MODULATORS OF DRUG DEPENDENCE PHENOMENA

Factors affecting morphine withdrawal syndrome and cocaine-intake in rodents

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MODULATORS OF DRUG DEPENDENCE PHENOMENA

Factors affecting morphine withdrawal syndrome and cocaine-intake in rodents

Onderzoek naar factoren die de expressie van het morfine-onthoudingssyndroom en cocaïne-inname in proefdieren beïnvloeden

(met een samenvatting in het Nederlands)

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"Iedere grootse plaats is alleen bereikbaar via een wenteltrap"
Sir Francis Bacon
Essays

Voor mijn ouders
Aan Robert

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Abbreviations

AC	adenylate cyclase
ACh	acetylcholine
BDZ	benzodiazepine
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dopamine
EAA	excitatory amino acid
EEG	electro encephalography
GABA	gamma-amino butyric acid
GLU	glutamate
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine (serotonin)
ICD-10	international classification diseases version 10
i.p.	intraperitoneal
i.v.	intravenous
LC	locus coeruleus
LSD	lysergic acid diethylamide
MDMA	3,4-methylenedioxymetamphetamine
NA	noradrenaline/norepinephrine
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
PCP	phencyclidine
s.c.	subcutaneous
THC	tetrahydrocannabinol
VEP	visual evoked potentials
VTA	ventral tegmental area
WHO	world health organization

Outline of the studies

This thesis compiles the experimental studies on several drugs, which modulate drug dependence phenomena in rodents. The main part of the studies is related to the morphine withdrawal (*chapters 3-7*), while a minor part is dealing with cocaine psychic dependence (*chapter 9*).

Part 1: ***Drug Dependence and Harmful Use of Drugs***

Chapter 1

In both men and animals, drug dependence phenomena have continuously been studied over the past decades. However, much confusion and discussion was (and is still) going on about terms such as dependence, abuse, addiction, etc. Therefore, these and other terms are firstly defined, while in the second part of this chapter, several classes of dependence-producing drugs are described. The characteristics of these drugs are discussed in respect to dependence, tolerance and withdrawal, and also some information related to their behavioral effects and mechanism of action is provided.

Part 2: ***Opioid Dependence***

Chapter 2

This chapter starts with a classification of opioids and their receptors and is followed by information related to morphine dependence phenomena, particularly tolerance and withdrawal syndrome.

Part 3: ***Morphine Withdrawal Syndrome - Experimental Studies***

Chapter 3

It has been shown that during morphine withdrawal, an enhanced release of several neurotransmitters occurs, including L-glutamate. The excitatory amino acid (EAA) L-glutamate, released presynaptically, activates the postsynaptically localized N-methyl-D-aspartate (NMDA)-glutamate receptors (Fig. 1). Since the opioid withdrawal syndrome is a reflection of neuronal and behavioral excitation, we studied the role of the excitatory glutamate system in the opioid withdrawal. We demonstrated that administration of NMDA receptor antagonists attenuated naloxone-precipitated withdrawal syndrome in morphine-dependent mice.

Chapter 4 and 5

Following the demonstration that NMDA receptor blocking agents attenuated morphine withdrawal syndrome (*chapter 3*), we postulated that endogenous compounds synthesized in response to stimulation of NMDA receptors may also play a role in the expression of opioid withdrawal syndrome. Among these compounds is the "new" peripheral and central neurotransmitter nitric oxide (NO). NMDA receptor mediated Ca^{2+} entry into the cell may stimulate Ca^{2+} dependent NO synthase (NOS) enzyme in some neurons, resulting in the formation of NO (Fig. 1). In order to examine the effect of this neurotransmitter on naloxone-precipitated withdrawal syndrome, we blocked the NO synthesis in both morphine-dependent mice (*chapter 4*) and rats (*chapter 5*) by NOS inhibitors. These two studies showed that the expression of the naloxone-precipitated withdrawal syndrome in morphine-dependent mice and rats can be affected by inhibition of NO synthesis. We suggest that these preclinical studies justify clinical trials of NOS inhibitors in drug-dependent subjects.

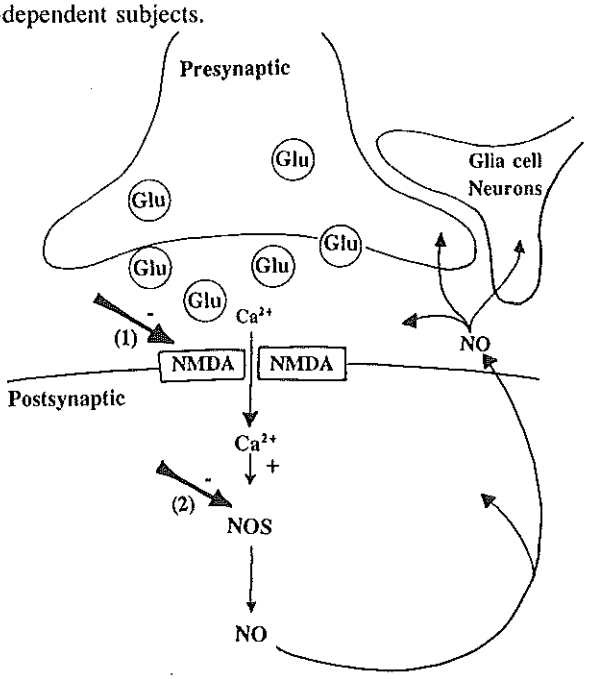

Fig. 1. Nitric oxide (NO) is produced following N-methyl-D-aspartate (NMDA) receptor activation and subsequent increase in intracellular Ca^{2+}. NO diffuses to adjacent glia and/or other neurons where it induces a wide variety of actions.

Glu = glutamate; $\xrightarrow{+}$ = stimulation; $\xrightarrow{-}$ = inhibition/blockade.

(1) Blockade of NMDA receptors by diverse NMDA receptor blocking agents. Results are described in *chapter 3*.

(2) Blockade of NO synthesis by NO synthase (NOS) inhibitors. Results are described in *chapters 4* and *5*.

Chapter 6

There are indications that some peptides released in cerebrospinal fluid (CSF) may modulate opioid withdrawal. In this study, we showed that the CSF of spontaneous morphine-abstinent donor rat precipitates in morphine-dependent recipient rat an opioid withdrawal syndrome. During this CSF-induced morphine withdrawal syndrome a decrease of the peak latency of visual evoked potentials was registered, indicating an enhanced central excitability. The CSF-induced morphine withdrawal syndrome is behaviorally less severe and electrophysiologically less prominent, but qualitatively identical to the naloxone-induced abstinence. However, in contrast to naloxone, the CSF from spontaneous morphine-abstinent rat does not exert a contraction of isolated morphine-dependent guinea-pig ileum. Chromatographic analysis of the CSF has shown that a putative "withdrawal substance" is present only in the CSF of spontaneous morphine-abstinent rats, but not in the CSF of naive or morphine-dependent rats. The "withdrawal substance" is a hydrophobic compound, without naloxone-like properties. However, further analysis of the biochemical structure and bioactivity are necessary.

Chapter 7

Studies, performed earlier at the Department of Pharmacology, Erasmus University Rotterdam, have shown that ibogaine may attenuate naloxone-precipitated withdrawal in morphine-dependent rats. It has been also claimed that ibogaine is an anti-addictive drug, presently undergoing a clinical trial in several countries. However, norharman is an endogenous physiological substance, with a biochemical structure related to ibogaine. Both drugs are indole derivatives with psychotogenic properties. Therefore, we performed a comparative study with these drugs in relation to the expression of opioid withdrawal syndrome. We have shown that norharman (parenteral administration) had a more prominent anti-withdrawal effect than ibogaine. It is known that norharman binds at the α-subunit of the gamma-amino butyric acid (GABA) receptor-complex. This physiological substance and the GABA receptor-complex might be a target for further elucidation of drug dependence phenomena.

Part 4: ***Drug Dependence induced by Psychostimulants***

Chapter 8

This chapter gives a general description of psychostimulants, with recent data mainly related to the cocaine dependence.

Part 5: ***Cocaine Dependence - Experimental Study***

Chapter 9

We examined the effect of ibogaine treatment on cocaine self-administration in rats. In this study ibogaine was selected for two reasons. Our earlier experiments have shown that ibogaine attenuates morphine withdrawal in rats. In addition, the non-controlled observations in humans demonstrated that ibogaine interrupts drug dependence on alcohol, amphetamine and nicotine. We have shown that ibogaine is a long-lasting interruptor of cocaine self-administration in cocaine-dependent rats. These preclinical studies justify a clinical trial of ibogaine in drug-dependent subjects, which presently takes place in several countries.

Concluding remarks and some suggestions for further research are given at the end of this thesis.

PART 1

Drug Dependence and Harmful Use of Drugs

Chapter 1

Definition of terms and classification of dependence-producing drugs

1.1. Terminology

In 1967, the World Health Organization (WHO) raised several criteria for drug addiction. The terms used in this thesis are in accordance with the definitions given by the WHO as proposed in their 28th report (1992), which in its turn, is in accordance with the International Classification Diseases (ICD-10) of mental and behavioral disorders (Hoffman, 1983).

Drug dependence:

replaced the term "drug addiction" and is defined as "a state, psychic and sometimes also physical, resulting from the interaction between a living organism and a drug, characterized by behavioral and other responses that always include a compulsion to take the drug on a continuous or periodic basis in order to experience its psychic effects, and sometimes to avoid the discomfort of its absence. Tolerance may or may not be present. A person may be dependent on more than one drug".

Harmful use:

replaced the term "abuse" and is defined as "a pattern of psycho-active drug use that causes damage to health, either mental or physical. Harmful use of drugs by an individual often has adverse effects on the drug user's family, community and society, in general".

The existence of a state of drug dependence is not necessarily harmful in itself, but may lead to the use of the drug(s) at dosage levels that produce deleterious physical or behavioral changes, constituting public health and social problems.

Tolerance:

is defined as "a reduction in the sensitivity to a drug following its repeated administration, in which increased doses are required to produce the same magnitude of effect previously produced by a smaller dose. This increase in dose may be necessitated by changes in the metabolism of the drug, or a cellular, physiological or behavioral adaptation to the effects of the drug".

Sensitization ("reverse-tolerance"):

describes the situation in which a constant drug dose elicits increasing effects (Nestler et al., 1993). It differs from tolerance since less drug is required to reinstate the initial effect.

Withdrawal syndrome:

is described as "after the repeated administration of certain dependence-producing

drugs, e.g. opioids, barbiturates and alcohol, abstinence can increase the intensity of drug-seeking behavior, because of the need to avoid or relieve withdrawal discomfort and/or produce physiological changes of sufficient severity to require medical treatment".

The withdrawal syndrome following cessation of hypnosedatives (Roelofs, 1985) or opioids (Martin and Eades, 1963) has a mainly excitatory character, which may culminate in an epileptic convulsion. In contrast, drug dependence induced by stimulants (Gawin and Kleber, 1986) or cannabinoids (Jones, 1983) give rise to a sedative withdrawal syndrome, which is less inconvenient and clinically less important.

Craving:

is defined as "the desire to experience the effect(s) of a previously used psycho-active substance". It has to be noted that not all drug craving is based on withdrawal, since craving can often occur in the absence of withdrawal (Markou et al., 1993).

Stimulus:

is defined as "an environmental event that produces a change in the behavior of an organism".

Response:

is defined as "the behavioral consequence of presenting a stimulus to an organism".

Positive reinforcer:

is defined as "a stimulus that increases the frequency of behavior that leads to its presentation". For example, if a hungry rat, placed in a box, presses a bar and is then given food, the animal will have a "positive" experience. The probability of a particular response (the bar press) has been increased through the immediate delivery of the "positive reinforcer" (the food). Things such as food, water, sex, and the opportunity to explore are usually considered as positive reinforcers (Houston, 1986). Also many dependence-producing drugs, such as cocaine, morphine, phencyclidine (PCP), barbiturates, ethanol and some volatile solvents serve as a positive reinforcer (Stolerman, 1992).

Negative reinforcer:

is defined as "stimulus that increases the frequency of a behavior that prevents or terminates its presentation". Generally speaking, noxious stimuli, such as shock are considered to be negative reinforcers.

Aversive stimulus:

is defined as "stimulus causing an organism to behave so as to minimize exposure to it (as in negative reinforcement or punishment procedures)".

Conditioning:

generally refers to relative simple learning situations, such as classical- and instrumental conditioning (Houston, 1986).

- *Classical/Pavlovian conditioning:*
  is defined as "procedures that present different stimuli in temporal proximity (contiguity), but where resulting responses have no reinforcing or aversive consequences". Well-known are the experiments performed by Pavlov, in which dogs were conditioned to salivate at the sound of a tone.
- *Instrumental/Operant conditioning:*
  is defined as "procedures where responses have reinforcing or aversive consequences and are instrumental (for example pressing a bar) in attainment of a goal (getting food or dependence-producing drugs)".

Reward:
is often defined similarly as reinforcement, but with some positive affective colouring, such as pleasure (Stolerman, 1992).

The most important animal models to study rewarding properties of drugs are:

- *Intracranial electrical self-stimulation in specific brain regions.* In this model electrodes are implanted in brain regions, with physiologically active dopaminergic (DA-ergic) systems (Fibiger and Phillips, 1988). The role of the DA-ergic system in respect to reinforcement is discussed in more detail (see *chapter 2* and *chapter 8*).

- *Place preference conditioning.* The apparatus used in this model consists of two different compartments (differences could be of visual-, tactile- or odour origin). During conditioning sessions, animals are allowed to access to only one compartment at a time. One compartment is repeatedly paired with drug injections and the other compartment with vehicle injections. During test sessions, the animals have access to the whole apparatus and the amounts of time spent in each compartment are usually recorded by a system of light beams and photo cells.

- *Self-administration model.* In this model a drug serves as a reinforcer of behaviour. The drugs are mostly obtained by an indwelling intravenously catheter (see technical details in *chapter 9*). Dependence-producing drugs (with exception of lysergic acid diethylamide (LSD) and cannabinoids) can serve as positive reinforcers in the self-administration model in rats and monkeys (Stolerman, 1992).

1.2. Dependence-producing drugs

ICD-10 recognizes the following psycho-active drugs or substances, which may produce drug-dependence:

- hypnosedatives
- cannabinoids
- hallucinogens
- tobacco
- volatile solvents
- opioids

- psychostimulants

In the following paragraphs of this chapter, *recent* experimental data relevant to the dependence-inducing properties of these drugs are briefly discussed.

1.2.1. Hypnosedatives

Drugs belonging to this group are ethanol (alcohol), benzodiazepines (BDZs) and barbiturates. In general, these compounds induce sleep and reduce anxiety.

A. Alcohol

Action on cellular level

There are indications that the binding place of alcohol is on the α-subunit section 6 (α6) of $GABA_A$ (γ-amino butyric acid) receptor-complex (Korpi and Seeburg, 1993). However, no substance is known, which might interfere with the binding place of alcohol. The importance of this subunit in respect to alcohol drug dependence has to be revealed in future.

If labelled membranes from neurons are exposed to intoxicating concentrations of alcohol, an increased "motion" within the membrane was observed (*"membrane fluidity theory"*: Goldstein, 1984). This disordering (fluidizing) effect of alcohol on the membrane may affect some receptors, such as the GABA or/and the NMDA (N-methyl-D-aspartate) receptors of the excitatory amino acid (EAA) glutamate. Accordingly, chronic alcohol treatments reduces $GABA_A$ function (Buck and Harris, 1991). GABA receptor systems (together with serotonin and noradrenaline) seemed to be involved in the decreased compulsivity of alcohol intake (Deitrich et al., 1989). During chronic alcohol use, there is an up-regulation of the NMDA receptors (probably due to NMDA receptor blockade) in the hippocampus, a brain area known to be associated with ethanol withdrawal seizure activity (Grant et al., 1990). Removal of alcohol induces a state of excessive EAA activation which may contribute to the alcohol withdrawal excitability (Grant et al., 1990; Michaelis et al., 1993). Alcohol use inhibits the production of nitric oxide (NO, Persson and Gustafsson, 1992), which could be a result of NMDA receptor blockade. However, further research is necessary to reveal whether chronic alcohol intake could alter NO production and bring some clarification in alcohol-related pathology.

Besides the GABA and EAA-NMDA receptor-complex, alcohol affects a variety of other neurotransmitter systems. Of particular importance is the fact that alcohol interferes with DA-ergic rewarding pathway, which is claimed to mediate positive reinforcement (Samson et al., 1990). It has been found that both systemic and locally-infused alcohol stimulate the release of dopamine (DA) in the nucleus accumbens (part of the meso-corticolimbic DA projection). Conversely, an alcohol withdrawal is associated with reduced release of DA in this pathway (Nutt and Peters, 1994). To some extent, it has

been demonstrated that DA receptor antagonists are able to block the reinforcing actions of alcohol (Nutt and Peters, 1994).

Ethanol also interacts with the endogenous opioid system. Acute administration of ethanol increased plasma levels of ß-endorphin in humans (Barret et al., 1987) and met-enkephalin in rat brain and pituitary (Seizinger et al., 1983). These findings might be of relevance, since opioid receptor antagonists tend to reduce alcohol consumption (Goldstein, 1984).

Tolerance

Repeated administration of alcohol results in tolerance for most of the effects of this drug (hypothermia, sedation, anxiolytic and motoric effects) in both humans (Tabakoff and Hoffman, 1988) and animals (Holloway et al., 1989). The acute tolerance for alcohol can be influenced by genetic selection, in a way that animals selected for higher ethanol preference demonstrate a greater acute tolerance than those selected for ethanol aversion (Waller et al., 1983).

Withdrawal syndrome

The physical abstinence syndrome in man, in severe form, develops after about 8 h. In the first stage, the main symptoms are tremor, nausea, sweating, fever and sometimes hallucinations. These symptoms last for about 24 h. This phase may be followed by tonic-clonic convulsions. Over the next 48 h, "delirium tremens" could develop, in which the patient becomes confused, agitated and often aggressive, and may suffer from severe hallucinations. However, not all components of withdrawal need to be present. The alcohol withdrawal (similarly to the diazepam withdrawal) is associated with anxiety (Roelofs, 1985).

Different treatments have been proposed in order to prevent the subject for the intake of alcohol or to attenuate a withdrawal syndrome after cessation of the alcohol use. Besides compounds inducing an aversive reaction, such as *disulfiram* (Goldstein, 1994) and *calciumcyanide* (Nagasawa et al., 1990), the use of *antagonists of opioid* or *NMDA receptors* has been recently suggested.

- *Opioid antagonists* tended to reduce alcohol consumption (Goldstein, 1984). Administration of naltrexone to alcohol addicts during detoxification process reduced craving and prevented single drinks from triggering binges (Volpicelli et al., 1992). A binge is a period of several hours during which large amounts of drugs are being consumed. Generally, a binge is followed by emotional distress ("coming down" or "crashing, Jaffe, 1990).
- *NMDA antagonists.* Some alcoholics become magnesium depleted, which accentuates the excessive NMDA stimulation during alcohol withdrawal (Mg^{2+}-ion is known to

block the NMDA-receptor). Therefore, many features of withdrawal can be blocked by magnesium sulphate infusions (Becker and Hale, 1993).

B. Benzodiazepines (BDZs)

Action on cellular level

BDZs facilitate the inhibitory GABA neurotransmission by increasing the permeability of a chloride ion channel in the CNS of animals (Young and Kuhar, 1979) and humans (Schoch et al., 1985). BDZs (like alcohol and barbiturates) interact with the $GABA_A$ receptor-complex. It is demonstrated that the α-subunit of the $GABA_A$ receptor is responsible for the binding of BDZs, in association with the γ-subunit. The functional importance of the δ- and the ε-subunits of the $GABA_A$ receptor-complex in respect to the mechanism of action of the BDZs, is still unclear (Giusti and Arban, 1993).

Tolerance

The clinical consequences of sedative effects of BDZs are partly counterbalanced by the development of tolerance to these effects. In clinical terms this means that patients frequently report diminution or disappearance of sedative effects despite continued use of the BDZ. Tolerance to the sedative effects is not accompanied by tolerance to the anti-anxiety effects of these drugs (Linnoila et al., 1983).

Withdrawal syndrome

Discontinuation of chronic use of BDZ could induce withdrawal signs in both animals and humans (Woods et al., 1987). The symptoms of the withdrawal syndrome in BDZ-dependent subjects are excessive sensitivity to light and sound, tremors, sweating, sleeplessness, abdominal discomfort, tachycardia, mild systolic hypertension and rarely seizures (Marks, 1978). After withdrawal, patients recover completely, but anxiety may occur (Shader and Greenblatt, 1993). The half-life of a BDZ is important in the expression and severity of the withdrawal syndrome. The abrupt cessation of various BDZs with short half-lives (Woods et al., 1992) is associated with rapid onset of withdrawal syndrome. Therefore, the BDZs with short half-lives should be stopped gradually rather than abruptly. It has been shown that the serotonin $5\text{-}HT_3$ antagonist ondansetron attenuates the BDZ withdrawal in animals (Oakley et al., 1988; Goudie and Leathley, 1990). However, this subject is controversial (Costall et al., 1988).

C. Barbiturates

Action on cellular level

Similar to alcohol and BDZs, the barbiturates have also binding site on the $GABA_A$ receptor-complex, which is claimed to be different from that of alcohol (Korpi and

Seeburg, 1993) and BDZs (Haefely, 1980). Recently, it was demonstrated that a barbiturate binding site is also present on the nicotinic acetylcholinergic receptors (nAChRs, De Armendi et al., 1993).

Tolerance

Tolerance to barbiturates develops to a marked degree and it is partly of a pharmacokinetic type. Repeated dosage of the drug is destroyed more rapidly (becomes somewhat less effective), because of the increased synthesis of hepatic cytochrome P450 and conjugating enzymes, which facilitate the biotransformation of barbiturates (Priest, 1980).

Withdrawal syndrome

A cessation of chronic use of barbiturates induces withdrawal syndromes sometimes accompanied with grand mal type convulsions or delirium tremens (Lockhart-Ewart and Priest, 1967). BDZs block the withdrawal seizures in subjects made dependent on barbiturates (Haefely, 1980).

1.2.2. Cannabinoids

(-)-Δ^9-Tetrahydrocannabinol (Δ^9-THC, also called Δ^1-THC according to different ring-numbering system) has been recognized for a long time as the major psycho-active component of marijuana (Gaoni and Mechoulam, 1964). The mechanism by which cannabinoids exert their behavioral effects in humans and animals, has recently been partially clarified.

Action on cellular level

Cannabinoid receptor. The cloning of central (Matsuda et al., 1990) and peripheral (Munro et al., 1993) cannabinoid receptors was performed recently. Autoradiographic studies showed a heterogenous distribution of the cannabinoid receptor in brain of a variety of mammalian species, including humans. Most of the cannabinoid receptors are located in the basal ganglia, hippocampus and cerebellum, but also in cerebral cortex and striatum (Herkenham et al., 1990,1991). It could be speculated that some of these anatomical sites correlate with observed pharmacological effects of marijuana, for example, cognitive impairment (hippocampus and cortex), ataxia (basal ganglia and cerebellum) and low toxicity (lack of receptors in brainstem) (Howlett et al., 1990; Martin et al., 1991). In the substantia nigra of humans, cannabinoid receptors are located on striatonigral terminals, which degenerate in Huntington's disease (Glass et al., 1993). These findings indicate that cannabinoids could be involved in locomotion and hyperkinetic/dystonic disorders, occurring in both Huntington's and Parkinson's disease.

