not been described previously for chronic NTX pre-treated animals. With respect to μ-opioid receptor knockout mice, the present findings are in agreement with those of Becker and co-workers who also reported comparable cocaine-induced locomotor activity for μ-opioid receptor knockout mice, which lack exons 2 + 3 of the μ-opioid receptor gene, and wild-type controls (Becker et al., 2002). Taken together, μ-opioid receptors are not required for the acute locomotor response to cocaine in mice.

Cocaine-induced behavioural sensitization

Opioid modulation of psychostimulant-induced behavioural sensitization has been described previously, at least for δ- and κ-opioid receptors. The δ-opioid receptor antagonist naltrindole and κ-opioid receptor selective agonists impaired the development of behavioural sensitization, which occurs after repeated intermittent treatment of rats with psychostimulant drugs (Heidbreder et al., 1993a; Heidbreder et al., 1993b; Heidbreder et al., 1995; Shippenberg & Heidbreder, 1995b; Shippenberg et al., 1996; Heidbreder et al., 1996; Shippenberg & Rea, 1997 but Vanderschuren et al., 2000). A role of μ-opioid receptors in psychostimulant sensitization was suggested first of all by impaired cocaine-induced sensitization after NTX co-administered with cocaine (Sala et al., 1995) and also by findings of two recent studies. Expression of mRNA encoding μ- and δ-opioid receptors was increased in the ventral tegmental area (VTA) of amphetamine sensitised rats (Magendzo & Bustos, 2003). Another study dealt with cocaine-induced behavioural sensitization in mice with a targeted deletion of exons 2 and 3 of the μ-opioid receptor gene. These μ-opioid receptor knockout mice developed a sensitized locomotor response to cocaine, although the temporal pattern of responding was different from wild-type mice (Yoo et al., 2003).

The present data show, that cocaine-induced behavioural sensitization is retained in exon 1 μ-opioid receptor knockout mice suggesting that μ-opioid receptors are not required for cocaine-induced behavioural sensitization. However, inherent to application of classic knockout strategies and hence lack of a specific gene from gestation, the possibility that for example δ-opioid receptors have compensated for the loss of μ-opioid receptors in this case can not be ruled out. Retained cocaine-induced behavioural sensitization for μ-opioid receptor knockout mice is not in agreement with previous studies, which suggested a role of μ-opioid receptors in psychostimulant sensitization (Sala et al., 1995; Magendzo & Bustos, 2003; Yoo et al., 2003). Therefore, a subsequent experiment was performed to elucidate the role of μ-opioid receptors in cocaine-induced sensitization. For this experiment, the μ-opioid receptor selective

antagonist CTOP, which is approximately 2000-fold more specific for μ- over δ-opioid receptors (Hawkins et al., 1989), was co-administered with cocaine. In agreement with the μ-opioid receptor knockout experiment, CTOP co-administration did not affect cocaine-induced behavioural sensitization. These findings indicate that μ-opioid receptors are not required for behavioural sensitization to the locomotor stimulant effects of cocaine to develop.

In the final experiment augmented behavioural sensitization to cocaine was observed in case of over-expression of predominantly μ-, but also δ-, opioid receptors as induced by chronic NTX pre-treatment. This is an interesting finding considering that the acute locomotor response to cocaine was not affected by chronic NTX pre-treatment, although at the 30 mg/kg dose the cocaine response appeared higher for mice that were pre-treated with NTX as opposed to placebo pre-treated controls. However, μ-opioid receptors seem not to be required for cocaine-induced behavioural sensitization to develop, as can be concluded from the μ-opioid receptor knockout and CTOP experiments, respectively. Therefore it appears unlikely that enhanced μ-opioid receptor levels in chronic NTX treated mice contribute to enhanced behavioural sensitization to the locomotor stimulant effects of cocaine in these mice. Rather, the augmented sensitized locomotor response to cocaine of chronic NTX pre-treated mice may be attributable to increased number of δ-opioid receptors, the subtype of opioid receptors which has indeed been implicated in cocaine-induced behavioural sensitization (Heidbreder et al., 1993a; Shippenberg & Heidbreder, 1995b; Heidbreder et al., 1996).

In contrast to retained cocaine-induced behavioural sensitization observed for μ-opioid receptor knockout mice, μ-opioid receptor knockout mice failed to self-administer cocaine (Lesscher et al., 2003b), suggesting that μ-opioid receptors are critically involved in cocaine reinforcement but are not required for cocaine-induced behavioural sensitization. For chronic NTX treatment, a striking parallel increase is apparent in the development of cocaine-induced sensitization and cocaine reinforcement, both of which are augmented after chronic NTX pre-treatment. Chronic treatment with NTX facilitated the initiation of cocaine self-administration in rats (Ramsey & Van Ree, 1990) and initiation of alcohol consumption has been shown to be potentiated by chronic NTX treatment in mice (Phillips et al., 1997). It appears that similar mechanisms might be involved in both phenomena. However, since chronic NTX treatment causes increases both in μ- and δ-opioid receptor levels (Lesscher et al., 2003a), the increased reinforcing effects of cocaine and the augmented cocaine-induced behavioural sensitization in chronic NTX pre-treated mice are likely to entail distinct mechanisms, that is through increased μ- and δ-opioid receptors, respectively.

In conclusion, the present findings show that μ-opioid receptors are not required for the acute locomotor response to cocaine nor for the development of cocaine-induced behavioural sensitization.

ACKNOWLEDGEMENTS

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CHAPTER 6

SELECTIVE REDUCTION OF DOPAMINE D3 RECEPTOR BINDING AND BEHAVIOURAL CHANGES IN μ-OPIOID RECEPTOR KNOCKOUT MICE

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SUBMITTED

ABSTRACT

Endogenous opioid systems have been implicated in drug reinforcement and drug seeking. Although the mechanism through which opioid systems modulate drug reinforcement and drug seeking, interactions of opioid systems with dopamine systems are likely involved. The present study was designed to investigate the dopamine system of mice with altered opioid receptor expression levels and with a reinforcement phenotype, *i.e.* the μ-opioid receptor knockout mice and mice with transient opioid receptor over-expression induced by chronic naltrexone (NTX) treatment. For both models, dopamine receptor expression and TH mRNA levels were determined. In addition, locomotor activity in a novel open field and climbing, behaviours which both involve dopamine, were assessed.

μ-Opioid receptor knockout mice, but not chronic NTX treated mice, displayed decreased dopamine D3 receptor binding. D1- and D2-like receptor binding were not changed in either model. Further, μ-opioid receptor knockout mice were less active than wild-type mice in the open field as opposed to chronic NTX treated mice, which displayed increased activity in the open field as compared to placebo controls. μ-Opioid receptor knockout mice and chronic NTX treated mice were not different from their controls in climbing behaviour. The differences in dopamine D3 receptor binding and open field behaviour of μ-opioid receptor knockout and chronic NTX treated mice suggest functional opioid control over dopamine systems. Moreover, the reduction in dopamine D3 receptors in μ-opioid receptor knockout mice is an interesting observation considering the increasing evidence that D3 receptors may be a target for treatment of drug addiction.

INTRODUCTION

Endogenous opioid systems have been shown to modulate drug reinforcement in animals (Herz, 1997; Van Ree et al., 1999). For example, opioid antagonists reduce cocaine and ethanol self-administration (De Vry et al., 1989; Froehlich et al., 1990; Kornet et al., 1991; Kuzmin et al., 1997a; Stromberg et al., 1998), primarily through μ-opioid receptors in the ventral tegmental area (VTA) (Ramsey et al., 1999). Although the mechanism through which endogenous opioid systems modulate drug reinforcement is unknown, opioid-dopamine interactions may be involved.

Anatomical and electrophysiology studies have provided evidence for opioid-dopamine interactions in the mesolimbic system with dopaminergic neurons in the VTA projecting to the nucleus accumbens, the prefrontal cortex and the striatum (Oades & Halliday, 1987). In the VTA principal dopamine-containing neurons and secondary non-dopaminergic neurons have been characterised, the latter being sensitive to μ-opioid agonists and presumably GABAergic interneurons (Gysling & Wang, 1983; Johnson & North, 1992b). Indeed, in the VTA μ-opioid receptors are expressed mainly by non-dopaminergic, presumably GABA containing neurons (Garzon & Pickel, 2001). Activation of μ-opioid receptors causes hyperpolarization of the secondary GABA containing neurons, thereby relieving inhibitory input to dopaminergic projection neurons (Johnson & North, 1992a), which in turn causes increased dopamine output in the nucleus accumbens. μ-Opioid receptors are also expressed in the nucleus accumbens where they are localised mainly on GABA-containing medium spiny neurons (Svingos et al., 1997). Although opioid agonists have been suggested to modulate dopamine release from the nucleus accumbens through local μ-opioid receptors (Yoshida et al., 1999), another study did not provide evidence for local modulation of accumbens dopamine release through μ-opioid receptors (Spanagel et al., 1992).

The aim of this study was to investigate opioid-dopamine interactions in relation to drug reinforcement. Two distinct mouse models were chosen for this study, which are characterised by altered opioid receptor expression and have a distinct reinforcement phenotype, *i.e.* μ-opioid receptor knockout mice and chronic naltrexone (NTX) treated mice. μ-Opioid receptor knockout mice, which lack the μ-opioid receptor gene from gestation, are impaired in drug reinforcement: self-administration or place preference for morphine, heroin, ethanol, cocaine, nicotine and Δ^9-tetrahydrocannabinol (Δ^9-THC) are reduced in μ-opioid receptor deficient mice (Matthes et al., 1996; Becker et al., 2000; Roberts et al., 2000; Hall et al., 2001; Ghozland et al., 2002; Becker et al., 2002; Contarino et al., 2002; Berrendero et al., 2002). Chronic treatment of mice with the opioid antagonist NTX results in an overall, but transient, upregulation of predominantly μ-opioid receptors, although to a lesser extent also of δ- and κ-opioid receptors, in brain (Yoburn et al., 1988; Lesscher et al., 2003a). Chronic NTX treatment further enhances the reinforcing efficacy of drugs of abuse: acquisition of cocaine self-administration was facilitated by chronic NTX treatment in rats (Ramsey & Van Ree,

1990) and chronic NTX exposed mice consumed more alcohol and displayed increased preference for alcohol (Phillips et al., 1997).

We hypothesised, based on available evidence for dopamine-opioid interactions, that in μ-opioid receptor knockout mice (ectopic lack of μ-opioid receptors) or in chronic NTX treated mice (transient opioid receptor over-expression), the dopamine system may have adapted, which might in turn contribute to the reinforcement phenotype of either model.

By quantitative *in vitro* autoradiography, binding to D1-like, D2-like and dopamine D3 receptors was determined. The D3 receptor was included in our analysis for its high abundance in the nucleus accumbens, a key area involved in reinforcement/drug seeking (Missale et al., 1998). Moreover, the D3 receptor has recently received considerable attention for its possible role in reinforcement/drug seeking (Pilla et al., 1999; Le Foll et al., 2000; Vorel et al., 2002; Di Ciano et al., 2003; Le Foll et al., 2003). In addition, TH mRNA levels were determined by *in situ* hybridisation. Further the behaviour of both μ-opioid receptor knockout mice and chronic NTX treated mice was assessed in two tasks, both known to involve dopamine, *i.e.* activity in an unfamiliar open field and spontaneous climbing (Costall et al., 1982; Sundstrom et al., 1990; Wolterink et al., 1990; Hooks & Kalivas, 1995).

MATERIALS AND METHODS

Animals

Male mice aged 2 - 3 months were group housed (2 - 4) in extended Macrolon© type I cages with water and food pellets available ad libitum. Environmental conditions were controlled (22°C and 50% humidity; lights on at 7:00 a.m. and lights off at 7:00 p.m., GDL Utrecht University). The experimental procedures were approved by the Ethical Committee for Animal Experiments of the University Medical Center Utrecht.

The μ-opioid receptor knockout mice used in this study have been described previously and were on a mixed 129Sv/C57Bl6 background (Schuller et al., 1999). No detectable binding of [^{3}H]DAMGO or μ-opioid receptor transcript was present in μ-opioid receptor knockout mice (Schuller et al., 1999). There is no evidence for compensatory changes in other opioid receptor subtypes: binding to δ-opioid receptor subtypes was comparable between genotypes and δ- and κ- and opioid receptor-like 1 (ORL-1) receptor mRNA levels were also unchanged (Schuller et al., 1999). Wild-type (+/+) and homozygous knockout (-/-) mice were obtained from heterozygous breeding. The mice used in the present study were on a C57Bl6/Jico background after 6-7 back-crossings to C57Bl6/Jico mice (Charles River, l′Arbresle, France). Mice were genotyped by Polymerase Chain Reaction on genomic DNA isolated from tail tips. The mutant product was 700 bp, the wild-type product 525 bp; the three primers used were outside the mutation site (5′ GAC TTT CCT GGC TGA TGC AAA CAA CCT 3′), within the

mutation site (5′ CAT GGT TCT GAA TGC TTG CTG CGG ACT 3′) and within the neomycin box (5′ CTA CCT GCC CAT TCG ACC ACC AA 3′).

For the chronic NTX experiments, male C57Bl/6JIco mice were obtained from Charles River (L'Arbresle, France). After transportation, the mice were allowed to acclimatise for at least one week before the experiment. Pellets containing 15 mg naltrexone or corresponding placebo pellets were implanted subcutaneous in the nape of the neck under isoflurane anaesthesia (2% / 53% N_2O / 45% O_2) (day 1). Naltrexone and placebo treated mice were housed together (2 of both per cage). On day 8 the pellet was removed (2% isoflurane / 53% N_2O / 45% O_2).

The mice were tested once and were naïve to the respective behavioural tasks. For autoradiography and *in situ* hybridisation experiments the chronic NTX / placebo treated mice were killed and brains were dissected 24 hours after pellet removal. Behavioural experiments for the chronic NTX mice were performed either 24 or 48 hours after the pellet was removed. At these time-points after removal of the pellet opioid receptors are still up-regulated (Tempel et al., 1982; Lesscher et al., 2003a). All experiments were performed during the light phase. Open field and climbing experiments were performed in reduced lighting conditions (50 and 30 Lux, respectively).

In vitro autoradiography for dopamine receptors

16 μm coronal sections were cut for nucleus accumbens, VTA and striatum according to the mouse brain atlas of Paxinos and Franklin (2001). The limbic regions, *i.e.* the nucleus accumbens and the VTA, were chosen for their role in drug reinforcement (Wise, 1998). The striatum is considered to be involved in habit learning and compulsive drug seeking at later stages of the addiction process (Robbins & Everitt, 2002). The sections, cut using a cryostat (Leica, Rijswijk, NL), were thaw-mounted on gelatine-subbed slides and stored at -80°C until use.

Autoradiography procedures were based on previous studies (Tarazi et al., 1998; Diaz et al., 2000). Sections were pre-incubated at room temperature (RT) for either 1 hour in 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ for D1-like and D2-like receptors or 3 times 5 minutes in 50 mM HEPES buffer, pH 7.5, containing 1mM EDTA and 0.1% BSA for D3 receptors. Subsequently the slides were incubated for 1 hour at RT in the before mentioned buffers containing 1.0 nM [^{3}H]SCH-23390 (in the presence of 40 nM ketanserin) or 1.0 nM [^{3}H]nemonapride (YM-09151-2) (in the presence of 0.5 μM DTG and 0.1 μM pindolol) for D1-like and D2-like receptors, respectively. For D3 receptor binding slides were incubated for 45 minutes with 0.2 nM [^{125}I]*trans*-7-OH-PIPAT in 50 mM HEPES buffer, pH 7.5, containing 1mM EDTA and 0.1% BSA. Non-specific binding was determined on adjacent sections in the presence of 1 μM cis-flupentixol, 10 μM S(-)-sulpiride or 1 mM dopamine for D1-like, D2-like and D3 receptors, respectively. Subsequently, the slides were washed either twice 5 minutes in ice-cold buffer (D1-like and

D2-like) or four times 2 minutes in ice-cold HEPES buffer containing 100 mM NaCl (D3). Thereafter slides were dried to air and exposed to Kodak Biomax MS film with Kodak Biomax Transcreens (Amersham, UK) for two (D1-like) or three weeks (D2-like) at -80°C. For D3 autoradiography slides were exposed to Kodak Biomax MR film (Amersham, UK) for 3 days.

In situ hybridisation for TH mRNA

For TH mRNA *in situ* hybridisation, 16 µm coronal sections were cut and thaw-mounted on Superfrost slides (Menzel, Germany). An 1151-bp TH cDNA fragment spanning nucleotides 14 – 1165 was subcloned into a PBS +/- vector (Promega, Leiden, NL). An antisense RNA probe was generated by *in vitro* transcription with 554 ng of linearised template DNA, 20 µCi [^{33}P]-UTP and 40 units T3 RNA polymerase. The sections were post-fixed in a 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.4) for 10 minutes at RT, washed twice in PBS for 5 minutes at RT and treated with 0.25% acetic anhydride in tri-ethanolamine (0.1 M, pH 8.0) for 10 minutes at RT. The sections were then rinsed in PBS for 5 minutes and in 0.83% NaCl for 5 minutes at RT. Subsequently the sections were dehydrated by immersing them in solutions with increasing concentrations of ethanol (50%, 70%, 80% and 100%) and dried to air. Hybridisation was performed in 50% deionised formamide, 10% dextran sulphate, 2×SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1x Denhardt's solution, 5 mM EDTA (pH 8.0), 10 mM phosphate buffer (pH 8.0) and 12.5 mg/ml tRNA, containing 0.8×106 cpm of the probe. The hybridisation mix was heated at 65°C for 5 minutes, transferred to ice and DTT was added to a final concentration of 2.5 M. Hybridisation was performed overnight at 55°C in a moist chamber, 100 µl hybridisation mix per slide. Coverslips were removed in 5×SSC at RT and the slides were briefly dipped in 2×SSC at RT, treated with RNAse A (2 mg/100 ml in 5 M NaCl, 1 M Tris, pH 8.0) for 15 minutes at 37°C and washed for 15 minutes at 37°C in 2×SSC. The slides were then washed twice in 2×SSC/50% formamide for 15 minutes at 60°C, twice in 2×SSC for 15 minutes at RT, dehydrated in graded ethanol concentrations and dried to air. Slides were apposed to Kodak Biomax MR films (Amersham, UK) for 11 days.

Image Analysis

Quantitative analysis of receptor binding and TH mRNA was performed using an MCID image analyser (Interfocus, Suffolk, UK). Specific binding was determined through subtraction of non-specific binding from total binding with the overlay-function (MCID analysis software). Binding or TH mRNA levels were determined by freehand drawing of the regions of interest according to the mouse brain atlas of Paxinos and Franklin (2001), *i.e.* striatum (D1-like, D2-like and D3; 1.10 mm from bregma), nucleus accumbens core and shell (D1-like, D2-like and D3; 1.10 mm from bregma) and VTA (D1-like and TH mRNA; -3.52

mm from bregma). Fmol/mg tissue equivalents of specific binding were determined from [^{3}H] or [^{125}I] microscales (Amersham, UK), which were laid down with each film. TH mRNA levels were expressed in counts per minute (cpm) as calculated from a standard curve of diluted hybridisation mix, which was laid down with the film. For each animal 2-3 measurements were made for each hemisphere. Since no significant differences were found between hemispheres, the data were pooled. Each measure thus represents a mean of 4-6 measurements per animal. N = 5-7, representing the number of animals per group.

