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**EFFECTS OF JOINT
COCAINE AND ETHANOL
ON THE BRAIN OPIOID
SYSTEMS**

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ABSTRACT

Concurrent abuse of cocaine and alcohol is common among human addicts, where 85% of all cocaine users have also been shown to meet the criteria for alcohol dependence. Concurrent abuse of cocaine and alcohol is associated with a more severe dependence and a more pronounced abstinence than using either drug alone. The neurobiological basis for the high frequency of concurrent use of cocaine and alcohol is not known, but the drug combination causes an increased and prolonged euphoria as compared to when either drug is taken alone which suggests that the two drugs may interact. Features shared by cocaine and ethanol are their ability to increase dopamine concentrations in the mesolimbic dopamine pathway. Closely connected to the mesolimbic dopamine system and involved in the effects of cocaine and ethanol is the endogenous opioid system. To investigate possible common mechanisms of concurrent cocaine and ethanol, the effects of separate as well as combined cocaine and ethanol on the endogenous opioid system and on the dopamine system were investigated.

An acute challenge of cocaine and ethanol in combination significantly increased the prodynorphin mRNA expression in the striatum and nucleus accumbens (NAcc), with a potentiated effect in the dorsolateral striatum. On the other hand, the combination of cocaine and ethanol down-regulated κ -opioid receptor mRNA levels in the striatum, NAcc, ventral tegmental area (VTA) and substantia nigra compacta with an additive effect in NAcc core. In addition, the combination of cocaine and ethanol produced a general decrease of κ -opioid receptor protein levels while increasing μ -opioid and ORL1 receptors throughout the brain. No effects on δ -receptors were detected in any of the treatment groups. These results show that initially both cocaine and ethanol affect prodynorphin and κ -opioid receptor mRNA expression as well as μ -, κ -opioid and ORL1 receptor levels.

Chronic ethanol administration and a subacute cocaine treatment significantly down-regulated κ -opioid receptor mRNA in the VTA and the NAcc separately and in combination, while two days of "binge" cocaine administration did not effect μ -opioid receptor mRNA expression in the NAcc. Further, pretreatment of ethanol caused a potentiated effect of cocaine-induced dopamine release in the NAcc, an effect that may be related to the increased euphoria produced by this drug combination in humans. The decreased expression of κ -opioid receptor mRNA levels in the NAcc and VTA after ethanol administration might influence the enhanced effect of cocaine-induced dopamine output in the NAcc after ethanol pre-treatment. This is supported by the data showing that blockade of κ -opioid receptors by locally applied nor-BNI increased dopamine release in the nucleus accumbens following chronic ethanol administration. Conversely, κ -opioid receptor stimulation with U50,488H had less impact on the dopamine release in ethanol pre-treated rats as compared to the control group.

Taken together, these studies show that ethanol potentiates the cocaine-induced dopamine release in the NAcc, in combination with alterations on the dynorphin/ κ -opioid receptor system. Together these alterations might influence the probability of a continued drug intake.

LIST OF PUBLICATIONS

- I. Rosin Å, Lindholm S, Franck J and Georgieva J. (1999) *Downregulation of kappa opioid receptor mRNA levels by chronic ethanol and repetitive cocaine in rat ventral tegmentum and nucleus accumbens*. Neuroscience Letters 275, 1-4.
- II. Rosin Å, van der Ploeg I and Georgieva J. (2000) *Basal and cocaine-induced opioid receptor gene expression in the rat CNS analysed by competitive reverse transcription PCR*. Brain Research 872, 102-109.
- III. Lindholm S, Rosin Å, Georgieva J and Franck J. (2001) *Ethanol administration potentiates cocaine-induced dopamine levels in the rat nucleus accumbens*. Brain Research 915, 176-184.
- VI. Rosin Å, Kitchen I and Georgieva J. (2003) *Effects of single and dual administration of cocaine and ethanol on opioid and ORL1 receptor expression in rat CNS: an autoradiography study*. Brain Research 978, 1-13.
- V. Rosin Å, Hurd Y and Georgieva J. (2005) *Acute ethanol and cocaine co-administration differentially affects brain prodynorphin and κ -opioid receptor mRNA expression*. Submitted to Brain Research.
- VI. Lindholm S, Rosin Å, Dahlin I, Georgieva J and Franck J. (2005) *Ethanol alters the effect of kappa receptor ligands on dopamine release in the nucleus accumbens*. Submitted to Physiology and Behaviour.