Endogenous ligand. Devane et al. (1992) demonstrated the existence of an endogenous cannabimimetic ligand, anandamide. The fact that anandamide could inhibit the N-type calcium channel current through the cannabinoid receptors (Mackie and Hille, 1992) could suggest a physiological role of this compound in the regulation of the release of other neurotransmitters (Mackie et al., 1993). Anandamide has only been tested in vivo in rodents and it was shown that the effects of this compound (hypomotility, hypothermia and nociception) have a rapid onset but shorter duration than other cannabinoids (Fride and Mechoulam, 1993; Crawley et al., 1993). Besides anandamide, other receptor selective agonists are: Δ^9-THC, CP 55940, WIN 55,212-2, levonantradol and nabilone. The activation of cannabinoid receptors is associated with a decrease of cyclic adenosine monophosphate (cAMP). A selective receptor antagonist is not known yet (The RBI handbook of receptor classification, 1994).

Tolerance

Tolerance to repeated use of marijuana has long been suspected, given the fact that experienced users are capable of consuming enormous quantities of the drug with few or no obvious ill effects (Cohen, 1976). Tolerance to cannabinoids in animals has also been reported (Carlini, 1968). Recently, it has been demonstrated that chronic administration of the selective cannabinoid receptor agonists Δ^9-THC and CP 55940, induced a receptor down-regulation. This indicates that tolerance to cannabinoids in vivo could occur (Oviedo et al., 1993).

Withdrawal syndrome

Discontinuation of cannabis after chronic heavy use induces a mild withdrawal syndrome in humans, characterized by irritability, restlessness, loss of appetite, sleeplessness, tremor, perspiration and sometimes nausea, vomiting and diarrhoea (Jones, 1983; Goldstein, 1994). In animals, withdrawal symptoms did not occur following cessation of cannabinoid use (McMillan et al., 1971).

1.2.3. Hallucinogens

The family of the hallucinogens is a very diverse one, with many naturally occurring and synthetic compounds with similar mind-altering effects.

Natural occurring compounds:

- *psilocin* is obtained from a fungus and is structurally related to serotonin (5-HT, Wasson, 1980).
- *mescaline* is derived from a Mexican cactus (*peyote*). Its structure is almost identical to that of amphetamine, which in its turn is closely related to that of DA (Jaffe, 1990).

Synthetic compounds:

- *LSD* is chemically related to 5-HT and is considered as one of the most potent hallucinogenic drugs (Schultes and Hofmann, 1979).
- *MDMA* (3,4-methylenedioxymethamphetamine, "ecstasy") belongs to the group of phenethylamines and is chemically related to amphetamines. In rats, MDMA causes massive destruction of 5-HT neurons (Rosecrans et al., 1988). The neurotoxic effect may be due not to MDMA itself, but rather to a product of the metabolism of MDMA in the body. Although there are not yet hard evidences that MDMA could cause brain damage in humans, it is striking that relatively many people die after MDMA intake (Henry et al., 1992). In the last years, a lot of phenethylamine derivatives are brought on the market. These derivatives, sold in the form of pills are mostly used during house-parties and in combination with alcohol are causing severe side-effects (respiration problems, hyperthermia) and could even lead to death.
- *PCP* (phencyclidine, "angel dust"), chemically resembling to ketamine, induces an increased locomotor activity, stereotyped movements and ataxia, although in animals depressant rather than stimulant effects predominate (Sanger and Jackson, 1989).

Action on cellular level

Many hallucinogens affect the serotonergic (5-HT-ergic) system in the brain, causing a massive discharge of 5-HT from the 5-HT-ergic neurons, followed by prolonged depletion of the neurotransmitter (Strassman, 1992).

LSD acts as a 5-HT antagonist in peripheral tissue, but in CNS it is believed mainly to work as an agonist. Neurophysiological studies show that LSD directly inhibits the firing of 5-HT-containing neurons in the raphe nuclei, apparently by activation of inhibitory autoreceptors of these cells (Aghajanian, 1981).

PCP interacts with NMDA receptors as a noncompetitive antagonist (Kemp et al., 1987). It was shown that following chronic infusion of PCP a significant decrease of D_2 receptors in rat striatum occurred (Spain et al., 1985). PCP and also MDMA are relatively selective neurotoxins, affecting mainly 5-HT neurons (Rosecrans et al., 1988).

Tolerance

LSD. Tolerance to the effects of LSD develops quite quickly, and there is cross-tolerance between this drug and most other hallucinogens. Animals trained to discriminate LSD respond almost identical to the presentation of psilocybin (Carlton, 1983).

PCP. Chronic PCP administration has been shown to produce tolerance to the behavioral actions of PCP (Nabeshima et al., 1985).

Withdrawal syndrome

LSD induces psychic- but not physical dependence (Stolerman, 1992).

PCP, in contrast to LSD, acts consistently as a primary reinforcer in animals experiments, inducing drug dependence (Carlton, 1983; Stolerman, 1992). Withdrawal of PCP after infusion for 7 days resulted in an abstinence syndrome in rats, comparable to that of opioids (piloerection, increased susceptibility to audiogenic sounds, weight loss). The first withdrawal signs occurred around 4 h after termination of infusion, and in the following 20 h, the abstinence syndrome subsides (Spain and Klingman, 1985). Buspirone is used for the treatment of PCP (and cocaine) withdrawal syndrome (Giannini et al., 1993).

1.2.4. Tobacco

Nicotine appears to be the only pharmacologically active substance in tobacco smoke, apart from carcinogenic tars. It is proved to be extreme difficult to induce animals to self-administer nicotine. This has led to the incorrect idea that nicotine is not addictive. However, a recent study demonstrated that stimulation of the mesolimbic DA system could be considered of major importance for the rewarding and dependence producing properties of nicotine (Nisell et al., 1994).

Action on cellular level

Nicotine affects several neurotransmitter systems, but its main effect is on central nAChRs. Several studies have revealed that nAChRs not only are present on cholinergic neurons (Clarke, 1993), but appear to be also located on a variety of pre- and postsynaptic sites of noncholinergic neurons (Rosecrans and Karan, 1993). This may indicate that several neuronal pathways are involved in the tobacco dependence phenomena. Recently, it has been shown that the presynaptic nicotinic binding site in mouse could be involved in the DA release (Grady et al., 1994). Systematically administered nicotine increases frontocortical 5-HT release, probably due to the activation of the nicotinic receptors on raphe neurons (Ribeiro et al., 1993).

Tolerance

An upregulation of brain nicotinic receptors during tolerance to nicotine was ascribed to receptor desensitization (Marks et al., 1993). Cross-tolerance with nicotine has been shown for alcohol (De Fiebre and Collins, 1993).

Withdrawal syndrome

A withdrawal syndrome occurs in both humans and experimental animals following the cessation of regular nicotine administration. Its main features are increased irritability,

impaired performance of psychomotor tasks, aggressiveness and sleep disturbance (Griffiths and Henningfield, 1982; Goldstein, 1994). The physical withdrawal syndrome disappears in 2-3 weeks, though craving for cigarettes persists for much longer. The withdrawal syndrome is much less severe than that produced by opioids and it can be alleviated not only by nicotine but also by amphetamine. This latter point suggests that the effect of nicotine may be partly due to catecholamine release in the brain, an hypothesis advanced for other dependence-producing drugs (Koob and Bloom, 1988).

Various therapeutic products have been developed in order to help the nicotine user to get rid of their addiction. This so called "nicotine replacement therapy" includes the followings:

- *Clonidine* is shown to decrease dose-dependently the tobacco withdrawal craving (Gourlay et al., 1994), perhaps by reducing the sympathetic arousal (Hughes, 1993).
- *Sertraline*, a 5-HT reuptake inhibitor, counteracts the hyperphagia and rapid weight gain associated with nicotine withdrawal, and might be a useful adjunct to smoking cessation (Levin et al., 1993).
- The *skin patches* are claimed to improve a smoking cessation both by reducing nicotine withdrawal effects and by reducing the reward of slips back to smoking (Levin et al., 1994).
- *Transdermal nicotine* is effective even given without psychotherapy, but does not consistently decrease postcessation weight gain, which is similar for the nicotine gum (Hughes, 1993). However, controversial results on the effectiveness of both nicotine gum and patch were reported (Tang et al., 1994).

Until now, no uniform therapy for helping nicotine-addicts is available. Recently, treatment with *antidepressant drugs* has started, but there are no results in respect to the treatment of nicotinic addiction come out yet.

1.2.5. Volatile solvents

Harmful use of volatile substances, also referred to as glue sniffing is defined as "the deliberate inhalation of a gas or of fumes given off from a substance at room temperature for its intoxicating effect" (cited by Chalmers, 1991). These category of drugs include a variety of chemical products such as petrol, anaesthetic gases, volatile nitrites, organic solvents, and are present in an array of household and commercial products, aerosols, fire extinguisher chemicals and natural gases (Chalmers, 1991).

Epidemiological study of deaths from harmful use of volatile substances in people under 18 years showed that 605 people died in United Kingdom in the period 1981-1990, and nearly as four times as many deaths occurred in the social lower class (Esmail et al., 1993).

Action on cellular level

There are indications that these drugs could act on GABA receptors in much the same way as alcohol does, however the precise mechanism of action is still unclear (Goldstein, 1994).

1.2.6. Opioids and Psychostimulants

The opioids and psychostimulants were used in our experimental studies and therefore, are discussed in more details (see *chapter 2* and *chapter 8*).

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PART 2

Opioid Dependence

Chapter 2

Opioids

2.1. Opioids and opioid receptors

The *opioids* includes four different groups of compounds:

- *"True opiates"* natural alkaloids derived from the opium poppy (*papaver somniferum*), such as morphine and codeine.
- *Semi-synthetic opioids*, structurally related to morphine (heroine).
- *Synthetic opioids*, structurally unrelated to morphine (fentanyl, methadone, pentazocine, etc).
- *Endogenous opioid peptides (ß-endorphin, Met- and Leu-enkephalin, dynorphin A and B)* were identified (Li et al., 1976; Hughes et al., 1975; Goldstein et al., 1979; Cone and Goldstein, 1982, respectively), following the discovery of stereospecific opioid binding site in the CNS (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973).

Opioid receptors are distributed throughout the mammalian CNS (Atweh and Kuhar 1977a,b,c), but could also be found in periphery (Cox, 1988). Three main receptor types were identified μ, κ, and δ (Martin et al., 1976; Lord et al., 1977; Chang et al., 1979). Recent studies have demonstrated the existence of two δ-opioid receptor subtypes (δ_1 and δ_2) (Jiang et al., 1991). Table 1. shows the proposed classification of opioid receptors with corresponding agonists and antagonists.

2.2. Opioid dependence

Neuronal pathways and neurotransmitters. It is claimed that psychic dependence to opioids and many other drugs is regulated by three main anatomically well-defined brain areas (Koob, 1992). These areas are the followings: *1. ventral tegmental area* (VTA), in which the cell bodies of the mesocorticolimbic dopamine (DA) system originate, *2. nucleus accumbens* (NAc), which receives projections from the VTA, *3. ventral pallidum*, which receives a major projection from the NAc.

Experimental studies showed that rats will self-administer opioids into the VTA, while opioid peptides injected into this brain region produce place preference (Di Chiara and North, 1992).

It has been shown that microinjection of the neurotoxin kainic acid, which destroys the cell bodies, but not fibres of passage, into the NAc markedly decreased an intravenous self-administration of both opioids and psychostimulants (Zito et al., 1985).

A similar effect was observed in the ventral pallidum following selective destruction of cell bodies by ibotenic acid (Hubner and Koob, 1987).

It was claimed that the DA neurons of the VTA are critical for opioid reinforcement (Bozarth and Wise, 1984). It seems that opioids excite DA neurons in the VTA, via μ-receptors located on GABA (γ-amino butyric acid)-releasing neurons. Opioids can induce a hyperpolarization of these GABA- neurons, by increasing the K^+ efflux. As a result, GABA release onto the DA cells is reduced and the firing rate of DA neurons is increased (Johnson and North, 1992; Fig.1). It is proposed that μ- and δ-receptors are implicated in mediating the reinforcing actions of opioids, while κ-receptors mediate their aversive actions (Di Chiara and Imperato, 1988; Spanagel et al., 1990).

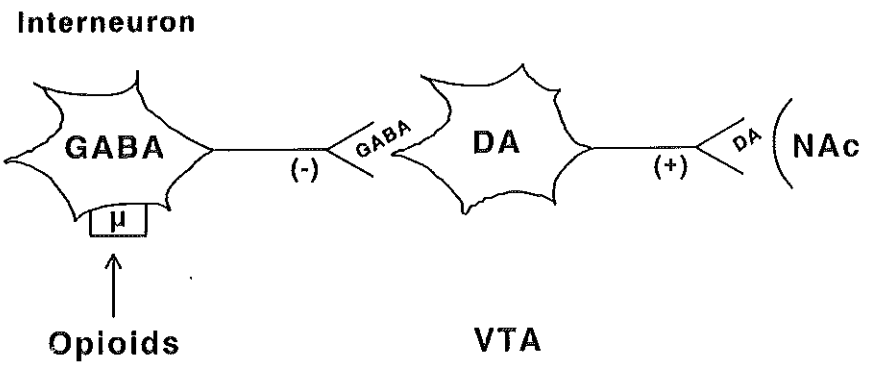

Fig. 1. Schematic illustration of the way in which DA-containing neurons in the ventral tegmental area (VTA) are excited by opioids. GABA-containing interneurons are hyperpolarized by opioids acting at μ-receptors. This results in decreased (-) GABA release and increased (+) firing and DA release of DA-containing neurons in the VTA towards the nucleus accumbens (NAc).

The level of other neurotransmitters, besides DA, is also deranged during opioid dependence. Several studies have been demonstrated that the excitatory amino acid (EAA) receptor system is involved in the process of opioid dependence. Morphine is known to inhibit the enzymes producing aspartic acid and glutamic acids (Koyuncuoglu et al., 1979, 1986) from asparagine and glutamine, respectively (Bielarczyk et al., 1986), resulting in the decreased level of EAAs. Accordingly, the chronic presence of opioid receptor agonists decreases a normal activation of NMDA receptors (Aanonsen and Wilcox, 1987; Tanganelli et al., 1991). Therefore, morphine dependence is associated with NMDA receptor up-regulation and/or supersensitivity (Koyuncuoglu et al., 1992a,b).

2.3. Withdrawal syndrome

Cessation of opioid agonist or administration of opioid receptor antagonist in opioid-dependent subjects induces a withdrawal syndrome. Although physical dependence occurs mainly following chronic exposure to an opioid drug, a withdrawal syndrome can be precipitated in man (Bickel et al., 1988) and various animals (Martin and Eades, 1961;

Way et al., 1969; Meyer and Sparber, 1977; Krystal and Redmond, 1983), following an acute opioid treatment as well.

Opioid physical dependence can be easily studied, since opioid withdrawal syndrome, induced by diverse opioid antagonists (Table 1.), can be abruptly abolished by opioid agonists (Wei et al., 1973a,b).

Table 1. Opioid receptor classification and corresponding drugs, which interfere with these receptors (adapted from The RBI handbook of receptor classification, by Kebabian and Neumeyer (eds.), 1994).

	OPIOID RECEPTORS			
		δ		
	μ	δ_1	δ_2	κ
Selective agonists	DAMGO Sufentanyl PL 017 Morphine	DPDPE DADLE	[D-Ala^2,Glu^4]-Deltorphin DSLET	U-69593 CI 977 U-50488
Selective antagonists	ß-FNA CTAP CTOP	BNTX DALCE Naltrindole ICI-174,864	Naltriben Naltrindole 5'-isothiocyanate Naltrindole ICI-174,864	*nor*-Binal-torphimine
Endogenous opioid peptides		[Leu^5]-Enkephalin [Met^5]-Enkephalin		Dynorphin-A
Universal opioid antagonists	ß-CNT Naloxone Naltrexone Cyprodime	ß-CNT Naloxone Naltrexone Cyprodime	ß-CNT Naloxone Naltrexone Cyprodime	ß-CNT Naloxone Naltrexone Cyprodime

DAMGO: [D-Ala^2,N-Me-Phe^4,Gly-ol^5]-Enkephalin
DPDPE: [D-Pen^{2,5}]-Enkephalin
DADLE: [D-Ala^2,D-Leu^5]-Enkephalin
DSLET: [D-Ser^2,Leu^5,Thr^6]-Enkephalin
DALCE: [D-Ala^2,Leu^5,Cys^6]-Enkephalin
ß-FNA: ß-Funaltrexamine
CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
BNTX: (E)-7-Benzylidenenaltrexone
ß-CNT: ß-Chlornaltrexamine

Behaviour. Heroin or morphine have in men a short half-life (2 to 3 h). The onset of withdrawal symptoms occurs within 8 to 16 h after the last dose, and the peak effect is around 2-3 days. Methadone has a longer half-life (15-20 h) and the onset of withdrawal symptoms is within 2-3 days after the last use. However, the peak effect is around 1-2 weeks, and some symptoms persist for months before resolution occurs (Zweben and

Payte, 1990). It has been demonstrated a long time ago (Himmelsbach et al., 1938, cited by Martin and Sloan, 1977) that the opioid withdrawal symptoms - nausea, vomiting, sweating, gooseflesh, diarrhoea, tremor, chills and fever - occurred predictably by discontinuing morphine administration in a person who had been maintained on a regular schedule for morphine injections with escalating dosage. Himmelsbach et al., (1938, cited by Martin and Sloan, 1977) developed a method of scoring the intensity of the withdrawal syndrome, placing emphasis on easily recognized objective disturbances rather than on subjective complaints.

The withdrawal syndrome in morphine-dependent rats includes whole-body shakes ("wet-dog" shakes), diarrhoea, escape jumps, teeth-chattering, salivation and irritability - aggression (Martin et al., 1963; Bläsig et al., 1973; Wei et al., 1973a). Later on, several other withdrawal signs have been specified, for example sniffing, grooming, rearing, gnawing, penile-licking, mastication, ptosis, writhing and rhinorrhoea (Acquas and Di Chiara, 1992; Maldonado and Koob, 1993; Gold et al., 1994).

In order to classify the severity of withdrawal syndrome, several scoring systems were proposed. Some researchers divided the withdrawal symptoms in counted and checked signs (Maldonado and Koob, 1993), or in dominant and recessive ones (Bläsig et al., 1973), while others provided signs with a weighting factor (Neal and Sparber, 1986). In our studies, described in *chapters 5-7*, the scoring system of Neal and Sparber (1986) has been used.

In table 2., are listed several withdrawal signs in morphine-dependent animals (rats and mice) in respect to their origin and involvement of specific neurotransmitters and/or receptor sites.

Neuronal pathways and neurotransmitters. The locus coeruleus (LC) is the main brain region playing an important role in the opioid withdrawal syndrome, but less in the opioid reinforcement. In contrast, the mesolimbic dopaminergic (DA-ergic) system mediates reinforcing properties of drugs, but is not extensively involved in drug withdrawal (Wise and Bozarth, 1987).

The LC is located on the floor of the fourth ventricle in the anterior pons. The small number of neurons provides widespread noradrenergic (NA-ergic) innervation to virtually all areas of the brain and spinal cord. Destruction of the LC decreased some opioid withdrawal signs, such as chewing and rearing in morphine-dependent rats (Maldonado and Koob, 1993). However, LC neurons recorded in slices from morphine-dependent rats do not exhibit a pronounced withdrawal hyperactivity (Christie et al., 1987), indicating that most of the withdrawal-induced activation of these cells observed *in vivo* is likely to be mediated by afferents to the LC. Studies revealed that the rostral medullary nucleus paragigantocellularis is the major excitatory input to LC neurons, acting primarily via

EAAs (Ennis and Aston-Jones, 1988; Hong et al., 1993).

The LC contains a high density of opioid receptors and it receives substantial direct enkephalin inputs. ß-Endorphin and dynorphin fibres are found in the LC area (Aston-Jones et al., 1993). Presynaptic opioid receptors located on terminals of central NA-ergic neurons, are probably responsible for the decreased release of NA (Arbilla and Langer, 1978) and the diminution of the LC terminal excitability that follows opioids exposure (Nakamura et al., 1982). *In vitro* studies revealed that opioids acting at μ receptor may increase K^+ efflux and inhibit Na^+ influx, which is followed by hyperpolarization of the LC neurons (Andrade et al., 1983).

Clonidine (α_2-agonist), a drug that decreases NA-ergic activity, blocks both opioid withdrawal symptoms and behaviour induced by electrical stimulation of the LC (Maldonado and Koob, 1993). It has been shown that clonidine, similarly to opioids, elicit a hyperpolarization in LC neurons (Aghajanian, 1978). Coapplication of clonidine and opioid agonists shows a response similar to that evoked by either agonist alone (Aghajanian and Wang, 1987). This finding implicates that both the α_2-adrenoceptor agonist and opioid agonists may affect K^+ efflux in the same way.

Recently, it has been demonstrated that K^+-channel openers can mimic the effects of morphine on neuronal K^+ currents, and as a consequence can act as substituents for morphine during withdrawal process (Robles et al., 1994).

Second messenger systems

Acute opioid action (Fig. 2). Opioid-induced inhibition of LC neurons via increasing the conductance of a K^+ channel and inhibition of a Na^+-dependent inward current (Aghajanian and Wang, 1987) is mediated by the pertussis toxin-sensitive G-proteins (Blume, 1978). Administration of opioids leads to activation of the K^+ channel by direct coupling of the opioid receptor to the K^+ channel via a G-protein. In contrast, inhibition of the Na^+-dependent current appears to be indirect. Namely, the Na^+ current is normally activated by a cAMP (cyclic adenosine monophosphate)-dependent protein kinase, either through direct phosphorylation of the Na^+ channel or by phosphorylation of some associated proteins (Wang and Aghajanian, 1990). The opioid inhibition of the Na^+ current appears to be mediated via inhibition of adenylate cyclase (AC) and reduced levels of cAMP. Reduced levels of cAMP decrease cAMP-dependent protein kinase activity and phosphorylation of the responsible channel/pump or closely related associated proteins. In addition to reduced firing rates (due to hyperpolarization), inhibition of cAMP pathway decreases catecholamine synthesis via reduced phosphorylation of tyrosine hydroxylase. Biochemical studies have confirmed that opioids inhibit AC activity in the LC (Duman et al., 1988) and cAMP-dependent protein phosphorylation (Guitart and Nestler, 1989).