Behavioural experiments

(1) Locomotor activity in an open field

The open field was a clear Plexiglas cylinder of 20 cm in diameter and 30 cm in height. The mice (8-10 per treatment or genotype) were placed in the cylinder and locomotor activity was measured for one hour. Using a camera-linked computerised tracking system with Ethovision Color-Pro 2.3 software (Noldus Information Technology, Wageningen, NL) the distance moved in the open field was measured in 5 minutes intervals.

(2) Climbing

The climbing cage consisted of wire-mesh netting walls and ceiling (mesh size 1.3 × 1.3 cm). The mice were placed in a climbing cage for 30 minutes. During this period the behaviour in the climbing cage was recorded on videotapes for subsequent analysis. The experimenter, blind to genotype or treatment, scored the time the mice spent climbing during the 30 minutes test using Observer Video-Pro 4.1 software (Noldus Information Technology, Wageningen, NL). Climbing was defined as 'all four paws holding on to the wire mesh'. N = 8 mice per treatment or genotype.

Statistical analysis

For statistical analyses SPSS10.1 was used. Data for dopamine D1-like, D2-like, D3-receptor binding were analysed separately by two-way ANOVA with area and genotype or NTX/placebo treatment as factors. When appropriate post-hoc Student's *t*-tests were performed. TH mRNA expression data was analysed by Student's *t*-tests with genotype or treatment as factor. Locomotor activity in the open field is expressed in distance moved in 5 minutes intervals. One-way ANOVA with repeated measurements was used for analysis of the open field data with genotype or NTX/placebo treatment as factor. Climbing was compared between groups with Student's *t* tests. Data are expressed as mean ± SEM. Statistical significance was accepted at $P < 0.05$.

RESULTS

In vitro autoradiography for dopamine receptors

Dopamine receptor binding for μ-opioid receptor knockout mice and chronic NTX treated mice are summarised in Tables 1 and 2, respectively.

Total binding of the D1-like antagonist [^{3}H]SCH23390 was not different between μ-opioid receptor knockout and wild-type mice ($F(1,47)=2.3$, $P = 0.14$). Specific binding to D1-like receptors was different between genotypes ($F(1,47)=8.8$, $P < 0.01$), although the change in specific D1-like binding was small (+8% relative to wild-type). Non-specific binding of [^{3}H]SCH23390, in presence of an excess of the non-radioactive D1-agonist cis-flupentixol, was also different between genotypes (-15% relative to wild-type, $F(1,47)=16.7$, $P < 0.001$).

No differences between wild-type and μ-opioid receptor knockout mice were observed for total binding of [^{3}H]nemonapride ($F(1,35)=0.1$, $P = 0.82$), specific D2 binding ($F(1,35)=0.0$, $P = 0.91$) or non-specific binding of [^{3}H]nemonapride ($F(1,35)=0.02$, $P = 0.89$).

Total binding of [^{125}I]*trans*-7-OH-PIPAT, a selective D3 ligand, was reduced in tissue of μ-opioid receptor knockout mice as compared to wild-type mice ($F(1,29)=7.6$, $P < 0.05$). Similarly, specific binding to dopamine D3 receptors was also significantly different between the genotypes ($F(1,29)=16.4$, $P < 0.001$) without regional differences as apparent from the lack of genotype×region interaction. The mean percentage change in specific binding to dopamine D3 receptors in μ-opioid receptor knockout mice relative to wild-type was –34% (Figure 1). Further, μ-opioid receptor knockout mice displayed increased non-specific binding of [^{125}I]*trans*-7-OH-PIPAT in presence of unlabelled dopamine (+13% compared to wild-type, $F(1,29)=5.6$, $P < 0.05$).

Chronic NTX treatment did not affect total binding of [^{3}H]SCH23390 ($F(1,46)=0.002$, $P = 0.96$) 24 hrs after removal of the pellet. Specific D1-like receptor binding was decreased in NTX treated mice as compared to placebo controls ($F(1,46)=17.2$, $P < 0.001$), although the overall effect of NTX treatment was small (-11.5%). There was variation between regions in the effects of chronic NTX upon specific binding of [^{3}H]SCH23390 to D1 receptors as apparent from a significant treatment×region interaction ($F(3,46)=3.0$, $P < 0.05$). Post-hoc analysis indicated that D1-like receptors were decreased in core and shell of the nucleus accumbens ($P = 0.01$ and $P < 0.05$, respectively) and in striatum ($P < 0.05$), but not in the VTA ($P=0.78$). Non-specific binding was also different between treatment groups (+47% compared to wild-type, $F(1,46)=11.1$, $P < 0.01$).

24 Hours after removal of the pellet, chronic NTX treated mice were not different from placebo controls in total [^{3}H]nemonapride binding ($F(1,35)=0.01$, $P = 0.94$), specific binding

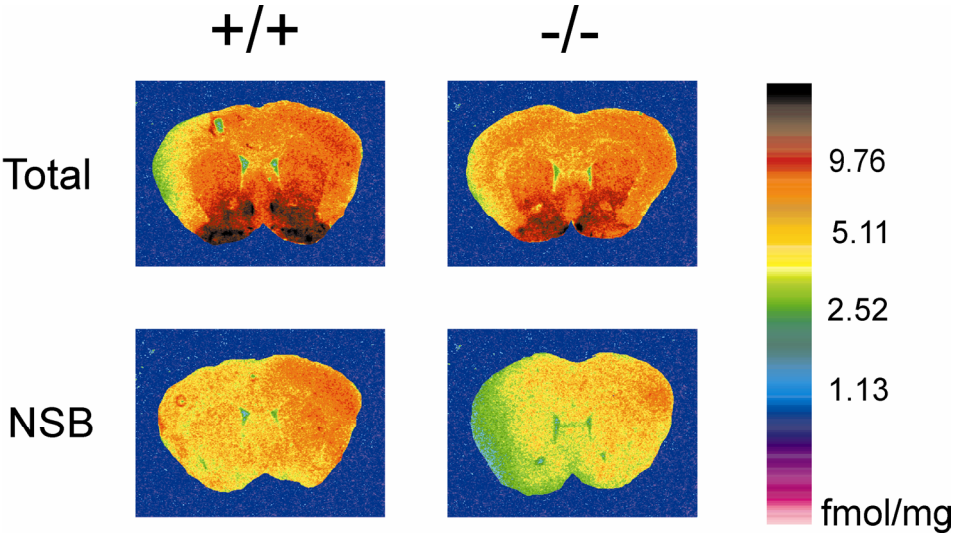

FIGURE 1

Computer-enhanced colour autoradiograms of dopamine D3 receptor binding in coronal sections from wild-type (+/+) and μ-opioid receptor knockout (-/-) mice. Images for +/+ and -/- groups were taken from the same film. Dopamine D3 receptor binding was determined by labelling coronal sections with the [^{125}I]7-OH-PIPAT (Total binding) and non-specific binding was determined by co-administration of 1 mM dopamine with [^{125}]7-OH-PIPAT. The colour bars show pseudo-colour interpretation of relative density of black and white film image calibrated in fmol/mg tissue.

to D2-like receptor sites ($F(1,35)=0.3$, $P = 0.60$) and non-specific binding of [^{3}H]nemonapride ($F(1,35)=0.27$, $P = 0.61$).

NTX treatment did not affect total binding of the D3 receptor ligand [^{125}I]*trans*-7-OH-PIPAT ($F(1,35)=0.37$, $P = 0.55$). Overall analysis did not reveal differences between chronic NTX and placebo treated mice in specific D3 receptor binding ($F(1,32)=2.0$, $P = 0.17$). Non-specific binding of [^{125}I]*trans*-7-OH-PIPAT was slightly increased in mice exposed to NTX ($F(1,35)=6.0$, $P < 0.05$).

In situ hybridisation for TH mRNA

TH mRNA expression levels for μ-opioid receptor knockout mice and chronic NTX treated mice and the appropriate controls are shown in Tables 1 and 2, respectively. There was no difference in TH mRNA in the VTA between genotypes ($P = 0.88$) nor did chronic NTX treatment affect TH mRNA expression ($P = 0.63$).

	Region	mm to bregma	+/+	-/-	% change	
D1-like	nucleus accumbens					
	core	1.10	212.1 ± 4.9	221.0 ± 9.7	3.8	
	shell	1.10	213.0 ± 3.6	221.1 ± 6.6	3.8	
	striatum	1.10	217.7 ± 5.9	242.1 ± 7.3	11.2	
	ventral tegmental area	-3.52	63.3 ± 4.9	71.4 ± 7.0	12.8	
D2-like	nucleus accumbens					
	core	1.10	55.2 ± 3.1	50.1 ± 3.7	-9.3	
	shell	1.10	59.2 ± 3.3	56.3 ± 3.5	-4.9	
	striatum	1.10	85.3 ± 4.1	94.6 ± 10.3	10.9	
D3	nucleus accumbens					
	core	1.10	8.1 ± 0.7	5.7 ± 0.7	-29.6	*
	shell	1.10	9.4 ± 0.7	7.4 ± 0.9	-21.3	
	striatum	1.10	3.8 ± 0.5	1.9 ± 0.3	-50.0	
TH mRNA	ventral tegmental area	-3.52	67.9 ± 6.4	69.2 ± 4.2	1.9	

TABLE 1

Specific binding to dopamine D1-like, D2-like and D3 receptors (fmol/mg) and TH mRNA (cpm) in wild-type (+/+) and homozygous μ-opioid receptor knockout mice (-/-). Data represent mean levels ± SEM per brain area (N = 5-7 per genotype). The mean percentage change (%) represents the change in specific binding in μ-opioid receptor knockout (-/-) tissue as compared to wild-type sections.

* $P < 0.001$, significant different from wild-type mice.

Genotype differences in non-specific binding of [^{3}H]SCH23390 caused false group differences in calculated specific D1-like receptor binding, which are therefore not highlighted here (see Discussion).

	Region	mm to bregma	placebo	NTX	% change
D1-like	nucleus accumbens				
	core	1.10	122.3 ± 3.9	106.8 ± 3.6	-12.7
	shell	1.10	131.9 ± 3.5	119.6 ± 4.0	-9.3
	striatum	1.10	136.1 ± 4.6	113.3 ± 5.7	-16.8
	ventral tegmental area	-3.52	32.9 ± 3.8	34.8 ± 6.1	5.8
D2-like	nucleus accumbens				
	core	1.10	99.6 ± 4.0	101.6 ± 3.9	2.0
	shell	1.10	102.8 ± 3.1	105.2 ± 3.7	2.3
	striatum	1.10	126.0 ± 8.0	127.7 ± 6.3	1.3
D3	nucleus accumbens				
	core	1.10	7.5 ± 0.3	6.6 ± 0.9	-12.0
	shell	1.10	8.5 ± 0.6	8.7 ± 0.8	2.4
	striatum	1.10	3.6 ± 0.6	2.3 ± 0.5	-44.4
TH mRNA	ventral tegmental area	-3.52	34.6 ± 2.8	36.2 ± 2.3	4.6

TABLE 2

Specific binding to dopamine D1-like, D2-like and D3 receptors (fmol/mg) and TH mRNA (cpm) in placebo and naltrexone (NTX) treated mice 24 hrs after pellet removal. Data represent mean levels ± SEM per brain area (N = 6-7 per group). The mean percentage change (%) represents the change in specific binding in tissue of naltrexone (NTX) treated mice as compared to placebo treated sections.
Treatment differences in non-specific binding of [^{3}H]SCH23390 caused false group differences in calculated specific D1-like receptor binding, which are therefore not highlighted here (see Discussion).

Behavioural experiments

(1) Locomotor activity in an open field

The locomotor activity of μ-opioid receptor knockout mice (Figure 2A) and chronic NTX treated mice 24 and 48 hrs after removal of the pellet (Figures 2B and 2C, respectively) was determined in an open field.

There was an overall effect of time on locomotor activity of μ-opioid receptor knockout and wild-type mice in the open field ($F(11,176)=13$, $P < 0.001$), indicative of adaptation of the mice to the open field. Further statistical analysis of the data revealed an overall effect of genotype ($F(1,16)=6.8$, $P < 0.05$), *i.e.* the μ-opioid receptor knockout mice were less active relative to the wild-type mice. There was no significant time×genotype interaction.

During the 60 minutes in the open field the chronic NTX and placebo mice 24 hrs after pellet removal adapted to the open field (effect of time $F(11,154)=12$, $P < 0.001$). Further, the analysis revealed a significant time×treatment interaction ($F(11,154)=2.0$, $P < 0.05$) indicative of effects of chronic NTX exposure upon the activity of the mice in the open field. More specifically, NTX treated mice were more active than placebo controls during intervals 35-40, 40-45 and 50-55 minutes (Student's *t*-tests, $P < 0.05$). In contrast, 48 hours after removal of the pellets chronic NTX treated mice were not different from their placebo controls in open field behaviour. There was a significant effect of time ($F(11,198)=19$, $P < 0.001$), but there was no overall effect of chronic NTX treatment nor a time×treatment interaction.

(2) Climbing

The percentage time the μ-opioid receptor knockout mice and chronic NTX treated mice spent climbing was determined during 30 minutes in metal wire climbing cages. The climbing behaviour thus determined for μ-opioid receptor knockout mice, chronic NTX treated mice (24 and 48 hrs post-removal) and the respective controls is depicted in Figures 3A, 3B and 3C, respectively.

μ-Opioid receptor knockout mice and wild-type mice were not different in the time they spent climbing during the 30 minutes in the climbing cage ($F(1,15)=0.001$, $P = 0.98$).

Climbing behaviour was not affected by chronic NTX treatment either. NTX and placebo treated mice were comparable in the percentage time they spent climbing 24 hrs ($F(1,15)=0.3$, $P = 0.60$) and 48 hrs after removal of the pellet ($F(1,15)=0.5$, $P = 0.49$).

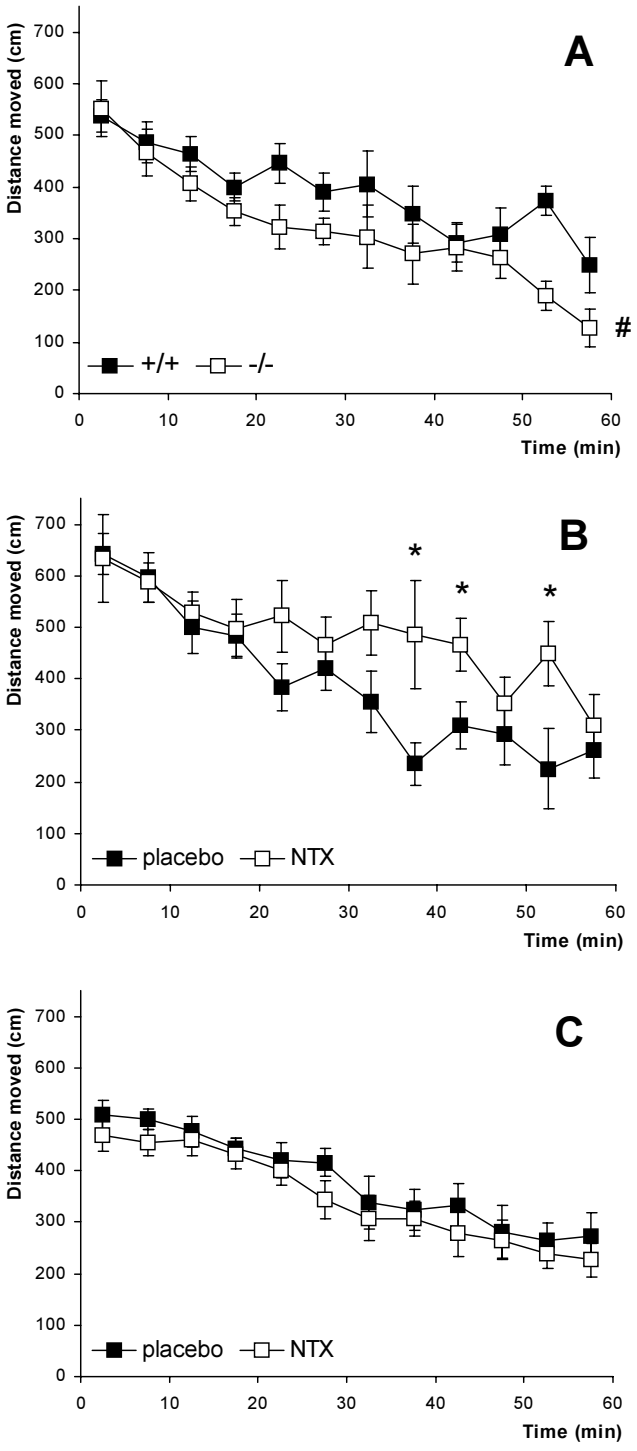

FIGURE 2

Locomotor activity of μ-opioid receptor knockout and chronic NTX treated mice in an unfamiliar open field. The locomotor activity during a 60 minutes trial is shown for (A) μ-opioid receptor knockout mice (-/-) and wild-type (+/+) littermates, (B) chronic NTX and placebo treated mice 24 hours after pellet removal and (C) chronic NTX and placebo treated mice 48 hours after surgical removal of the pellets. Data are expressed as mean ± SEM distance moved (cm) in the open field in 5 minutes intervals (N = 8-10).

# $P < 0.05$, significant from +/+ mice, overall genotype effect; * $P < 0.05$, significant from placebo treated controls.