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LIST OF ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CI-977	((-)- <i>N</i> -Methyl- <i>N</i> -[7-(1-pyrrodinyl)-1-oxospiro [4, 5]dec-8-yl]-4-benzofuranacetamide
DAMGO	D-Ala ² -Methyl-Phe ⁴ -Gly-ol ⁵ enkephalin
DELT	D-Ala ² , Asp ⁴ -deltorphan
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DOPAC	3, 4-dihydroxyphenylacetic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV
GABA	γ -aminobutyric acid
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
G-protein	Guanine nucleotide binding-protein
HPLC	High performance liquid chromatography
ICD-10	International classification of Diseases 10
i.e	Id est
i.p.	Intraperitoneal
Leu-Enkephalin	Leucine-Enkephalin
Met-Enkephalin	Methionine-Enkephalin
mRNA	messenger Ribonucleic acid
NAcc	Nucleus accumbens
NMDA	<i>N</i> -methyl-D-aspartate
Nor-BNI	Nor-binaltorphimine
ORL1	Opioid receptor-like 1
POMC	Proopiomelanocortin
PCR	Polymerase chain reaction
Ro-64,6198	[(1 <i>S</i> ,3 <i>aS</i>)-8-(2,3,3 <i>a</i> ,4,5,6-Hexahydro-1 <i>H</i> -phenalen-1-yl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one]
RT-PCR	Reverse transcription polymerase chain reaction
S.E.M.	Standard error of the means
SNC	Substantia nigra pars compacta
SNR	Substantia nigra pars reticulata
TRK-820	(-)-17-Cyclopropylmethyl-3,14beta-dihydroxy-4,5alpha-epoxy-6beta-[<i>N</i> -methyl-3-trans-3-(3-furyl) acrylamido] morphinan hydrochloride
U50-488H	{ <i>Trans</i> -(\pm)-3,4-Dichloro- <i>N</i> -methyl- <i>N</i> -[2-(2-pyrrolidinyl)cyclohexyl]benzenacetamide}
U69, 593	(5alpha,7alpha,8beta)-(-)- <i>N</i> -methyl-[7-(1-pyrrolidinyl) -1-oxaspiro(4,5)dec-8-yl]benzeneacetamide
VMS	Ventromedial striatum
VTA	Ventral tegmental area
WHO	World Health Organization

1 INTRODUCTION

The combined use of cocaine and alcohol is one of the most common drug-combination today (83). People with an alcohol dependency are more likely to become cocaine abusers (97, 254). In fact, 26-63% of alcohol dependent patients were also found to be cocaine dependent (173). Likewise, many cocaine users are abusing alcohol as well, where 62% of cocaine dependent patients have concurrent alcohol dependence (235). Cocaine users drink alcohol during their cocaine abstinent periods in order to reverse the negative consequences of cocaine withdrawal such as dysphoria and anxiety (154) and alcohol is also used when the abusers can not afford cocaine. In addition, cocaine is often used simultaneously during alcohol intoxications (97) since cocaine and ethanol in combination have been reported to prolong and increase the cocaine-induced euphoria (199, 62, 164). Moreover, it has been hypothesized that cocaine decreases the withdrawal symptoms associated with alcohol (79). The combination of cocaine and ethanol is more toxic than either drug alone with a significant increase in the incidence of medical emergencies and sudden death (229). Concurrent ingestion of cocaine and ethanol increases the heart-rate to a higher extent than after the use of cocaine alone (63). The co-abuse of cocaine and alcohol is also associated with increased hepatotoxicity (12, 191) and cardiotoxicity (93). The mechanism behind the high prevalence for concurrent use of cocaine and alcohol is not clear.