Table 2. Some withdrawal signs observed in rats and/or mice in respect to their origin (central) and the involved neurotransmitter/receptor sites.

withdrawal sign	brain area/brain nucleus	neurotransmitter receptor-sites
"wet-dog" shakes	forebrain[24]; lower diencephalon /brain stem[6]; area diencephalon /mesencephalon[27]; anterior hypothalamus[15]; thalamus[6]; medial thalamus[23,27]; nucleus raphe magnus[6]; amygdala[8]; globus pallidus[23]; substantia nigra[5]; locus coeruleus[17]; hippocampus[6]; nucleus accumbens[21]	5-HT[6]; DA[21]; ACh[27] μ-receptor site[5]
jumping	medial thalamus[23]; raphe nuclei[15]; central/dorsal amygdala[8,23]; globus pallidus[23]; locus coeruleus[15]; periaquaductal gray[22]	σ- and PCP-receptor site[28]
rearing	locus coeruleus[15,17]; periaquaductal gray[15]; nucleus accumbens[20]	μ-receptor site[25]; DA[20]
locomotion	area diencephalon/ mesencephalon[27]; hypothalamus lateral[1,12] - medial[27]; amygdala[8]; substantia nigra[4,15]; locus coeruleus[17]; periaquaductal gray[7,15]; nucleus accumbens[7]	μ-receptor site[25]; DA[20,21];DA-ACh[21]
teeth-chattering	basal ganglia[24]; substantia nigra - pars reticulata[5,15]; nucleus accumbens[21]	DA[21]
chewing	amygdala - striatum[8,23]; substantia nigra[10]; locus coeruleus[17]; nucleus accumbens[15]	DA[14,19]; ACh[9]
grooming	medial/lateral hypothalamus[1,13]; nucleus accumbens[20]; cerebellum[3]	DA[20,26]
penile erection	hypothalamus - hippocampus[2,11]; olfactory bulb[11]; hypothalamus paraventricular nuclei[2]; amygdala[8]; spinal cord[11]	μ-receptor site[16]; ACh[11]
gastro-intestinal activity	medial thalamus[23]; amygdala anterodorsal/ centrobasolateral[8,23]; striatum[8] - caudal[23]; substantia nigra[5]; nucleus accumbens[21]	5-HT[18]; DA[21]
irritability on touch	periaquaductal gray[22]; substantia nigra[5]	

1. Adams et al., *Physiol & Behav* 53: 1127, 1993; **2.** Argiolas et al., *Reg Pept* 45: 139, 1993; **3.** Ball et al., *Physiol Behav* 13: 123, 1974; **4.** Baumeister et al., *Neuropharmacol* 28: 1151, 1989; **5.** Baumeister et al., *Neuropharmacol* 31: 835, 1992; **6.** Bedard and Pycock, *Neuropharmacol* 16: 663, 1992; **7.** Bozarth and Wise, *Science* 224: 516, 1984; **8.** Calvino et al., *Brain Res* 177: 19, 1979; **9.** Gunne et al., *Psychopharmacol* 134, 1972; **10.** Jones et al., *Brain Res* 560: 163, 1991; **11.** Krane et al., *New Eng Med* 321: 1648, 1989; **12.** Lammers et al., *Brain Res* 449: 294, 1988; **13.** Lammers et al., *Brain Res* 449: 311, 1988; **14.** Levin et al., *Pharmacol Biochem Behav* 34: 43, 1989; **15.** Maldonado et al., *J Pharmacol Exp Ther* 261: 669, 1992; **16.** Maldonado et al., *Neuropharmacol* 31: 1231, 1992; **17.** Maldonado and Koob, *Brain Res* 605: 128, 1993; **18.** Neal and Sparber, *J Pharmacol Exp Ther* 236: 157, 1986; **19.** Patrick et al., *Eur J Pharmacol* 231: 243, 1993; **20.** Pei et al., *Eur J Pharmacol* 230: 63, 1993; **21.** Pothos et al., *Brain Res* 566: 348, 1991; **22.** Stinus et al., *Neurosci* 37: 767, 1990; **23.** Tremblay and Charton, *Neurosci Lett* 23: 137, 1981; **24.** Tseng et al., *Neuropharmacol* 14: 247, 1975; **25.** Ukai et al., *Brain Res* 557: 77, 1991; **26.** Van Wimersma Greidanus et al., *Eur J Pharmacol* 173: 227, 1989; **27.**Wei et al., *J Pharmacol Exp Ther* 185: 108, 1973; **28.** Yukhananov et al., INRC-abstract p41, 1994.

Chronic opioid action. Following chronic opioid administration, the LC neurons develop tolerance to the prior described acute inhibitory actions as neuronal firing rates recover toward control levels (Aghajanian, 1978; Christie et al., 1987). It was suggested that during chronic exposure to opioids, long term adaptations in intracellular messenger proteins could occur, which could be involved in the process of cellular tolerance, dependence and withdrawal. It has been shown that chronic administration of opioids increases the levels of G-proteins (Nestler et al., 1989) and stimulates the AC (Duman et al., 1988), cAMP-dependent protein kinase (Nestler and Tallman, 1988) in the neurons of the LC. Tyrosine hydroxylase is also activated (Guitart et al., 1990), which is the rate-limiting enzyme, involved in the biosynthesis of catecholamine neurotransmitters (Fig. 2).

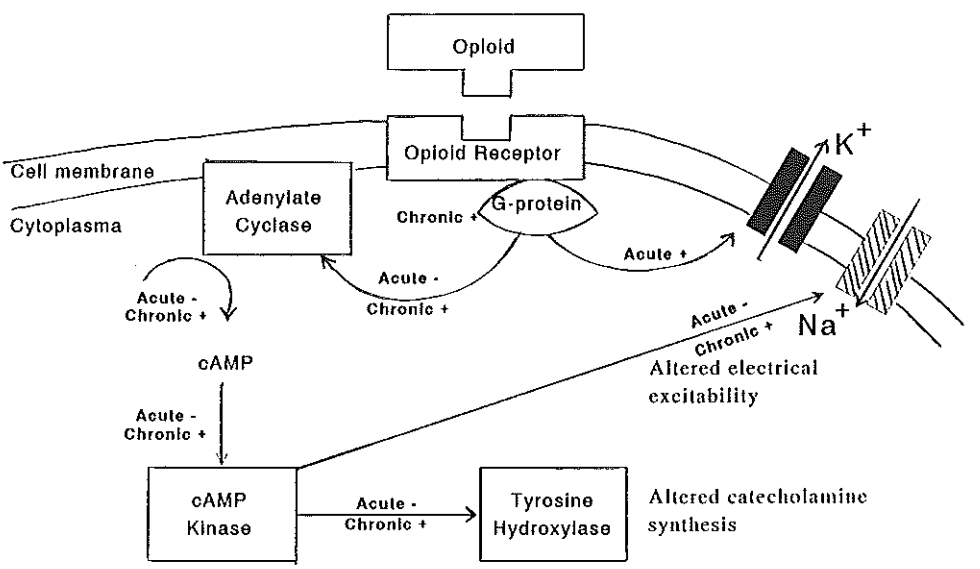

Fig. 2. Opioid actions in the locus coeruleus (LC). *Acute* administration of opioids inhibited (-) both adenylate cyclase and cAMP-dependent protein phosphorylation. *Chronic* administration of opioids increased (+) the levels of G-proteins and stimulated (+) the activity of adenylate cyclase, cAMP-dependent protein kinase and tyrosine hydroxylase.

In the chronic opioid-dependent state, the combined presence of opioids and the up-regulated cAMP pathway would return the LC firing rate to control levels. Removal of the opioids would leave the up-regulated cAMP pathway unopposed, leading to withdrawal excitation of neuronal activity. Excitation of the LC neurons during withdrawal is necessary for producing many of the behavioral signs of opioid abstinence (Rasmussen et al., 1990; Maldonado and Koob, 1993).

In conclusion, all these findings indicate that upregulation of the cAMP pathway is a likely mechanism of opioid dependence in the LC. It is probably not the only mechanism, but this up-regulated cAMP pathway represent one of the examples in which a behavioral component (physical opioid dependence) can be correlated with biochemical and electrophysiological adaptations occurring in the neurons of the LC.

Following the chronic exposure to opioids, alterations on for example the molecular level (gene expression) have demonstrated (Nestler et al., 1993). However, these changes are not discussed in this thesis.

Treatment of opioid withdrawal syndrome. The expression of the morphine withdrawal syndrome in men/animals could be inhibited by **opioids** and **non-opioids.** Some examples are the followings:

- **Opioids.** High doses (30 fold higher) of opioids terminated the precipitated withdrawal stimulus in animals (Holtzman, 1985) and men (Zweben and Payte, 1990), while the administration of enkephalinase inhibitors attenuated the expression of morphine withdrawal behaviour in rats and mice (Dzoljic, 1986; Dzoljic et al., 1986).
- **Non-opioids.**

- *Clonidine* is effective in the treatment of the opioid withdrawal in humans (Gold et al., 1978; Kleber et al., 1980). In morphine-dependent rats, clonidine eliminated "wet-dog" shakes, diarrhoea and teeth-chattering and prevented the release of DA in the NAc (Romandini et al., 1984; Pothos et al., 1991).
- *5-HT Reuptake blockers* (d-fenfluramine) attenuated opioid withdrawal jumping in rats (Cervo et al., 1981), a sign which is not influenced by clonidine.
- *K^+-Channel openers* (cromakalim and diazoxide) can mimic the effects of morphine on neuronal K^+ currents, and could act as substituents for morphine in the withdrawal syndrome (Robles et al., 1994).
- *Ca^{2+}-Channel blockers* (verapamil, nimodipine, flunarizine) reduced several signs of naloxone-precipitated withdrawal such as diarrhoea, ptosis and jumping in morphine-dependent rats (Bongianni et al., 1986; Baeyens et al., 1987).
- *EAA receptor antagonists* (discussed in *chapter 3*).
- *Nitric oxide synthase (NOS) inhibitors* (discussed in *chapters 4-5*).
- *Ibogaine and norharman* (discussed in *chapter 7*).

2.4. Tolerance

Biochemical changes following tolerance

Opioid receptor system. It has been suggested that chronic opioid treatment alters the opiate receptor density in CNS (Collier, 1965). However, this subject is controversial.

Some authors have reported a decrease in the number of μ binding sites in tolerant animals (Rogers and El-Fakahany, 1986; Bhargava and Gulati, 1990; Abdelhamid and Takemori, 1991), while others showed no changes (Klee and Streaty, 1974; Nishino et al., 1990) or even an increase of μ-receptor binding sites (Pert and Snyder, 1976; Brady et al., 1989).

Second messenger systems. There seemed to be some common mechanism underlying dependence (discussed on page 41) and tolerance. The up-regulated cAMP system likely contributes to tolerance by making it more difficult for opioids to inhibit cAMP system and corresponding increase of the Na^+-dependent inward current. It is also possible that the upregulated cAMP system could result in greater levels of opioid receptor density through phosphorylation of the receptor. This hypothesis is based on observations that brief exposure to met-enkephalin desensitizes the μ-opioid receptor in the LC and the evidence that agents which activate the cAMP pathway promote this desensitization (Harris and Williams, 1991). By promoting desensitization, the upregulated cAMP system in the tolerant state could lead to a reduced ability of opioids to activate acutely G-proteins and the K^+ channel. In rats chronically treated with morphine, μ-receptors couple less well to G-proteins (Christie et al., 1987; Tao et al., 1993). This uncoupling of receptors and G-proteins may also contribute to the occurrence of opioid tolerance.

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PART 3

Morphine Withdrawal Syndrome - Experimental Studies

Chapter 3

Excitatory amino acid receptor antagonists and naloxone-precipitated withdrawal syndrome in morphine-dependent mice

Abstract - The effects of excitatory amino acid (EAA) receptor antagonists MK-801 (non-competitive NMDA receptor antagonist), DNQX (competitive non-NMDA receptor antagonist) and 5,7-DCKA (antagonist of glycine site of NMDA receptor) have been examined on the naloxone (4 mg/kg, i.p.)-precipitated withdrawal jumping behaviour in morphine-dependent mice. The results indicate that withdrawal jumping behaviour in morphine-dependent mice was attenuated by all three EAA receptor antagonists, MK-801, DNQX and 5,7-DCKA. However, MK-801, DNQX and 5,7-DCKA inhibited the jumping behaviour in a relatively narrow dose range.
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Central excitatory amino acids (EAAs) with corresponding receptors have been the focus of much attention in order to clarify neuronal development, long-term potentiation, kindling, epilepsy, learning or memory (Cotman and Iversen, 1987). In addition, there is evidence of the involvement of EAA in drug dependence phenomena. It has been shown that MK-801 blocks alcohol withdrawal seizures in the rat (Morrisett et al., 1990). It seems that chronic alcohol treatment uregulates the number of N-methyl-D-aspartate (NMDA) receptors in the hippocampus (Grant et al., 1990), which might explain both the seizures in alcohol withdrawal and anticonvulsant activity of NMDA receptor antagonists.

Furthermore, recent data indicate that the non-selective antagonist of EAA receptors, kynurenic acid (Krystal et al., 1990) attenuated naloxone-precipitated withdrawal in rats. Similarly, the non-competitive (MK-801) and competitive (LY274614) NMDA antagonists suppressed the behavioural signs of withdrawal in morphine-dependent rats (Koyuncuoglu et al., 1992; Rasmussen et al., 1991; Trujillo and Akil, 1991). Evidently, the functional activity of NMDA receptors may have a modulatory effect on drug dependence phenomena in the rat. The role of other EAA receptors, besides the NMDA receptor, in morphine dependence remains unclarified.

The aim of this study is to examine the role of various EAA receptor antagonists in naloxone-precipitated withdrawal in morphine-dependent mice. We used the non-competitive NMDA receptor antagonist MK-801 (Wong et al., 1988), the non-NMDA receptor antagonist DNQX (Honoré et al., 1988) and 5,7-DCKA, a selective antagonist of NMDA receptor-

associated strychnine-insensitive glycine binding site (Baron et al., 1991). An additional reason for using 5,7-DCKA was that glycine receptor antagonism may produce motor effects different from the competitive NMDA receptor antagonists (Koek and Colpaert, 1990).

Materials and Methods

Animals

Male Swiss mice weighing 25-35 g were used in all experiments. The animals were housed singly in polyethylene cages with food and water ad libitum. Artificial light was supplied in a 12-h light-dark cycle.

Morphine dependence

In general, the experimental model for the opioid withdrawal in mice was followed (Way et al., 1969; Kosersky et al., 1974). Chronic morphine dependence in mice was induced by morphine pellets (25 mg morphine base/mouse, s.c.) implanted on the back of the animal under ether anaesthesia. The morphine withdrawal was precipitated by administration of naloxone (4 mg/kg, i.p.), 72 h after the implantation of the pellet. The withdrawal severity was quantified by counting the frequency of jumping from a circular platform (30 cm high, 12 cm diameter). The general behaviour of drug-treated naive and morphine-dependent mice was observed and registered.

The animal was pretreated with vehicle or one EAA receptor antagonist, 30 min prior to naloxone. The pretreated animal was placed on the platform and observed for the following 30 min (in time intervals of 5 min). At the end of the 30-min period, the animal was given naloxone and placed again on the platform in order to be observed in a similar way, for the following 30 min.

Experimental protocol

Morphine-dependent mice were divided into five groups, pretreated intraperitoneally with vehicle (saline, 0.5 ml, n=5; DMSO, 0.5 ml, n=5), MK-801 (1-80 μg/kg, n=78), DNQX (0.63-10 mg/kg, n=40) or 5,7-DCKA (5-160 mg/kg, n=35). In all these animals, the withdrawal jumping behaviour was precipitated by administration of naloxone (4 mg/kg, i.p.), 30 min after drug pretreatment. In order to observe the behavioural effect of EAA antagonist in morphine-dependent mice, naloxone was replaced by saline. These three additional groups (n=14-36) were pretreated (30 min before saline) with various doses of EAA receptor antagonists. Each animal was used only once.

Drugs

The following drugs were used: MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*] cyclohepten 5,10-imine maleate, Research Biochemicals Inc. USA], DNQX (6,7-dinitroquinoxaline 2,3-dione, Tocris Neuramin) and 5,7-DCKA (5,7-dichlorokynurenic acid, Brunschwig Chemie). Two compounds, MK-801 and DNQX, were dissolved in distilled water. The pH of MK-801 was adjusted to 7-8, while the pH of DNQX was adjusted to 9 in order to solubilise the compound completely. 5,7-DCKA was dissolved in 10% DMSO (dimethylsulfoxide) and adjusted to pH 7-8. In the control experiments the vehicle solutions were adjusted to the corresponding pH value of drugs. All drug solutions were administered

i.p. and given in an equal volume (0.5 ml/injection).

Statistics

Data are expressed as medians. The effects of drug and vehicle treatment were evaluated statistically using the non-parametric Kruskal-Wallis one-way analysis of variance, followed by the Mann-Whitney *U*-test, with a level of $P<0.05$ being considered significant (Glantz, 1989).

Results

MK-801

In our preliminary experiments we observed that administration of MK-801, in a dose range of 0.1-10 mg/kg (i.p.), induced a pronounced locomotor dysfunction in both naive and morphine-dependent mice, consisting of wild running (hyperlocomotion), jumping, ataxia and convulsion. The incidence of these locomotor disturbances was dose related, while the higher doses of MK-801 (1-10 mg/kg) induced mainly ataxia and convulsions. However, concentrations of MK-801 below 0.1 mg/kg did not affect a normal behaviour of naive or morphine-dependent mice.

In order to avoid the influence of disturbed locomotion on the withdrawal jumping in mice, we used MK-801 in a dose range of 1-80 µg/kg (i.p.) which did not affect locomotion in naive or morphine-dependent mice. MK-801 significantly attenuated the naloxone precipitated jumping behaviour in a dose range of 5-20 µg/kg, i.p. (Fig. 1). The higher doses of MK-801 (40-80 µg/kg, i.p.) were without significant effect on withdrawal jumping behaviour of mice.

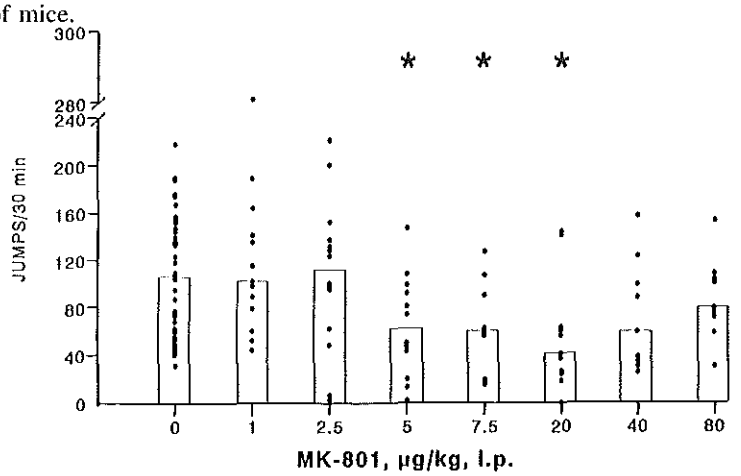

Fig. 1. Effect of MK-801 (µg/kg, i.p., 30 min before naloxone) on jumping behaviour precipitated by naloxone (4 mg/kg, i.p.) in morphine-dependent mice. Histograms represent medians and dots indicate individual animal scores (n=9-14 in MK-801-treated groups, control group = 36).
* Significant difference ($P<0.05$) from the value in vehicle (saline)-pretreated animals.

DNQX

DNQX (20-80 mg/kg, i.p.) caused convulsions (observation period 60 min after drug administration) and/or death (observation period 72 h after drug administration) in morphine-dependent mice. However, in naive mice neither convulsions nor death has been observed (Table 1). Therefore, in our further experiments we selected lower concentrations of DNQX (0.63-10 mg/kg, i.p.), which did not induce convulsions in morphine-dependent or naive mice. DNQX reduced naloxone-precipitated withdrawal jumping in mice in a dose range of 1.25-5 mg/kg, i.p. Similarly to MK-801, the dose-response curve of DNQX was also U-shaped (Fig. 2).

DNQX (mg/kg, i.p.)	Morphine-dependent mice (n=4)		Naive mice (n=4)	
	Lethality	Convulsions	Lethality	Convulsions
20	2/4	1/4	0/4	0/4
40	2/4	2/4	0/4	0/4
80	4/4	3/4	0/4	0/4

Table 1. Lethal (observation period 72 h after drug administration) and convulsant (observation period 60 min after drug administration) effect of DNQX (20-80 mg/kg, i.p.) on morphine-dependent and naive mice.

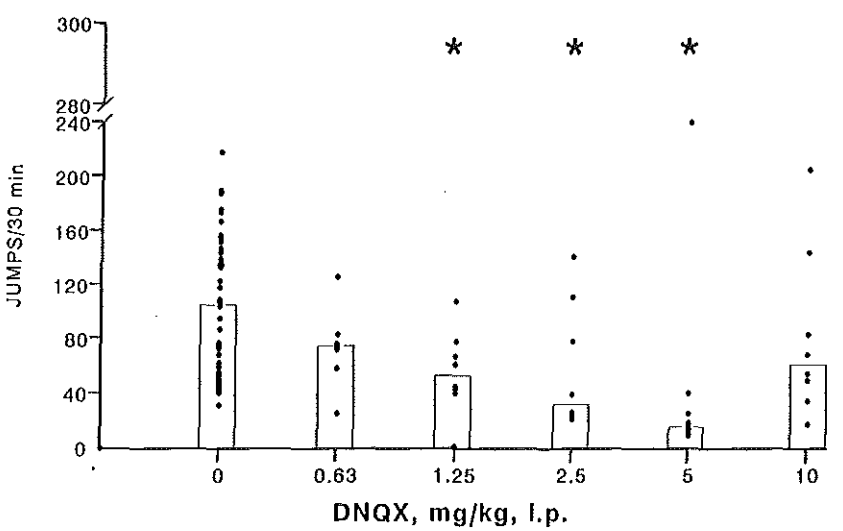

Fig. 2. Effect of DNQX (mg/kg, i.p., 30 min before naloxone) on jumping behaviour precipitated by naloxone (4 mg/kg, i.p.) in morphine-dependent mice. Histograms represent medians and dots indicate individual animal scores (n=6-8 in DNQX-treated groups, control group = 36).
* Significant difference ($P<0.05$) from the value in the vehicle (saline)-pretreated animals.

5,7-DCKA

Administration of 5,7-DCKA in a relatively wide dose range (5-160 mg/kg, i.p.) did not affect the usual behavioral pattern of either naive or morphine-dependent mice. However, 5,7-DCKA in a dose range of 20-40 mg/kg, i.p. significantly attenuated the naloxone-precipitated jumping behaviour in morphine-dependent mice (Fig. 3). The maximal effect was seen after treatment with 40 mg/kg 5,7-DCKA, while higher doses (80-160 mg/kg) were ineffective.

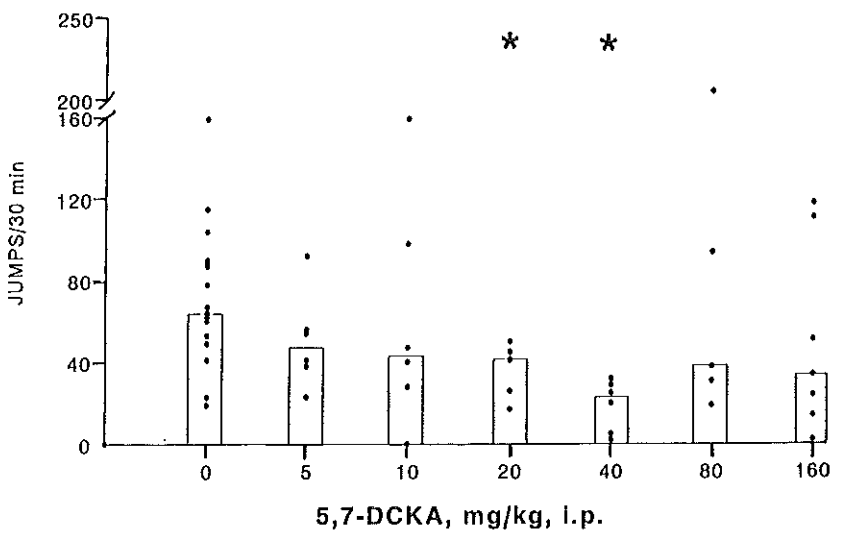

Fig. 3. Effect of 5,7 DCKA (mg/kg, i.p., 30 min before naloxone) on jumping behaviour precipitated by naloxone (4 mg/kg, i.p.) in morphine-dependent mice. Histograms represent medians and dots indicate individual animal scores (n=5-7 in 5,7-DCKA-treated groups, control group = 16).
* Significant difference ($P<0.05$) from the value in vehicle (DMSO)-pretreated animals.