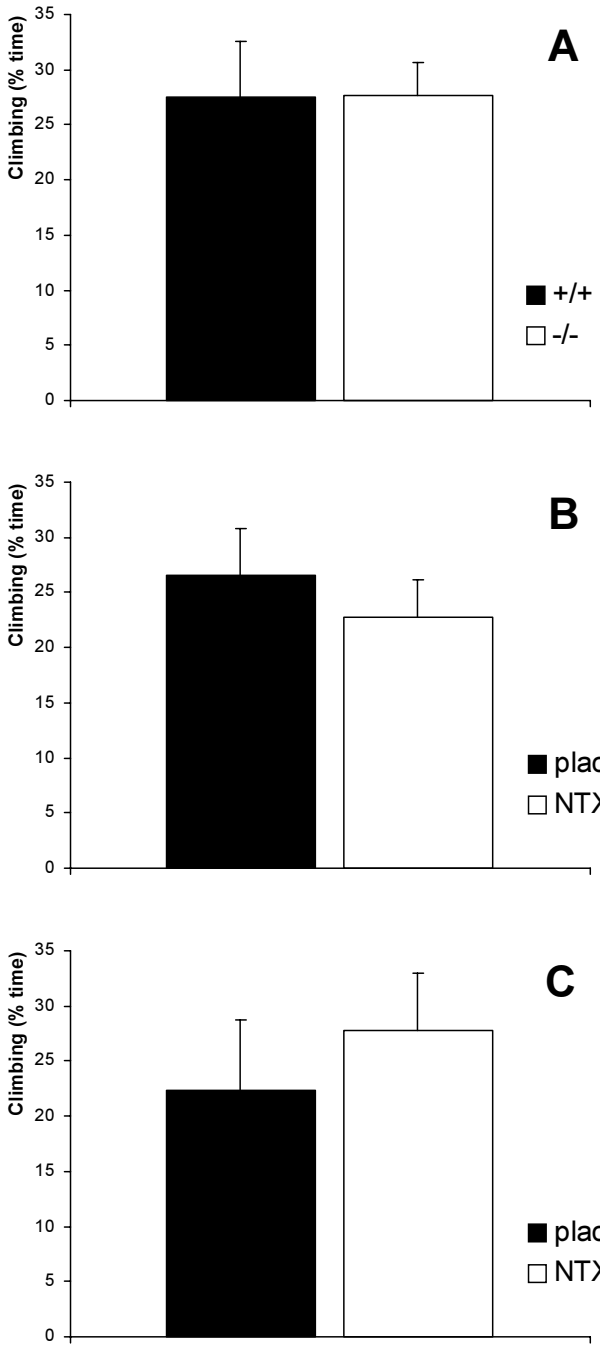

FIGURE 3
Climbing behaviour of μ-opioid receptor knockout and chronic NTX treated mice. The percentage of the time the mice spent climbing during the 30 minutes trial is shown for (A) μ-opioid receptor knockout mice (-/-) and wild-type (+/+) littermates, (B) chronic NTX and placebo treated mice 24 hours after pellet removal and (C) chronic NTX and placebo treated mice 48 hours after surgical removal of the pellets. Data are expressed as mean ± SEM percentage (%) of the time spent climbing (N = 8).

DISCUSSION

Endogenous opioid systems, and particularly μ-opioid receptors, have been implicated in drug reinforcement (Herz, 1997; Van Ree et al., 1999). Interactions between opioid and dopamine systems may contribute to opioid modulation of drug reinforcement. We hypothesised, based on anatomical and functional evidence for opioid-dopamine interactions, that alterations in opioid receptor expression might lead to concurrent changes in dopamine parameters which may in turn contribute to altered drug reinforcement. This was explored using two models with distinct opioid receptor levels and reinforcement phenotypes, *i.e.* μ-opioid receptor knockout mice, which are characterised by impaired drug reinforcement and chronic NTX treatment, which is known to enhance drug reinforcement. The latter model is further characterised by transient over-expression of μ- and to a lesser extent also δ- and κ-opioid receptors (80%, 39% and 11% increase, respectively, (Lesscher et al., 2003a)) and hence is a valuable tool in this study. Decreased dopamine D3 receptor binding in tissue of μ-opioid receptor knockout mice in addition to reduced locomotor activity of these mice and increased activity in the open field 24 hrs after cessation of chronic NTX treatment indeed suggest that alterations, although subtle, in dopamine systems emerge in case of absence or over-expression of (μ-)opioid receptors.

Dopamine receptor binding

μ-Opioid receptor knockout mice displayed decreased dopamine D3 receptor binding in nucleus accumbens and striatum. This is an interesting finding because dopamine D3 receptors are potential targets in treatment of drug addiction (Le Foll et al., 2000). Both the partial D3 receptor agonist BP897 and the selective D3 receptor antagonist SB-277011-A reduced cue-controlled cocaine seeking (Pilla et al., 1999; Di Ciano et al., 2003). Also, BP897 and SB-277011-A reduced nicotine and amphetamine cue-conditioned hyperactivity (Vorel et al., 2002; Aujla et al., 2002; Le Foll et al., 2002; Le Foll et al., 2003). BP897 and SB-277011-A did not affect responding for cocaine on an FR1 schedule of reinforcement, suggesting that D3 receptors are in fact not involved in drug reinforcement (Pilla et al., 1999; Di Ciano et al., 2003). Taken these facts into account, it is unlikely that the reduction in dopamine D3 receptors in nucleus accumbens and striatum of μ-opioid receptor knockout mice as described here contributes to their reduced sensitivity to drug reinforcement as compared to wild-type mice (Matthes et al., 1996; Becker et al., 2000; Roberts et al., 2000; Hall et al., 2001; Ghozland et al., 2002; Becker et al., 2002; Contarino et al., 2002; Berrendero et al., 2002). Reduced dopamine D3 receptor binding in μ-opioid receptor knockout mice might contribute to a drug seeking phenotype of μ-opioid receptor deficient mice, which is yet to be investigated. No overall change in dopamine D3 receptor binding was apparent for the chronic NTX treated mice.

The present autoradiography data further revealed small differences in calculated specific dopamine D1 receptor binding between genotypes and treatment groups. However, total binding of [^{3}H]SCH23390 did not reflect this difference in specific binding. Rather, non-specific binding of [^{3}H]SCH23390 in presence of an excess of cis-flupentixol was different between groups and caused apparent changes in calculated specific binding to dopamine D1-like receptors. Clearly, these data do not point to altered dopamine D1-like receptor expression in chronic NTX treated mice nor in μ-opioid receptor knockout mice as compared to their respective controls, but rather point to changes in binding of [^{3}H]SCH23390 to as yet undetermined non-D1 sites. Increased dopamine D1 and D2 receptor mRNA expression has been described for μ-opioid receptor knockout mice (Park et al., 2001). The lack of effect of μ-opioid receptor deficiency upon dopamine D1- and D2-like receptor binding suggests that the altered mRNA levels as reported by Park et al. may not reflect protein levels.

Open field and climbing behaviour

Interestingly, opposing effects of μ-opioid receptor gene knockout and chronic NTX treatment were observed upon locomotor activity in an unfamiliar open field, but no differences were found for climbing behaviour. Compared to wild-type mice, μ-opioid receptor knockout mice were hypoactive upon exposure to the novel environment, which is in agreement with previous studies (Matthes et al., 1996; Tian et al., 1997; Hall et al., 2003a). In contrast, chronic NTX treated mice were more active as compared to placebo treated controls during the hour in the unfamiliar open field 24 hrs after removal of the NTX pellet. Important to note, chronic NTX treated mice were no longer different from placebo treated controls at 48 hrs of withdrawal from NTX. The transient character of chronic NTX effects upon locomotor activity may be related to the relatively short-lived changes in opioid receptor levels after withdrawal from NTX, returning to placebo-like levels after 6 days withdrawal from NTX exposure (Tempel et al., 1982). The respective reduction and increase in locomotor activity of μ-opioid receptor knockout mice and chronic NTX treated mice may reflect reduced and increased (novelty-induced) dopamine levels.

Opioid-dopamine interactions

The adaptations in the dopamine system described here for mice, which either lack μ-opioid receptors from gestation or are characterised by transient over-expression of (μ-)opioid receptors, confirm opioid control over dopamine systems.

With regard to the μ-opioid receptor knockout mice, both a decrease in novelty-induced locomotor activity and a reduction in dopamine D3 receptor binding were noted. The exact mechanisms underlying these adaptations are not known, but reduced dopamine levels are likely involved. Indeed, dopamine depletions in accumbens and striatum after 6-OHDA lesions of the medial forebrain bundle have been associated with reduced locomotor activity (Sundstrom et al., 1990) and with reductions in D3 receptor binding and mRNA (Wolterink et

al., 1990; Vos et al., 1995; Levesque et al., 1995; Guillin et al., 2001). Moreover, activation of μ-opioid receptors causes hyperpolarization of GABA containing neurons in the VTA, thereby relieving inhibitory input to dopaminergic projection neurons (Johnson & North, 1992a). In absence of μ-opioid receptors, such disinhibition can not occur and therefore increased inhibitory input to dopamine neurons and concurrent reductions in dopamine levels in the nucleus accumbens and striatum might be expected for μ-opioid receptor knockout mice. However, microdialysis revealed no change in basal extracellular dopamine levels in the nucleus accumbens of μ-opioid receptor knockout as compared to wild-type mice (Tang et al., 2002) nor did we find changes in TH mRNA expression in these mice. Differences between genotypes in novelty-induced dopamine levels can however not be ruled out.

Hyperactivity of chronic NTX treated mice, 24 hrs after withdrawal from NTX, might involve enhanced (novelty-induced) dopamine levels in nucleus accumbens and/or striatum. However, previous studies have shown that basal dopamine levels, dopamine synthesis or dopamine metabolite levels were not different between chronic NTX and placebo treated rats 24 hrs after withdrawal from NTX or placebo treatment (Bardo et al., 1988; Ahtee et al., 1990).

In conclusion, the present findings confirm opioid control over dopamine systems. It is important to take into account the interaction between opioid and dopamine systems considering the role of both systems in mediating the effects of drugs of abuse. Further, the reduction in dopamine D3 receptors may suggest a drug seeking phenotype of μ-opioid receptor knockout mice, which is yet to be investigated.

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CHAPTER 7

RECEPTOR SELECTIVE CHANGES IN μ-, δ- AND κ-OPIOID RECEPTORS AFTER CHRONIC NALTREXONE TREATMENT IN MICE

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ABSTRACT

Chronic treatment with the opioid antagonist naltrexone (NTX) induces functional supersensitivity to opioid agonists, which may be explained by receptor upregulation induced by opioid receptor blockade. In the present study the levels of opioid receptor subtypes through the brain of mice were determined after chronic NTX treatment using quantitative *in vitro* autoradiography. This is the first complete mapping study in mice for μ-, δ- and κ-opioid receptors after chronic NTX exposure. Treatment with naltrexone clearly induced upregulation of μ- (mean 80%) and, to a lesser extent, of δ-opioid receptors (mean 39%). The upregulation of μ- and δ-opioid receptors was evident throughout the brain, although there was variation in the percentage change across brain regions. In contrast, consistent upregulation of κ-opioid receptors was observed in cortical structures only and was not so marked as for μ- and δ-opioid receptors. In non-cortical regions κ-opioid receptor expression was unchanged. Taken together, the present findings suggest opioid receptor subtype selective regulation by chronic NTX treatment in mice.

INTRODUCTION

Chronic exposure to opioid antagonists is known to result in supersensitivity to opioid agonists. For example, chronic treatment with naltrexone (NTX) has been shown to enhance the analgesic, lethal and respiratory depressant potency of opioids (Tempel et al., 1985; Yoburn et al., 1986b; Yoburn et al., 1995; Diaz et al., 2002). Further, chronic treatment with NTX alters reward sensitivity in rats. Morphine-induced place preference and the acquisition of cocaine self-administration were enhanced in rats pre-treated with NTX (Bardo & Neisewander, 1987; Ramsey & Van Ree, 1990). In addition, increased heroin-induced facilitation of intracranial self-stimulation has been observed after chronic NTX treatment (Schenk & Nawiesniak, 1985).

Several studies have shown upregulation of opioid receptors, particularly of the μ- but also the δ-subtype, after chronic NTX treatment using whole brain homogenates (Tempel et al., 1985; Yoburn et al., 1986b; Yoburn et al., 1988; Yoburn et al., 1989; Cote et al., 1993; Unterwald et al., 1995; Yoburn et al., 1995; Castelli et al., 1997; Duttaroy et al., 1999). Zukin et al. (1982) described increased [^{3}H]etorphine binding in homogenates of limbic structures. Autoradiography revealed highest increases in opioid receptor binding in the hypothalamus, hippocampus, substantia nigra, amygdala and basal ganglia (Tempel et al., 1984; Morris et al., 1988; Unterwald et al., 1998; Diaz et al., 2002).

The upregulation of opioid receptors in the brain may underlie the effects induced by chronic opioid antagonist treatment. NTX treatment is one of the pharmacotherapies to treat heroin or alcohol addicts (Kreek et al., 2002). It is therefore of major interest to learn about the effects of chronic NTX treatment upon opioid receptors, which may have implications for drug dependent patients after cessation of NTX treatment (Yoburn et al., 1986b). Moreover, chronic NTX treatment may be used as a model to gain insight into the role of opioid receptors in various processes. For this purpose, however, a detailed description of opioid receptor upregulation throughout the brain is required. Previous autoradiographic studies either described opioid receptor binding for a limited number of regions or dealt with μ- and not δ- and κ-opioid receptor binding. In addition, all autoradiographic studies dealing with opioid receptor regulation by chronic NTX treatment have used rats. Taken together a detailed account of the effects of chronic exposure to NTX upon μ-, δ- and κ-opioid receptor binding across the brain is lacking, especially for mice. Data regarding the effects of NTX in mice is essential, particularly in light of extensive new data on opioid systems from gene knockout mice (Kieffer & Gaveriaux-Ruff, 2002). The aim of the present study was therefore to determine in mice μ-, δ- and κ-opioid receptor levels in different regions in the brain after chronic naltrexone treatment. Quantitative autoradiography was applied using radioligands selective for the μ-, δ- or κ-opioid receptors.

MATERIALS AND METHODS

Animals and treatment

Male C57/Bl6 mice, 2 months of age, were obtained from Charles River (L'Arbresle, France) and were housed in groups of 4 mice in extended Macrolon© type I cages with water and food pellets available ad libitum. Environmental conditions were controlled (22°C and 50% humidity; lights on at 7:00 h and lights off at 19:00 h). After transport the mice were allowed to acclimatise for at least one week before the experiment. The experimental procedures were approved by the Ethical Committee for Animal Experiments of the University Medical Center Utrecht.

Pellets containing 15 mg naltrexone or corresponding placebo pellets were implanted subcutaneously in the nape of the neck under halothane anaesthesia (5% / 95% O_2) (day 1). Naltrexone and placebo treated mice were housed together (2 of both per cage). On day 8 the pellet was removed (5% halothane / 95% O_2). 24 Hours after pellet removal the mice were killed by cervical dislocation. Brains were rapidly dissected, frozen in crushed dry ice and stored at –80°C until use.

Autoradiographic procedures

The autoradiographic procedures used have been described previously (Kitchen et al., 1997). Briefly, 20 µm coronal sections were cut 300 µm apart using a cryostat (Leica, Rijswijk, The Netherlands) and thaw-mounted on gelatin-subbed slides. The tissue was left to dry at –20°C in air-tight boxes containing $CaSO_4$ until further processing.

Tissue was pre-incubated for 30 minutes in 50 mM Tris-HCl pH 7.4 containing 0.9 % NaCl. Thereafter the sections were incubated in 50 mM Tris-HCl pH 7.4 containing either 5 nM [^{3}H]DAMGO (µ-opioid receptors), 8 nM [^{3}H]deltorphin-1 (δ-opioid receptors) or 2.5 nM [^{3}H]CI-977 (κ-opioid receptors). Ligand concentrations approximated 3-4 × K_D. Non-specific binding was determined on adjacent sections in presence of 1 µM naloxone (µ- and κ-opioid receptors) or 10 µM naloxone (δ-opioid receptors). After incubation the slides were rinsed three times in ice-cold 50 mM Tris-HCl pH 7.4 and subsequently dried to cold air.

Slides were apposed to [^{3}H]Hyperfilm (Amersham, UK) for three, four or six weeks for µ-, δ- and κ-opioid receptors, respectively. Slides of both naltrexone and placebo treatment groups were apposed to the same film (2 mice/group/film). Films were developed using Kodak D19 developer and fixed using Kodak Rapid Fixer (Sigma, UK).

Image Analysis

Quantitative analysis of receptor binding was carried out by video-based computerised densitometry using an MCID image analyser (Imaging Research, Canada). Specific binding

was determined by subtraction of the non-specific binding from the total binding, using the overlay function. Specific binding is expressed in fmol/mg tissue, as derived from calibrated [^{3}H] microscale standards (Amersham, UK) laid down with each film. For each region, quantified measures were taken from both hemispheres. Measures of binding therefore represent a duplicate determination for each brain region. Cortical areas, olfactory tubercle and hippocampus were analysed by sampling 5-8 times with a box tool. All other regions were analysed by freehand drawing of anatomical areas. Structures were identified according to the mouse brain atlas of Paxinos and Franklin (2001). The N value refers to the number of mice per treatment group.

Statistical Analysis

For statistical analysis of the data SPSS 10.1 was used. The data for the three opioid receptor subtypes were analysed separately. Since binding was determined for both hemispheres, paired-sampled Student's *t* tests were performed to determine hemisphere effects. Because there were no significant differences between hemispheres, the data for left and right measurements were pooled. The mean values per area per animal were used in further analyses. An overall analysis (two-way ANOVA) was performed with treatment and area as factors. In addition, separate analyses were carried out for cortical regions, limbic structures and regions involved in movement or in pain/sensory functions. Data are represented as mean ± SEM; significance was accepted at $P < 0.05$.

RESULTS

The mean values of the quantitative μ-, δ- and κ-opioid receptor binding for placebo and chronic naltrexone treated mice are summarised in Tables 1, 2 and 3 respectively. To enable comparison of opioid receptor regulation between neuroanatomical and functional distinct systems, the data are categorised by cortical regions, limbic structures, regions involved in movement or in pain/sensory functions and regions such as hypothalamus and thalamus (for details see Tables 1, 2 and 3). Representative images for μ-, δ- and κ-opioid receptor autoradiography are shown in Figure 1. Non-specific binding was low and did not differ between placebo and naltrexone treated mice.

μ-Opioid receptor autoradiography: specific [^{3}H]DAMGO binding

Quantitative analysis of μ-opioid receptors revealed upregulation of μ-opioid receptors after chronic NTX treatment (Table 1). The mean percentage change of [^{3}H]DAMGO binding across all regions was 80%; changes in μ-opioid receptor levels per region ranged from 22 to 308%. There was a significant overall effect of chronic NTX treatment upon μ-opioid receptor densities ($P < 0.001$). Further, two-way ANOVA revealed a significant treatment by area interaction ($P < 0.001$), indicative of interregional differences in upregulation.