Cocaine and ethanol are psychoactive substances, affecting moods, cognition and behavior. Cocaine is causing a feeling of increased self-confidence, alertness, well-being and indefatigability, as well as an intense euphoria (284). The cocaine user experiences a very short-lasting rush of intense pleasure and the person is soon craving a return to the initial euphoria, explaining why cocaine is usually abused in an intermittent pattern with many doses of cocaine during a short period of time ("binges"). Heavy "binges" leads to a complete exhaustion often followed by a period of abstinence in order to recover from the lack of sleep and food. Cocaine abstinence is mainly characterized by depression and anxiety (68), which often leads the user back to another "binge" period. The most common way of ingesting cocaine is through nasal inhalation but cocaine can also be injected intravenously or smoked (284). Ethanol has been described as a central nervous system depressant but that refers to large doses of ingested ethanol. Small doses of ethanol instead cause an initial euphoria as well as other stimulating effects (207). At first glance, cocaine and ethanol seem to have different properties but they share the effect (together with most drugs of abuse) of creating a feeling of reward that could cause a desire to experience that drug again. The sense of reward or positive reinforcement (behaviors associated with a drug tend to be repeated) that are caused by drugs of abuse are also experienced by natural rewards such as eating, drinking and mating (125). The reward and positive reinforcement have been linked to a specific brain circuit that has been named the brain reward system. Hence, the reward system seems to be stimulated in association with activities that serve to promote the survival of the individual or the species and drugs of abuse mimic the way natural rewards act (131). In the brain reward system dopamine has been suggested to be an important neurotransmitter, as seen in experimental studies where food, water, mating (203, 293, 7, 278, 8) and drugs of abuse (111, 52) increase dopamine release. Rodents can be trained to self-administer most drugs that are abused by humans and blockade of dopamine neurotransmission in the brain reward pathway reduce or abolish self-administration behavior (225, 153) supporting the idea that dopamine is

essential in mediating reward sensations caused by drugs of abuse. In addition to dopaminergic activity, there are several other neuroactive substances involved in mediating reward and reinforcement of drugs of abuse, including the endogenous opioid system (136, 95). The endogenous opioid system has been connected with drug abuse and dependence after the finding that opiate-derived drugs like morphine and heroin mediate their effects through binding opioid receptors in the brain. Like opiates, the endogenous opioids exert a reinforcing action and are self-administered by experimental animals (10, 274). The dopamine and the endogenous opioid system are closely linked anatomically and functional interactions have been proven by studies showing that opioid peptides can modulate dopaminergic activity (53, 252) and vice versa (256). Thus, although the impact of dopamine in mediating the reinforcement and reward of drugs of abuse is large, other systems such as the endogenous opioid system may also be critically involved through indirect modulations of the dopaminergic activity in the brain reward system. Therefore, experimental studies of these common substrates for cocaine and ethanol reinforcement might provide information about possible interactions between cocaine and ethanol in the brain reward system. Such information can help to explain the high frequency concurrent cocaine and ethanol use that might further delineate factors that could be used in clinical practice.