Discussion

Our present findings demonstrate that antagonists of various glutamate receptors, such as MK-801 (non-competitive NMDA receptor antagonist), DNQX (competitive non-NMDA receptor antagonist) and 5,7-DCKA (antagonist of glycine site of NMDA receptors), attenuated the jumping withdrawal behaviour in morphine-dependent mice. These results are consistent with previous works showing that the non-selective EAA antagonist kynurenic acid (Krystal et al., 1990) and selective non-competitive and competitive NMDA receptor antagonists MK-801 and LY274614, respectively, suppressed the withdrawal signs in morphine-dependent rats (Koyuncuoglu et al., 1992; Rasmussen et al., 1991; Trujillo and Akil, 1991). However, in addition to NMDA receptors, this study indicates an involvement of the

glycine site of NMDA receptors and non-NMDA receptors in drug withdrawal as well.

Each of these three substances has a U-shaped dose-effect curve. Although unusual, a U-shaped dose-effect curve should not be considered as an exceptional phenomenon in the research of drug withdrawal. For example, buprenorphine in lower doses (0.01-0.5 mg/kg) precipitated abstention symptoms in morphine-dependent mice, while the higher doses (1-50 mg/kg) were less active or completely inactive (Lizasoain et al., 1991). The reason for the U-shaped dose effect curve is not known, but it could be assumed that a new mechanism(s) activated by higher drug concentrations may induce effects that are different from those produced by administration of lower doses. As regards the substances used in this study, it is known that higher doses of EAA receptor antagonists (in contrast to the lower doses), exerted a prominent excitatory/proconvulsant effect in naive animals (Jurson and Freed, 1990; Schoepp et al., 1990).

An additional relevant point is that morphine withdrawal in humans and animals is associated with an increase of neuronal and behavioral excitation (Wise and Bozarth, 1987). It seems that a further increase of central neuro-excitability induced by higher doses of drugs creates a new situation, which is presumably not favourable for an attenuation of morphine withdrawal. This might explain a failure of the higher doses of EAA receptor antagonists to attenuate morphine withdrawal and corresponding U-shaped doses-response curves. As a matter of fact, it should be expected that a further increase of neuro-excitability, due to elevated concentrations of EAA receptor antagonists, might even aggravate a withdrawal syndrome. Recent experiments support this idea, since administration of 0.1 mg/kg of MK-801 attenuated naloxone-precipitated withdrawal in rats, while a higher dose (0.3 mg/kg) increased the severity of the same abstention syndrome (Koyuncuoglu et al., 1992).

The mechanism of the suppressing effect of EAA antagonists on morphine withdrawal remains to be elucidated. There is evidence that the noradrenergic system plays an important role in opioid withdrawal. It has been reported that opioid withdrawal is associated with noradrenaline (NA) release (Laverty and Roth, 1980) and increased activity of noradrenergic cells in the locus coeruleus (Aghajanian, 1978; Valentino and Wehby, 1989). Several studies, in vitro and in vivo, indicate that NMDA antagonists may decrease NA release (Jones et al., 1987; Pittaluga and Raiteri, 1992) or activity (Burgard et al., 1989; Dahl and Sarvey, 1990; Löscher et al., 1991). In this respect, it is of importance that MK-801 significantly decreased the levels of NA and adrenaline in the amygdala of naive rats (Löscher et al., 1991). This might be of relevance, since the amygdala was implicated in emotion-related behaviour (Coulombe and White, 1978) and fear response (Hitchcock and Davis, 1986). An anxiolytic activity of NMDA antagonists, observed in naive mice (Trullas et al., 1989) and rats (Kehne et al., 1991), might play a role in the attenuation of withdrawal jumping in mice, since anxiety is a symptom of opioid withdrawal in humans and animals (Lal and Emmett-Oglesby, 1983).

An observation that relatively high doses of DNQX promote convulsions in morphine-dependent mice, while the naive mice remained unaffected, deserves a comment. Recent data indicate that morphine dependence is associated with upregulation and/or supersensitivity of NMDA receptors (Marek et al., 1991). Other EAA receptors were not examined. A derangement of these, and possibly other EAA receptors, may explain a different reactivity to EAA receptor antagonists in morphine-dependent and naive mice.

In conclusion, the attenuating effect of EAA receptor antagonists on opioid withdrawal in mice might be due to complex changes in the activity of neurotransmitters and corresponding behavioral alterations in the addicted subjects. For further study the role of the NMDA glycine site is of particular interest, since drugs acting on this site are devoid of muscle-relaxant properties and possess significant anxiolytic effects.

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Chapter 4

Inhibitory effect of nitric oxide (NO) synthase inhibitors on naloxone-precipitated withdrawal syndrome in morphine-dependent mice

Abstract - The effect of intraperitoneally administered nitric oxide (NO) synthase inhibitors has been examined on the naloxone-precipitated withdrawal syndrome in morphine-dependent mice. L-NAME (30-200 mg/kg) and L-NOARG (7.5-50 mg/kg) induced a significant decrease of naloxone-precipitated withdrawal jumping and diarrhoea. However, L-NMMA (3.5-100 mg/kg), considered as a less potent NO synthase (NOS) inhibitor, did not significantly affect the withdrawal signs in mice. Although a specificity of NOS inhibitors is not fully established, these results indicate that inhibition of NO synthesis in the central nervous system and periphery may significantly affect the morphine withdrawal phenomena. Accordingly, this study suggests an involvement of NO in morphine withdrawal syndrome.
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The cessation of chronic use of central depressant drugs is associated with excitatory withdrawal signs. This could suggest an involvement of excitatory neurotransmitters in drug dependence phenomena. Accordingly, a recent study indicates that kynurenic acid, a non-selective antagonist of excitatory amino acid (EAA) receptors, attenuated a naloxone-precipitated withdrawal in rats (Rasmussen et al., 1991a). Similarly, other studies demonstrated that the non-competitive N-methyl-D-aspartate (NMDA) antagonist, MK-801, and the competitive NMDA antagonist, LY274614, suppressed the behavioral signs of withdrawal in morphine-dependent rats (Koyuncuoglu et al., 1992; Rasmussen et al., 1991b; Trujillo and Akil, 1991). We also observed that antagonists of various EAA receptors (NMDA-antagonist MK-801, non NMDA-antagonist DNQX and antagonist of glycine site of NMDA receptor 5,7-DCKA) attenuated the naloxone-precipitated withdrawal syndrome in morphine-dependent mice (Cappendijk et al., 1993).

Concerning nitric oxide (NO), it has been suggested that this compound is produced enzymatically in postsynaptic structures, in response to activation of central EAA receptors (Garthwaite et al., 1989; Knowles et al., 1989). Thus, there is a possibility that anti-withdrawal effect of antagonists of NMDA receptors might be due to the decrease of NO synthesis. In order to explore the proposed mechanism of action of antagonists of EAA transmission on drug withdrawal, we studied the effect of several NOS inhibitors N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), N^G-nitro-L-arginine (L-NOARG) and N^G-

monomethyl-L-arginine acetate (L-NMMA) on naloxone-precipitated withdrawal in morphine-dependent mice.

Materials and Methods

Morphine dependence

Chronic morphine dependence was induced in male Swiss mice (25-35 g) by implantation of pellet (25 mg morphine base/mouse, according to the method of Kosersky et al., 1974) under ether anaesthesia. The animals were housed individually in a room maintained on a 12-h light-dark cycle (lights on 08.00 h) with food and water ad libitum. The morphine withdrawal was precipitated by administration of naloxone (4 mg/kg, i.p.), 72 h after pellet implantation. The withdrawal severity was quantified by counting the frequency of jumping from a circular platform (30 cm high, 12 cm diameter). The presence of diarrhoea was checked.

Experimental protocol

The mice were divided into four groups, pretreated intraperitoneally with vehicle (distilled water, n=21), L-NAME (7.5-400 mg/kg, n=49), L-NOARG (3.5-100 mg/kg, n=36), or L-NMMA (3.5-100 mg/kg, n=30) 30 min prior to naloxone. Based on our preliminary experiments and other studies (Moore et al., 1991; Morgan et al., 1992), we selected biologically active doses of these drugs, which do not alter the locomotor activity. Regarding the penetration of NOS inhibitors in CNS, it is known that these substances are lipophilic - particularly L-NAME, and therefore, may pass easily through various lipid membranes, including blood-brain barrier (Gardiner et al., 1990; Morgan et al., 1992; Rees et al., 1990).

A drug pretreated animal was placed on a platform and observed for the following 30 min. At the end of the 30-min period, naloxone was administered and withdrawal jumping behaviour was counted and withdrawal diarrhoea was checked, for the following 30 min. The pH of drug solutions was adjusted to 7-8 and all drug solutions were given in an equal volume (0.5 ml/injection, i.p.).

Statistics

Data are expressed as mean ± SEM, while the control group (animals pretreated with vehicle) was taken as 100%. The effects of drug and vehicle treatment were evaluated statistically using the non-parametric Kruskall-Wallis one-way analysis of variance, followed by the Mann-Whitney *U*-test, with a level of $P<0.05$ being considered significant (Glantz, 1989).

Results

Data in Fig. 1A show a significant and dose-related decrease of the withdrawal jumps following administration of L-NAME (30-200 mg/kg, i.p.). Similarly, L-NOARG (7.5-50 mg/kg, i.p.) induced a significant decrease of naloxone-precipitated withdrawal jumps (Fig. 1B).

However, further increase of concentrations of L-NAME (400 mg/kg, i.p.) and L-NOARG (100 mg/kg) failed to decrease a withdrawal jumping behaviour (Figs. 1A and 1B). In contrast, L-NMMA (3.5-100 mg/kg) did not significantly decrease the naloxone-precipitated withdrawal jumping in mice (Fig. 1C).

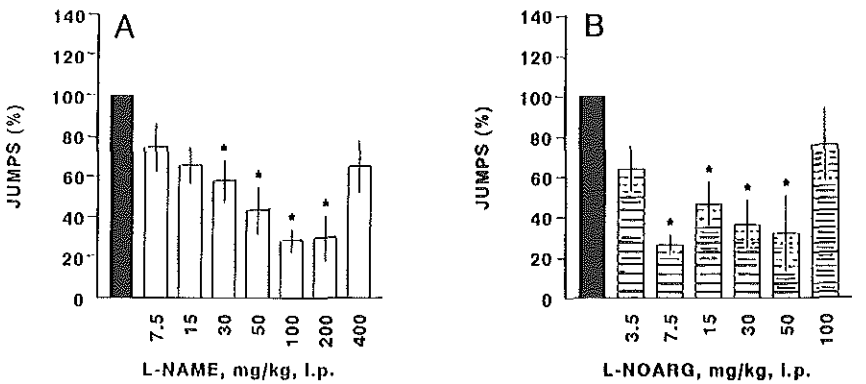

Fig. 1A-C. Naloxone-precipitated withdrawal jumps in the morphine-dependent mice. Results are shown as mean jumps ± SEM during 30 min following naloxone (4 mg/kg, i.p.) administration. The numbers of jumps in the control group (■) pretreated with vehicle (n=21) was expressed as 100%. This group is the same in all three figures. Other animals were pretreated with L-NAME (□, Fig. 1A, 7.5-400 mg/kg, i.p., 30 min prior naloxone; n=7, each dose group), or with L-NOARG (▤, Fig. 1B, 3.5-100 mg/kg, i.p., 30 min prior naloxone; n=6, each dose group), or with L-NMMA (▥, Fig. 1C, 3.5-100 mg/kg, i.p., 30 min prior naloxone; n=6, each dose group. Data in these figures were analyzed by the non-parametric Kruskall-Wallis ANOVA, one-way followed by the Mann-Whitney *U*-test.
* Significance at level of $P<0.05$.

Withdrawal diarrhoea was present in 20 of the in total 21 tested control animals. L-NAME and L-NOARG showed a dose-related inhibitory effect on the withdrawal diarrhoea. Sufficiently higher concentrations of L-NAME (50 mg/kg) and L-NOARG (100 mg/kg) completely abolished the morphine withdrawal diarrhoea in mice. However, L-NMMA in a dose range of 3.5-100 mg/kg had no significant effect on withdrawal diarrhoea (Table 1).

Dose (mg/kg, i.p.)	L-NAME n=7	L-NOARG n=6	L-NMMA n=6
3.5	NT	5 / 6	5 / 6
7.5	7 / 7	5 / 6	6 / 6
15	6 / 7	2 / 6	4 / 6
30	3 / 7	5 / 6	5 / 6
50	0 / 7	2 / 6	5 / 6
100	2 / 7	0 / 6	5 / 6
200	1 / 7	NT	NT
400	0 / 7	NT	NT

Table 1. Naloxone-precipitated withdrawal diarrhoea in morphine-dependent mice, pretreated with NO synthase (NOS) inhibitors. The results are presented as a number of animals with withdrawal diarrhoea versus total number of observed mice in the corresponding dose group. In the control group of morphine-dependent mice pretreated with vehicle (n=21), the naloxone (4 mg/kg, i.p.)-precipitated withdrawal diarrhoea was observed in 20 animals. NT = not tested dose; n = number of animals in each dose group.

Discussion

The results of this study indicate that the NOS inhibitors, L-NAME and L-NOARG, significantly attenuated the naloxone-precipitated withdrawal jumping and diarrhoea in morphine-dependent mice. However, L-NMMA in the dose range used in this study had no effect on withdrawal jumping behavior or diarrhoea in mice. This might probably be due to the fact that L-NMMA compared to the other NOS inhibitors is a significantly less potent drug (Garthwaite, 1991; Lambert et al., 1991).

In addition, we proposed that the anti-withdrawal effect of antagonists of EAA receptors is mediated by decreased activity of NO. This hypothesis was supported by results of this study, since the blockade of the NO synthesis attenuated the withdrawal syndrome in morphine-dependent mice. Administration of both single and continuous injection of L-NAME (100 mg/kg, s.c.; 12 mg/rat/day, respectively) to morphine-dependent rats also showed an attenuation of the naloxone-induced withdrawal syndrome (Adams et al., 1993). Accordingly, NO donor isosorbide dinitrate induced a quasi morphine-abstinence syndrome and aggravated the opioid withdrawal symptoms (Adams et al., 1993).

It is of interest to note that the higher dose of L-NAME (400 mg/kg, i.p.) and L-NOARG

(100 mg/kg, i.p.) failed to attenuate the naloxone-precipitated withdrawal jumping. A similar phenomenon has been observed with high doses of EAA receptor antagonists (Koyuncuoglu et al., 1992; Cappendijk et al., 1993). The reason for this effect is not known, but an involvement of additional mechanism(s) activated by higher drug concentrations should be considered. For example, it is known that higher doses of EAA receptor antagonists exert a prominent excitatory/proconvulsant effect in animals (Jurson and Freed, 1990; Schoepp et al., 1990). This may aggravate a morphine withdrawal syndrome, since it is composed of mainly excitatory psychomotoric symptoms.

Pharmacological characteristics of NOS inhibitors are not yet fully clarified. The reason for decrease of withdrawal jumping in mice following NOS inhibitors is also not known. However, some reasonable possibilities could be suggested. NO has a substantial effect on presynaptic neurotransmitter release (Garthwaite, 1991). A derangement of this transmitter release, following administration of NOS inhibitors might significantly affect withdrawal syndrome. Noradrenaline could be one of the transmitters involved in the anti-withdrawal effect of NOS inhibitors. NO increases tyrosine hydroxylase and potentiates presynaptic catecholamine release (O'Sullivan and Burgoyne, 1990). A possible decrease of the central catecholamine release, following treatment with NOS inhibitors might have an attenuating effect on morphine withdrawal. Several studies provided evidence that the central noradrenergic system is hyperactive during opioid withdrawal syndrome (Tseng et al., 1975; Aghajanian, 1978). In addition, it is indicated that serotonin is also involved in the mechanisms which lead to compulsive jumping during naloxone-precipitated withdrawal (Cervo et al., 1981). However, the precise relationship between NO and serotonin release is not known.

The diarrhoea associated with morphine withdrawal is of both central and peripheral origin (Burks et al., 1988). A decrease in withdrawal diarrhoea following administration of NOS inhibitors L-NAME and L-NOARG might predominantly be due to decrease of peripheral NO, since the muscle relaxation involved in the peristalsis is mediated by NO, synthesized in the neurons of the myenteric plexus (Snyder and Bredt, 1992). In addition, it has recently been demonstrated that alkyl esters of L-arginine (such as L-NAME) have anti-muscarinic properties (Buxton et al., 1993). Thus, it could be suggested that at least a part of the decrease in withdrawal diarrhoea might due to peripheral atropine-like activity of NOS inhibitors.

The NOS inhibitors used in this study are vasoconstrictors. However, there is no evidence that higher blood pressure attenuates opioid withdrawal. The blood pressure lowering agents - clonidine and NO donor isosorbide dinitrate - had an opposite effect on the opioid abstinence. Clonidine attenuated (Dionyssopoulos et al., 1992), while isosorbide dinitrate aggravated opioid withdrawal (Adams et al., 1993). Evidently, an alteration in blood pressure does not account for the anti-withdrawal effects of NOS inhibitors.

In conclusion, this study indicates an involvement of NO in the withdrawal syndrome,

since NOS inhibitors had a prominent attenuating effect on withdrawal jumping and diarrhoea. Furthermore, these results might support our hypothesis that the anti-withdrawal effect of EAA receptor-blocking drugs, observed by us and other authors, could be mediated by inhibition of NO synthesis. This could be further supported by the fact that the NO donor isosorbide dinitrate aggravates an opioid withdrawal (Adams et al., 1993). Although the mechanism of anti-withdrawal action of NOS inhibitors remains unknown, the complex changes in the central presynaptic neurotransmitter release, particularly decrease of noradrenaline, should be considered. In addition to the central NO, a decrease of peripheral NO synthesized in the myenteric plexus of gastro-intestinal tract and/or anti-muscarinic effect of NOS inhibitors might be of importance for attenuation of withdrawal diarrhoea.

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Chapter 5

Comparative study of normotensive and hypertensive nitric oxide synthase inhibitors on morphine withdrawal syndrome in rats

Abstract - The effects of the normotensive, mainly centrally active nitric oxide synthase (NOS) inhibitor, 7-nitro indazole and the hypertensive compound N^G-nitro-L-arginine, which blocks both the endothelial and central NOS, have been examined for their effects on naloxone-precipitated withdrawal syndrome in morphine-dependent rats. Both drugs attenuated the same withdrawal signs (teeth-chattering, penile licking, diarrhoea, chewing, wet-dog shakes, grooming), while other signs remained unaffected (rearing, jumping, ptosis, rhinorrhoea, irritability on touch). These findings indicate that mainly central (but not endothelial) nitric oxide is involved in the expression of some opioid withdrawal symptoms.
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Activation of NMDA (N-methyl-D-aspartate) receptors stimulates synthesis of nitric oxide (NO, Garthwaite, 1991), while the blockade of these receptors attenuates naloxone-precipitated withdrawal syndrome in morphine-dependent animals (Trujillo and Akil, 1991; Cappendijk et al., 1993a). This may implicate involvement of NO in the expression of morphine withdrawal syndrome. This idea received support from several experiments showing an attenuating effect of NO synthase (NOS) inhibitors on opioid withdrawal in mice (Kolesnikov et al., 1993; Cappendijk et al., 1993b; Majeed et al., 1994) and rats (Adams et al., 1993; Kimes et al., 1993). In addition, it has been suggested that NOS inhibitors block the development of morphine tolerance and dependence (Kolesnikov et al., 1993; Majeed et al., 1994). The NOS inhibitors used in all these studies inhibited the activity of both peripheral (endothelial) and central NOS. However, recently 7-nitro indazole (7-NI), which is a selective inhibitor for brain NOS, lacking effects on endothelial NOS and blood pressure (Moore et al., 1993a,b), has become available.

It is known that opioid withdrawal signs are affected by both central and peripheral factors (Neal and Sparber, 1986; Maldonado et al., 1992). In order to specify the role of central and peripheral NO in morphine-dependent rats, we compared the effects of 7-NI and N^G-nitro-L-arginine (L-NOARG) on morphine withdrawal syndrome in rats. L-NOARG inhibits both the central and the endothelial NOS and is a potent hypertensive agent. Hypertension is mainly due to the inhibition of endothelial synthesis and a

corresponding reduction of vasodilatory NO (Iwata et al., 1992). This study shows that anti-withdrawal effect of NOS inhibitors is mainly due to a decrease in the activity of central NO.

Materials and methods

Animals

Male Wistar rats (TNO Zeist), weighing 290-330 g were housed in groups and had free access to food and water. The room was maintained on a 12-h light/dark cycle (lights on 08.00 h), with constant temperature (21° C) and humidity (55%).

Morphine dependence

Drug dependence in a rat was induced by s.c. implantation of 3 pellets, containing 25 mg morphine base/pellet, on the back of the animal under ether anaesthesia. The withdrawal syndrome was precipitated by administration of naloxone (4 mg/kg, i.p.) 72 h after pellet implantation (Cappendijk et al., 1993a). The observer was "blind" to the drug treatment procedure. The withdrawal symptoms were monitored for 30 min following injection of naloxone and scored according to the weighting factors described by Neal and Sparber (1986). In short, the signs observed during a mild withdrawal syndrome were assigned with 1 (chewing, diarrhoea, grooming, rearing, irritability on touch). Weighting factor 2 was given to the withdrawal signs, teeth-chattering, wet-dog shakes, penile licking, ptosis and jumping. The sign rhinorrhoea, observed during severe withdrawal was assigned a 3.

Experimental protocol

L-NOARG (7.5-100 mg/kg, i.p., n=35; Research Biochemical Incorporation, England) or vehicle (distilled water, i.p., n=12) were administered 30 min prior to naloxone. 7-NI (6.25-50 mg/kg, i.p., n=28; Sigma) or vehicle (arachis oil, i.p., n=9) were given 5 min prior to naloxone. These doses of L-NOARG and 7-NI have an inhibitory effect on NOS (Klatt et al., 1994; Moore et al., 1993b) and biological effect, for example on nociception (Babbedge et al., 1993; Moore et al., 1993a). The time interval between the administration of 7-NI or L-NOARG and naloxone was chosen to ensure a maximal inhibitory effect of these drugs on NOS (Klatt et al., 1994; Moore et al., 1993b) during observation of withdrawal signs.