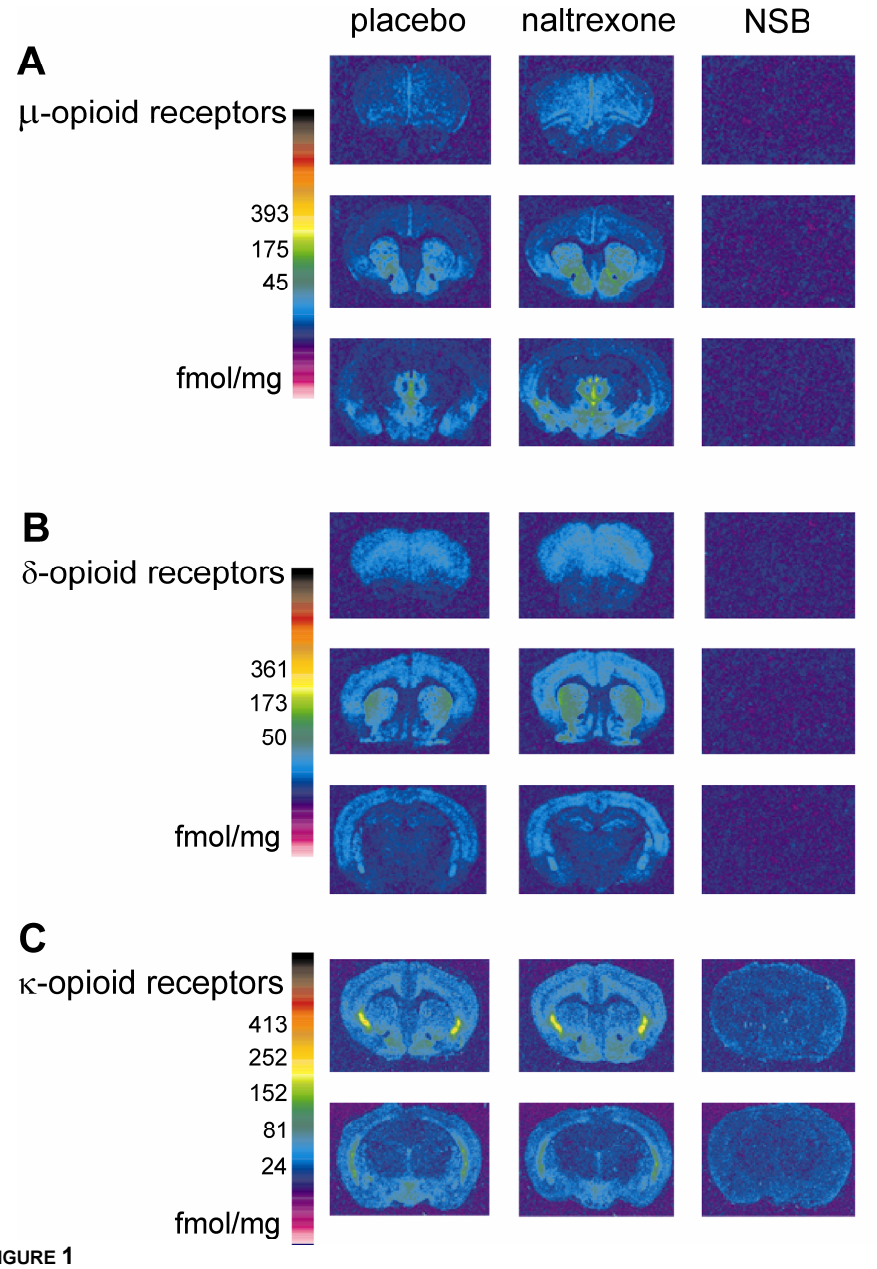

FIGURE 1

Computer-enhanced colour autoradiograms of coronal sections from placebo and NTX treated mice. Images for placebo and NTX treatment groups were taken from the same film. Panel (A) represents μ-opioid receptor binding of [^{3}H]DAMGO. In panel (B) images are shown of δ-opioid receptors, labelled with [^{3}H]deltorphin-1. Panel (C) represents images for κ-opioid receptor binding with [^{3}H]CI-977. The colour bars show pseudo-colour interpretation of relative density of black and white film image calibrated in fmol/mg tissue. The autoradiograms for non-specific binding (NSB) are taken from placebo treated mice; non-specific binding was very low and was not different between placebo and NTX treated groups.

Separate analyses for distinct neuroanatomical and functional systems revealed significant increases in μ-opioid receptor expression in cortical regions (102.1%, $P < 0.001$), limbic structures (64.5%, $P < 0.001$), regions involved in motor activity (58.6%, $P < 0.01$) and in pain/sensory related structures (62.5%, $P < 0.001$). The data suggest an overall upregulation of μ-opioid receptors after chronic NTX treatment, which was highest in cortical regions and was less pronounced in a small number of regions, *e.g.* septum and substantia nigra compacta.

δ-Opioid receptor autoradiography: specific [^{3}H]deltorphin-1 binding

δ-Opioid receptor binding was increased with a mean percentage change of 39% across all regions; the percentage change per area varied from 1 to 109% (Table 2). There was a main effect of chronic NTX exposure upon [^{3}H]deltorphin-1 binding ($P < 0.001$) and a significant treatment by area interaction ($P < 0.001$), indicative of regional differences in δ-opioid receptor upregulation. Further analyses for separate functional systems revealed significant increases of δ-opioid receptors in cortical regions (34.4%, $P < 0.001$), limbic regions (43.2%, $P < 0.001$) and regions involved in motor activity (38.8%, $P < 0.001$) or in pain/sensory functions (39.6%, $P < 0.001$). The data for δ-opioid receptor binding suggest an overall upregulation of δ-opioid receptors after chronic NTX pre-treatment. It should be noted that the upregulation of δ-opioid receptors was less pronounced in some regions, *e.g.* amygdala, medial geniculate nucleus, ventral tegmental area and hypothalamus.

κ-Opioid receptor autoradiography: specific [^{3}H]CI-977 binding

Across all regions the mean percentage change in κ-opioid receptor binding was 11% (Table 3). Statistical analysis of the [^{3}H]CI-977 binding revealed a significant effect of chronic naltrexone treatment ($P < 0.01$) but no significant treatment by area interaction. Further analysis of the data revealed significant increases in κ-opioid receptor expression in cortical regions (24.7%, $P < 0.001$). There was no significant change in κ-opioid receptor binding in limbic regions (2.5%) and regions involved in motor activity (6.5%) or in pain/sensory functions (-4.1%). Thus chronic NTX-induced upregulation of κ-opioid receptors was restricted to cortical regions.

TABLE 1 Quantitative analysis of μ-receptor binding in placebo and chronic NTX treated mice (N = 6)

Region		Bregma	[^{3}H]DAMGO binding (fmol/mg)		Change (%)
			Placebo	Chronic NTX	
Cortical regions					
Motor	Superficial	2.10	10.1 ± 2.8	19.3 ± 3.6	92
	Deep		16.2 ± 2.8	32.2 ± 6.5	98
Prelimbic	Superficial	2.10	47.5 ± 7.2	90.1 ± 12.5	90
	Deep		43.3 ± 4.6	74.8 ± 7.1	73
Infralimbic	Superficial	2.10	53.4 ± 6.0	89.8 ± 8.1	68
	Deep		52.8 ± 5.6	80.3 ± 6.2	52
Orbital	Superficial	2.46	35.1 ± 4.0	77.2 ± 7.0	120
	Deep		33.6 ± 4.9	68.8 ± 4.5	105
Rostral somatosensory	Superficial	1.10	6.2 ± 1.0	18.7 ± 5.3	200
	Deep		14.8 ± 3.5	33.8 ± 3.7	128
Cingulate	Superficial	1.10	26.0 ± 5.1	49.9 ± 7.6	92
	Deep		32.9 ± 4.5	55.2 ± 8.2	68
Caudal somatosensory	Superficial	-1.70	5.9 ± 1.9	16.6 ± 3.0	182
	Deep		10.4 ± 3.2	23.5 ± 3.8	127
Auditory	Superficial	-2.54	16.4 ± 7.9	23.3 ± 1.6	42
	Deep		24.1 ± 5.9	35.3 ± 3.7	46
Visual	Superficial	-2.54	3.3 ± 0.9	13.3 ± 1.3	308
	Deep		11.3 ± 3.1	21.3 ± 2.2	89
Retrosplinial	Superficial	-2.54	16.1 ± 2.0	26.4 ± 1.0	64
	Deep		8.9 ± 1.6	16.7 ± 1.3	89
Entorhinal	Superficial	-3.64	18.7 ± 2.9	31.3 ± 4.5	67
	Deep		30.7 ± 3.6	45.2 ± 3.7	47
Limbic regions					
Olfactory tubercle		1.10	9.3 ± 3.7	22.3 ± 3.7	140
Nucleus accumbens	Core	1.10	160 ± 12.7	250 ± 18.7	57
	Shell		131 ± 11.4	207 ± 12.3	57
Medial Septum		0.74	44.3 ± 3.4	58.2 ± 4.9	31
Lateral Septum		0.74	21.6 ± 4.2	26.4 ± 3.4	22
Ventral Pallidum		0.02	63.9 ± 9.0	121 ± 12.6	89
Stria Terminalis		-0.22	57.0 ± 9.0	92.8 ± 8.9	63
Bed Nucleus of the Stria		-0.22	92.5 ± 7.5	144 ± 11.0	55
Amygdala	Basolateral	-1.46	131 ± 10.1	200 ± 13.0	53
	Basomedial		104 ± 7.3	180 ± 7.9	73
	Medial		100 ± 16.6	154 ± 9.7	53
Hippocampus		-2.54	10.2 ± 1.8	15.8 ± 3.3	55
Dentate Gyrus			9.2 ± 0.9	16.5 ± 2.0	80
Ventral tegmental area		-3.28	52.7 ± 4.4	91.7 ± 2.8	74
Motor regions					
Caudate putamen		1.10	123 ± 16.7	183 ± 25.5	49
Globus Pallidus		-0.22	14.7 ± 1.6	28.8 ± 4.2	95
Substantia Nigra	Compacta	-3.28	45.4 ± 6.2	57.9 ± 3.5	27
	Reticularis		10.7 ± 1.9	17.4 ± 1.6	63
Sensory / pain regions					
Red Nuclei		-3.40	18.2 ± 3.0	33.6 ± 3.6	84
Superficial gray of superior collicus		-3.64	110 ± 8.8	164 ± 8.1	49
Intermediate gray of superior collicus		-3.64	74.9 ± 6.1	118 ± 9.8	57
Medial geniculate nucleus		-3.40	51.9 ± 5.1	85.7 ± 5.9	65
Periaqueductal gray		-3.64	68.5 ± 8.0	107 ± 6.8	56
Habenula		-1.70	250 ± 17.1	334 ± 3.4	34
Thalamus		-1.70	133 ± 10.2	223 ± 12.4	67
Zona Incerta		-1.46	101 ± 7.7	155 ± 7.0	54
Hypothalamus		-1.70	77.4 ± 6.7	137 ± 6.0	78
Ventromedial			90.4 ± 11.7	129 ± 5.2	43
Interpeduncular nucleus		-3.28	327 ± 42.1	465 ± 15.3	42
Pontine nucleus		-3.88	N.D.	N.D.	

N.D. = not detectable

TABLE 2 Quantitative analysis of δ-receptor binding in placebo and chronic NTX treated mice (N = 6)

Region		Bregma	[^{3}H]deltorphin-1 binding (fmol/mg)						Change (%)
			Placebo			Chronic NTX			
Cortical regions									
Motor	Superficial	2.10	69.1	±	4.6	95.4	±	5.0	38
	Deep		74.8	±	6.2	107	±	4.0	42
Prelimbic	Superficial	2.10	101	±	7.3	127	±	1.5	25
	Deep		98.9	±	5.9	133	±	3.0	34
Infralimbic	Superficial	2.10	84.9	±	8.9	112	±	6.9	31
	Deep		80.3	±	4.9	110	±	6.8	37
Orbital	Superficial	2.46	75.9	±	5.8	93.6	±	2.8	23
	Deep		72.0	±	7.0	90.2	±	2.7	25
Rostral somatosensory	Superficial	1.10	75.8	±	3.3	99.6	±	0.6	31
	Deep		75.9	±	3.5	95.0	±	3.3	25
Cingulate	Superficial	1.10	85.4	±	6.9	116	±	7.3	36
	Deep		84.8	±	5.4	117	±	7.8	38
Caudal somatosensory	Superficial	-1.70	67.3	±	3.6	94.1	±	5.8	40
	Deep		61.1	±	4.2	80.9	±	3.9	32
Auditory	Superficial	-2.54	69.8	±	2.6	91.2	±	6.2	31
	Deep		68.0	±	2.0	85.9	±	5.4	26
Visual	Superficial	-2.54	71.3	±	3.7	97.7	±	3.0	37
	Deep		62.2	±	2.4	89.6	±	5.2	44
Retrosplinial	Superficial	-2.54	53.4	±	2.3	72.4	±	3.3	36
	Deep		57.0	±	2.2	79.5	±	4.8	39
Entorhinal	Superficial	-3.64	30.5	±	3.0	39.7	±	3.7	30
	Deep		40.1	±	5.7	61.9	±	4.1	54
Limbic regions									
Olfactory tubercle		1.10	93.3	±	8.8	148	±	5.3	59
Nucleus accumbens	Core	1.10	83.5	±	1.5	121	±	5.1	45
	Shell		84.4	±	7.6	125	±	5.7	48
Medial Septum		0.74	28.6	±	3.7	39.7	±	2.2	39
Lateral Septum		0.74	10.3	±	2.4	19.1	±	2.2	86
Ventral Pallidum		0.02	68.7	±	6.4	97.6	±	7.6	42
Stria Terminalis		-0.22	18.6	±	2.3	22.6	±	2.2	22
Bed Nucleus of the Stria		-0.22	20.1	±	1.6	29.2	±	2.0	45
Amygdala	Basolateral	-1.46	109	±	7.7	130	±	11.0	19
	Basomedial		41.2	±	2.6	61.6	±	7.5	49
	Medial		33.6	±	2.3	38.9	±	2.1	16
Hippocampus		-2.54	34.7	±	3.7	52.1	±	3.1	50
Dentate Gyrus			46.1	±	4.2	77.7	±	5.5	69
Ventral tegmental area		-3.28	14.1	±	2.8	16.3	±	3.6	16
Motor regions									
Caudate putamen		1.10	135	±	5.1	177	±	7.7	31
Globus Pallidus		-0.22	109	±	5.7	152	±	4.3	39
Substantia Nigra	Compacta	-3.28	11.5	±	1.3	15.3	±	1.8	33
	Reticularis		8.4	±	1.7	12.9	±	1.2	53
Sensory / pain regions									
Red Nuclei		-3.40	13.1	±	1.6	17.8	±	0.7	36
Superficial gray of superior colliculus		-3.64	5.2	±	1.1	9.5	±	1.8	82
Intermediate gray of superior colliculus		-3.64	9.6	±	1.8	12.9	±	1.4	34
Medial geniculate nucleus		-3.40	16.4	±	1.7	19.1	±	2.1	16
Periaqueductal gray		-3.64	15.6	±	2.0	20.3	±	1.2	30
Habenula		-1.70	6.8	±	0.7	11.6	±	1.1	71
Thalamus		-1.70	34.6	±	2.0	44.5	±	3.8	29
Zona Incerta		-1.46	25.0	±	2.2	35.2	±	2.4	41
Hypothalamus		-1.70	21.5	±	1.5	25.1	±	2.5	17
Ventromedial			23.4	±	3.8	23.7	±	4.2	1
Interpeduncular nucleus		-3.28	29.3	±	11.2	36.4	±	10.7	24
Pontine nucleus		-3.88	35.2	±	10.4	73.7	±	6.9	109

TABLE 3 Quantitative analysis of κ-receptor binding in placebo and chronic NTX treated mice (N = 6)

Region		Bregma	[^{3}H]CI-977 binding (fmol/mg)						Change (%)
			Placebo			Chronic NTX			
Cortical regions									
Motor	Superficial	2.10	10.8	±	1.3	19.3	±	2.6	79
	Deep		14.5	±	2.5	22.3	±	3.0	53
Prelimbic	Superficial	2.10	19.9	±	4.1	26.8	±	2.1	35
	Deep		31.0	±	5.1	40.9	±	4.3	32
Infralimbic	Superficial	2.10	18.8	±	4.7	26.4	±	3.0	40
	Deep		22.8	±	8.5	26.2	±	3.5	15
Orbital	Superficial	2.46	16.9	±	4.3	24.3	±	2.8	43
	Deep		18.9	±	2.7	21.2	±	1.1	12
Rostral somatosensory	Superficial	1.10	15.2	±	2.9	19.4	±	3.5	28
	Deep		16.1	±	2.0	23.2	±	4.0	44
Cingulate	Superficial	1.10	12.6	±	1.8	17.8	±	3.0	41
	Deep		26.6	±	3.2	34.5	±	3.4	30
Caudal somatosensory	Superficial	-1.70	16.5	±	1.5	20.3	±	4.3	23
	Deep		14.8	±	2.3	20.1	±	2.5	36
Auditory	Superficial	-2.54	16.6	±	0.8	15.8	±	3.9	-5
	Deep		16.2	±	1.9	18.7	±	3.2	16
Visual	Superficial	-2.54	16.6	±	1.9	17.0	±	3.3	3
	Deep		13.2	±	2.5	15.1	±	2.6	15
Retrosplinial	Superficial	-2.54	12.2	±	2.0	10.6	±	2.2	-13
	Deep		12.2	±	2.2	10.2	±	2.7	-16
Entorhinal	Superficial	-3.64	14.1	±	3.8	13.1	±	3.7	-7
	Deep		13.1	±	1.7	18.3	±	3.1	39
Limbic regions									
Olfactory tubercle		1.10	23.3	±	4.2	36.3	±	5.7	56
Nucleus accumbens	Core	1.10	43.8	±	4.8	40.5	±	5.2	-7
	Shell		42.3	±	3.0	42.7	±	4.3	1
Medial Septum		0.74	11.3	±	1.5	9.0	±	2.1	-20
Lateral Septum		0.74	10.3	±	1.4	9.8	±	1.9	-5
Ventral Pallidum		0.02	35.6	±	3.2	39.4	±	4.4	11
Stria Terminalis		-0.22	24.5	±	3.7	22.9	±	2.7	-7
Bed Nucleus of the Stria		-0.22	30.1	±	3.9	34.7	±	2.6	15
Amygdala	Basolateral	-1.46	85.6	±	6.6	84.2	±	8.6	-2
	Basomedial		20.3	±	3.7	15.9	±	4.0	-22
	Medial		68.3	±	6.8	60.5	±	4.4	-12
Hippocampus		-2.54	6.5	±	1.0	8.0	±	1.0	23
Dentate Gyrus			5.8	±	1.6	5.5	±	1.4	-5
Ventral tegmental area		-3.28	34.4	±	4.6	37.4	±	5.1	9
Motor regions									
Caudate putamen		1.10	24.2	±	1.2	24.1	±	2.2	-1
Globus Pallidus		-0.22	12.5	±	1.2	13.9	±	1.1	11
Substantia Nigra	Compacta	-3.28	22.1	±	2.7	23.2	±	2.4	5
	Reticularis		19.6	±	2.6	21.6	±	1.5	11
Sensory / pain regions									
Red Nuclei		-3.40	8.5	±	2.6	10.8	±	2.3	28
Superficial gray of superior colliculus		-3.64	15.4	±	2.7	10.0	±	3.0	-35
Intermediate gray of superior colliculus		-3.64	10.1	±	2.6	9.2	±	3.0	-9
Medial geniculate nucleus		-3.40	7.0	±	2.8	6.7	±	2.7	-5
Periaqueductal gray		-3.64	35.0	±	2.3	35.0	±	1.4	0
Habenula		-1.70	5.1	±	1.2	4.8	±	2.0	-6
Thalamus		-1.70	14.6	±	1.6	16.2	±	2.0	11
Zona Incerta		-1.46	36.3	±	4.2	33.5	±	4.8	-8
Hypothalamus		-1.70	35.9	±	1.5	36.4	±	3.3	1
Ventromedial			35.3	±	2.2	35.4	±	3.0	0
Interpeduncular nucleus		-3.28	19.5	±	4.2	12.7	±	3.9	-35
Pontine nucleus		-3.88	N.D.			N.D.			