1.1 DRUG DEPENDENCE

Drug dependence is a major health problem affecting large segments of the society. The transition from controlled use to drug dependence develops in a manner that is both complex and unclear, for example why do not all people using a drug of abuse become dependent to these substances? It has been suggested that the initial use of a particular drug is related to its ability to produce a sense of well-being and euphoria but genetic and environmental factors might contribute to the subjective experience of the first drug intake (69). For some individuals, drug use might grow into abuse. Abuse is a state that is defined as controlled harmful drug intake that is continued despite negative effects (i.e. physical hazards or failure to fulfill obligations at work, school or home), according to the American classification systems for psychiatry disorders, Diagnostic and Statistical Manual of Mental Disorders (DSM IV; Association 1994) (4). The “harmful” use of a drug is also defined in the World Health Organization’s international classification of diseases (ICD-10, 1992) (292). Drug dependence is the final condition where the individual needs the drug of abuse to be able to function within normal limits. This condition can develop gradually after a period of drug exposure and is manifested by three or more of the following symptoms, occurring at any time in the same 12-month period (DSM-IV), occurring together for at least one month or together in a 12-month period (ICD-10, 1992); 1. Tolerance 2. Withdrawal symptoms 3. Impaired control 4. Neglect of other activities and increased drug-related activities 5. Continued use despite problems 6. Compulsions (ICD-10 only).

Tolerance, the first symptom of drug dependence is usually described as a need for increased amounts of the drug in order to achieve the desired response. In animal models tolerance can be seen as reduced locomotor activity after a chronic treatment with cocaine as compared to the initial drug-induced locomotor response (240). The second parameter of drug dependence is withdrawal which can cause physical withdrawal symptoms characteristic for the substance abused when the drug is no longer present in the body. For example alcohol withdrawal produces tremor and autonomic hyperactivity. In addition, withdrawal from chronic drug abuse is associated with anxiety and depression. In order to avoid or

relieve withdrawal symptoms individuals might continue to use the drug. The first two parameters are important influences for the motivation to drive humans or animals to continue the use of drugs. The third parameter includes difficulties in controlling the drug intake where the drug dependent individual consumes larger amounts or uses the drug of abuse over longer periods than first intended. The fourth symptom indicates that drug-related activities such as searching for the drug, consuming the drug and recovering from the effects of drug use are of higher priority than other social activities. The fifth parameter implies that the desire for the drug overshadows everything including harmful physiological consequences that have been caused by the use of that drug. The sixth parameter is only included in the ICD-10 (WHO) criteria for drug dependence and emphasizes that drug dependent individuals feel a strong desire to take the drug.

Drugs that have high abuse potential in humans correspond well with the drugs that have positive reinforcing effects in animal models (130). The availability of animal models of drug dependence has provided helpful tools to study factors involved in acquisition, maintenance, withdrawal and relapse of drug dependence. These factors can carefully be extracted in laboratory-controlled situations by using methods such as the self-administration paradigm, drug discrimination paradigm and conditioned place preference. The neurobiological mechanisms involved in the positive reinforcing effects of drugs and the negative reinforcing effects of drug abstinence can be elucidated. In addition, the environmental, behavioral, and neurobiological factors that contribute to individual differences in vulnerability to drug addiction can be explored with animal models. Thus, animal experiments can provide information about behavioral and neuropharmacological mechanisms underlying drug dependence.

1.2 MECHANISMS UNDERLYING DRUG DEPENDENCE

Animal experiments have identified several different molecular mechanisms that might explain the initiation and the maintenance of drug dependence. First, the initial effects of a drug are caused by the drugs binding to target molecules leading to an activation of neurons in the brain reward system that is associated with reward and positive reinforcement, an activation that might be strong enough to induce self-administration behavior in animal experiments. The continued use of a drug can lead to counter-adaptive or mal-adaptive molecular and cellular changes in order to maintain the homeostasis in the brain (132, 185). These alterations develop in neurons located in “within systems”, i.e. systems that are directly linked to the acute positive reinforcing actions in the brain as well as changes in “between systems”, i.e. systems that are not directly linked to the acute positive reinforcing effects of the drug (131). The mal-adaptive changes caused by maintained use of a drug have been suggested to cause sensitization, where drug intake results in an enhanced behavioral or neurochemical response. The sensitized brain systems have been proposed to make the drug increasingly desirable (increasing the incentive value), which might lead to an increased motivation of wanting the drug (226) and might therefore play a role in the initial phase of drug dependence (132). The counter-adaptive changes within the reward circuit are long lasting and counteracts the initial changes caused by a drug of abuse in order to maintain normal functions at a given drug dose. These changes might eventually result in the physiological and behavioral changes associated with drug dependence such as tolerance, withdrawal, craving (increase in drug-seeking behavior) and relapse. Tolerance might develop from counter-adaptive changes in brain systems related to reward leading to the diminished effects of the drug and withdrawal symptoms are also caused by