Solutions of L-NOARG and 7-NI were given i.p. in a volume of 2.2 ml/injection. Naloxone was given i.p. in a volume of 1 ml/kg animal. The pH of drug solutions and vehicles were adjusted to 7-8. Each animal was used only once.

Statistics

The data were evaluated by using the non-parametric Kruskal-Wallis one-way analysis of variance, followed by the Mann-Whitney *U*-test, with a level of $P<0.05$ being considered significant.

Results

The results show that no differences were observed with respect to the frequency of the withdrawal signs, between the controls treated with distilled water or arachis oil. Therefore, both controls were considered as a single group. The results illustrated in Fig. 1A show that both 7-NI (12.5-50 mg/kg, i.p.) and L-NOARG (15-100 mg/kg, i.p.) attenuated significantly the severity of withdrawal syndrome (P=0.0001, P=0.0003, respectively) compared to the control group. Between 7-NI and L-NOARG treatment no differences were observed in the expression of the withdrawal syndrome (P=0.37). The withdrawal signs, teeth-chattering, penile licking, diarrhoea, chewing, wet-dog shakes and grooming (Fig. 1 B-G) were significantly attenuated by both 7-NI and L-NOARG. However, the effect of 7-NI was predominantly dose-related, while L-NOARG induced a U-shape dose-response curve in three out of six withdrawal signs. Other withdrawal signs, rearing, jumping, ptosis, rhinorrhoea and irritability on touch were not significantly altered by any of the NOS inhibitors used in this study (Fig. 1 H-L).

Discussion

This study demonstrated that both NOS inhibitors, 7-NI and L-NOARG induced a significant decrease of severity of the naloxone-precipitated withdrawal syndrome. The fact that some withdrawal symptoms were attenuated by both NOS inhibitors, implicates that these signs are predominantly affected by decreased concentrations of central NO. However, this does not exclude an additional involvement of peripheral NO.

The exact mechanism involved in the role of central NO in the withdrawal syndrome remains unknown. However, some possibilities related to the specific withdrawal signs may be considered:

Activation of the NO system stimulates release of several neurotransmitters, such as acetylcholine (ACh, Lonart et al., 1992; Prast and Philippu, 1992), noradrenaline (NA, Lonart et al., 1992), and dopamine (DA, Lonart et al., 1993). The occurrence of some withdrawal signs has been ascribed to the increased release of specific neurotransmitters. For example, wet-dog shakes were ascribed to activity of serotonin (5-HT) and NA. Accordingly, lesion of the locus coeruleus or administration of 5-HT blocking agents reduced wet-dog shakes (Bedard and Pycock, 1977; Maldonado and Koob, 1993); grooming was related to stimulation of dopamine D_1 receptors (Van Wimersma Greidanus et al., 1989), while the chewing response was elicited by stimulation of cholinergic system and reduced by anticholinergic drugs (Gunne et al., 1982). Thus, an inhibition of the NO system by NOS inhibitors and the corresponding decrease of neurotransmitter release may contribute to the attenuation of morphine withdrawal syndrome.

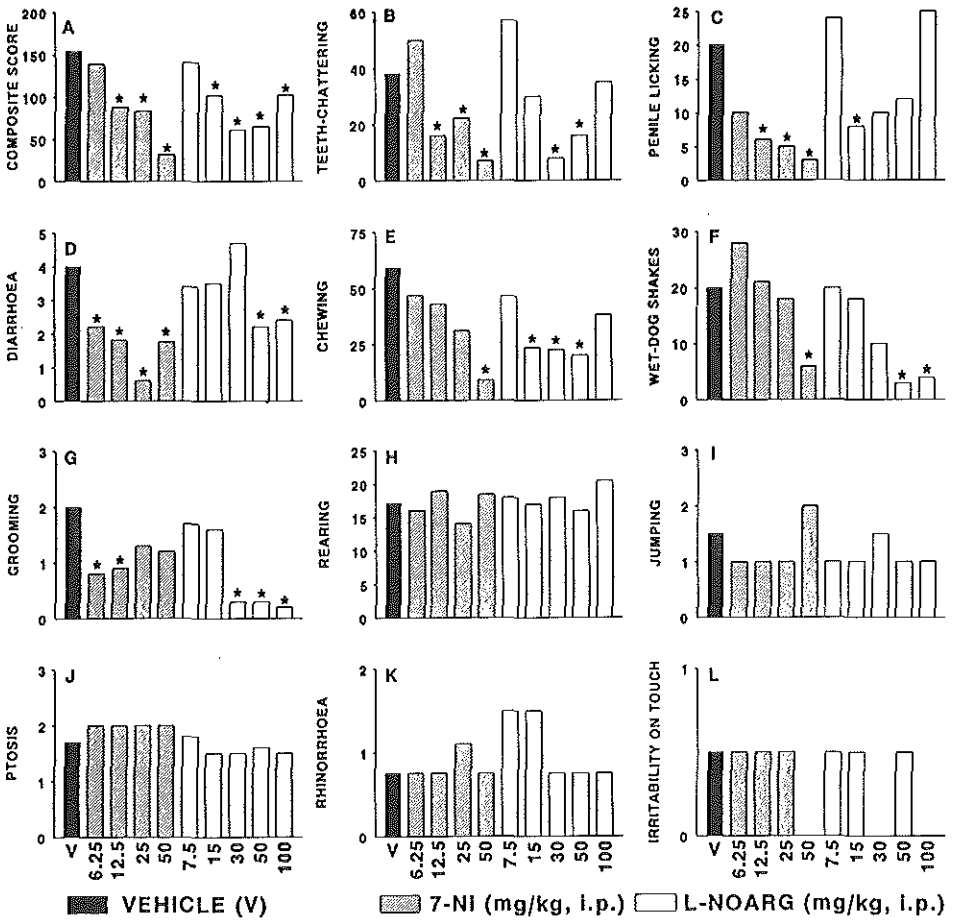

Fig. 1. Effect of the nitric oxide synthase (NOS) inhibitors 7-nitro indazole (7-NI) and N^G-nitro-L-arginine (L-NOARG) on the severity of naloxone (4.0 mg/kg, i.p.)-precipitated withdrawal syndrome (A) and various withdrawal signs (B-L) in morphine-dependent rats. Animals were pretreated with 7-NI (▨, 6.25-50 mg/kg, i.p., 5 min prior naloxone; n=7, each dose group) or with L-NOARG (□, 7.5-100 mg/kg, i.p., 30 min prior naloxone; n=7, each dose group). The control animals, pretreated with vehicles (■, arachis oil, n=9, or distilled water, n=12) did not show differences in the severity of naloxone-precipitated withdrawal syndrome. Therefore, controls were considered as a single, vehicle-treated group (V, n=21). Data in fig. 1A are expressed as composite score, determined by counting the number of all observed withdrawal signs, during the 30-min period of abstinence. The withdrawal signs were scored according to the method described by Neal and Sparber (1986) and all data were expressed as median values. * Significance at level of $P < 0.05$. Note that both NOS inhibitors attenuated the severity of the withdrawal syndrome and some of the withdrawal signs.

In this study, NOS inhibitors also attenuated penile licking. Spinal cholinergic and NO-generating systems are known to be involved in the sensory regulation of the sympathetic and parasympathetic outflow to the penis (Krane et al., 1989; Zhuo et al., 1993). It seems that attenuation of the penile licking by 7-NI might be due to both a supraspinal insufficiency of the NO system (responsible for psychogenic erection) and decreased spinal release of ACh (responsible for the reflexogenic erection). In addition, the L-NOARG-induced attenuation of penile licking might be due to peripheral inhibition of NOS in non-adrenergic and non-cholinergic nerve terminals, which innervate the corpus cavernosum (Rajfer et al., 1992). The morphine withdrawal syndrome, therefore, could be used as an appropriate animal model to study the erectile mechanism.

Diarrhoea occurring during morphine withdrawal was usually considered as a peripheral effect (Maldonado et al., 1992), with some involvement of the CNS (Warhurst et al., 1984). The fact that both 7-NI and L-NOARG attenuated withdrawal diarrhoea favours the central component. However, an additional involvement of peripheral NO, following administration of L-NOARG could not be excluded, since the neurons of the myenteric plexus synthesize NO, which participates in the relaxation phase of peristalsis (Bredt et al., 1990).

Several other withdrawal symptoms (jumping, rearing, ptosis, rhinorrhoea and irritability on touch) were not altered by NOS inhibitors. This might indicate that NO is not involved in the expression of these signs.

An additional point of interest is the fact that attenuation of morphine withdrawal induced by L-NOARG is limited to a certain dose range. For some of the signs, the highest dose of L-NOARG failed to attenuate the naloxone-precipitated withdrawal symptoms. A similar phenomenon has been observed with high doses of NMDA receptor blockers (Koyuncuoglu et al., 1992; Cappendijk et al., 1993a) and NOS inhibitors (Cappendijk et al., 1993b) used for attenuation of withdrawal behaviour in rats and mice. The reason for this U-shaped dose-response curve is not known, but involvement of some additional mechanism(s) activated by higher drug concentrations should be considered. For example, a conversion of L-NOARG to L-arginine (Hecker et al., 1990) associated with accumulation of L-arginine and corresponding self-inhibition might be one of the explanations. The attenuating effect of 7-NI on withdrawal syndrome is predominantly dose-related.

In conclusion, this study indicates that central NO is involved in the expression of some (but not all) opioid withdrawal signs. Accordingly, an attenuation of the withdrawal syndrome, induced by NOS inhibitors is unrelated to an inhibition of endothelial NOS and increased blood pressure. We suggest that the anti-withdrawal effect of NOS inhibitors is due to a decrease of central NO levels and related decrease of neurotransmitters. This neurotransmitter derangement may affect the corresponding withdrawal signs in morphine-

dependent subject.

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Chapter 6

"Withdrawal substance" in cerebrospinal fluid of morphine-abstinent rats

Abstract - The behavioral, electrophysiological (visual evoked potentials, VEP) and *in vitro* effects of cerebrospinal fluid (CSF) taken from the donor rat have been investigated in the recipient rat and guinea-pig isolated ileum. The CSF of spontaneous morphine-abstinent donor rat precipitated in morphine-dependent recipient rat an opioid withdrawal syndrome, which was characterized by a decrease in the VEP peak latency N3 and amplitudes N2-P3 and P3-N3. The CSF-induced withdrawal syndrome was behaviorally less severe and electrophysiologically less prominent, but qualitatively identical to the naloxone-induced abstinence. However, in contrast to naloxone, the CSF from spontaneous morphine-abstinent rat did not contract the morphine-dependent isolated guinea-pig ileum. Chromatographic analysis of CSF samples from naive, morphine-dependent or morphine-abstinent rats reveal distinct fractions, containing an active component present only in CSF of morphine-abstinent rats. The estimated relative molecular mass of this active component was around 50 kDa and the short retention time on the reversed-phase column suggests the high hydrophobicity. The results indicate that spontaneous morphine-abstinent donors synthesize and release certain quantity of putative "withdrawal substance" in the CSF, which is without naloxone-like properties. This further suggests, that the CSF- and naloxone-precipitated withdrawal in the morphine-dependent recipients are mediated by activation of different neuronal mechanisms.
Part of these data are published in Regulatory Peptides 1: S227-S228, 1994.

It was shown that cerebrospinal fluid (CSF) from morphine-abstinent donor rats, precipitates an opiate withdrawal syndrome in morphine-dependent recipient rats (Malin et al., 1987). These authors found an increased level of octapeptide F-8-F-NH_2 (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2)-like immunoreactivity in CSF of morphine-dependent rats (Malin et al., 1990a). The octapeptide F-8-F-NH_2 precipitates withdrawal syndrome in morphine-dependent rats, but not in the naive ones (Malin et al., 1990b). However, the role of this peptide in the CSF during morphine dependence and withdrawal remains unclear.

Opioid withdrawal is an excitatory syndrome, characterized by psychomotor activation, in both animals and men. The behavioral activation implicates an increase of neuronal excitability during morphine abstinence. This has been supported by finding of

increased cell firing during opioid withdrawal (Aghajanian, 1978). Accordingly, a similar behaviour and increase of neuronal excitability should be expected following administration of CSF from morphine-abstinent donor rat into morphine-dependent recipient rat.

In order to test this hypothesis, we examined the electrophysiological effects of CSF withdrawn from abstinent rats, by monitoring the peak latencies and amplitudes of visual evoked potentials (VEP) during CSF-precipitated withdrawal syndrome in rats. The effect of CSF from spontaneous morphine-abstinent rats was also studied in naive rats, morphine-dependent rats and morphine-dependent isolated guinea-pig ileum. In addition, we analyzed CSF samples from morphine-abstinent, morphine-dependent and naive rats by high-performance liquid chromatography (reversed-phase and gel-filtration techniques).

Materials and methods

Animals

Male Wistar rats (TNO, Zeist), weighing 200-300 g were housed in groups and had a free access to food and water. The room was maintained on 12 h light-dark cycle (lights on 8.00 h), with constant temperature (21° C) and humidity (55%).

Procedures

Surgical procedure

All animals were anaesthetized with pentobarbital (60 mg/kg, i.p.). The donor rats were implanted with a chronic cannula, placed into the cisterna magna in order to withdraw CSF from conscious animals (Bouman and Van Wimersma Greidanus, 1979). In recipient rats, receiving CSF from donor rats, a cannula was placed into the lateral ventricle (Paakkari, 1980). Rats, involved in the VEP experiments were all recipient rats and, in addition to the lateral ventricle cannula, they were implanted with stainless steel screw electrodes over the right and left visual cortex (7 mm posterior to the bregma and 3 mm lateral to the midline). A reference electrode was placed in the frontal sinus. The electrodes were soldered to a miniature socket and attached to the skull with dental acrylate. In the recovery period (7 days), all operated animals were housed individually with food and water *ad libitum*. At the end of the experiments, the placement of lateral ventricle cannula was confirmed by injection of methylene blue.

Morphine dependence and abstinence

Morphine dependence was induced by treating animals with morphine for 8 days (twice daily, 9h and 17h). A starting dose of morphine was 10 mg/kg/injection, which increased daily to 20, 20, 40, reaching a final dose of 80 mg/kg/injection on the 5th day. Rats treated with distilled water (1.0 ml/kg) for 8 days (twice daily, 9h and 17h), formed a group of naive animals. Morphine-dependent rats were considered as spontaneous morphine-abstinent, 6 h following the last morphine injection.

The control experiments were performed in naive and morphine-dependent rats on the 7th day of drug treatment. The behavioral signs similar to withdrawal symptoms occurred

sporadically following administration of vehicle or artificial CSF. On the next day (8th day of drug treatment), the animals were treated with naloxone or CSF from naive, morphine-dependent or morphine-abstinent donor rats. The behaviour of the animals on the 7th or 8th day of drug treatment was monitored for 30 min following drug administration and scored by a person "blinded" to the experimental procedure.

The withdrawal signs were scored according to the weighting factors described by Neal and Sparber (1986). In short, the signs observed during a mild withdrawal syndrome were assigned with 1 (chewing, diarrhoea, grooming, rearing, irritability on touch). The weighting factor 2 was given to the withdrawal signs, teeth-chattering, wet-dog shakes, penile licking, ptosis and jumping. The sign rhinorrhoea, observed during severe withdrawal was assigned a 3.

Visual Evoked Potentials (VEP)

A postoperative recovery period of 7 days was followed by habituation of the recipient to the recording procedure. The method used for recording of VEP is described in the previous study of Dzoljic et al., (1994). Briefly, the animal was placed in a test chamber and after connecting the electrodes, a flash stimulus was induced at 1 per 7 s for 10 min. The habituation period lasted 3 days. This approach was selected, since it has been shown that under these conditions VEP discharges stabilize after several days (Bigler, 1975). The flash light was generated by a Grass S44 stimulator in a frequency of 0.14 Hz. Brain responses were amplified with a Grass model 79 B, connected to an analog-digital convertor (Lab Master, Scientific solutions Inc., Ohio, USA), triggered by the Grass S44 stimulator after every flash light. A computer connected to the analog-digital convertor performed the averaging of 25 VEP over a 800 ms epoch after every flash light and printed the results. Stimulation was performed only in animals with open eyes. The VEP parameters (peak latency and amplitude) were recorded in a total of six sessions, namely 5, 15 and 30 min before drug administration (self-control) and in the same time intervals after drug administration.

In vitro experiments

Male guinea-pigs (n=5, 600-900 g) were killed by a blow on the head. A 40 cm long segment of the small intestine was rapidly removed and placed in Krebs solution (room temperature). The terminal section of the guinea-pig ileum was used after discarding the portion of 10 cm closest to the ileo-caecal junction (Munro, 1953). The ileum was cut in eight 3-cm long segments. These segments were gently and thoroughly washed free of faecal matter by flushing Krebs solution through the lumen. Each streap of ileum was set up in a 8 ml organ bath containing Krebs solution and bubbled with 95% O_2 and 5% CO_2. Every 15 min the bath was perfused with fresh warm Krebs solution. The temperature and pH of the Krebs solution were maintained at 37°C and 7.4, respectively. The ileum was fixed at a resting tension of 1 g and allowed to equilibrate for 30 min. No drug was added in this time period. The spontaneous activity of the ileum was recorded isometrically. In order to induce morphine dependence, the ileum was exposed to morphine (1 μM) for 2 h (Cruz et al., 1991). The pieces of ileum not treated with morphine were considered as naive ileum. Exposure of ileum to CSF for 5 min was followed (after washing) by naloxone (0.1 μM). Naloxone remained in the bath also for 5 min. The CSF was made artificially or withdrawn from the donor rats (naive, morphine-dependent or morphine-

abstinent) on the 8th day of drug treatment.
The contraction of the ileum was defined as the peak tension observed within 1 min after drug administration. In order to check the contractility of smooth muscle, each ileum was exposed to methacholine (0.1 μM) at the end of experiment. Only experiments with morphine-dependent ileum responding to methacholine and naloxone were taken as valid.

High-Performance Liquid Chromatography (HPLC)

Pooled samples of CSF (approximately 240-300 μl total volume) taken from naive, morphine-dependent or morphine-abstinent rats were analyzed using the SMART micropurification system (Pharmacia Biotech., Uppsala, Sweden). The system was operated as described in previous reports (Nyberg et al., 1991; Renlund et al., 1993). Briefly, a reversed-phase column μRPC C2/C18 (2.1 x 10 mm) and a gel-filtration column Superdex 75 (3.2 x 300 mm) were used in this study. The CSF samples were filtered through a nonsterile 45 μm filter (Ultrafree-MC, Millipore, Bedford, MA, USA) and injected into the system. The reversed-phase column was eluted with a 30 min linear gradient from 0-60% acetonitrile, supplemented with 0.1% trifluoroacetic acid. The flow-rate was maintained at 50 μl/min and one-min fractions were collected. The size separations (100 μl sample injected) were conducted using 20 mM Tris-HCl buffer of pH 7.4 as the eluent. The collected material was stored at -80° C until assayed.

Drugs

Morphine hydrochloride (OPG, Utrecht) and naloxone hydrochloride (Research Biochemical Incorporation, England) were dissolved in distilled water. The composition of solutions (expressed in mM) was as follows: Krebs buffer - NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 5.55; Artificial cerebrospinal fluid - NaCl 138; KCl 3.3; CaC_2 2.2; $MgCl_2$ 1.15; $NaHCO_3$ 2.1; NaH_2PO_4 0.6; urea 2.16; glucose 3.38. The volume of CSF administered i.c.v. was 80 ± 5 μl per recipient rat.

Statistical analysis

The data in relation to withdrawal behaviour and electrophysiological study were statistically evaluated by using the non-parametric Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney *U*-test, with a level of $P<0.05$ being considered significant.

Results

Behaviour

Naive rats - recipients of CSF (Fig. 1A)

Administration of artificial CSF (80 ± 5 μl, i.c.v.) into naive animals (n=15) treated with vehicle for 7 days, did not alter the normal behaviour, characterized with occasional appearance of grooming, digging, scratching and rearing. On the following day (8th day of the vehicle treatment) these animals, randomly divided into three groups, received the CSF

(80 ± 5 µl, i.c.v.) taken from the naive rats (n=5), morphine-dependent (n=4) or spontaneous morphine-abstinent rats (n=6). The behaviour of animals in all three groups receiving various samples of CSF remained unaltered.

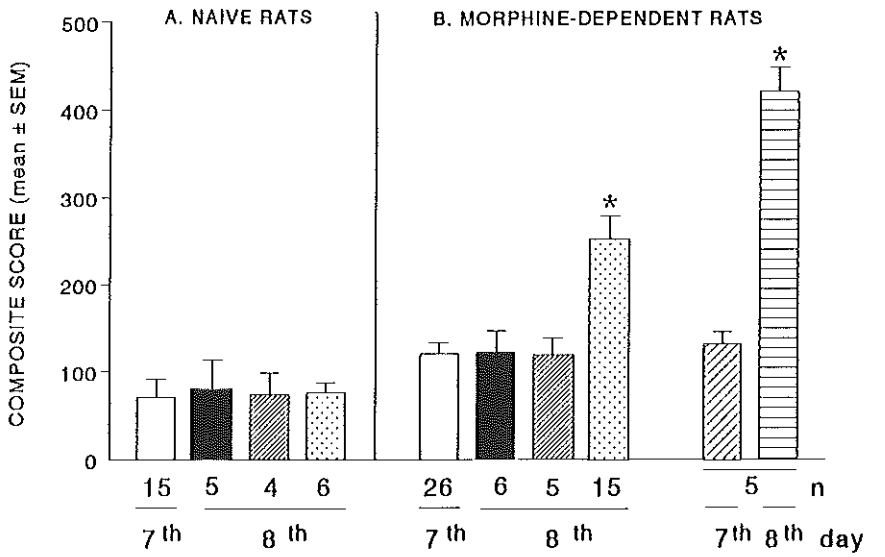

Fig. 1. Effect of CSF (80 ± 5 µl, i.c.v.) on the behaviour of naive (A) and morphine-dependent (B) rats. Both groups of animals were injected with artificial cerebrospinal fluid (□, i.c.v.) on the 7th day of drug (vehicle/morphine) treatment. The following day (8th day), the animals were treated with cerebrospinal fluid (CSF) taken from the naive (■), morphine-dependent (▨) or spontaneous morphine-abstinent rats (▩). An additional group of morphine-dependent rats was treated with vehicle (▨, 1.0 ml/kg, i.p., on the 7th day of morphine treatment) and naloxone (▤, 4.0 mg/kg, i.p. on the following day). Numbers beneath the bars represent the number of animals (n) and the day of treatment with vehicle or morphine. Data are expressed as composite score (mean ± SEM) by counting the number of all behavioral signs occurring in the naive and morphine-abstinent rats. The behaviour was monitored for 30 min. The behavioral signs were scored according to the method of Neal and Sparber (1986). * indicates significant differences ($P<0.05$) between the control and drug treatments. Note that CSF from the spontaneous morphine-abstinent rats (▩) induced in morphine-dependent recipient rats (but not in the naive ones) a withdrawal syndrome, which is significantly less severe than the naloxone-induced withdrawal (▤).