N.D. = not detectable

DISCUSSION

Chronic NTX is known to induce supersensitivity to opioid agonists. This study provides a quantitative mapping for regional changes in the main opioid receptor subtypes throughout the brain in mice treated chronically with NTX. This is the first full quantitative mapping of μ-, δ- and κ-opioid receptors in chronic NTX treated mice. There was a clear upregulation of μ-opioid receptors and a lower but significant increase in δ-opioid receptors throughout the brain. In contrast, upregulation of κ-opioid receptors was restricted to cortical structures.

Ligand binding assays on whole brain homogenates of mice and rats showed chronic NTX-induced upregulation of μ- and δ-opioid receptors, with a mean percentage change of 43 to 90% and 20 to 70%, respectively (Tempel et al., 1985; Yoburn et al., 1986b; Danks et al., 1988; Yoburn et al., 1988; Yoburn et al., 1989; Cote et al., 1993; Unterwald et al., 1995; Yoburn et al., 1995; Castelli et al., 1997; Kest et al., 1998; Duttaroy et al., 1999). For κ-opioid receptor binding either no change or an increase in κ-opioid receptor binding by 30% were described (Tempel et al., 1985; Yoburn et al., 1995). Although these studies provided evidence for chronic NTX-induced upregulation of opioid receptors, they do not provide insight into regional changes in opioid receptor densities.

In the present study opioid receptor regulation induced by chronic NTX in mice was studied using quantitative autoradiography.

- μ-opioid receptor autoradiography

Without exception upregulation of μ-opioid receptors was observed for all regions. The data from this study show inter-regional variation in upregulation, but provide no evidence for upregulation of μ-opioid receptors in specific functional neuroanatomical systems. For example, μ-opioid receptor densities were enhanced in limbic structures but also in basal ganglia and structures involved in pain and sensory-motor functions, such as the periaqueductal gray, red nuclei and colliculi. Although different authors suggested there were regional differences, they disagree on exactly which areas are sensitive to chronic NTX-induced μ-opioid receptor upregulation. In fact, comparison of the different previous studies on this matter, which all used rats, reveals that the data to support regional differences are rather weak. Zukin et al. (1982) suggested high increases in opioid receptors in the limbic system whereas others found relatively low increases in μ-opioid receptors in limbic structures compared to other areas (Tempel et al., 1984; Morris et al., 1988). Further, Diaz et al. described highest increases in [^{3}H]DAMGO binding in caudate putamen and nucleus accumbens, while Unterwald et al. found highest increments in μ-opioid receptors in central grey and hypothalamus (Unterwald et al., 1998; Diaz et al., 2002). The data from the present mapping study suggest that chronic NTX exposure induces a general upregulation of μ-opioid receptors in mice, although the percentage increase may be highest in some cortical regions.

- δ-opioid receptor autoradiography

There was an upregulation of δ-opioid receptors after treatment with NTX, although the mean percentage change was lower as compared to the μ-opioid receptor data. This finding corresponds well to data of homogenate binding assays for chronic NTX treatment in mice (Yoburn et al., 1986b; Yoburn et al., 1988; Yoburn et al., 1989; Yoburn et al., 1995; Duttaroy et al., 1999) and an autoradiographic study by Morris et al. (1988). Although there was variation between regions, upregulation of δ-opioid receptor binding was not restricted to a specific functional neuroanatomical system, which contrasts with earlier work by Morris et al. (1988). In summary, chronic NTX treatment results in a general increase in δ-opioid receptor binding across the brain.

- κ-opioid receptor autoradiography

The nature of the differences between placebo and NTX treated mice for κ-opioid receptor binding did not mirror those for μ- and δ-opioid receptors. Cortical regions showed upregulation, whereas in non-cortical regions there was no change in binding compared to placebo treated controls. It can be concluded, that 15 mg NTX pellets implanted subcutaneously for 1 week causes an upregulation of κ-opioid receptors in merely cortical structures in mice. Morris et al. (1988) described effects of chronic naloxone treatment in rats upon κ-opioid receptor binding, but this was found for the high dose (3.0 mg/kg/hr) and not the low dose (0.5 mg/kg/hr). Apparently dependent on the dose, naloxone, and probably also NTX, can induce upregulation of κ-opioid receptors.

In this study, a single time-point after chronic NTX treatment is addressed: 24 hours after NTX was removed, based upon studies that showed functional opioid supersensitivity in mice after chronic NTX treatment. At this time-point most of the NTX is eliminated ($T_{½}$ = 4.6 hr, Yoburn et al., 1986a; 23 hr post-removal approximately 97% is eliminated). Tempel et al (1982) studied the time course of [^{3}H]etorphine binding after chronic NTX treatment in rats. [^{3}H]etorphine binding was enhanced by chronic NTX after 8 days of NTX treatment, which is the time-point of pellet removal in the present study. During NTX withdrawal Tempel et al (1982) showed that receptor levels declined and reached baseline levels by day 6 after removal of the pellets.

Quantitative autoradiography cannot discriminate between changes in B_{max} and K_D values. However, whole brain homogenate binding assays consistently showed increases in B_{max} without any changes in K_D for μ-, δ- and κ-opioid receptors (Yoburn et al., 1989; Giordano et al., 1990; Cote et al., 1993; Unterwald et al., 1995; Yoburn et al., 1995; Castelli et al., 1997; Duttaroy et al., 1999). It is therefore likely that the results described here in fact represent increases in B_{max} as opposed to changes in affinity.

The mechanism of opioid receptor upregulation induced by chronic NTX is not known, although different theories have been addressed. Opioid receptor upregulation is probably not due to increased transcription of the opioid receptor gene or related to altered opioid receptor mRNA stability (Unterwald et al., 1995; Jenab et al., 1995; Castelli et al., 1997; Duttaroy et al., 1999). Danks and Rothman (Danks et al., 1988; Rothman et al., 1989) observed μ- and δ-opioid receptor upregulation induced both by chronic morphine and naltrexone, which they suggested might be explained by agonist- and antagonist-induced release of 'anti-opiates', *i.e.* endogenous peptides such as cholecystokinin-8, α-MSH, dynorphin, β-endorphin and Met-enkephalin, which participate in opioid receptor upregulation. Data concerning G-protein changes are contradictory; one study reported increased sensitivity of the newly synthesised or unmasked opioid receptors to guanyl nucleotides (Tempel et al., 1985), although in another study no changes in G-protein mRNA levels after chronic exposure to NTX were observed (Rubino et al., 1994). Further, mechanisms such as changes in receptor protein stability, changes in receptor turnover or degradation and unmasking of 'silent' receptors have been proposed (Castelli et al., 1997). Unterwald et al. (1995) suggested that naltrexone might inhibit normal downregulation of opioid receptors, presumably by preventing endogenous opioids to bind. Finally, discrepancies between quantitative μ-opioid receptor immunoreactivity and quantitative μ-opioid receptor autoradiography led to suggest that the percentage of active receptors may be increased without a change in the total number of receptors (Unterwald et al., 1998).

The data of the present study clearly show chronic NTX-induced upregulation of μ- and δ-opioid receptors while κ-opioid receptor expression was enhanced in cortical but not in non-cortical regions. The pattern of upregulation induced by chronic naltrexone treatment (μ > δ > κ) as described here does not parallel the ratio in affinity of naltrexone for μ-, δ- and κ-opioid receptors (μ > κ > δ, Kieffer, 1995; Gutstein & Akil, 2001). This suggests that the affinity of naltrexone to a receptor is not predictive of the extent of naltrexone-induced supersensitivity of that receptor. It is further interesting to note that μ- and κ-opioid receptors are thought to have different, even opposing functions (for review see Pan, 1998; Narita et al., 2001). For example, μ-opioid agonists induce antinociception and tolerance, μ-opioid agonists are rewarding (Van Ree et al., 1999), μ-agonists impair memory, cause euphoria, increase dopamine release from the nucleus accumbens (Di Chiara & Imperato, 1988b) and are proconvulsant. In contrast, κ-opioid receptor agonists have anti-μ-opioid actions, *i.e.* they block morphine analgesia, reduce morphine tolerance and they improve μ-opioid agonist-induced memory impairment. Moreover κ-opioid agonists induce μ-opioid opposing effects (but see Van Ree et al, 1999): they induce dysphoria, are aversive, improve memory processes, act as anticonvulsants and decrease dopamine release from the nucleus accumbens. The present data suggest that μ- and κ-opioid receptors are also differentially regulated by chronic NTX.

In conclusion, the present full quantitative mapping study of μ-, δ- and κ-opioid receptors revealed clear effects of chronic NTX exposure upon opioid receptors in mice. Chronic NTX induced overall increases in μ- and, although to a lesser extent, in δ-opioid receptor binding. Changes in κ-opioid receptors were restricted to cortical regions. The findings described here suggest opioid receptor subtype selective regulation mechanisms.

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CHAPTER 8

ENDOGENOUS CANNABINOIDS ARE NOT INVOLVED IN COCAINE REINFORCEMENT AND DEVELOPMENT OF COCAINE-INDUCED BEHAVIOURAL SENSITIZATION

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SUBMITTED

ABSTRACT

The endogenous cannabinoid system is a relatively recently discovered system consisting of cannabinoid CB1 receptors, which are expressed both in the periphery and in the central nervous system, peripheral cannabinoid CB2 receptors and endogenous cannabinoids, that is anandamine and 2-arachidonyl glycerol. The cannabinoid CB1 receptors have recently been implicated in rewarding aspects of not only the cannabinoid drug Δ^9-tetrahydrocannabinol (Δ^9-THC), but also of other drugs of abuse, including cocaine. The present study was designed to further investigate the role of CB1 receptors in reward-related effects of cocaine. Using the CB1 receptor selective antagonist SR141716A, the involvement of CB1 receptors in cocaine reinforcement was determined by intravenous cocaine self-administration. In addition, the effects of the CB1 receptor selective antagonist SR141716A upon the development of cocaine-induced behavioural sensitization were investigated.

SR141716A did not affect cocaine reinforcement nor did it affect the development of behavioural sensitization to the locomotor stimulant effects of cocaine. These findings suggest that CB1 receptors are not involved in acute cocaine reinforcement nor in cocaine-induced behavioural sensitization.

INTRODUCTION

The endogenous cannabinoid system consists of cannabinoid type 1 receptors (CB1), CB2 receptors, which are restricted to the periphery, and the endogenous cannabinoids anandamide and 2-arachidonyl glycerol (2-AG) (Childers & Breivogel, 1998). Cannabinoid CB1 receptors have been implicated in the rewarding aspects not only of the Cannabis Sativa derived compound Δ^9-tetrahydrocannabinol (Δ^9-THC, Maldonado & Rodriguez, 2002), but also of opiates such as heroin and morphine (Chaperon et al., 1998; Navarro et al., 2001; Solinas et al., 2003; De Vries et al., 2003). Moreover, endogenous cannabinoids may be involved in reward-related effects of other drugs of abuse such as alcohol (Arnone et al., 1997; Freedland et al., 2001; Gallate & McGregor, 1999; Serra et al., 2001; Lallemand et al., 2001 but Vacca et al., 2002; Colombo et al., 2002; Wang et al., 2003), nicotine (Cohen et al., 2002), 3,4-methylenedioxymethamphetamine (MDMA) (Braida & Sala, 2002) and cocaine (Chaperon et al., 1998; De Vries et al., 2001 but Fattore et al., 1999).

Here we further investigated the role of CB1 receptors in acute and chronic reward-related effects of cocaine. For this purpose we used the selective CB1 receptor antagonist SR141716A (Rinaldi-Carmona et al., 1995). First, the involvement of CB1 receptors in the initiation of intravenous cocaine self-administration in drug-naive mice was studied. Initiation of cocaine self-administration is predominantly determined by the positive reinforcing properties of cocaine and is not or less influenced by effects of repeated drug administration (Van Ree et al., 1999). In a yoked-controlled self-administration paradigm the effects of acute CB1 receptor blockade with SR141716A upon cocaine reinforcement were determined. In the second part of this study, the involvement of CB1 receptors in chronic effects of cocaine was determined. Prolonged exposure to cocaine, or other drugs of abuse, leads to sensitization of brain systems, which mediate incentive salience or 'drug wanting' (Robinson & Berridge, 2000). Here behavioural sensitization to the locomotor stimulant effects of cocaine, which is assumed to involve the same neural substrates, was studied for involvement of CB1 receptors. SR141716A or placebo was co-administered with repeated intermittent cocaine or saline sensitization injections for 11 subsequent days.

MATERIALS AND METHODS

Animals

Male C57Bl/6Jico mice (Charles River, l'Arbresle, France) aged 2-3 months were group housed (2-4) in extended Macrolon© type I cages with water and food pellets available ad libitum. Environmental conditions were controlled (22°C and 50% humidity; lights on at 7:00 a.m. and lights off at 7:00 p.m., GDL Utrecht University). The experimental procedures were

approved by the Ethical Committee for Animal Experiments of the University Medical Center Utrecht.

Effects of SR141716A upon intravenous cocaine self-administration

This experiment was designed to determine the effects of the CB1 antagonist SR141716A upon intravenous (i.v.) cocaine self-administration in drug-naive mice. As described previously (Kuzmin et al., 1997a; Kuzmin et al., 1997b), mice were tested in pairs, one active and one yoked control, in identical 8×8×8 cm test cages made from non-transparent material (RITEC, St. Petersburg, Russia). Each cage has a frontal nose-poking hole supported with infrared sensors interfaced to a computer. Mice were partially immobilized by fixing their tails, which protruded through the vertical slot in the back wall, to the horizontal surface using tape. A 10 minutes pre-test was performed at least 2 hours prior to the self-administration session. The mice were subsequently paired according to basal nose-poke responding.

SR141716A was dissolved in a mixture of 0.5% Tween/Ethanol in saline, which was also used as the control solution (placebo). 30 Minutes prior to the self-administration session, the mice received either a placebo or a 1 mg/kg SR141716A injection intraperitoneally (i.p.) (Chaperon et al., 1998; Serra et al., 2001; De Vries et al., 2001). During the self-administration session (30 minutes) each nose-poke response by the active mouse resulted in a contingent i.v. injection of 1.6 µl of a cocaine solution (0.2, 0.4 or 0.8 µg cocaine per infusion) in the lateral tail vein of both the active and the yoked control mouse. Nose-pokes by the yoked control mice were counted but had no programmed consequences. The data are expressed as total nose poke responses or total cocaine intake (mg/kg bodyweight) during the 30 minutes experiment. N = 6-8 per dose per type (active or yoked) per treatment.

Effects of SR141716A upon cocaine-induced behavioural sensitization

This experiment was designed to determine the effects of the CB1 antagonist SR141716A upon the development of behavioural sensitization induced by cocaine. 16 Mice received daily saline injections and another 16 mice received 20 mg/kg cocaine once daily for 11 subsequent days. These saline and cocaine groups were subdivided into two groups of 8 mice each, which received either placebo (0.5% Tween/Ethanol in saline, i.p.) or SR141716A (1 mg/kg, i.p.) 30 minutes prior to the daily saline or cocaine injection (co-administration). All injections during the 11 days of sensitization were administered from the home cage. 72 Hrs after the last cocaine or saline injection, the mice were transported and allowed to acclimatise to the experimental room for at least 1 hour. Then the mice were placed in the open field, which was a clear Plexiglas cylinder of 20 cm in diameter and 30 cm in height. During the first hour in the open field the locomotor activity was measured to monitor the adaptation of the mice to the novel environment. Subsequently all mice received a saline injection (i.p.) after which they were returned to the open field and monitored for another hour. Thereafter all mice received a cocaine challenge (10 mg/kg i.p.) and their locomotor activity in the open field was

determined during 30 minutes. The distance moved in the open field was measured in 5 minutes intervals using a camera-linked computerised tracking system with Ethovision software (Noldus, Wageningen, NL). N = 8 mice per group, except for the placebo/cocaine treatment group. One mouse was discarded from this group because it escaped from the open field during the hour after saline injection.

Since the results of this initial experiment suggested a slight reduction in behavioural sensitization (see results section), an additional experiment was performed for verification. For this experiment, 72 mice were used. No SR141716A/saline control was included because the initial experiment (see above) revealed no effect of SR141716A upon acute cocaine- induced locomotor activity. In this case, 24 mice received daily saline injections and 48 mice received 20 mg/kg cocaine once daily for 11 subsequent days. All saline treated mice received placebo injections (0.5% Tween/Ethanol in saline) 30 minutes prior to saline administration. The cocaine group was subdivided in two groups of 24 mice each: a placebo group and a SR141716A group (1 mg/kg in 0.5% Tween/Ethanol in saline). Placebo or SR141716A were administered 30 minutes prior to saline or cocaine injections. 72 Hrs after the last sensitization injection, the mice were tested in the open field as described. In this experiment the mice were allowed to adapt to the open field for 1.5 hr after which saline was injected and either 1, 3 or 10 mg/kg cocaine challenge doses were given at 2 hr after placement in the open field. N = 8 per group.

Statistical analysis

For cocaine self-administration, the number of nose pokes for the 10 minutes pre-test and the nose poke responding during the 30 minutes cocaine self-administration session were analysed by three-way ANOVAs using SPSS 10.1 software. The independent factors were type (active or yoked), dose (µg/infusion) and treatment (placebo or SR141716A). The intake data of the active mice were analysed by two-way ANOVA with dose and pre-treatment as factors.

Open field activity is expressed as distance moved in 5 minutes intervals. Separate analyses of variance (ANOVAs) with repeated measurements were used to analyse the data for the adaptation to the open field, the period after saline injection as well as the 30 minutes after cocaine challenge. Distance moved was the variable and co-administration (placebo or SR141716A) and treatment (saline or cocaine) were the independent factors. When appropriate, one-way ANOVA's with repeated measurements were performed. In the second sensitization experiment with 1, 3 or 10 mg/kg cocaine challenge doses no SR141716A/saline group was included because there was no effect of SR141716A co-administration upon the acute locomotor response to the cocaine challenge in the initial experiment. Because the SR141716A/saline group was absent, separate analyses were performed (1) for the saline and cocaine treated mice within the placebo co-administration group with treatment (saline or cocaine) and dose (1, 3 or 10 mg/kg) as factors and (2) for the cocaine treated mice, which received either placebo or SR141716A co-administration, with co-administration (placebo or

SR141716A) and dose (1, 3 or 10 mg/kg) as the independent factors. All data are expressed as mean ± SEM and statistical significance was accepted at $P < 0.05$.

RESULTS

Effects of SR141716A upon intravenous cocaine self-administration

Overall analysis of the nose-pokes during the 10 minutes pre-test revealed no effect of SR141716A treatment nor were there differences between active and yoked control mice or between cocaine doses (Table 1), showing that during the initial pre-test all mice were equally active in their nose-poke responding.