long-term neuroadaptations leading to physical symptoms such as anxiety, dysphoria and craving when the drug is absent. Craving can be linked to both negative reinforcement (negative withdrawal effects that can only be reversed by renewed intake of the drug) or to sensitization of the incentive phase (“wanting the drug”) leading to increased motivation for drug seeking and intake (226). Craving can be triggered by natural cues that are associated with the drug (a phenomenon called conditioning), stress or a priming dose of the drug but also by another drug of abuse (58, 257, 27, 275). The different phases (i.e. initiation, maintenance and relapse) of a drug dependent cycle are all thought to develop from different neurochemical changes in the brain, making the identification of these changes very important.

A neural substrate for reward and reinforcement has been identified in animals. The brain is provided with a reward system mediating a sense of well-being, lust and euphoria, first discovered by Olds and Milner in 1954 where direct electrical stimulation of different brain areas was powerfully rewarding in mice (192). The physiological aspect of this system is believed to reinforce basic behaviors, such as eating, drinking and sexual behavior (125) where drugs of abuse such as ethanol, cocaine, nicotine and heroin usurp the reward system (131), thus initiating a sense of well-being, lust and euphoria. More specific studies of the brain reward system have shown that the mesocorticolimbic dopamine system is a key component (279).

1.3 DOPAMINE

1.3.1 Dopamine pathways

The mesocorticolimbic dopamine system is anatomically based in the ventral tegmental area with projection neurons to both limbic (subcortical) structures and cortical structures (44, 266, 11), Figure 1. The mesocorticolimbic dopamine system has been divided into a mesolimbic dopamine system and a mesocortical dopamine system based on the projection fields of the ventral tegmental area neurons. The mesolimbic dopamine system includes dopamine innervation from the ventral tegmental area to limbic areas such as the nucleus accumbens, the olfactory tubercle, the septal area, the amygdaloid complex and the bed nucleus of the stria terminalis while the mesocortical dopamine system includes innervations to cortical areas such as the prefrontal cortices and the cingulate cortex (266, 11).

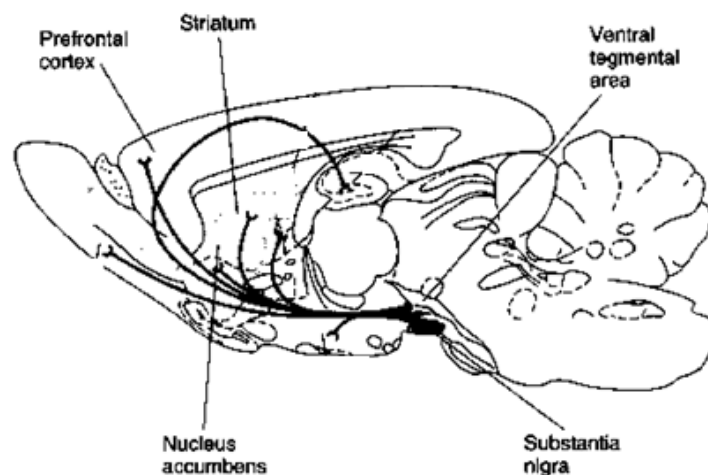


Figure 1. A schematic drawing of the mesocorticolimbic and the nigrostriatal dopamine systems originating in the midbrain.

The mesolimbic dopamine system has been implicated in emotions and reward (281) while the mesocortical dopamine system regulates higher motor execution of behavior, motivation and cognition (243). The other major group of dopamine neurons is anatomically based in the substantia nigra and projects to the striatum and is therefore referred to as the nigrostriatal pathway (5). The nigrostriatal dopamine system is involved in regulation of motor functions.