Morphine-dependent rats - recipients of CSF or treated with naloxone (Fig. 1B)

Administration of artificial CSF into dependent animals (n=26, on the 7th day of morphine treatment) did not change the behaviour. On the following day (8th day of morphine treatment) these animals, randomly divided into three groups, received CSF obtained from the naive (n=6), morphine-dependent (n=5) or spontaneous morphine-abstinent (n=15)

donor rats. No behavioral changes were observed in the morphine-dependent rats, receiving CSF from the naive or morphine-dependent donors. However, morphine-dependent recipients treated with CSF taken from spontaneous morphine-abstinent rats exhibited a significant increase in the expression of withdrawal syndrome. In order to compare the severity of CSF- and naloxone-precipitated withdrawals, an additional group of dependent rats (n=5) was treated with vehicle (1.0 ml/kg, i.p., on the 7th day of morphine treatment) and naloxone (4.0 mg/kg, i.p. on the following day). It was found that naloxone-precipitated withdrawal was significantly more severe than the withdrawal induced by CSF.

Visual Evoked Potentials (VEP)

Naive rats - recipients of CSF (Fig. 2)

Artificial CSF or CSF taken from naive (n=5) or spontaneous morphine-abstinent donor rats (n=5) and administered into naive recipient rats (15 animals equally divided in three groups for each particular sample of CSF) did not change their peak latencies (Fig. 2A) or amplitude values (Fig. 2B).

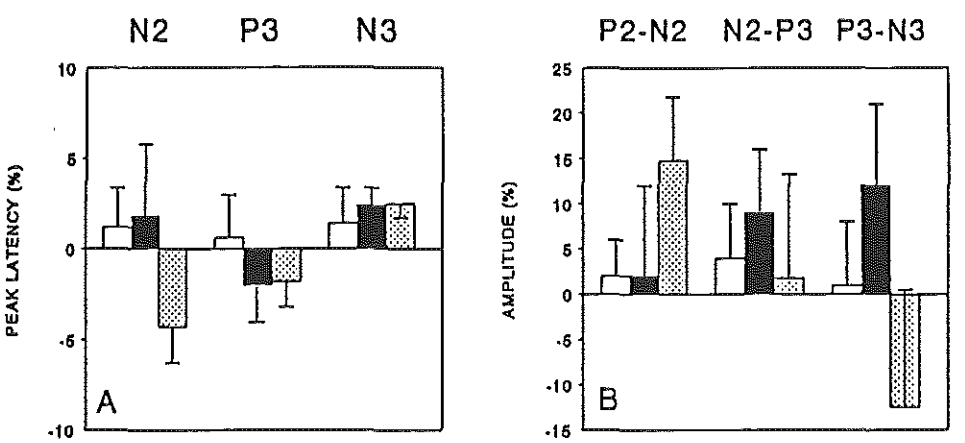

Fig. 2. Effect of CSF (80 ± 5 µl, i.c.v.) on the peak latencies (A) and amplitude values (B) of VEP in naive recipient rats. The artificial CSF (□) or CSF withdrawn from the naive (■, n=5) or spontaneous morphine-abstinent (▨, n=5) donor rats were administered into three groups of naive recipient rats (5 animals for each different sample of CSF). The 0%-line, taken as self-control indicates the average of peak latencies and amplitude values, measured in three sessions (5, 15 and 30 min) before administration of CSF. Data are expressed as % ± SEM of altered peak latencies and amplitude values compared to self-control. Note that none of the CSF samples altered VEP parameters (latency and amplitude) in naive recipient rats.

Morphine-dependent rats - recipients of CSF (Fig. 3)

Artificial CSF or CSF taken from the naive donors (n=5) were administered to morphine-dependent rats (10 animals equally divided into two groups for each particular sample of CSF). No significant changes in the peak latencies (Fig. 3A) or amplitudes (Fig. 3B) were observed. However, CSF taken from spontaneous morphine-abstinent donor rats (n=6) significantly decreased the peak latency N3 (Fig. 3A), and amplitude values of N2-P3 and P3-N3 (Fig. 3B) in morphine-dependent recipients (n=6).

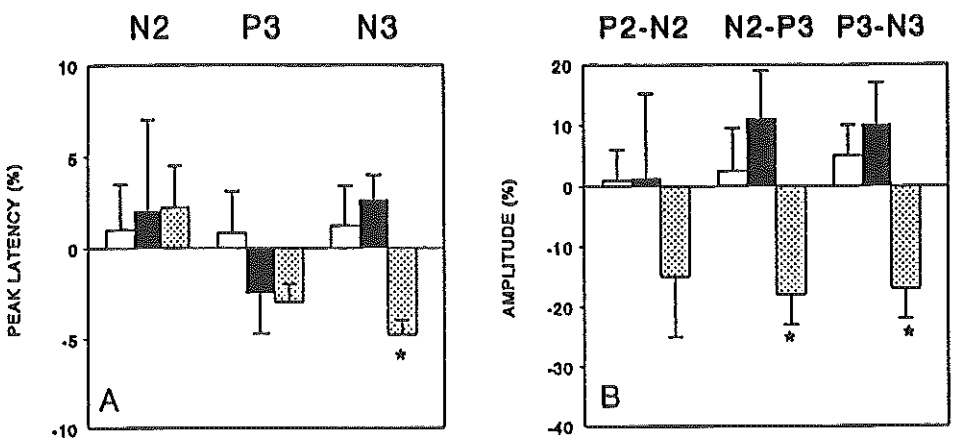

Fig. 3. Effect of CSF (80 ± 5 µl, i.c.v.) on peak latencies (A) and amplitude values (B) of VEP in morphine-dependent recipient rats. CSF made artificially (□) or CSF withdrawn from the naive (■, n=5) or spontaneous morphine-abstinent (▤, n=6) donor rats were administered into morphine-dependent recipient rats (5-6 animals for each different sample of CSF). The 0%-line, taken as self-control indicates the average of peak latencies and amplitude values, measured in three sessions (5, 15 and 30 min) before administration of CSF. Data are expressed as % ± SEM of altered peak latencies and amplitude values compared to self-control. Significant changes of peak latencies and amplitude values versus self-control are indicated by * ($P<0.05$). Note that CSF from spontaneous morphine-abstinent donors (▤) induced a significant decrease of peak latency N3 and amplitude values (N2-P3, P3-N3) of VEP in morphine-dependent rats.

Morphine-dependent rats - treated with vehicle and naloxone (Fig. 4)

Morphine-dependent animals (n=9) treated with vehicle, did not show changes in peak latencies (Fig. 4A) or amplitude values (Fig. 4B). However, the administration of naloxone to the same animals (n=9) on the next day (8^{th} day of the morphine treatment) induced a significant decrease of the peak latencies P3 and N3 (Fig. 4A) and the amplitude values N2-P3 and P3-N3 (Fig. 4B).

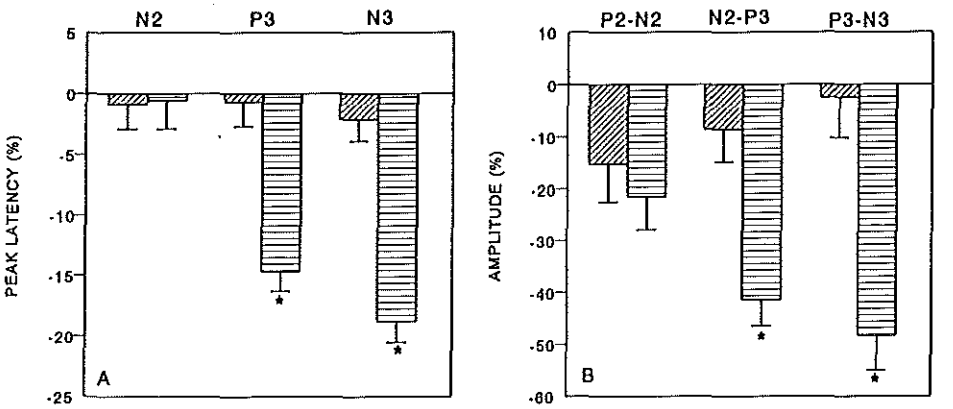

Fig. 4. Effect of vehicle (distilled water, 1.0 ml/kg, i.p.) and naloxone (4.0 mg/kg, i.p.) on the peak latencies (A) and amplitude values (B) of visual evoked potentials (VEP) in morphine-dependent rats (n=9). Vehicle (▨) or naloxone (▤) were administered on the 7th and 8th day of morphine treatment, respectively. The 0%-line, taken as self-control indicates the average of peak latencies and amplitude values, measured in three sessions (5, 15 and 30 min) before administration of CSF. Data are expressed as % ± SEM of altered peak latencies and amplitude values compared to self-control. Significant changes of peak latencies and amplitude values versus self-control are indicated by * ($P<0.05$). Note that naloxone induced a significant decrease of peak latencies (P3 and N3) and amplitude values (N2-P3 and P3-N3) of VEP in morphine-dependent rats.

In vitro experiments

Guinea-pig ileum (Fig. 5)

Administration of naloxone (0.1 μM) to the bath with morphine-dependent ileum (n=5) was followed by clear contractions (Fig. 5B), while the tonus of the naive ileum (n=5) remained unaltered (Fig. 5A). However, the artificial CSF or CSF taken from the naive (n=3), morphine-dependent (n=3) or spontaneous morphine-abstinent rats (n=4) did not affect the basal tonus of isolated naive or morphine-dependent guinea-pig ileum (Fig. 5A and 5B). Methacholine (0.1 μM) induced a contraction of both, naive and morphine-dependent ileum (Fig. 5A and 5B).

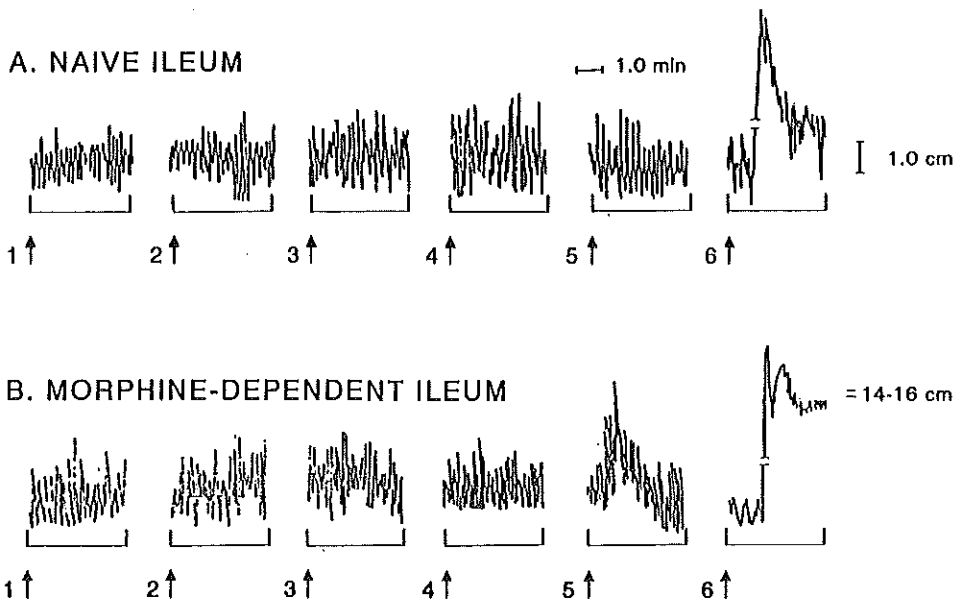

Fig. 5. Effect of CSF (80 ± 5 µl, 1↑, 2↑, 3↑, 4↑), naloxone (0.1 µM, 5↑) and methacholine (0.1 µM, 6↑) on naive and morphine-dependent guinea-pig ileum *in vitro*. The CSF added into the bath was made artificially (1↑) or withdrawn from the naive (2↑), morphine-dependent (3↑) or spontaneous morphine-abstinent (4↑) donor rats. Note that no any sample of CSF (1↑, 2↑, 3↑, 4↑) had an effect on muscle tonus, while naloxone (5↑) induced a contraction only in the morphine-dependent ileum. Methacholine (6↑) induced a contraction in both, naive and morphine-dependent ileum.

High-Performance Liquid Chromatography (HPLC)

Chromatographic analysis of CSF samples, both using reversed-phase and gel-filtration techniques reveal distinct fractions, containing a putative "withdrawal substance", which was present only in CSF from the morphine-abstinent animals. In general, distinct UV-patterns (taken at 280, 254 and 214 nm) were observed in CSF from naive, morphine-dependent and morphine-abstinent animals. The estimated relative molecular mass of the active component was around 50 kDa and its short retention time on the reversed-phase column suggests the high hydrophobicity.

Discussion

Behaviour

Administration of CSF samples taken from morphine-abstinent rats (donors) into the lateral ventricle of morphine-dependent rats (recipients), precipitated a withdrawal syndrome, which could not be observed in the naive recipient rats. The CSF-induced withdrawal syndrome was less severe, but qualitatively identical to the naloxone-precipitated withdrawal syndrome. Artificial CSF or CSF from the naive or morphine-dependent animals did not induce withdrawal syndrome in no any group of recipient animals. These results confirmed the earlier data (Malin et al., 1987) related to the withdrawal induced by CSF from morphine-abstinent rats.

Visual Evoked Potentials (VEP)

Peak latencies: The CSF taken from the spontaneous morphine-abstinent donor rat induced a decrease of peak latency N3 in morphine-dependent recipient, which indicates a stimulation of central neurotransmission. The CSF-induced decrease of peak latency was less prominent compared to the effect of naloxone. This is a good reflection of similar differences observed in respect to the severity between the withdrawal syndromes induced by CSF and naloxone. The development of N3 component is a result of massive discharge of lateral geniculate units (Bigler, 1975), while the components P2, N2 and P3 represent a diffuse activity between thalamus, midbrain and cortex (Creel et al., 1974). The peaks N3 and P3 reflect an arousal level in the brain (Joseph et al., 1981). A decrease of peak latencies during naloxone- or CSF-precipitated withdrawal suggest an increase of neuronal excitability in the mentioned brain areas.

VEP amplitude values: The CSF taken from the spontaneous morphine abstinent donor rats and naloxone induced a decrease of amplitude values of several peaks (N2-P3 and P3-N3) in the morphine-dependent recipients. Decrease of amplitude values reflects a neuronal depression, which is rather unexpected finding, since naloxone-precipitated withdrawal was described as a state of psychomotor stimulation (Wise and Bozarth, 1987). The naloxone-induced decrease of VEP amplitudes contrasted also to the suggested epileptogenic properties of naloxone, since this opioid receptor antagonist induced an increase of photically evoked discharges in the naive conscious rats (Shearer et al., 1984). However, the possibility that excitation of some inhibitory neurons may lead to depression of other neurons in the visual pathways of morphine-dependent rat, might explain these controversies. Furthermore, an anticonvulsant effect of naloxone has also been demonstrated (Carter-Snead III and Bearden, 1982).

In vitro experiments

These experiments showed that CSF taken from the spontaneous morphine-abstinent rats

failed to induce a contraction of the isolated morphine-dependent guinea-pig ileum. This contrasted to the naloxone-induced contraction of the isolated morphine-dependent guinea-pig ileum. It further indicates that CSF taken from the spontaneous morphine-abstinent rat does not interfere with opioid receptors. It is of importance to note that these negative results with CSF *in vitro,* support the idea that the putative CSF withdrawal-precipitating substance is without properties of a competitive opioid receptor antagonist. It seems that the octapeptide F-8-F-NH_2 is also without naloxone-like properties (Allard et al., 1989).

High-Performance Liquid Chromatography (HPLC) study

The bioactive component partially isolated in this study, seems to be different from the octapeptide F-8-F-NH_2, due to the higher hydrophobicity and much lower molecular mass of the latter (Kivipelto et al., 1989; Labrouche et al., 1993). We can not, however, exclude at this moment, that such a component might bound to a larger protein, affecting its chromatographic and spectral properties. Attempts to determine this factor in CSF of morphine-abstinent rats as well as the examination of bioactivity of these fractions are in progress.

Concluding, this study shows that a "withdrawal substance", not yet chemically defined is synthesized and released in CSF during the development of spontaneous morphine abstinence. This substance is formed in sufficiently high concentrations to induce a withdrawal in morphine-dependent recipient rats. Regarding to the fact that total CSF volume in a 300 g rat is about 580 µl (Lai et al., 1983) and that total CSF volume of rats is replaced completely within 10-25 min (Bouman and Van Wimersma Greidanus, 1979), the release of this substance during withdrawal has to be very abundant. The CSF taken from the spontaneous morphine-abstinent rats decreased the VEP peak latencies and amplitude values, which is identical to the corresponding effects of naloxone in the morphine-dependent rats. However, data from the *in vitro* study indicate that CSF from spontaneous morphine-abstinent rats does not exert a naloxone-like activity on morphine-dependent guinea-pig ileum. More studies are necessary in order to clarify the chemical and bioactive properties of the "withdrawal substance".

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Chapter 7

The inhibitory effect of norharman on morphine withdrawal syndrome in rats: comparison with ibogaine

Abstract - Norharman (20 mg/kg, i.p.) and ibogaine (40 mg/kg, i.p.) significantly attenuated naloxone (4 mg/kg, i.p.)-precipitated withdrawal syndrome in morphine-dependent rats. Several withdrawal signs, such as teeth-chattering, chewing, penile licking and diarrhoea, were decreased by both norharman and ibogaine. In addition, norharman reduced also the withdrawal grooming and rearing. It is concluded that both norharman and ibogaine are inhibitors of withdrawal syndrome in morphine-dependent rats.
Published in Behavioural Brain Research 65: 117-119, 1994.

Norharman (ß-carboline) is an endogenous substance in brain and other tissues in rats and humans (Honecker and Coper, 1978; Greiner and Rommelspacher, 1984). Recently, elevated plasma levels of norharman were detected in chronic alcoholics (Rommelspacher et al., 1991) and heroin addicts (Stohler et al., 1993). These data favour the involvement of norharman in drug dependence processes. A substance structurally related to norharman is ibogaine. Both, norharman and ibogaine are indole derivatives with psychotogenic/ hallucinatory properties (Farnsworth, 1968; Airaksinen and Kari, 1981). It has been shown that ibogaine attenuates morphine withdrawal (Dzoljic et al., 1988; Glick et al., 1992) and interrupts drug dependence (Glick et al., 1991; Cappendijk and Dzoljic, 1993). These facts justify a further elucidation of the effects of these two substances in drug dependence phenomena. In order to make a comparison between these two chemically and behaviorally (psychotogenic/hallucinatory) similar substances, we studied the effects of both drugs, norharman and ibogaine on naloxone-precipitated withdrawal in morphine-dependent rats.

Materials and methods

Animals

Male Wistar rats (TNO Zeist), weighing 290-330 g were housed in groups and had a free access to food and water. The room was maintained on a 12-h light/dark cycle (lights on 08.00 h), with constant temperature (21° C) and humidity (55%).

Experimental protocol

Morphine dependence was induced by implantation of a morphine base pellet (75 mg/rat, s.c., n=30) on the back of rats the animal under ether anaesthesia (Cappendijk et al.,

1993). The morphine-dependent animals were used only once. Morphine-dependent rats were divided into three groups, pretreated intraperitoneally with vehicle (distilled water, n=10), norharman (20 mg/kg, n=10) or ibogaine (40 mg/kg, n=10). The selected doses of norharman and ibogaine are biologically active, shown by previous studies (Morin, 1984; Cappendijk and Dzoljic, 1993).

Morphine withdrawal syndrome

The withdrawal syndrome in morphine-dependent animals was precipitated by naloxone (4 mg/kg, i.p.), given 30 min after vehicle, norharman or ibogaine. The naloxone treatment occurred 72 h following pellet implantation. The observer was "blind" to the treatment order and registered the withdrawal symptoms during 30 min following injection of naloxone. The withdrawal signs were scored according to the weighting factors described by Neal and Sparber (1986). In short, the signs observed during a mild withdrawal syndrome were assigned with 1 (diarrhoea, chewing, grooming, irritability on touch, rearing), whereas the sign rhinorrhoea, observed during severe withdrawal, was assigned a 3. All other withdrawal signs, teeth-chattering, wet-dog shakes, penile licking, ptosis and jumping were assigned by a weighting factor 2.

Drugs

Norharman (Sigma, England) and ibogaine (Sigma, England) were administered in volume of 2.2 ml/injection. Naloxone HCl (Sigma Chemical Co., St Louis, MO) was given in volume of 1 ml/kg. The pH of drug solutions and vehicles were adjusted to 7-8. All drugs were dissolved in distilled water.

Statistics

Data were evaluated by using the non-parametric Kruskall-Wallis one-way analysis of variance, followed by the Mann-Whitney *U*-test, with a level of $P<0.05$ being considered significant (Glantz, 1989).

Results

A decreased locomotion and exploratory behaviour was observed in norharman (20 mg/kg, i.p.)-treated naive (n=6) and morphine-dependent (n=10) animals. This effect lasted 5-20 min. In contrast, ibogaine (40 mg/kg, i.p.) induced within 4 min tremor and excitatory behaviour (jumping or violent locomotion on touch). The behavioral effects, induced by norharman or ibogaine disappeared within 30 min.

Norharman and ibogaine significantly attenuated the naloxone-precipitated withdrawal syndrome in rats (Fig. 1A). Related to the specific symptoms, both norharman and ibogaine attenuated teeth-chattering, chewing, penile licking and diarrhoea (Fig. 1B, D, F, G). Grooming and rearing response were reduced by norharman only (Fig. 1C, E).

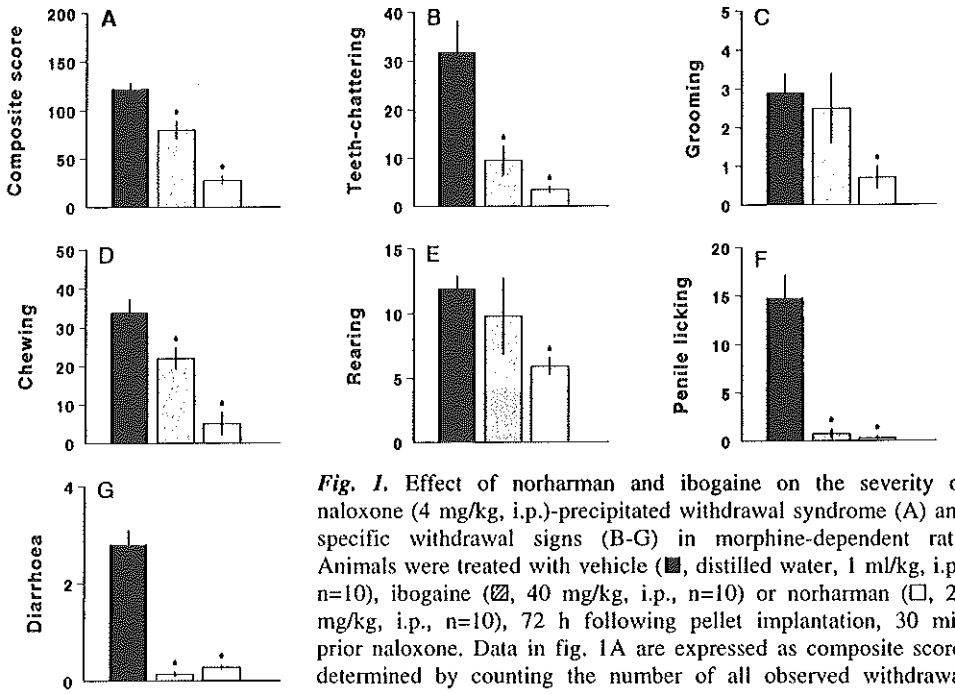

Fig. 1. Effect of norharman and ibogaine on the severity of naloxone (4 mg/kg, i.p.)-precipitated withdrawal syndrome (A) and specific withdrawal signs (B-G) in morphine-dependent rats. Animals were treated with vehicle (■, distilled water, 1 ml/kg, i.p., n=10), ibogaine (▨, 40 mg/kg, i.p., n=10) or norharman (□, 20 mg/kg, i.p., n=10), 72 h following pellet implantation, 30 min prior naloxone. Data in fig. 1A are expressed as composite score, determined by counting the number of all observed withdrawal signs, during the 30-min period of abstinence. All data were expressed as mean ± SEM. * Significant decrease of withdrawal syndrome or signs (Mann-Whitney *U*-test, $P<0.05$) compared to the control group. Note that norharman and ibogaine attenuated the severity of withdrawal syndrome and frequency of withdrawal signs.