During the cocaine self-administration session, the active mice responded with significantly more nose-pokes than the yoked control mice did (Figure 1A, overall analysis, effect of type $F(1,73)=10$, $P < 0.01$). Although the number of nose poke responses was not dependent upon the unit cocaine dose, total cocaine intake by the active responding mice increased with rising amounts of cocaine administered per infusion (Figure 1B, effect of dose $F(2,40)=13$, $P < 0.001$). These findings demonstrate reliable i.v. cocaine self-administration in this experiment. With respect to involvement of endogenous cannabinoids, acting through CB1 receptors, there was no effect of SR141716A treatment upon the number of nose-pokes nor was there an interaction between SR141716A treatment and type, thus indicating that SR141716A treated mice responded for cocaine in i.v. self-administration as placebo treated controls did. Moreover, analysis of the total cocaine intake, which is considered indicative of the reinforcing efficacy of cocaine (Van Ree et al., 1978; Van Ree et al., 1999), revealed no effect of SR141716A upon total cocaine intake during the 30 minutes self-administration session.

		Cocaine (µg/infusion)		
		0.2	**0.4**	**0.8**
placebo	Active	51.0 ± 11	69.1 ± 6.1	59.3 ± 4.4
	Yoked	42.7 ± 13	63.5 ± 4.0	55.0 ± 8.7
SR141716A	Active	74.0 ± 13	57.7 ± 12	59.0 ± 9.2
	Yoked	62.3 ± 18	49.8 ± 2.9	73.9 ± 7.2

TABLE 1

Nose poke responding during the 10 minutes pre-test for placebo and SR141716A -treated mice. Data is expressed as mean ± SEM nose pokes during 10 minutes per treatment per type (active or yoked) per dose.

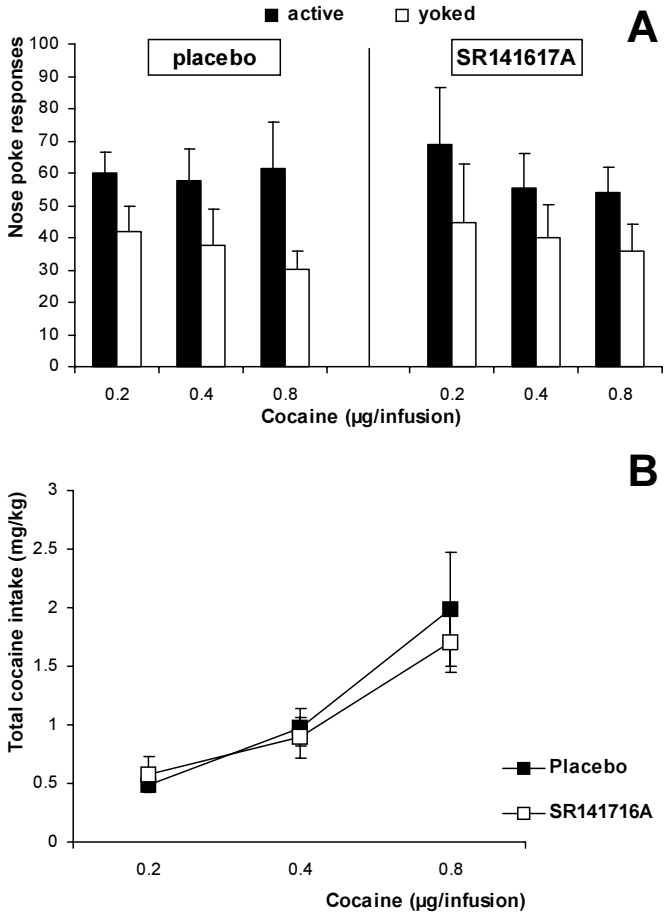

FIGURE 1

Effects of SR141716A upon cocaine self-administration in C57Bl/6JIco mice. (A) Total number of nose-poke responses during the 30 minutes session as a function of cocaine dose (µg per infusion) is shown for the active responding mice (right panel) and the yoked controls (left panel). The data represent the mean nose poke responses ± SEM. In (B) the total cocaine intake (mg/kg) by the active responding mice, which received either placebo or 1 mg/kg SR141716A 30 minutes prior to the self-administration session, is shown as a function of the unit cocaine dose (µg/infusion). The data represent the mean intake (mg/kg) ± SEM. N = 6-8 per group.

Effects of SR141716A upon cocaine-induced behavioural sensitization

The time-course of open field activity of the mice tested for behavioural sensitization to the locomotor stimulant effects of cocaine and the effects of SR141716A co-administration upon cocaine-induced behavioural sensitization is shown in Figure 2. During the first hour in the open field, the mice adapted to the open field as was reflected by a reduction in their activity over time ($F(11,297)=26$, $P < 0.001$). Cocaine treatment or SR141716A co-administration did

not affect basal activity of the mice during the first hour in the open field. Subsequent to the saline injection, the mice further reduced their activity ($F(11,297)=6.5$, $P < 0.001$) and no cocaine or SR141716A effects were observed.

Repeated intermittent administration of cocaine (20 mg/kg) resulted in behavioural sensitization (Figure 2) as was evident from a significant effect of cocaine treatment upon the locomotor response to a 10 mg/kg cocaine challenge ($F(1,27)=10$, $P < 0.01$) and a significant time × treatment (saline or cocaine) interaction ($F(5,135)=20$, $P < 0.001$). Overall analysis of the data of the last 30 minutes period further revealed a significant co-administration (placebo or SR141716A) × treatment (saline or cocaine) interaction ($F(1,27)=5.7$, $P < 0.05$), although there was no time × co-administration × treatment interaction ($F(5,135)=1.5$, $P = 0.2$). These findings suggest an effect of the cannabinoid CB1 receptor antagonist SR141716A upon cocaine-induced behavioural sensitization. Post-hoc analyses revealed significant effects of cocaine treatment for the mice, which received placebo co-administration during the intervals 0-5, 5-10 and 10-15 minutes after cocaine challenge ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively). For the mice, which received SR141716A co-administration with the repeated intermittent cocaine injections, cocaine sensitization was only apparent during the first 5 minutes interval after cocaine challenge ($P < 0.05$).

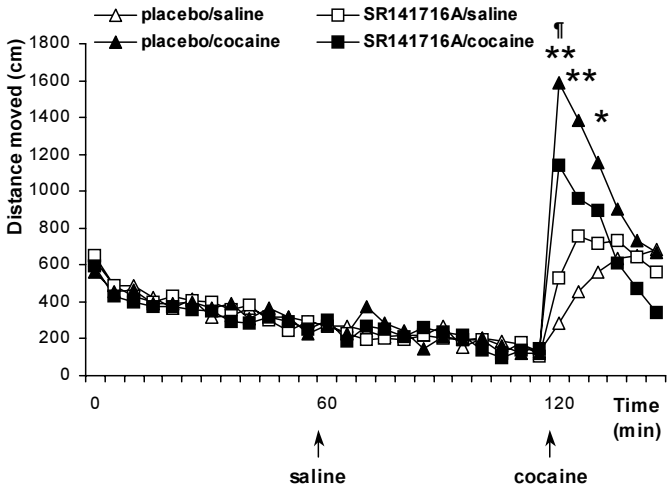

FIGURE 2

Effects of SR141716A upon the development of cocaine-induced behavioural sensitization. SR141716A or placebo were injected 30 minutes prior to repeated intermittent saline or cocaine (20 mg/kg) injections for 11 days (co-administration). The time-course of the activity in the open field, 72 hrs after the last sensitization injection, is shown with 1 hour adaptation to the open field, followed by 1 hour in the field after an i.p. saline injection and 30 minutes after an i.p. injection of a 10 mg/kg cocaine challenge. The data represent the mean total distance moved in cm during 5 minutes intervals ± SEM. N = 7-8 per group. * $P < 0.01$, ** $P < 0.001$, significant difference between placebo/saline and placebo/cocaine treatment groups; ¶ $P < 0.05$, significant difference between SR141716A/saline and SR141716A/cocaine treated subjects.

To verify and extend these findings, the effects of SR141716A upon cocaine-induced sensitization were further explored in an additional experiment with three different cocaine challenge doses (1, 3 or 10 mg/kg, Figure 3). No SR141716A/saline group was included because in the initial experiment no effects of SR141716A upon the acute locomotor response induced by cocaine were observed (see above).

Separate analysis of the mice within the placebo co-administration group revealed an overall effect of time during the first 1.5 hrs in the open field ($F(17,714)=36$, $P < 0.001$) and no effect of saline versus cocaine treatment. The activity of the mice reduced further during the minutes subsequent to saline injection ($F(5,200)=3.3$, $P < 0.01$) and was not different between the planned cocaine challenge dose groups. Analysis of the activity after cocaine challenge injection of mice, which received placebo co-administration, revealed a significant time × cocaine challenge dose (1, 3 or 10 mg/kg) interaction ($F(10,210)=4.0$, $P < 0.001$), a significant time × treatment interaction (saline or cocaine, $F(5,210)=8.7$, $P < 0.001$) and a significant time × treatment × dose interaction ($F(10,210)=3.9$, $P < 0.001$) thus showing challenge dose-dependent cocaine-induced behavioural sensitization in this experiment.

Subsequent analysis for the cocaine-treated groups, which was performed in order to assess the effect of SR141716A co-administration upon cocaine-induced behavioural sensitization, revealed again a significant effect of time upon locomotor activity during the first 1.5 hrs in the open field ($F(17,714)=52$, $P < 0.001$) with comparable locomotor activity of mice which received either SR141716A or placebo co-administration and no difference between cocaine dose groups. A significant time effect was observed after saline injection at 90 minutes after placement in the open field ($F(5,190)=7.3$, $P < 0.001$). Analysis of the locomotor activity in the open field after cocaine challenge injection revealed a significant time × dose interaction ($F(10,210)=5.4$, $P < 0.001$). However, SR141716A did not affect the development of cocaine-induced behavioural sensitization, as there was no significant effect of SR141716A co-administration upon the cocaine challenge-induced locomotor response ($F(1,42)=1.6$, N.S.) nor were there a significant dose × co-administration ($F(2,42)=0.4$, N.S.), time × co-administration ($F(5,210)=1.2$, N.S.) or time × dose × co-administration ($F(10,210)=1.6$, N.S.) interaction effects upon the cocaine challenge-induced locomotor response.

DISCUSSION

Endogenous cannabinoid systems have been implicated in the rewarding aspects not only of the *Cannabis Sativa* derived compound Δ^9-THC, but also of opiates, alcohol and psychostimulants. Here we investigated the involvement of cannabinoid CB1 receptors in cocaine reinforcement and cocaine-induced behavioural sensitization. Neither cocaine self-administration in drug-naive mice nor cocaine-induced behavioural sensitization was affected by co-administration of the CB1 receptor selective antagonist SR141716A. The present data suggest that CB1 receptors are not required for cocaine reinforcement or for sensitization to the locomotor stimulant effects of cocaine.

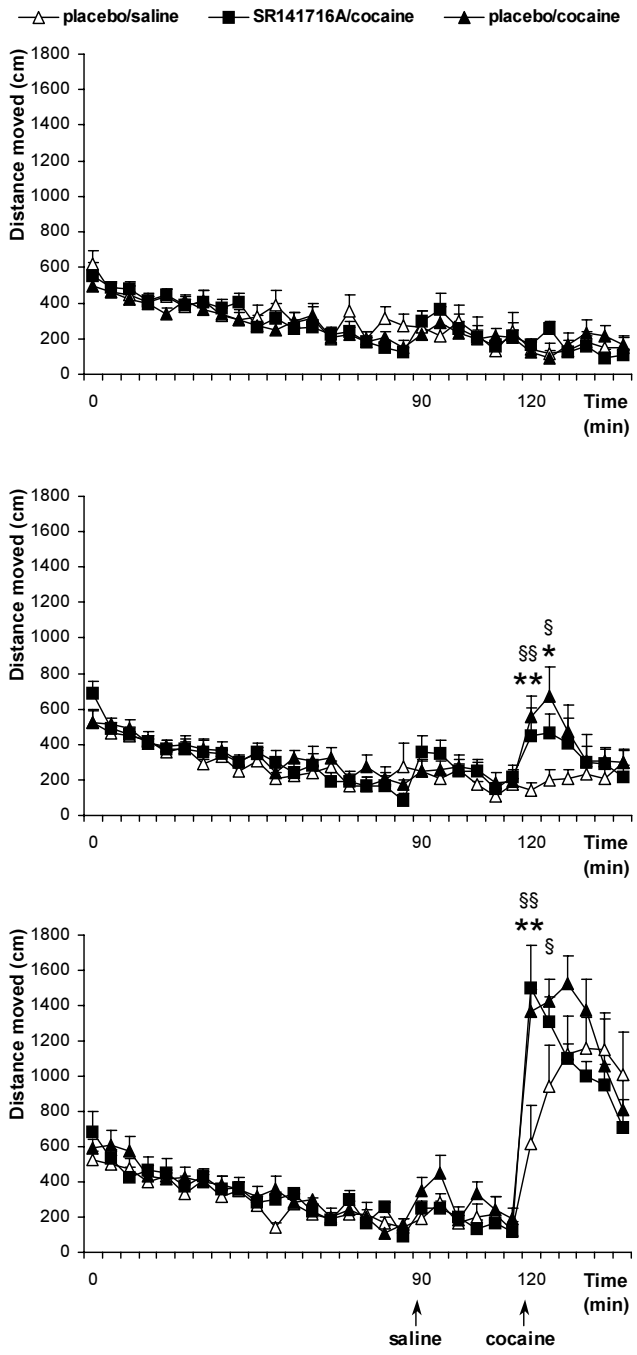

FIGURE 3

Effects of SR141716A and placebo co-administration upon the development of behavioural sensitization induced by repeated intermittent treatment with saline or cocaine (20 mg/kg) for 11 days.

The activity in the open field, 72 hrs after the last sensitization injection, is shown for 1.5 hour adaptation to the open field, 30 minutes after an i.p. saline injection and 30 minutes after an i.p. injection of (A) 1 mg/kg cocaine, (B) a 3 mg/kg cocaine challenge and (C) 10 mg/kg cocaine.

The data represent the mean total distance moved in cm during 5 minutes intervals ± SEM. N = 8 per group. * $P < 0.05$, ** $P < 0.01$, significant difference between placebo/ saline and placebo/cocaine treated mice; § $P < 0.05$, §§ $P < 0.01$, significant difference between placebo/ saline treated mice and cocaine treated mice (placebo + SR141716A).

The selective CB1 receptor antagonist SR141716A did not affect cocaine self-administration by drug naive mice, thus showing that CB1 receptors are not involved in cocaine reinforcement. Previous studies have shown that comparable doses of SR141716A did not affect cocaine self-administration in rats during maintenance (Fattore et al., 1999; De Vries et al., 2001) and cocaine self-administration by cannabinoid CB1 receptor knockout mice was normal (Cossu et al., 2001). In contrast, SR141716A impaired cocaine-induced conditioned place preference (CPP) (Chaperon et al., 1998), although such a role of CB1 receptors in cocaine-induced CPP was not confirmed by another group using CB1 knockout mice (Martin et al., 2000a). Further, SR141716A was shown to reduce both cocaine and cue-induced relapse to cocaine seeking (De Vries et al., 2001). The present findings demonstrate that SR141716A co-administration with repeated intermittent saline or cocaine treatment was without effect on the development of cocaine-induced behavioural sensitization in mice. This finding is in agreement with a previous study, which described normal cocaine-induced sensitization in CB1 receptor knockout mice (Martin et al., 2000a).

When reviewing the literature dealing with cannabinoid modulation of drug reward it is striking that depressants such as opiates or alcohol involve CB1 receptor activity while psychostimulants do not, at least with regard to their reinforcing efficacy. For example, mice treated with cannabinoid antagonists or CB1 receptor knockout mice consistently displayed reduced opiate self-administration (Ledent et al., 1999; Cossu et al., 2001; Navarro et al., 2001; Solinas et al., 2003; De Vries et al., 2003), impaired opiate-induced CPP (Martin et al., 2000a; Braida et al., 2001) and impaired morphine-induced behavioural sensitization (Martin et al., 2000a), although normal morphine-induced CPP for CB1 knockout mice (Rice et al., 2002) and normal behavioural sensitization to morphine after SR141716A co-administration (Norwood et al., 2003) were also reported. Similarly, most studies dealing with alcohol reward reported reducing effects of SR141716A or CB1 receptor gene knockout upon either alcohol preference or intake (Arnone et al., 1997; Gallate & McGregor, 1999; Serra et al., 2001; Lallemand et al., 2001; Freedland et al., 2001; Hungund et al., 2003; Poncelet et al., 2003) whilst others reported no effect of either SR141716A (Colombo et al., 2002; Wang et al., 2003) or CB1 receptor knockout (Racz et al., 2003). In contrast, studies on cocaine self-administration, which include the present study, consistently demonstrate that the endogenous cannabinoid system is not involved in cocaine reinforcement (Fattore et al., 1999; Cossu et al., 2001; De Vries et al., 2001) or amphetamine reinforcement (Cossu et al., 2001). Yet, cannabinoid CB1 receptor blockade impaired cocaine-induced CPP (Chaperon et al., 1998) and reinstatement of cocaine self-administration (De Vries et al., 2001), possibly suggesting that the endogenous cannabinoid system might modulate conditioned responses to cocaine rather than cocaine reinforcement. Thus, depressant drugs such as opiates and alcohol appear sensitive to cannabinoid modulation, while reinforcing effects of psychostimulants do not involve CB1 receptor activity.

In conclusion, the cannabinoid CB1 receptor antagonist SR141716A did not affect cocaine self-administration or cocaine-induced behavioural sensitization, thus demonstrating that cocaine reinforcement and sensitization to the locomotor stimulant effects of cocaine do not involve cannabinoid CB1 receptors.

ACKNOWLEDGEMENTS

Sanofi-synthelabo Recherche (Montpellier, France) kindly provided a sample of the CB1 antagonist SR141716A, which is gratefully acknowledged.

CHAPTER 9

GENERAL DISCUSSION

The sensitivity to the reinforcing effects of drugs of abuse constitutes a likely vulnerability factor for drug dependence. The overall objective of the studies described in this thesis was to investigate the role of μ-opioid receptors in cocaine reinforcement and the underlying neurobiological mechanisms. In this chapter the findings of this thesis will be discussed accordingly.

μ-OPIOID RECEPTORS AND COCAINE REINFORCEMENT

The studies described in **Chapters 3 and 5** of this thesis demonstrate that μ-opioid receptor knockout mice were impaired in cocaine self-administration while, on the other hand, no differences between μ-opioid receptor knockout mice and wild-type controls were observed in cocaine-induced locomotor activity or cocaine-induced behavioural sensitization. These findings show (1) that μ-opioid receptors are specifically involved in cocaine reinforcement, and (2) that the mechanisms involved in cocaine reinforcement are divergent from those involved in acute cocaine-induced locomotor activity and sensitization to the locomotor stimulant effects of cocaine, at least with regard to μ-opioid receptor involvement.