1.3.2 Dopamine release

In the above described dopamine systems, dopamine is synthesized in the neurons and stored in vesicles until the midbrain dopamine neurons are activated, dopamine is then released into the synaptic cleft through vesicle fusion with the cell membrane by a Ca^{2+} -dependent mechanism (38). The dopamine transporter present at the terminals reabsorbs synaptic dopamine into the pre-synaptic neurons. Midbrain dopamine neurons are tonically active with single spike action potentials resulting in stable background levels of extracellular dopamine but they also respond to behaviorally relevant stimuli with phasic dopamine release induced by burst firing (80, 81, 77, 76). Dopamine neurons respond to unexpected events, where primary rewards such as food and water are the most effective activators of the neurons (175). It has been suggested that dopamine neurons respond to stimuli that are behaviorally salient and requires a behavioral response of the animal (172, 65).

The activity of the dopamine system is regulated by tonic inhibition of dopamine neurons that arises from γ -aminobutyric acid (GABA) containing neurons in the ventral tegmental area and the substantia nigra (2). There are also GABAergic feedback loops from the nucleus accumbens and the striatum projecting to the ventral tegmental area and the substantia nigra (272, 122). The activity of dopamine neurons and dopamine release is further modulated by afferent input to the midbrain dopamine cell bodies and to the terminal fields. Several neurotransmitter systems contribute to this modulation, including excitatory amino acids, acetylcholine, noradrenaline, serotonin, and neuropeptides (280, 2).

1.3.3 Dopamine receptors

The released dopamine that is not removed by the dopamine transporter binds to both pre- and post-synaptic receptors initiating a series of events. There are two general classes of dopamine receptors, the dopamine D1-like receptors (including D1 and D5 receptor subtypes) and the dopamine D2-like receptors (D2, D3, D4 receptor subtypes) (246). Both of the dopamine receptor subtypes belong to the G-protein coupled seven transmembrane receptors but they differ in properties such as pharmacological profile, localization and mechanisms of action. The D1-like receptors are mainly located postsynaptically and when stimulated increase the formation of cyclic adenosine monophosphate (cAMP) (251), which in turn causes cellular responses. The D2-like receptors on the other hand inhibit adenylate cyclase activity and are located postsynaptically and presynaptically, where they presynaptically act as autoreceptors inhibiting dopamine release and synthesis (38). Dopamine receptors of the D1 and the D2 subtypes are both located in the nucleus accumbens (273) where they play an important role in mediating the rewarding properties and increasing the motivational value of a drug or a drug-associated stimulus (289, 215). Lower levels of D2 receptor subtypes in the striatum have been observed in both alcohol and cocaine users (285, 286) probably representing a neuroadaptive change related to excessive dopamine release associated with a prolonged drug-use. Furthermore, this down-regulation of D2 receptor subtypes

in the nucleus accumbens has been linked to an increased craving for alcohol (92). D1 and D2 receptor subtypes are also located in the striatum, the substantia nigra and ventral tegmental area (273, 2). Interestingly, D3 receptor subtypes are mainly located in the nucleus accumbens and have been suggested to be involved with the reinforcing actions as well as with the reinstatement of cocaine-seeking behaviors (290, 54).

1.3.4 Functional aspects of dopamine

There are several distinct types of behaviors related to the dopamine network, including motivated behavior such as reward and attention and motor control (243, 281).