Discussion

This study is the first demonstration that norharman significantly attenuated a naloxone-precipitated withdrawal syndrome in rats. Ibogaine, in accordance with previous data (Dzoljic et al., 1988; Glick et al., 1992), also reduced naloxone-precipitated withdrawal syndrome (Fig. 1A). However, the data indicate that norharman and ibogaine induced a similar (but not identical) decrease of opioid withdrawal symptoms.

Although the mechanism of action of norharman and ibogaine is not known, an involvement of the opioid system may be considered, since both drugs have an agonist action on opioid receptors. Norharman acts as a partial μ-agonist (Airaksinen and Kari, 1981), while ibogaine is an agonist at κ-receptors (Deecher et al., 1992). The binding activity of both drugs to central opioid receptors with possible displacement or preventing the binding of naloxone to opioid receptors may lead to an antiwithdrawal effect. In

periphery, μ and κ-agonists can depress acetylcholine release from the cholinergic neurons of myenteric plexus (Burks et al., 1988). This effect may contribute to the decreased intensity of withdrawal diarrhoea, induced by norharman and ibogaine.

The other neurotransmitter system which could be involved in the decreased expression of the opioid withdrawal is the glutaminergic system. Glutamate antagonists may prevent morphine abstinence in mice and guinea pigs (Tanganelli et al., 1991; Cappendijk et al., 1993). Consistent with this finding, morphine is able to block the glutamate-mediated excitation in the monkey (Willcockson et al., 1986) and in the mouse (Aanonsen and Wilcox, 1987). The fact that both norharman and ibogaine also have morphine-like properties (Airaksinen and Kari, 1981; Deecher et al., 1992) favour the hypothesis that blockade of the glutamate-mediated transmission could contribute to the attenuation of the excitatory character of withdrawal syndrome. This idea has been supported by Dowson et al. (1975), showing that harmala alkaloids inhibit the transmission at the glutamate-mediated neurons.

In conclusion, the present experiments show that norharman and ibogaine attenuate the opioid withdrawal syndrome and favour an idea of an inhibitory role of both drugs in the expression of morphine abstinence. Although an involvement of the opioid- and or glutamate-neurotransmitter system could be considered as a main underlying mechanism for the attenuation of withdrawal syndrome, the precise mechanisms of action of norharman and ibogaine remain unclear. However, of particular importance would be a further clarification of the role of norharman as a physiological modulator of morphine withdrawal phenomena.

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PART 4

Drug Dependence induced by Psychostimulants

Chapter 8

Psychostimulants

Compounds, producing excitement, euphoria, reduced sensitivity of fatigue and increased motor activity, belong generally to the psychostimulants. These drugs could be divided into three categories: amphetamines, methylxanthines and cocaine.

8.1. Amphetamines and methylxanthines

- *Amphetamines.* Repeatedly taken amphetamine may induce, over the course of a few days, a state of "amphetamine psychosis" in men (Caplehorn, 1990) and animals (Balfour, 1990; Lillrank et al., 1991). Human amphetamine users report visual and auditory hallucinations, accompanied by paranoid symptoms. In both men and animals, aggressive behaviour may occur, and at the same time repetitive stereotyped behaviour could develop. When the intake of drugs is stopped there is usually, after a few days, a period of deep sleep and on awakening, the subject feels extremely lethargic, depressed, anxious, and is often hungry (Swerdlow et al., 1991).

  Tolerance develops rapidly to the sympathomimetic and anorectic effects of amphetamine, but much more slowly to the other effects, such as locomotor stimulation and stereotyped behaviour (Lillrank et al., 1991).

  Repeated administration of amphetamine to experimental animals may lead to behavioral sensitization, a process in which the dopaminergic (DA-ergic) system seems to be involved (Segal and Kuczenski, 1992).
- *Methylxanthines* are constituents of various beverages (tea, coffee, cocoa etc.). The main components are caffeine and theophylline, both having common stimulant effects on the CNS. Compared to the amphetamines, the methylxanthines produce less locomotor stimulation, and do not induce euphoria, stereotyped behaviour, or a psychotic state (Swerdlow et al., 1986). Tolerance develops to a small extent, but much less than with amphetamines (Denaro et al., 1991).

Cocaine was used in our experimental study and therefore, more extensively data will be discussed.

8.2. Cocaine

Administration route and metabolism. Cocaine is an alkaloid, derived from the plant *erythroxylon coca.* Two chemical forms of cocaine exist, *hydrochloride salt* and *free base.* The salt ("snow" or "coke") dissolved in water, can be taken by vein or in the nose. The

free base ("crack") is smoked (Siegel, 1985). In the earlier days coca leaves were chewed, but it appeared that the effects of cocaine occurred later and were less intense compared to the intranasally route. The route of administration determines the rate and peak of blood levels achieved. It takes for cocaine 5-10 seconds by smoking, 30-120 seconds intravenously, and 1-3 minutes intranasally to reach and produce the onset of effects in the brain. Unlike heroin, which tends to be used on daily basis, cocaine (and amphetamine), is characterized by drug consumption in heavy binges (Bozarth and Wise, 1985).

Cocaine is detoxified by liver and plasma esterase enzyme system. Two water soluble metabolites, benzoylecgonine and ecgonine methyl ester, are excreted in the urine (Stewart et al., 1979) and are useful markers of cocaine use. Benzoylecgonine could be detected in the urine for as many as 22 days after the last cocaine intake (Weiss and Gawin, 1988). The plasma half-life of cocaine varies from 30-80 min (Prakash and Das, 1993).

Transporter molecules and neurotransmitters. It has been shown that cocaine primarily acts on monoaminergic systems, by blocking the reuptake of dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT, Taylor and Ho, 1978). Recently, it has been demonstrated that cocaine inhibits several monoamine transporter molecules in the mammalian brain, but particular attention was paid to the inhibition of the DA transporter (Hitri et al., 1994). The neurotransmitter transporters terminate synaptic transmission by rapid sodium-dependent reaccumulation of released neurotransmitter in the presynaptic terminal. Not only cocaine is acting by this type of mechanism, also antidepressants and neurotoxins that induce Parkinsonism are shown to act in this way (Meister, 1993).

DA-ergic system. The inhibition of DA reuptake has been demonstrated in several brain nuclei, such as nucleus accumbens (NAc, Bradberry and Roth, 1989; Kalivas and Duffy, 1990), medial prefrontal cortex (Maisonneuve et al., 1990), ventral tegmental area (VTA, Bradberry and Roth, 1989) and striatum (Church et al., 1987). Recently, it has been shown that stereotypy induced by cocaine is mediated by a DA-ergic activation of a glutaminergic system within the striatum (Karler et al., 1994). In general, the DA-ergic seems to be involved in the behavioral effects of cocaine. D_1 receptor antagonists block cocaine-induced increase of locomotor activity, stereotypy, and decrease of food intake (Spealman, 1990). However, the cocaine/amphetamine induced increase of locomotor activity in mice was blocked by the NA antagonist prazosin, which implies that the underlying mechanism is more complex (Snoddy and Tessel, 1985).

NA-ergic and 5-HT-ergic system. It was demonstrated, that cocaine blocks the uptake of 5-HT and NA in the dorsal raphe (Cunningham and Lakoski, 1990) and in locus coeruleus (Hadfield and Nugent, 1983; Reith et al., 1986; Lacey et al., 1990). The inhibited firing of these neurons is probably mediated by activation of presynaptic α2-adrenoceptors (Suprenant and Williams, 1987) and 5-HT_{1A} receptors (Cunningham and Lakoski, 1990).

8.3. Cocaine dependence

Similarly to the opioids, the mesocorticolimbic DA-ergic pathway seems to play a major role in the process of reinforcement of cocaine (Woolverton and Johnson, 1992). The 5-HT- and/or NA-ergic pathways seemed not to be involved in reinforcing process (Fibiger et al., 1992). Lesions of the mesocorticolimbic DA-ergic system, induced by 6-hydroxydopamine, produced a selective termination of cocaine self-administration in rats (Caine and Koob, 1994), while the administration of 5-HT and NA receptor antagonists was without any effect on the cocaine intake in rats (Fibiger et al., 1992). Microdialysis studies in rats during cocaine self-administration have shown an increased release of DA within the NAc (Hurd et al., 1989) and in the amygdala (McGregor et al., 1994), while the NA and 5-HT levels remained unaffected (McGregor et al., 1994). The involvement of the D_2-receptors in cocaine reinforcement was demonstrated by D_2-agonists bromocriptine (Hubner and Koob, 1990) and lisuride (Pulvirenti and Koob, 1994), which reduced the intravenous cocaine self-administration intake in rats.

8.4. Withdrawal syndrome

Human and animal studies have shown that there are behavioral consequences of termination of exposure to cocaine (Woolverton and Johnson, 1992). The initial phase of the withdrawal (hours-days) is termed the "crash". During the crash an intense depression, fatigue, hypersomnia, hyperphagia, and drive for repeated cocaine use are present. The later phase (weeks to months) is characterized by mood lability, depression, anhedonia, low energy, sleep disturbances, suspiciousness and anxiety (Gold, 1983; Gawin and Kleber, 1986).

Neurochemically, it appears that a functional reduction of DA neurotransmission may be one important component of cocaine withdrawal. A significant reduction in DA overflow in the NAc of rats withdrawing from unlimited access to cocaine self-administration has been shown (Weiss et al., 1992).

Treatment of cocaine dependence. Having in mind a role of DA in cocaine dependence, several DA receptor agonists in the treatment of cocaine dependence have been used:

- *Bromocriptine*, agonist at D_2 receptor, was shown to reduce the intake of i.v. cocaine self-administration in rats (Hubner and Koob, 1990). In humans, the results are controversial. Some studies showed that bromocriptine reduced symptoms of cocaine withdrawal, such as dysphoria (Giannini et al., 1987), while others reported no effect of bromocriptine on craving and no alterations of the subjective effects of cocaine, such as "rush" or "good feeling" (Kumor et al., 1989).
- *Lisuride*, agonist at D_2 receptor, reduced cocaine intake in rats (Pulvirenti and Koob, 1994). In humans, lisuride is involved in the normalization of the disturbed sleep

pattern occurring during cocaine withdrawal, however, it did not modify subjective ratings of craving and mood (Gillin et al., 1994).

- *Amantadine* (Weddington et al., 1991) and *pergolide* (Malcolm et al., 1991) have been used with some success in treatment of cocaine human addicts.

Besides the DA agonists as adjuncts for the treatment of cocaine dependence two other categories of drugs have been suggested:

- *Opioid antagonists.* Animal and human studies have revealed that naltrexone, but also the partial opioid agonist/antagonist buprenorphine may have therapeutic value in cocaine addiction (Kosten et al., 1989; Mello et al., 1993).
- *Catalytic antibodies* have been developed and these compounds combine with cocaine and in the same time destroy the molecules. The antibodies are injected into the bloodstream and could protect a person (at least partially) from the effects of cocaine by destroying the drug more rapidly than do the enzymes already present in the blood. However, these antibodies are not effective by oral administration (Tramontano et al., 1986).

8.5. Tolerance and sensitization

Both tolerance and sensitization to the behavioral effects of chronic administration of cocaine have been demonstrated in man and animal (Post and Contel, 1983). Whether sensitization or tolerance would occur, seems to depend on the method of drug administration (dose, duration and interval). For example, tolerance was induced following continuous infusion of cocaine (Reith et al., 1987), whereas sensitization was observed after intermittent injection of cocaine (King et al., 1994).

It has been suggested, that tolerance induced by continuous cocaine administration is associated with supersensitivity of D_2 autoreceptor and 5-HT_{1A} receptors, but not by changes in 5-HT_{1B} receptor sensitivity (King et al., 1994).

One of the most interesting aspects of sensitization to cocaine is, that it is a relatively long lasting process (Post et al., 1987), like the process of kindling and long-term potentiation. Such long lasting changes imply that cocaine "experience" can induce structural modifications in synaptic architecture that are responsible for the strengthened synaptic circuitry that may underlie behavioral sensitization to cocaine. It seems that glutaminergic neurotransmission, particularly that mediated by the NMDA receptor subtype, is thought to play an important role. Accordingly, the NMDA channel blocker MK-801 blocks the sensitization to cocaine and amphetamine (Karler et al., 1989), indicating a role of the NMDA (glutamate) system in cocaine abuse.

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PART 5

Cocaine Dependence - Experimental Study

Chapter 9

Inhibitory effects of ibogaine on cocaine self-administration in rats

Abstract - In order to determine the potential anti-addictive properties of ibogaine, we used the cocaine self-administration model in rats. The results indicate that a single injection of ibogaine (40 mg/kg i.p.) produced a significant decrease of cocaine intake, which remained unaltered for more than 48 h. Since the half-life time of ibogaine is short, this might suggest the involvement of one or several active metabolites of ibogaine in cocaine intake. Repetitive administration of ibogaine on three consecutive days also induced a pronounced decrease of cocaine intake. However, a more prominent inhibitory effect on cocaine intake was observed in animals treated repeatedly with ibogaine (40 mg/kg i.p.), once each week for 3 consecutive weeks. These results indicate that ibogaine or its metabolite(s) is a long-lasting interruptor of cocaine dependence, which supports similar observations from uncontrolled clinical studies.
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Ibogaine, an indole alkaloid found in the root bark of the African shrub *Tabernanthe iboga*, has been used in Gabon (West Central Africa) in low doses as a stimulant (combat fatigue, hunger and thirst) and in high doses for its hallucinogenic properties (religious rituals).

Recent animal studies and non-controlled observations in humans indicate that ibogaine may significantly affect drug dependence phenomena such as drug withdrawal and intake of addictive drugs. Accordingly, it has been demonstrated that ibogaine (i.c.v.) attenuated many (but not all) symptoms of naloxone-precipitated withdrawal in morphine-dependent rats (Dzoljic et al., 1988). A similar anti-withdrawal effect of ibogaine has been observed in morphine-dependent monkeys (Aceto et al., 1989) and rats (Glick et al., 1992).

Related to the intake of addictive drugs, it has been shown that ibogaine pretreatment decreases intravenous morphine self-administration in rats for several days (Glick et al., 1991). These results of animal experiments are in accordance with the long-lasting interruption of heroin abuse by ibogaine in humans (Lotsof, 1985). Ibogaine is also claimed to interrupt cocaine and amphetamine abuse and it was suggested that series of four treatments may be effective for several years (Lotsof, 1986). Other claims are that ibogaine attenuates alcohol and nicotine/tobacco dependency syndromes (Lotsof, 1989, 1991).

The aim of the present experiments was to determine whether an interrupting effect of ibogaine on cocaine intake could be demonstrated in cocaine-dependent animals. We

examined the effects of single and repeated injections of ibogaine on the cocaine self-administration model in rats.

Materials and methods

Animals

Male wistar rats. (TNO Zeist) were used, weighing 200-250 g at the start of the experiments. The animals were housed in groups with water and food ad libitum. Artificial light was supplied on a 12-h light/dark cycle.

Operation procedure

All animals were anaesthetized with sodium pentobarbital (60 mg/kg i.p.) and surgically implanted with a chronic i.v. jugular catheter (0.5 mm inside diameter, 1.0 mm outside diameter, polyethylene tubing). The catheter was passed subcutaneously to a small incision at the back of the neck. After the operation the animals were housed individually with food and water ad libitum. Two days before the start of the experiments (i.e. 5-6 days after operation), the animals were brought to the test room and were deprived of food in order to obtain a weight reduction of about 20%. Weight reduction was introduced in order to facilitate acquisition of self-administration (Takahashi et al., 1978). A reversed 12-h light/dark cycle (lights out 8.00 - 20.00 h) was maintained during the whole experiment.

Apparatus

The experiments were performed in operant conditioning chambers. Cocaine infusions (1.2 mg/kg), consisting of 0.25 ml fluid (pH 7.30-7.35) delivered in 20 s, occurred when the reinforcement lever was depressed. During the infusion, the stimulus light was turned off and pressing the same lever had no programmed consequences.

Test-procedure

Following 5-6 days of postoperative recovery, the rats were connected to an infusion pump (Braun Perfusor Secura MRD) by polyethylene tubing and a fluid swivel, which permitted unlimited movement of the animal during the session. Session length was 3 h each day (during the dark period of the cycle), 5 days per week with 2 days of no testing (during weekends, between each 5-day block of testing). The study of the effect of ibogaine began when the baseline rate of cocaine self-administration stabilized (< 10% variation between 3 consecutive sessions) after 12-16 days (sessions). These animals were randomly divided into vehicle and ibogaine-treated groups. The experiments lasted about 6 weeks (including the first 2 weeks used for stabilization of cocaine intake).

Experimental groups

Vehicle (1.0 ml/kg i.p.) or ibogaine was given 30 min prior to self-administration testing and the behaviour of animals was monitored for the subsequent 3 h.

Single administration of ibogaine (10-40 mg/kg i.p., n= 6-7 per dose). Our preliminary experiments showed that administration of 80 mg/kg ibogaine caused severe locomotor disturbances (ataxia, jumping when touched and tremor for about 60 min). Therefore, in further experiments, this dose was omitted and 40 mg/kg ibogaine was constantly used. This

dose had less prominent and shorter lasting behavioral effects than the higher dose (see Results).

Repetitive administration of ibogaine (40 mg/kg i.p.). In one group of animals (n=5) ibogaine was administered once on each of 3 consecutive days, while the other group (n=5) received ibogaine once at the beginning of each of 3 consecutive weeks.

Drugs

Cocaine hydrochloride (OPG, Utrecht, Netherlands) was dissolved in saline and the pH was adjusted to 7.30-7.35. Ibogaine hydrochloride (kindly donated by H. Lotsof, NDA, New York) was dissolved in distilled water.

Data analysis

Responses were summed over the 3-h test period and subjected to two-way analysis of variance (ANOVA) with repeated measurements on days. Individual comparisons of means were made with Student's *t*-test (between baseline and treated groups and between vehicle and ibogaine-treated groups) with significance at $P<0.05$ level.

Results

Single administration of ibogaine (10-40 mg/kg i.p. n= 6-7 per dose)

Behaviour. Administration of ibogaine in cocaine-dependent rats induced within 4 min stiffness of the hind legs, tremor, ataxia and hypersensitivity (jumping or violent locomotion when touched). The severity of this behavioral syndrome was dose dependent and, in the case of the highest dose of ibogaine (40 mg/kg), the effect lasted for a maximum of 30 min. Thereafter, animals showed normal behaviour and were used for the self-administration procedure.

Cocaine intake. The baseline cocaine intake was 5.0 ± 0.5 mg/kg (Fig. 1). A single injection of 40 mg/kg ibogaine produced a significant depression of cocaine intake, while 10 and 20 mg/kg were ineffective (Fig. 1). The inhibitory effect of a single administration of ibogaine on cocaine intake became more prominent on the next day and remained below the control level for the 24 h following (48 h after drug administration, Fig. 1). Further studies were performed with the 40 mg/kg dose of ibogaine.

Repeated (three) administration of ibogaine (40 mg/kg i.p.)

Ibogaine administered on each of 3 consecutive days. Compared to the baseline (5.3 ± 0.4), administration of vehicle (1.0 ml/kg i.p. n=5) on each of 3 consecutive days did not induce significant changes in cocaine intake (Fig. 2). However, a significant decrease of the cocaine intake (n=5) occurred on the second day of ibogaine treatment. After the third injection of ibogaine, the inhibitory effect on cocaine intake lasted for the next 24 h (Fig. 2). This effect on cocaine intake was not significantly different from that of a single injection of ibogaine,

but was shorter (24 h versus 48 h).

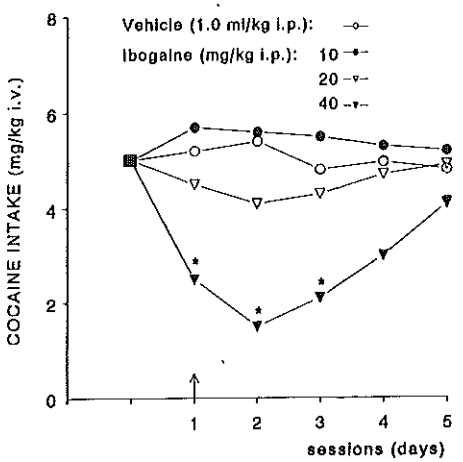

Fig. 1. Effect of a single dose of ibogaine (10-40 mg/kg i.p. n= 6-7 per dose) on cocaine intake in rats. The baseline cocaine intake (■ 5.0±0.5 mg/kg) was calculated as the average rate of three consecutive sessions (<10% variation) preceding treatment with vehicle (distilled water 1.0 ml/kg i.p. n=7) or ibogaine. Vehicle (↑) or ibogaine (↑) were administered 30 min before the session started. The data are expressed as means of cocaine intake per session. * Indicates a significant decrease of cocaine intake (ANOVA and *t*-test P<0.05) compared to baseline intake and vehicle-treated group. Note that a single injection of ibogaine (40 mg/kg) exerted a long-lasting (48 h) inhibition of cocaine intake.

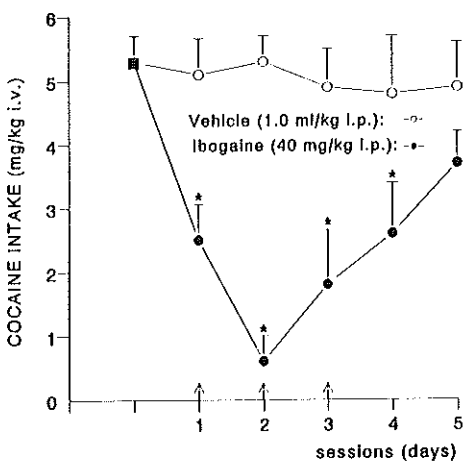

Fig.2. Effect of repeated administration of ibogaine (40 mg/kg i.p. n=5, given once on each of three consecutive sessions) on cocaine intake in rats. The baseline cocaine intake (■ 5.3±0.4 mg/kg) was calculated as the average rate of three consecutive sessions (<10% variation) preceding treatment with vehicle (distilled water 1.0 ml/kg i.p. n=5) or ibogaine. Vehicle (↑) or ibogaine (↑) was administered 30 min before the session started. The data are expressed as means ± SEM cocaine intake per session. * Indicates a significant decrease of cocaine intake (ANOVA and *t*-test P<0.05) compared to baseline intake and vehicle-treated group. Note that each injection of ibogaine significantly decreased cocaine intake.