Previous studies demonstrated, by local administration of the opioid antagonist naltrexone, that particularly opioid receptors in the ventral tegmental area (VTA) account for opioid modulation of cocaine reinforcement (Ramsey et al., 1999). In the VTA of wild-type mice, μ-opioid receptors are present on GABAergic neurons (Figure 1A; Garzon & Pickel, 2001; Garzon & Pickel, 2002). Opioids can activate μ-opioid receptors in the VTA, which involves intracellular signalling through the ERK1/2 pathway (**Chapter 4**), causing hyperpolarization of the GABAergic neurons (Johnson & North, 1992a; Johnson & North, 1992b). These GABAergic neurons are thought to be local interneurons, although GABAergic projection neurons from the VTA to amongst others the nucleus accumbens and prefrontal cortex have also been described (Van Bockstaele & Pickel, 1995; Steffensen et al., 1998; Carr & Sesack, 2000). The local GABAergic interneurons in the VTA synapse onto dopaminergic neurons that form a major output pathway from the VTA (Johnson & North, 1992a). In μ-opioid receptor knockout mice, the inhibitory GABAergic input onto dopamine neurons in the VTA was increased in a cocaine free state, as is evident from increased spontaneous inhibitory postsynaptic currents (IPSC's) measured from dopamine neurons (**Chapter 3**, Figure 1C).

Interestingly, actively self-administered, but not passively administered, cocaine was positively correlated with pro-opiomelanocortin (POMC) mRNA levels in the arcuate nucleus (**Chapter 3**), that is POMC mRNA levels increased as total active cocaine intake increased. POMC is the precursor of the μ-opioid receptor selective endogenous opioid peptide β-endorphin. Indeed, β-endorphin levels increase in response to cocaine and also after administration of amphetamine and alcohol, at least in the nucleus accumbens (Olive et al., 2001; Roth-Deri et al., 2003; Marinelli et al., 2003). Also, *in vivo* autoradiography revealed that opioid levels are increased after cocaine self-administration (Gerrits et al., 1999). Active self-administration is a measure for cocaine reinforcement, which requires μ-opioid receptor activation (**Chapter 3**). The positive correlation of active cocaine self-administration with

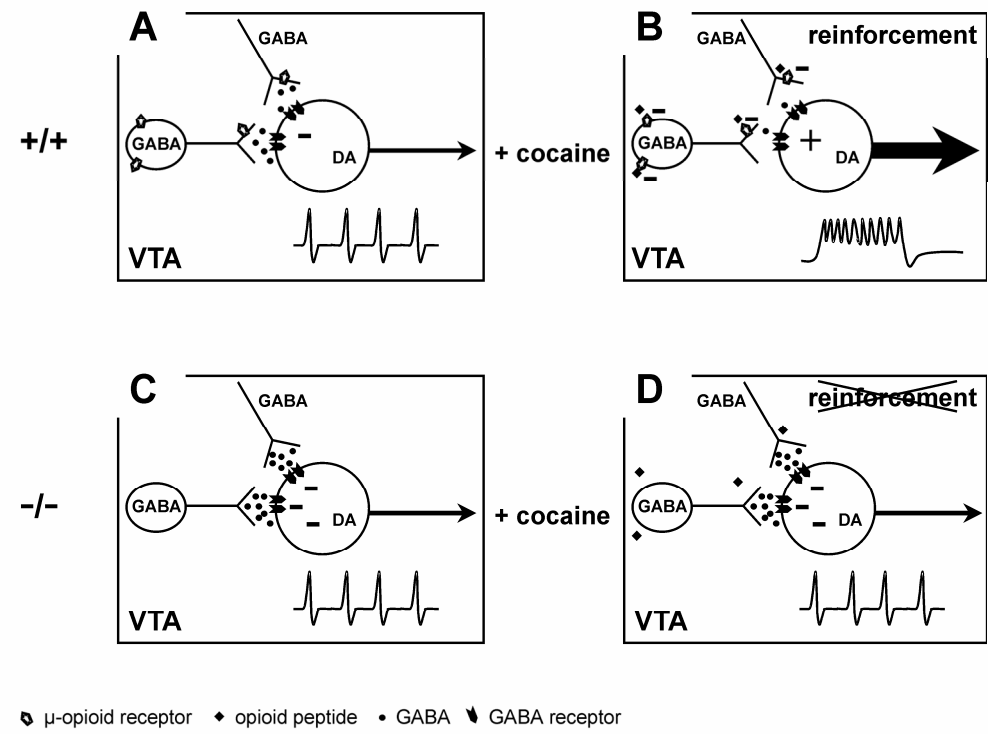

FIGURE 1

Proposed mechanism for μ-opioid receptor mediated cocaine reinforcement in the VTA. The cocaine-free state of the VTA of wild-type (+/+) and knockout (-/-) mice, obviously with differences in μ-opioid receptors and consequent changes in GABAergic transmission are shown in (A) and (C), respectively. Addition of cocaine causes endogenous opioid peptides to be released, which through activation of μ-opioid receptors cause hyperpolarization of GABA neurons and consequently disinhibition of dopamine neurons, facilitation of burst firing of dopamine neurons and enhancement of dopamine output. As a result, cocaine is reinforcing to wild-type (+/+) mice (B). In contrast, endogenous opioid peptides, released in response to cocaine, can not activate μ-opioid receptors in the μ-opioid receptor knockout (-/-) animals. As a consequence, disinhibition of dopamine neurons does not occur, dopamine output is not enhanced and cocaine is not reinforcing in μ-opioid receptor knockout mice (D).

POMC mRNA, the precursor of β-endorphin, therefore suggests that β-endorphin, through interactions with μ-opioid receptors, may account for opioid modulation of cocaine reinforcement (Figure 1B). However, other endogenous opioids such as the novel μ-opioid receptor selective endomorphins 1 and 2 (Zadina et al., 1997) or enkephalins, which also have affinity for μ-opioid receptors, may also be involved. It is through inhibition of the GABAergic neurons that μ-opioid receptors cause disinhibition of dopamine neurons in the VTA, thereby presumably contributing to augmented dopamine release from the nucleus accumbens in response to different drugs of abuse (Di Chiara & Imperato, 1988a) and to burst

firing of dopamine neurons (Schultz et al., 1997; Cooper, 2002), which may ultimately lead to cocaine reinforcement (Figure 1B).

POMC mRNA levels were not different between μ-opioid receptor knockout mice and wild-type controls (Zhou et al., 2002), suggesting that similar levels of β-endorphin, the major endogenous μ-opioid receptor ligand, are released in response to cocaine in both genotypes. However, in case of μ-opioid receptor knockout mice, this or other endogenous opioids obviously cannot activate a μ-opioid receptor. Consequently, the GABA neurons cannot be hyperpolarized and disinhibition of dopamine neurons cannot occur as in wild-type mice. We propose that impaired disinhibition of dopamine neurons together with the increased GABAergic inhibitory input onto the dopamine neurons contributes to impaired cocaine self-administration by μ-opioid receptor knockout mice (**Chapter 3**). There is no evidence for altered basal firing frequency of dopamine neurons *in vitro* in the VTA of μ-opioid receptor knockout mice (**Chapter 3**) nor are there indications for altered dopamine release from the nucleus accumbens, both under basal conditions and in response to alcohol (Tang et al., 2002). Rather, the threshold for burst firing of dopamine neurons might be augmented in these mice, which is yet to be investigated (Figure 1D).

DRUG REINFORCEMENT

μ-Opioid receptors and drug reinforcement

This thesis provides evidence for an important role of μ-opioid receptors in cocaine reinforcement. Previous studies substantiate the involvement of the μ-opioid receptor in drug reinforcement across pharmacological classes. For instance, μ-opioid receptor knockout mice do not self-administer morphine (Becker et al., 2000) and consume less alcohol (Roberts et al., 2000; Hall et al., 2001; Becker et al., 2002).

Thus, drugs of abuse from different pharmacological classes have μ-opioid receptor mediated modulation of their reinforcing efficacy in common. This is interesting considering the different primary targets that are used by different drugs of abuse. Opiates interact with opioid receptors (Snyder & Pasternak, 2003), cocaine acts as a dopamine transporter blocker (Ritz et al., 1987), amphetamine interacts with the vesicular monoamine transporter (Pifl et al., 1995), alcohol is considered to act through interactions with ligand-gated ion channels (Soderpalm et al., 2000), nicotine acts through nicotinic acetylcholine receptors (Corrigall et al., 1992; Picciotto et al., 1998) and cannabinoids act through cannabinoid receptor interactions (Gardner & Vorel, 1998; Childers & Breivogel, 1998). It is likely that the actions of different classes of drugs converge to a common system. μ-Opioid receptors in the VTA may form part of such a common system, which is relevant for drug reinforcement.

How does μ-opioid receptor modulation of drug reinforcement relate to the mesolimbic dopamine system? It appears likely, that μ-opioid receptors modulate drug reinforcement by affecting dopamine output of the mesolimbic system. For, as outlined previously in this

chapter, μ-opioid receptors in the VTA cause, by disinhibition of dopamine neurons, enhanced dopamine output of the mesolimbic system.

Yet, the importance of the mesolimbic dopamine system in drug reinforcement is a matter of debate. For example, opiate reinforcement does not require intact dopamine input to the nucleus accumbens (Gerrits & Van Ree, 1996) and haloperidol or the D1 receptor antagonist SCH23390 could not abolish the initiation of heroin self-administration (Pettit et al., 1984; Van Ree & Ramsey, 1987; Gerrits et al., 1994, see **Chapter 1**). Apparently, opiates can support self-administration independent of dopamine. There is an interesting parallel in the effects of opiates and dopamine upon GABAergic medium spiny neurons in the nucleus accumbens, which form the main output neurons of the nucleus accumbens (Tzschentke & Schmidt, 2000). Opiates, dopamine and also psychostimulants depress excitatory postsynaptic currents (EPSC's) in the nucleus accumbens as measured from medium spiny neurons in the nucleus accumbens (Pennartz et al., 1992; Harvey & Lacey, 1996; Nicola et al., 1996; Martin et al., 1997; Hoffman & Lupica, 2001). Inhibition of these GABAergic medium spiny neurons, and not so much dopamine, may therefore be an important common effect of drugs of abuse that may be required for drug reinforcement. The relevance of medium spiny neuron inhibition in the nucleus accumbens for reinforcement could be subject of future research. Obviously, the model outlined here is simplified; other brain regions, such as the ventral pallidum, are also likely involved in drug reinforcement.

Cannabinoid CB1 receptors and drug reinforcement

In **Chapter 8** of this thesis the involvement of cannabinoid CB1 receptors in behavioural effects of cocaine was investigated. No effects were observed of the CB1 receptor antagonist SR141716A upon cocaine reinforcement nor was cocaine-induced behavioural sensitization affected by SR141716A treatment. When reviewing the available literature dealing with CB1 receptor involvement in reward-related effects of drugs of abuse, in combination with the present findings, a discrepancy between CB1 involvement in opiate and alcohol but not psychostimulant reinforcement is apparent.

Other studies also suggest that the endogenous cannabinoid system is not involved in cocaine reinforcement (Fattore et al., 1999; Cossu et al., 2001; De Vries et al., 2001) or amphetamine reinforcement (Cossu et al., 2001), although cannabinoid CB1 receptor blockade impaired cocaine-induced CPP (Chaperon et al., 1998) and reinstatement of cocaine self-administration (De Vries et al., 2001). In contrast to these findings, other studies reported CB1 receptor mediated modulation of opiate reinforcement (Ledent et al., 1999; Cossu et al., 2001; Navarro et al., 2001; Solinas et al., 2003; De Vries et al., 2003) and CB1 modulation of alcohol reinforcement (Arnone et al., 1997; Serra et al., 2001; Lallemand et al., 2001), although others found no effect of SR141716A upon alcohol preference or intake (Colombo et al., 2002). With respect to drug-induced behavioural sensitization, CB1 receptor knockout mice were impaired in morphine-induced behavioural sensitization (Martin et al., 2000a), although behavioural sensitization to morphine was not affected by SR141716A co-administration (Norwood et al.,

2003). Thus, the reinforcing effects of and sensitized responses to depressant drugs such as opiates and alcohol are sensitive to cannabinoid modulation while CB1 receptors appear not to modulate psychostimulant reinforcement or behavioural sensitization.

Little is known about the mechanisms, which may explain the discrepancy in CB1 receptor involvement in the effects of different drugs of abuse. A likely site of CB1 receptor mediated involvement in drug reinforcement is the VTA. Interestingly, strikingly similar mechanisms of action of CB1 receptors in the VTA were observed as for μ-opioid receptors: CB1 receptors inhibit GABA neurons in the VTA resulting in disinhibition of dopamine neurons and causing enhanced dopamine output to the nucleus accumbens (Szabo et al., 2002). Tanda and co-workers however described non-reciprocal cannabinoid-opioid interactions in dopamine release from the nucleus accumbens. They reported that the μ-opioid receptor antagonist naloxonazine, administered in the VTA, reduced both the cannabinoid and heroin-induced increase in dopamine release from the nucleus accumbens. In contrast, intra-VTA administered SR141716A only inhibited the effects of cannabinoids upon dopamine release but failed to affect the response to heroin. It thus appears that CB1 receptors are not required for μ-opioid receptor mediated effects in the VTA, thus suggesting that VTA CB1 receptors might not be involved in CB1 receptor effects upon opiate reinforcement. Future studies could use local injections of antagonists to pinpoint the site and mechanism of interaction between μ-opioid and CB1 receptors in modulation of drug self-administration and explore the differential involvement of CB1 receptors in opiate/alcohol but not psychostimulant reinforcement.

μ-OPIOID RECEPTORS AND VULNERABILITY FOR DRUG DEPENDENCE

What are the implications for human drug addiction of the important role of μ-opioid receptors in drug reinforcement? Drug reinforcement is a key factor in vulnerability for drug dependence. Therefore, involvement of μ-opioid receptors in the sensitivity to drug reinforcement across pharmacological classes suggests that variations in μ-opioid receptors, either genetic or environmental in nature, may contribute to an individual's vulnerability for drug dependence.

Genetic variation in the human μ-opioid receptor gene

Single nucleotide polymorphisms (SNP's) make up for genetic variations between individuals. They occur everywhere in the genome and can affect the expression or function of genes. A number of SNP's in the human μ-opioid receptor gene have been identified and studied for association with drug addiction in humans. An example is the relatively extensively studied nucleotide substitution at position 118 (A118G), predicting an Asp40Asn amino acid change (Bergen et al., 1997; Bond et al., 1998). Different studies suggested a significant association of the A118G variant with opiate dependence (Szeto et al., 2001; Tan et al., 2003) or with alcohol dependence (Town et al., 1999; Schinka et al., 2002). The frequency of the A118G

variant is lower in drug dependent subject groups. However, other studies failed to show a significant association of the A118G variant of the μ-opioid receptor gene with either opiate (Bond et al., 1998; Franke et al., 2001; Shi et al., 2002) or alcohol dependence (Bergen et al., 1997; Sander et al., 1998; Gelernter et al., 1999; Franke et al., 2001; Rommelspacher et al., 2001). Such genetic studies may define genetic predisposition and associated risk for drug addiction in individuals. Moreover, they can contribute to delineate the importance of specific genes in the neurobiological mechanisms of addiction.

Environmental factors, endogenous opioids and reinforcement

In contrast to genetic, intrinsic factors, environmental influences may also affect the functionality of the endogenous opioid system and could thereby contribute to the reinforcing efficacy of drugs of abuse and hence, the vulnerability for drug dependence. In this section, prenatal morphine, emotional stress and play deprivation will be outlined briefly as examples of such environmental factors, which facilitate drug reinforcement, presumably through changes in endogenous opioid systems.

(1) *Prenatal morphine* treatment leads to increased μ-opioid receptor binding in rats (Vathy et al., 2003). Interestingly, prenatal morphine treatment also causes rats, as adults, to be more sensitive to the reinforcing effects of both heroin and cocaine (Ramsey et al., 1993). (2) Another example is *emotional stress*, which is a witness stress: the emotional stressed animal can see, hear, smell but not touch the physically stressed subject, that receives uncontrollable footshocks (Takahashi et al., 1987). Cocaine and morphine self-administration was facilitated by emotional stress in rats and mice (Ramsey & Van Ree, 1993; Kuzmin et al., 1996) and also intracranial self-stimulation was facilitated by emotional stress (Bespalov, unpublished data). By means of autoradiography and naloxone administration, changes in the endogenous opioid system were demonstrated after emotional stress (Van den Berg et al., 1998; Pijlman, unpublished data). (3) Further, isolation of rats during postnatal weeks 4-5 in which rats normally display high levels of play behaviour, which involves increased endogenous opioid peptide release (Panksepp & Bishop, 1981), has been associated with adaptations in opioid peptide and opioid receptor levels (Vanderschuren et al., 1995; Van den Berg et al., 1999). Recent findings revealed, that *play deprivation* caused facilitation, although modest, of the acquisition of cocaine self-administration in adults rats (Gerrits, unpublished data).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study revealed that μ-opioid receptors have an important role in cocaine reinforcement, suggesting that variations in μ-opioid receptors might contribute to vulnerability to develop drug dependence. Factors that affect the function of the μ-opioid receptor should be considered to influence drug dependence. The genetic make-up of the μ-opioid receptor gene and developmental and environmental factors affecting its expression could be part of a neurobiological process underlying vulnerability for drug dependence. An important next step

will be to confirm that variations in μ-opioid receptors indeed contribute to liability to develop drug dependence.

Repeated and extended self-administration sessions in models that mimic human addiction more are required in preclinical studies. Such models of extended access, described by different groups, are for example characterized by escalating and irregular patterns of drug intake and would be valuable tools in this respect (Ahmed & Koob, 1998; Tornatzky & Miczek, 2000; Mantsch et al., 2001). Important to consider in this context is the notion that, based on previous studies which for instance described a rightward shift in cocaine self-administration after naloxone treatment (Kuzmin et al., 1997a), μ-opioid receptors probably modulate drug reinforcement but may not be required as such for drugs to act as reinforcers. Therefore, μ-opioid receptor knockout mice may eventually acquire cocaine self-administration. If true, it will also be interesting to investigate the reinstatement behaviour of μ-opioid receptor knockout mice, considering the reduction in dopamine D3 receptor levels observed for these mice (**Chapter 6**).

Clearly the present findings suggest further human research to determine the role of μ-opioid receptor variations in susceptibility to drug dependence. Since variations in μ-opioid receptors may be genetic in nature or may be induced by environmental factors, such as traumatic life events, attempts should be made to differentiate between variations in μ-opioid receptors caused by genetic and environmental factors. Moreover it is interesting to consider gene × environment interactions in connection to vulnerability for drug dependence.

Although the main output of the VTA is formed by dopamine projections to amongst others the nucleus accumbens, GABA projections to nucleus accumbens and prefrontal cortex have also been described to originate in the VTA (Steffensen et al., 1998). These projections have been poorly studied in relation to addiction processes and it will be interesting to focus more on these projections and to investigate their role in drug reinforcement. Also, it is interesting to consider in future studies the role of nucleus accumbens medium spiny neurons in drug reinforcement.

Finally, future studies could also focus on cannabinoid CB1 receptors, which appear to differentially modulate opiate and alcohol as opposed to psychostimulant reinforcement. Knowledge of the mechanisms through which cannabinoid CB1 receptors interfere with opiate and alcohol reinforcement may provide more insight into the distinct mechanisms of action of depressant and psychostimulant drugs.

The findings described in this thesis suggest a role of μ-opioid receptors, and neurobiological mechanisms associated to μ-opioid receptors, in vulnerability for drug dependence. This knowledge may lead to improvement of strategies in prevention and treatment of addiction.