The mesocorticolimbic dopamine system is considered to play an important role in reward-related functions. Firstly, most drugs of abuse cause an initial increase of dopamine release in the nucleus accumbens (111, 52), while withdrawal from these drugs decreases dopamine release (230, 276, 56, 57). Secondly, self-administration of drugs is attenuated when dopamine neurotransmission in the nucleus accumbens is inhibited, which has been shown either by blocking the binding of dopamine to dopamine receptors or via neurotoxic lesions of dopaminergic cells in the nucleus accumbens (51). The dopamine system has been suggested to respond when the rewarding stimulus is novel or if it does not match previous experience and the nucleus accumbens might work as an integrator assessing the value of a reinforcer, amplifying reward that were better than expected and dampening expected stimuli or less important ones (124). There is a link between drug reinforcement and the ability of a drug to induce locomotor activity, as proposed by the psychomotor stimulant theory of addiction (282). According to this theory, the ability of a drug to induce reinforcing actions can be predicted by its ability to produce acute motor-activating effects and both properties are associated with enhanced dopamine transmission in the mesocorticolimbic dopamine system (282). The behavioral activities might be initiated in association with or in anticipation of a rewarding stimulus in order to obtain the reward where the nucleus accumbens might assess the value of a reinforcer leading to a goal-directed behavior (237). Even though the precise contribution of dopamine signaling in reward is unclear, continuous administration of most drugs of abuse causes molecular, cellular, structural and functional adaptations (104) in the mesocorticolimbic dopamine system and/or in systems connected to this circuit. These changes might be, at least partly responsible for processes involved in drug dependence such as sensitization, tolerance and vulnerability to relapse.

The nigrostriatal dopamine neurons are involved in regulation of motor functions. The locomotor behavior is controlled by the direct (striato-nigral) pathway that stimulates motor performances and by the indirect (striato-pallidal) pathway that decreases movement (84). A balance in the activity of the direct and the indirect pathways is required for normal motor functioning and dopamine controls the balance between those two pathways (84). Parkinson's disease in humans is characterized by an impaired initiation of actions as a result of decreased dopamine input to striatum (20, 98). On the other hand, increased concentrations of dopamine or stimulation of dopamine receptors with a dopamine receptor agonist will lead to increased motor activity such as choreic movements, tics or stereotypic behavior (85). Stereotypic behavior is a repetitive motor action that can be induced by high doses of psychostimulant drugs and initiation of this behavior seems to be involved with dopamine transmission in the striatum

(127) and nucleus accumbens core (208). The increase in drug-induced locomotor activity has been linked to the nucleus accumbens (126). Thus, the increase in dopamine in the nucleus accumbens and striatum caused by cocaine and other psychostimulants seems to disturb the balance of the direct and indirect pathways leading to increased locomotor activity, an effect that is enhanced upon repeated treatment (283).

Despite the well-established changes in dopamine transmission after drug administration the neurobiology of drug dependence is complex and involves several neurotransmitter systems. One such system is the endogenous opioid system that has been shown to modulate the activity of mesolimbic dopamine neurons (53, 252). The endogenous opioids have been proposed to be involved in many aspects of the drug dependence cycle (69) and therefore the endogenous opioid system could be a common substrate for drugs of abuse, including cocaine and ethanol.

1.4 THE ENDOGENOUS OPIOID SYSTEM

Opium has been used for a long time for its addictive properties, which lead to the suggestion that all opiate alkaloids, such as opium, morphine and heroin mimic substances already present in the brain. This was supported in the early 1970s by the demonstration of opiate binding sites in the brain (201, 247, 260), a finding that was followed by the discovery of endogenous opioid activity in brain tissues (133, 261). There is convincing evidence for three major classes of opioid receptors, designated μ -, δ - and κ - opioid receptors (161, 150) and different classes of opioid peptides; the endorphins, (14), enkephalins (100) and dynorphins (75). In addition, an opioid like receptor 1 (ORL1) with structural similarities to classical endogenous opioid receptors (67, 177) and its ligand nociceptin has been discovered (169, 222).

1.4.1 Endogenous opioid receptors

The occurrence of at least three opioid receptor subtypes was suggested by early pharmacological studies (161) and later confirmed by the cloning of three different opioid receptor genes. The cloning showed significant sequence homologies for the three opioid receptors and that the three receptors all belong to the family of seven transmembrane G-protein coupled receptors (60, 128, 26, 174). All opioid receptors mediate their actions through inhibition of adenylate cyclase activity, inwardly rectifying K^+ conductance, inhibition of high-voltage-activated Ca^{2+} channel currents and impediment of neurotransmitter release (40). Subtypes of all three receptors have also been indicated (50) as well as a μ/δ -receptor complex (234). The distribution of the opioid receptors in the mesolimbic and nigrostriatal systems shows that the ventral tegmental area is rich in μ -opioid receptors with low densities of δ - and κ -opioid receptors while the nucleus accumbens and striatum contain high levels of all three receptors (121).