Ibogaine administered at the beginning of each of 3 consecutive weeks. The baseline cocaine intake (4.9 ± 0.5) was not significantly affected by vehicle (1.0 ml/kg i.p. n=5) administered at the beginning of each of 3 consecutive weeks (Fig. 3). However, a significant decrease of

cocaine intake was observed following each ibogaine injection. Compared to that after the first injection of ibogaine, the decrease of cocaine intake was more sustained after the second and third administration of ibogaine (Fig. 3).

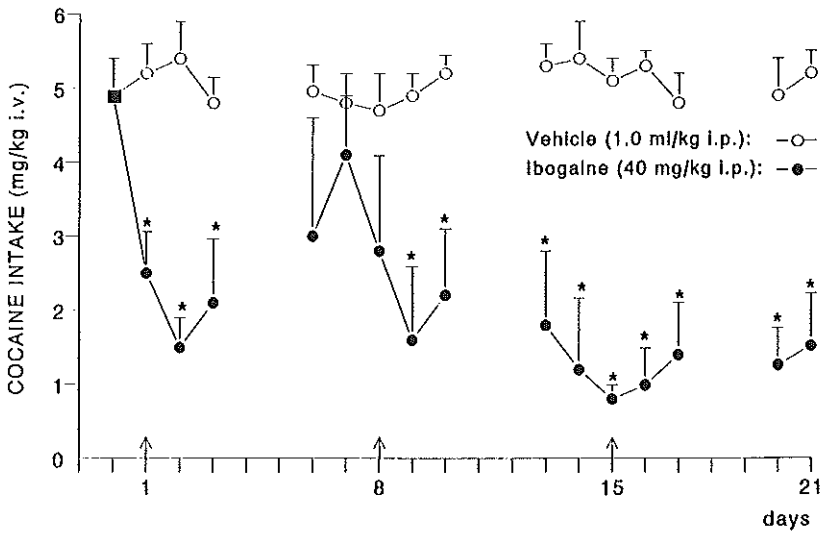

Fig. 3. Effect of repeated administration of ibogaine (40 mg/kg i.p. n=5, given once at the beginning of each of 3 consecutive weeks) on cocaine intake in rats. The baseline cocaine intake (■, 4.9±0.5 mg/kg) was calculated as the average rate of 3 consecutive sessions (<10% variation) preceding treatment with vehicle (distilled water 1.0 ml/kg i.p. n=5) or ibogaine. Vehicle (↑) or ibogaine (↑) was administered 30 min before the session started. The data are expressed as means ± SEM cocaine intake per session. The animals were not tested during weekends. * Indicates a significant decrease of cocaine intake (ANOVA and *t*-test $P<0.05$) compared to the baseline intake and the vehicle-treated group. Note a gradual and long-lasting decrease of cocaine intake following second and third injection of ibogaine.

Discussion

A single dose or repeated doses (on each of 3 consecutive days) of ibogaine (40 mg/kg i.p.) in rats induced a decrease of cocaine intake lasting 1-2 days. This effect could be potentiated and prolonged by three injections of ibogaine, given once each week (but not once each day). This was rather surprising, as the half-life time of ibogaine in rodents is about 1 h, and a day after administration, the ibogaine levels in the body were undetectable (Dhahir,

1971, cited by Glick et al., 1991). This might indicate that the depression of cocaine intake could be ascribed to an active and long-lasting metabolite(s) of ibogaine or to irreversible interruption of the biological mechanism of cocaine dependence.

Related to the mechanism of anti-addictive properties of ibogaine several possibilities could be considered:

Disturbed locomotion

Ibogaine enhanced the amphetamine-induced increase of motor activity (Maisonneuve and Glick, 1992). Additional disturbances of motility, such as tremor and ataxia observed in this and other studies (Glick et al., 1991), might further affect the self-administration of cocaine. However, this possibility is unlikely, since in our experiments the ibogaine-induced locomotor disturbances such as ataxia and tremor lasted about 30 min, while the anti-addictive effect of single dose of this drug remained for at least 2 days. A long-lasting effect of ibogaine (several days) on morphine intake in rats was also observed in other studies (Glick et al., 1991). Ibogaine pretreatment of rats (40 mg/kg i.p. 19 h prior) had no effect on the increased locomotion induced by various doses of cocaine (5, 10 and 40 mg/kg), while the locomotion after administration of 20 mg/kg cocaine was potentiated for only 1 hour (Maisonneuve et al., 1992). Evidently, an effect of ibogaine on motor activity in rats is of marginal importance for understanding the long lasting anti-addictive properties of ibogaine.

Dopaminergic system

The rewarding effects of drugs of abuse have been associated with their ability to increase dopamine release, particularly in the nucleus accumbens (Di Chiara and Imperato, 1988). It is of importance to note that ibogaine reduced the cocaine-induced dopamine release in the nucleus accumbens (Broderick et al., 1992). Thus, an anti-addictive effect of ibogaine might be explained by its inhibitory effect on dopaminergic neurotransmission, which seems of importance for rewarding processes. However, the interaction between ibogaine and dopamine neurotransmission has not been shown conclusively, mainly due to controversial data. For example, a recent study indicated that ibogaine (40 mg/kg i.p.) potentiates the cocaine-induced increase in extracellular dopamine levels in striatum and nucleus accumbens (Maisonneuve et al., 1992). Thus, in contrast to the previous data, this might indicate a stimulatory effect of ibogaine on the reinforcing properties of cocaine.

Serotonergic system

Stimulation of the serotonergic system by the 5-HT uptake inhibitor, fluoxetine, attenuates cocaine self-administration in animals (Richardson and Roberts, 1991). It has been shown that ibogaine inhibits the enzymic oxidation of 5-HT in the periphery (Barrass and Coult, 1972). However, it is not known whether such a relationship exists in the CNS. There seems to be

no direct evidence that an ibogaine-induced derangement of 5-HT systems might affect the rewarding properties of cocaine. This possibility remains to be examined.

Central neuronal excitability

Ibogaine increases arousability (Schneider and Sigg, 1957), which might affect behaviour. The proconvulsant effect of ibogaine (40 mg/kg i.p.) lasting several hours that we observed in our EEG study (unpublished data), supports the idea that ibogaine significantly affects the responsiveness of central neurons. A proconvulsant state is probably incompatible with self-administration behaviour. However, it is less clear why cocaine intake is decreased in the absence of a proconvulsant EEG pattern, more than 48 h after ibogaine administration.

In conclusion, these experiments indicate that ibogaine inhibits cocaine intake in rats. This effect could be potentiated by repeated injections of ibogaine, once each week. Although the mechanism of action of ibogaine remains to be established, the present results suggest the presence of an anti-addictive and long-lasting metabolite(s) of ibogaine or its irreversible/long-lasting derangement of an addictive mechanism in cocaine-dependent animals.

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Concluding remarks

The role of several modulators was examined on morphine and cocaine dependence phenomena in rodents. The results of these studies were presented in the experimental parts of this thesis. The conclusions of these studies with possible clinical relevance were briefly pointed out. Finally, some suggestions for further research were given.

- *Antagonists of excitatory amino acid receptors (NMDA-type) and inhibitors of NO synthesis attenuated the expression of morphine withdrawal syndrome in both rats and mice.*

We concluded that NO is an important neurotransmitter in respect to several withdrawal signs. This might be due to a facilitatory effect of NO on the release of various brain neurotransmitters and corresponding changes in the transmitter balance and functional activity of central neurons. We are suggesting that these preclinical studies of NO synthase inhibitors justify clinical trials of NO synthase inhibitors in drug-dependent subjects.

- *A putative "withdrawal substance" released in the cerebrospinal fluid (CSF) of spontaneous morphine-abstinent donor rats induces a withdrawal syndrome in morphine-dependent recipient rats. This putative "withdrawal substance" is hydrophobic and has no naloxone-like properties.*

The presence of a "withdrawal factor" is a challenge for the further biochemical characterization of this substance and CSF of drug-dependent subjects. The question arises whether a "withdrawal substance" is a consequence or causally related to drug withdrawal phenomena.

- *Norharman prevented the expression of the naloxone-induced withdrawal syndrome. A somewhat similar (although less prominent) effect was observed in ibogaine treated morphine-dependent rats.*
- *Ibogaine treatment of cocaine-dependent rats significantly decreased the cocaine-intake.*

It is of importance to note that norharman as a physiological substance exerts anti-withdrawal properties. Although the chemical structure of ibogaine is similar to norharman, these two substances may have a different mechanism of action. Ibogaine acts as a competitive NMDA antagonist, which might be an underlying mechanism of action of this alkaloid. The mechanism of action of norharman might be more related to the GABA receptor-complex on which this physiological substance has a binding place.

Suggestions for further research

- The biochemical analysis of the CSF of drug-dependent subjects and further study of bioactive properties of the "withdrawal substance" found in the CSF of spontaneous morphine-abstinent rats is one of the forthcoming aims to study.

- It is of importance to examine the synthesis and release of central NO and other neurotransmitters during morphine dependence and withdrawal syndrome, in the absence and presence of NOS inhibitors, in order to elucidate the role of NO in corresponding behavioral changes, occurring during opioid withdrawal.

- There are indications that eicosanoids, the metabolic products of arachidonic acid may contribute to the mechanism of opioid withdrawal diarrhoea. Therefore, the interactions between disturbed production of arachidonic acid metabolites and the severity of naloxone-precipitated withdrawal diarrhoea in morphine-dependent animals is one of the subjects which deserves an attention to be studied.

- The glutamate-NMDA-NO system has a profound influence on many neurotransmitters, including the endogenous opioid peptides. We have already demonstrated that the glutamate-NMDA-NO system is involved in the expression of morphine withdrawal signs. The dopamine release induced by enkephalins, depends on involvement of glutamatergic transmission via NMDA receptors. Therefore, the interaction between the endogenous opioid peptides or exogenous opioids and EAA-glutamate system deserves to be studied.

- It has been demonstrated that in the plasma of alcoholics and heroin addicts the level of norharman is increased. Further research in respect to the mechanism of action of norharman (the role of the GABA receptor-complex) and its effects in other types of drug dependence (alcohol, benzodiazepines, etc.) is worthwhile to study.
  The ability of ibogaine to modify drug seeking behaviour in the self-administration animal model, suggests a possible use of ibogaine in the treatment of human drug dependence and thereby warranting further study on its mechanism(s) of action.

- The impression could be raised that drug dependence phenomena could be totally explained as pharmacological processes. However, the social interactions are very important factors, which play a prominent role in human drug dependence phenomena. However, in most of the performed animal studies, social interactions have not

been included. To my opinion, it is worthwhile to study drug dependence phenomena in animals housed in groups versus solitary housed animals.

Samenvatting

Dit proefschrift beschrijft de effecten van verschillende factoren op drug-afhankelijkheidsverschijnselen bij muizen en ratten. Er is met name gekeken naar factoren die het morfine-onthoudingssyndroom (*hoofdstukken 3-7*) en het cocaïne-innamepatroon (*hoofdstuk 9*) kunnen beïnvloeden.

Deel 1: ***Drug-afhankelijkheid en schadelijk druggebruik***

Hoofdstuk 1

Ondanks het feit dat drug-afhankelijkheidsverschijnselen al sinds jaren bestudeerd worden, is het moeilijk gebleken om goede omschrijvingen te geven van begrippen die met deze verschijnselen te maken hebben. Termen zoals afhankelijkheid ("*dependence*"), schadelijk druggebruik ("*abuse - harmful use*"), onthoudingssyndroom, drug-zoekend gedrag (zuchtigheid, "*craving*") en beloning ("*reinforcement*") etc., worden dan ook in het eerste gedeelte van dit hoofdstuk gedefinieerd. De term drug-afhankelijkheid wordt voornamelijk geassocieerd met drugs en gedefinieerd als de behoefte aan continue inname van één of meer stoffen. Drugs die afhankelijkheid kunnen veroorzaken behoren tot de zogenoemde *psycho-actieve* stoffen. In het tweede gedeelte van dit hoofdstuk worden verschillende klassen van psycho-actieve stoffen behandeld. Recentelijk uit experimenten verkregen gegevens worden besproken. De eigenschappen van verschillende klassen van psycho-actieve drugs worden benadrukt, voor wat betreft gedragseffecten en het werkingsmechanisme op cellulair niveau tijdens drug-afhankelijkheid en drug-onthouding.

Deel 2: ***Opioid-afhankelijkheid***

Hoofdstuk 2

In dit hoofdstuk worden allereerst de opioiden en hun receptoren geclassificeerd, gevolgd door specifieke informatie voor wat betreft morfine-afhankelijkheidsverschijnselen, waaronder tolerantie en onthoudingssyndroom.

Deel 3: ***Morfine-onthoudingssyndroom - dierexperimenteel onderzoek***

Hoofdstuk 3

Het is aangetoond dat tijdens het morfine onthoudingssyndroom een verhoogde afgifte van verschillende neurotransmitters, waaronder het excitatoire aminozuur L-glutamaat, plaatsvindt. L-Glutamaat, presynaptisch vrijgemaakt, activeert de postsynaptisch gelegen N-methyl-D-aspartaat (NMDA) glutamaatreceptoren (zie Figuur 1, *Outline of the studies*).

We hebben de rol van het excitatoire glutamaat-systeem bestudeerd tijdens het opioid-onthoudingsproces. Specifieke NMDA-receptorantagonisten zijn aan morfine-afhankelijke muizen toegediend, voordat, door middel van naloxon, onthoudingsgedrag geïnduceerd werd. In deze studie is aangetoond dat toediening van verschillende NMDA-receptorblokkers een verlaagde expressiviteit van het opioid-onthoudingsgedrag in muizen induceert.

Hoofdstuk 4 en 5

Nadat een verlaagde expressie van opioid-onthoudingsgedrag aangetoond was door blokkade van NMDA-receptoren (*hoofdstuk 3*), is door ons gepostuleerd dat stoffen die postsynaptisch gesynthetiseerd worden na stimulatie van de NMDA-receptoren ook een rol zouden kunnen spelen in de expressie van het morfine-onthoudingssyndroom. Eén van de mogelijke stoffen is de relatief "nieuwe" perifere en centrale neurotransmitter stikstofmonoxide ("*nitric oxide*" - NO). De door NMDA-receptoren gereguleerde calciuminflux in het postsynaptische gedeelte van het neuron zou het calcium-afhankelijke NO-synthase (NOS) enzym stimuleren, wat resulteert in de synthese van NO (zie Fig. 1, *Outline of the studies*). Om het effect van deze neurotransmitter op het naloxon-geïnduceerde morfine-onthoudingsgedrag te onderzoeken, hebben we de NO-synthese geblokkeerd met behulp van NOS-blokkers. Dit is gedaan bij zowel morfine-afhankelijke muizen (*hoofdstuk 4*) als morfine-afhankelijke ratten (*hoofdstuk 5*). Uit beide studies bleek dat een significante verlaging van de expressie van het morfine-onthoudingsgedrag optrad. Er mag geconcludeerd worden dat NO een rol speelt bij de expressie van het opioid-onthoudingssyndroom. Verlaging van de NO-synthese zou bij kunnen dragen aan een verstoorde centrale en perifere transmissie van andere neurotransmitters, wat op zichzelf consequenties zou kunnen hebben voor gedragskenmerken tijdens het optreden van het onthoudingssyndroom.

Deze resultaten, uit preklinische experimenten met de NOS-remmers, rechtvaardigen klinische proeven met stikstofmonoxide blokkers in drug-afhankelijke mensen.

Hoofdstuk 6

Er zijn aanwijzingen dat peptiden die opioid-onthouding zouden kunnen moduleren, vrijkomen in de hersenvloeistof ("*cerebrospinal fluid*" - CSF) van ratten. Wij hebben aangetoond dat toediening van CSF, verkregen van spontane morfine-abstinente donorratten, een opioid-onthoudingssyndroom induceert in morfine-afhankelijke ontvangerratten. Tijdens dit CSF-geïnduceerde onthoudingssyndroom is een verlaging van de pieklatentie bij visueel opgewekte potentialen geregistreerd, wat wijst op een stijging van centrale zenuwexcitabiliteit. Dit CSF-geïnduceerde onthoudingssyndroom is gedragsmatig en electrofysiologisch minder uitgesproken dan de naloxon-geïnduceerde abstinentie, maar kwalitatief zijn beide verschijnselen identiek. In tegenstelling tot naloxon veroorzaakt de

CSF van spontane morfine-abstinente dieren echter geen contractie van het geïsoleerde morfine-afhankelijke cavia ileum. Chromatografische analyse van de CSF van spontane morfine-abstinente ratten wees uit dat een mogelijke "onthoudingssubstantie", alleen aanwezig in CSF van spontane morfine-abstinente ratten, hydrofobe eigenschappen bezit.

Het is echter duidelijk geworden dat verdere biochemische analyse en karakterisering van deze "onthoudingssubstantie" noodzakelijk zijn.

Hoofdstuk 7

Eerder onderzoek, uitgevoerd op de afdeling Farmacologie, Erasmus Universiteit Rotterdam, heeft aangetoond dat intracerebroventriculaire toediening van het alkaloïde ibogaïne de expressie van het naloxon-geïnduceerde onthoudingsgedrag in morfine-afhankelijke ratten vermindert. Er worden aan ibogaïne anti-verslavende eigenschappen toegeschreven. In niet-gecontroleerde humane studies is namelijk aangetoond dat ibogaïne afhankelijkheid van alcohol, amfetamine en nicotine kan onderbreken. Norharman is een endogene fysiologische stof, die qua structuur sterk met ibogaïne overeenkomt. Beide stoffen zijn indol-derivaten met psychotogene eigenschappen. We hebben om deze redenen een vergelijkende studie uitgevoerd, teneinde het effect van beide stoffen op de expressie van het opioid-onthoudingsgedrag te bestuderen. Wij concludeerden dat parenterale toediening van norharman een meer prominent anti-onthoudingseffect heeft in vergelijking tot ibogaïne.

Recent onderzoek heeft aangetoond dat één van de bindingsplaatsen van norharman zich op het gamma-aminoboterzuur (GABA) receptorcomplex bevindt. Het is dan ook belangrijk om norharman en het GABA-receptorcomplex nader te bestuderen om een beter inzicht in mogelijke therapeutische eigenschappen en werkingsmechanisme van deze fysiologische anti-onthoudingsstof te verkrijgen.

Deel 4: ***Drug-afhankelijkheid geïnduceerd door psychostimulantia***

Hoofdstuk 8

Dit hoofdstuk geeft een algemene en korte omschrijving van psychostimulantia, gevolgd door meer specifieke en recente gegevens gerelateerd aan cocaïne-afhankelijkheid.

Deel 5: ***Cocaïne-afhankelijkheid - dierexperimenteel onderzoek***

Hoofdstuk 9

We hebben het effect van ibogaïne op de cocaïne-inname bij ratten onderzocht met behulp van het zogenaamde zelf-injectiemodel. De keuze van ibogaïne was gebaseerd op eerder gedane experimenten waarin aangetoond is dat ibogaïne de expressie van morfine-

onthoudingsgedrag in ratten vermindert. In dit experimentele onderzoek is aangetoond dat ibogaïne een langdurig inhiberend effect op de cocaïne-inname bij ratten heeft.

Deze preklinische studie rechtvaardigt een klinische proef met mensen als proefpersoon, die op dit moment in verschillende landen uitgevoerd wordt.

Samenvattend leiden de uitgevoerde studies tot de volgende conclusies en commentaren.

- *Antagonisten van excitatoire aminozuurreceptoren (NMDA type) en blokkers van het NO-synthase (NOS) enzym verlagen de expressie van het morfine-onthoudingsgedrag zowel bij muizen als ratten.*

Wij suggereren dat NO een belangrijke neurotransmitter is voor wat betreft de ontwikkeling van een aantal specifieke onthoudingsverschijnselen. Dit zou te maken kunnen hebben met het feit dat NO de afgifte van verscheidene centrale neurotransmitters vergemakkelijkt, waardoor hiermee samenhangende veranderingen in de transmitterbalans en de functionele activiteit van centrale neuronen optreden. Deze preklinische studies met de NOS-blokkers rechtvaardigen de klinische proeven met deze blokkers in drug-afhankelijke mensen.

- *Een mogelijke "onthoudingssubstantie" die vrijkomt in de hersenvloeistof van spontaan morfine-abstinente ratten induceert in morfine-afhankelijke ratten een onthoudingssyndroom en versterkt centrale zenuwexcitabiliteit. Deze substantie heeft geen naloxon-achtige eigenschappen en is hydrofoob.*

De aanwezigheid van een "onthoudingsfactor" is een uitdaging om de verdere biochemische karakterisering van hersenvloeistof van verslaafde subjecten (zowel dierexperimenteel als humaan) uit te voeren. De vraag rijst of deze "onthoudingssubstantie" een factor is die modulerende effecten op onthoudingsverschijnselen heeft, of een gevolg is van drug-afhankelijkheid zonder invloed te hebben op het onthoudingssyndroom.

- *Norharman voorkomt de expressie van het naloxon-geïnduceerde onthoudingsgedrag in morfine-afhankelijke ratten. Een ietwat gelijksoortig (maar minder prominent) effect werd geobserveerd in morfine-afhankelijke ratten, die voorbehandeld waren met ibogaïne.*
- *Ibogaïne-voorbehandeling verlaagt significant de cocaïne-inname van cocaïne-afhankelijke ratten in het zelf-injectiemodel.*

Het is belangrijk te vermelden dat de chemische structuur van ibogaïne vergelijkbaar is met die van de endogene fysiologische stof norharman. Hoewel deze twee stoffen een chemische overeenkomst bezitten, zouden ze een verschillend werkingsmechanisme kun-

nen hebben. Het is aangetoond dat ibogaïne zich gedraagt als een competitieve NMDA-antagonist, een gegeven dat verder onderzocht dient te worden. Het werkingsmechanisme van norharman zou waarschijnlijk meer gerelateerd kunnen te worden aan het GABA-receptorcomplex, daar norharman hier een bindingsplaats op heeft. Het is van belang om norharman en het GABA-receptorcomplex nader te bestuderen teneinde een beter inzicht in het werkingsmechanisme en mogelijke therapeutische eigenschappen van deze fysiologische stof te verkrijgen.

List of publications

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1. *Published in journals:*

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Curriculum vitae

Susanne Cappendijk was born on 17 September 1966 in Kloosterzande. After finishing secondary school (Gymnasium ß) in 1985, she studied biology at the University of Utrecht. During this study she was involved in two research projects, namely "Hormone synthesis in male and female eels" (Department of Endocrinology, headed by Prof. Dr. P.G.W.J. van Oordt) and "The influence of cocaine self-administration on ß-endorphins in rat cerebrospinal fluid" (Department of Pharmacology, Rudolf Magnus Institute, under guidance of Prof. dr. J.M. van Ree). She attained her masters degree in Biology in June 1990. From September 1990 till the end of 1994 she worked as a Ph-D student (under guidance of Dr. M.R. Dzoljic) at the Department of Pharmacology Erasmus University Rotterdam (headed by Prof. Dr. P.R. Saxena) in co-operation with the Addiction Research Institute Rotterdam. In April 1995, she joins the group of Prof. Dr. L. Terenius at the Department of Clinical Neurosciences, Division of Drug and Alcohol Research, Karolinska Institute Stockholm, Sweden.