Chapter 10

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A

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CHAPTER 11

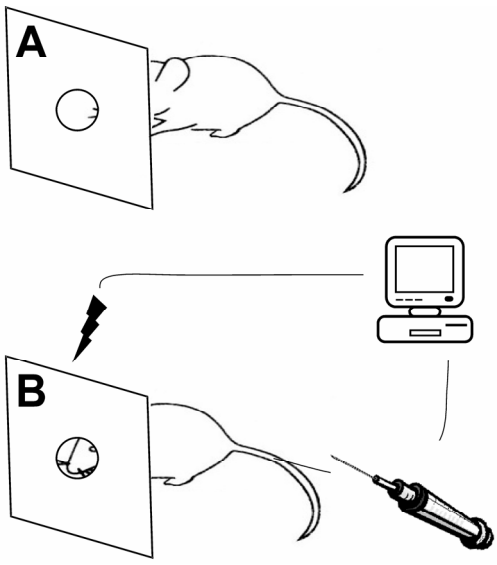
SAMENVATTING

Drugsverslaving

Drugsverslaving is een wereldwijd gezondheidsprobleem dat gekarakteriseerd wordt door dwangmatig drugsgebruik en een hoge mate van terugval na afkicken. Voorbeelden van verslavende stoffen zijn morfine en heroïne (opiaten), cocaïne en amfetamine (psychostimulantia), alcohol, nicotine en Δ^9-tetrahydrocannabinol (Δ^9-THC, de actieve stof in marihuana). Deze verslavende stoffen behoren tot verschillende farmacologische klassen, wat wil zeggen dat ze elk een ander primair werkingsmechanisme hebben.

Circa 5-15% van diegenen die ooit een verslavende stof gebruikt hebben ontwikkelt daadwerkelijk problematisch gebruik van die stof. Drugsgebruik leidt dus niet altijd tot drugsverslaving. Er bestaan individuele verschillen in de gevoeligheid voor verslaving en vermoedelijk bestaan er dus risicofactoren voor problematisch drugsgebruik en drugsverslaving. Verslavende stoffen hebben gemeen dat ze positief bekrachtigende (reinforcing) eigenschappen bezitten. Positieve reinforcement is een maat voor de facilitering van gedrag dat nodig is om bijvoorbeeld de verslavende stof te verdienen of te verkrijgen. De gevoeligheid voor de reinforcing effecten van verslavende stoffen is waarschijnlijk een belangrijke bepalende factor voor het risico op herhaling van drugsgebruik, hetgeen uiteindelijk tot afhankelijkheid van de verslavende stof, ofwel tot drugsverslaving, kan leiden.

Reeds in de jaren zeventig werd een proefdiermodel ontwikkeld waarmee deze positieve reinforcing effecten van verslavende stoffen kunnen worden gemeten: de intraveneuze zelftoediening. In dit model leert bijvoorbeeld een muis om zichzelf een verslavende stof zoals cocaïne direct in de bloedbaan toe te dienen, door bijvoorbeeld zijn neus in een opening in de wand te steken (nose-poke). De straal die door de nose-poke wordt doorbroken wordt door een computer geregistreerd. Vervolgens wordt een signaal doorgegeven naar een pomp, die via een in de staartvene aangebrachte naald en canule zorgt voor een intraveneuze injectie in de staartvene van de muis (zie Figuur 1).



FIGUUR 1
Intraveneuze zelftoediening. De muis in **A** zit in de zelftoedieningsopstelling maar laat geen nose-poke respons zien en krijgt dus ook geen injectie. De muis in **B** steekt zijn neus door de opening, wat door de computer wordt geregistreerd. De pomp wordt geactiveerd en de muis krijgt een injectie. De snelheid waarmee de stof in het bloed komt is via intraveneuze injectie zeer snel, waardoor de muis het effect van de stof zal associëren met de nose-poke respons.

Voorgaande studies hebben, onder meer met behulp van intraveneuze zelftoediening door proefdieren, inzicht verschaft in de neurobiologische mechanismen van verslaving (**hoofdstuk 1**) en in de rol van specifieke genen in verslavingsprocessen (**hoofdstuk 2**). Er zijn in dit verband meerdere systemen beschreven, waaronder het dopamine-, het opioid- en het cannabinoid-systeem.

Hoewel het dopamine-systeem als belangrijke speler in verslavingsprocessen wordt gezien is dit systeem niet noodzakelijk voor de reinforcing effecten van alle verslavende stoffen. Zo leidt beschadiging van dopamine neuronen met behulp van het toxine 6-hydroxydopamine of blokkade van dopamine receptoren met dopamine antagonisten wel tot een verlaging van intraveneuze zelftoediening van psychostimulantia, maar niet van opiaten of alcohol. Met andere woorden, dopamine is wel noodzakelijk voor de reinforcing effecten van psychostimulantia, maar is niet vereist voor de reinforcing effecten van opiaten en alcohol.

Een ander belangrijk systeem in verslavingsprocessen is het endogene opioid systeem, dat bestaat uit de lichaamseigen opioiden β-endorphine, enkephaline en dynorphine, die respectievelijk binden aan μ-, δ- en κ-opioid receptoren. Blokkade van opioid receptoren met behulp van de opioid receptor antagonisten naltrexone of naloxone, verlaagt zelftoediening van cocaïne en alcohol door ratten, muizen en apen. Hiermee lijkt het opioid systeem wel een centrale rol te kunnen spelen in de bekrachtigende werking van verschillende farmacologische klassen van verslavende stoffen. Een andere belangrijke vinding was die van Ramsey en collegae (1999). Zij lieten, met behulp van lokale injecties van naltrexone in verschillende hersengebieden, zien dat met name de opioid receptoren in het ventrale tegmentum (VTA), een kleine structuur in het midbrein, belangrijk zijn voor de reinforcing effecten van cocaïne. Omdat naltrexone en naloxone relatief selectief zijn voor μ-opioid receptoren en omdat de VTA relatief rijk is aan μ-opioid receptoren, wordt verondersteld dat voornamelijk μ-opioid receptoren in belangrijke mate betrokken zijn bij cocaïne reinforcement, hetgeen het onderwerp is van de in dit proefschrift beschreven studies.

Doelstelling

Het doel van de studies beschreven in dit proefschrift is om de rol van de μ-opioid receptor in cocaïne reinforcement te bepalen en om inzicht te verkrijgen in de daaraan ten grondslag liggende mechanismen.

μ-opioid receptoren en gedragseffecten van cocaïne

Voor het bestuderen van de rol van de μ-opioid receptor in de reinforcing effecten van cocaïne werd in **hoofdstuk 3** gebruik gemaakt van muizen waarin het gen voor de μ-opioid receptor blijvend is uitgeschakeld, 'uitgeknockt' (zie **hoofdstuk 2**). Met behulp van deze 'μ-opioid receptor knockout muizen', die dus functionele μ-opioid receptoren missen, is een cruciale rol van μ-opioid receptoren in cocaïne reinforcement aangetoond. Vergeleken met wild-type controle muizen bleek de zelftoediening van cocaïne door μ-opioid receptor knockout muizen

verlaagd: het aantal nose-pokes voor cocaïne injecties was significant lager. Dit duidt op verlaagde gevoeligheid voor de reinforcing effecten van cocaïne in afwezigheid van μ-opioid receptoren. De μ-opioid receptor blijkt dus een belangrijke en specifieke rol te spelen in cocaïne reinforcement. Dit betekent dat variatie in μ-opioid receptoren inderdaad een risicofactor kan zijn voor de ontwikkeling van cocaïne verslaving.

Vervolgens is onderzocht of de μ-opioid receptor ook andere effecten van cocaïne moduleert. Naast de reinforcing effecten verhoogt cocaïne ook de motorische activiteit. In **hoofdstuk 5** werd de rol van de μ-opioid receptor in de acute motorische effecten van cocaïne bestudeerd. Hiervoor werd opnieuw gebruik gemaakt van de μ-opioid receptor knockout muis. Daarnaast werden ook muizen gebruikt die juist gekenmerkt worden door een verhoogde expressie van opioid receptoren. Deze muizen werden gedurende 1 week met de opioid receptor antagonist naltrexone (NTX) behandeld, waardoor de opioid receptoren geblokkeerd werden. Vervolgens werd deze behandeling met NTX gestaakt. In reactie op chronische blokkade van opioid receptoren trad er compensatie op in de vorm van meer opioid receptoren. Dit werd met behulp van *in vitro* receptor binding aangetoond, zoals in **hoofdstuk 7** beschreven. Noch de afwezigheid (μ-opioid receptor knockout) noch een toename van het aantal μ-opioid receptoren (chronisch NTX) bleek van invloed op de acute cocaïne-geïnduceerde motorische respons. Hieruit kan geconcludeerd worden dat de μ-opioid receptor niet belangrijk is voor acute motorische effecten van cocaïne.

Naast de betrokkenheid van het μ-opioid receptor systeem bij acute effecten van cocaïne werd tevens de rol van de μ-opioid receptor in chronische effecten van cocaïne bestudeerd. Verschillende studies laten zien dat er, bij langdurige blootstelling aan verslavende stoffen, adaptaties optreden in de hersenen die vermoedelijk bijdragen aan dwangmatig drugsgebruik en het daadwerkelijk verslaafd raken. Langdurige blootstelling aan cocaïne leidt eveneens tot een versterkte motorische respons op cocaïne, een effect dat wordt toegeschreven aan veranderingen in dezelfde hersengebieden als die verantwoordelijk zijn voor dwangmatig drugsgebruik. Om deze reden is de versterkte motorische respons op cocaïne, ofwel cocaïne-geïnduceerde gedrags-sensitizatie een interessant fenomeen. In **hoofdstuk 5** werd daarom de rol van de μ-opioid receptor in cocaïne-geïnduceerde gedragssensitizatie onderzocht. De μ-opioid receptor knockout muizen ontwikkelden sensitizatie zoals wild-type muizen dat deden. Bovendien had ook de specifieke μ-opioid receptor antagonist CTOP geen effect op de ontwikkeling van cocaïne-geïnduceerde sensitizatie. Daarentegen lieten muizen, die chronisch behandeld waren met NTX, een versterkte cocaïne-geïnduceerde gedragssensitizatie zien. Het lijkt onwaarschijnlijk, gezien de vindingen uit het experiment met de μ-opioid receptor knockout muizen en die uit het experiment met CTOP, dat de versterkte sensitizatie in chronisch NTX behandelde muizen toe te schrijven is aan een verhoogd aantal μ-opioid receptoren. Het is mogelijk, dat δ-opioid receptoren, die onder invloed van chronisch NTX ook in aantal toenemen (**hoofdstuk 7**), hebben bijgedragen aan de versterkte cocaïne-geïnduceerde gedragssensitizatie in 'chronisch NTX muizen'.

Neurobiologische mechanismen

De overige experimenten beschreven in dit proefschrift gaan in op het neurobiologisch mechanisme via welke μ-opioid receptoren cocaïne reinforcement moduleren. Het is bekend dat cocaïne een verhoogde afgifte van endogene opioiden, zoals β-endorphine, veroorzaakt. In **hoofdstuk 3** is een interessant experiment beschreven, waarbij gebruik werd gemaakt van het zelftoedieningsmodel. Zoals uitgelegd kan een muis zichzelf cocaïne toedienen door middel van nose-poke responsen. Ter controle werd altijd een tweede muis gekoppeld aan dezelfde pomp als deze 'actieve' muis. Omdat stoffen als cocaïne ook de motorische activiteit kunnen verhogen is een dergelijke controle van belang. In tegenstelling tot de 'actieve' muis had deze tweede, 'passieve', muis geen controle over de cocaïne die hij kreeg toegediend, maar kon deze wel nose-poken, wat een maat is voor activiteit. Op deze manier werd het effect van 'actief' en 'passief' toegediende cocaïne op de expressie van POMC, de voorloper van het μ-opioid receptor selectieve endogene opioid β-endorphine, bestudeerd en vergeleken. De 'actieve' en 'passieve' muizen werden 30 minuten na beëindiging van een zelftoedieningssessie gedood. Vervolgens werd de expressie van POMC mRNA bepaald in de hersenen van deze dieren. Er werd een positief verband tussen cocaïne inname en POMC mRNA expressie gevonden, echter alleen voor 'actieve' en niet voor 'passieve' cocaïne inname. Hoe meer cocaïne actief werd toegediend hoe meer POMC mRNA tot expressie kwam. Aangezien actieve zelftoediening een maat is voor cocaïne reinforcement geven deze bevindingen aan dat endogene opioiden inderdaad betrokken zijn bij cocaïne reinforcement.

Voorts werd de aandacht gericht op μ-opioid receptoren in het ventrale tegmentum (VTA), waar een sleutelrol voor opioid-modulatie van cocaïne reinforcement is weggelegd. In **hoofdstuk 3** is gekeken naar veranderingen in signaaloverdracht in de VTA van μ-opioid receptor knockout muizen, die mogelijk de verlaging in cocaïne zelftoediening zouden kunnen verklaren. μ-Opioid receptoren bevinden zich in de VTA op GABA neuronen. Activering van μ-opioid receptoren leidt tot een verlaging van de activiteit van deze GABA neuronen, die op hun beurt een remmend effect uitoefenen op dopamine neuronen in de VTA. Deze dopamine neuronen projecteren onder meer naar de nucleus accumbens en de prefrontale cortex. Dopamine neuronen in de VTA van μ-opioid receptor knockout muizen, die zoals gezegd geen functionele μ-opioid receptoren bezitten, bleken sterker geremd te worden door GABA neuronen. Dit betekent dat de activiteit van het zogenaamde mesolimbische dopamine systeem, met projecties vanuit de VTA naar onder meer de nucleus accumbens en prefrontale cortex, is verlaagd. Deze verandering in signaaloverdracht in de VTA zou daarom inderdaad kunnen bijdragen tot de verlaagde cocaïne reinforcement in deze knockout muizen.

Het endogene opioid systeem interacteert dus met het dopamine systeem, zoals reeds eerder door andere onderzoekers werd aangetoond. Dit riep vragen op over mogelijke veranderingen in het dopamine systeem die opgetreden zouden kunnen zijn in afwezigheid van de μ-opioid receptor, zoals dat bij de μ-opioid receptor knockout muis het geval is, of in het geval van overexpressie van opioid receptoren in het geval van chronisch NTX behandelde muizen (**hoofdstuk 7**). In **hoofdstuk 6** werden de dopamine receptor niveaus bepaald met behulp van

in vitro receptor binding. Dopamine receptoren zijn ingedeeld in 'D1-like' en 'D2-like' receptoren. Er werd geen effect gevonden van afwezigheid of over-expressie van opioid receptoren op de expressie van beide hoofdklassen van dopamine receptoren. Verder onderzoek wees uit dat de dopamine D3 receptor, een D2-subtype, verlaagd was in de nucleus accumbens en het striatum van μ-opioid receptor knockout muizen. Dit is een interessante vinding omdat uit recent onderzoek in proefdieren is gebleken dat D3 receptor blokkade terugval na afkicken kan verlagen. Mogelijk zijn de μ-opioid receptor knockout muizen eveneens afwijkend in terugval in drugsgebruik na een periode van onthouding, hetgeen onderwerp moet zijn van toekomstig onderzoek.

In **hoofdstuk 6** is eveneens gekeken naar dopamine-gemedieerd gedrag van de μ-opioid receptor knockout en chronisch NTX behandelde muizen. In een open veld waren de μ-opioid receptor knockout muizen minder actief terwijl chronisch NTX behandelde muizen juist actiever waren ten opzichte van hun controles in een open veld. Dit kan duiden op verschillen in het dopamine systeem. Dit onderstreept het belang van goede controles, met name voor activiteit, in cocaïne zelftoediening.

Het cannabinoid systeem en cocaïne

Tot slot is in **hoofdstuk 8** gekeken naar de rol van het endogene cannabinoid systeem in het moduleren van cocaïne reinforcement en van cocaïne-geïnduceerde gedragssensitizatie. Er werd geen effect op cocaïne zelftoediening waargenomen van blokkade van cannabinoid CB1 receptoren door middel van de CB1 antagonist SR141716A. Verder had SR141716A ook geen effect op cocaïne-geïnduceerde gedragssensitizatie. Uit deze bevindingen kan geconcludeerd worden dat CB1 receptoren niet betrokken zijn bij cocaïne reinforcement en bij lange-termijn adaptaties die zouden kunnen bijdragen tot het ontwikkelen van cocaïneverslaving.

Conclusie

De belangrijkste bevinding van dit proefschrift is dat de μ-opioid receptor een belangrijke en specifieke rol speelt in cocaïne reinforcement. Op basis daarvan en in combinatie met overige studies, die laten zien dat de μ-opioid receptor ook belangrijk is voor alcohol en opiaat reinforcement, zou variatie in μ-opioid receptor niveaus inderdaad een risicofactor kunnen vormen voor het ontwikkelen van cocaïneverslaving. Momenteel wordt veel aandacht besteed aan varianten van het μ-opioid receptor gen in relatie tot drugsverslaving bij de mens. De resultaten beschreven in dit proefschrift onderstrepen het belang van verder onderzoek naar μ-opioid receptoren met betrekking tot het risico voor het ontwikkelen van drugsverslaving.

CURRICULUM VITAE

Heidi Lesscher werd op 10 januari 1976 geboren te Almelo. In 1994 behaalde zij haar VWO diploma aan het Lyceum de Grundel te Hengelo, waarna zij met de studie Biofarmaceutische Wetenschappen begon binnen het Leiden/Amsterdam Center for Drug Research (LACDR) aan de Universiteit Leiden.

Haar eerste wetenschappelijke stage werd uitgevoerd bij de afdeling Medische Farmacologie van het LACDR. Hier deed zij onder begeleiding van drs. J.O. Workel en prof. dr. E.R. de Kloet onderzoek naar lange-termijn effecten van maternale deprivatie. Aansluitend deed zij bij SmithKline Beecham (Harlow, Groot-Brittannië), onderzoek naar de gedrags-effecten en localisatie van PrRP, het ligand van een nieuwe G-eiwit gekoppelde receptor. Hierin werd zij begeleid door dr. D.N.C. Jones en dr. J.J. Hagan. In 1999 werd het doctoraal diploma in de Biofarmaceutische Wetenschappen behaald.

In 1999 is Heidi Lesscher aangesteld als assistent in opleiding bij de afdeling Farmacologie & Anatomie van het Rudolf Magnus Instituut voor Neurowetenschappen. De resultaten van het promotie-onderzoek dat zij aldus heeft uitgevoerd onder begeleiding van dr. M.A.F.M. Gerrits, prof. dr. J.P.H. Burbach en prof. dr. J.M. van Ree, staan beschreven in dit proefschrift. Een gedeelte van dit onderzoek werd uitgevoerd aan de Universiteit van Surrey (Guildford, Groot-Brittanië) in samenwerking met dr. A. Bailey en prof. dr. I. Kitchen van de Pharmacology Group van deze universiteit. Vanaf maart 2004 zal Heidi Lesscher als postdoc werkzaam zijn bij de Neurology Department van het Ernest Gallo Clinic and Research Center, University of California San Francisco bij prof. dr. E.R. Messing.

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