1.4.2 Endogenous opioid peptides

The opioid receptors represent targets for the endogenous opioid peptides, which are synthesized from enzymatic cleavage of three precursor molecules, pro-opiomelanocortin (POMC), proenkephalin and prodynorphin (183, 37, 120), Table 1.

TABLE 1
Precursor molecules, major opioid peptides and relative affinity for opioid receptors

Precursor molecule	Major opioid peptide	Relative affinities for opioid receptor
Proopiomelanocortin	β -Endorphin 1-31	$\mu=\delta$
Proenkephalin	Met-Enkephalin	$\mu<\delta$
	Leu-Enkephalin	$\mu<\delta$
Prodynorphin	Dynorphin A	κ
	Dynorphin B	κ
	Leu-Enkephalin	$\mu<\delta$

From POMC the opioid β -endorphin is generated but also several nonopioid peptides such as the stress hormone adrenocorticotropin and β - and γ -melanocyte-stimulating hormones. Proenkephalin gives rise to leucine (Leu)- and methionine (Met)-enkephalin, metorphamide and Met-enkephalin-Arg⁶-Phe⁷. Prodorphin can generate several opioid peptides including α - and β -neoendorphin, Leu-enkephalin, dynorphin A and dynorphin B. POMC biosynthesis mainly occurs in the pituitary, arcuate nucleus of the hypothalamus and the nucleus tractus solitarius, while proenkephalin and prodorphin are synthesized widely throughout the central nervous system (3). With regard to the mesolimbic and the nigrostriatal dopamine systems, the nucleus accumbens and the ventral tegmental area receive endorphinergic input from the arcuate nucleus of the hypothalamus and dynorphin and enkephalin peptides are enriched in the striatum (3). There seems to be some preference for the different endogenous opioid ligands for the different receptors. β -endorphin binds with about equal affinity to the μ - and δ -opioid receptor, enkephalin shows preference for the δ -opioid receptor and dynorphin for the κ -opioid receptor (39), Table 1.

1.4.3 The endogenous opioid system and dopamine

Several animal studies and a body of anatomical evidence have demonstrated the importance of the endogenous opioid system in regulating dopamine transmission in the mesolimbic dopamine system, Figure 2. The β -endorphin neurons in the hypothalamus that project to the ventral tegmental area stimulate μ - and possibly δ -receptors on inhibitory GABA neurons (119). Stimulation of μ - and δ -receptors in the ventral tegmental area removes GABAergic inhibition of dopamine cells leading to an increased dopamine release in the nucleus accumbens (49) Activation of κ -receptors decreases dopamine release in the nucleus accumbens (53, 252) possibly via hyperpolarization of dopamine terminals through presynaptically located κ -receptors in the nucleus accumbens (259) but possibly also through κ -receptors located directly on the soma of dopamine cells in the ventral tegmental area (158). In addition to a decreased dopamine release, a recent report has shown that acute stimulation of κ -receptors increases dopamine clearance in the nucleus accumbens (262). Not only does the endogenous opioid system affect the dopamine system, but the reverse is also true. Dopamine has been suggested to have a tonic excitatory influence on the expression of dynorphin peptides via activation of D1 receptor subtypes and at the same time to inhibit the expression of enkephalin peptides through D2 receptor stimulation. This can be shown in experiments where depletion of dopamine neurons decreases dynorphin gene expression and increases that of enkephalin in striatum (296, 147) and these effects are reversed by D1 or D2 receptor activation leading to increased levels of dynorphin expression or decreased levels of enkephalin, respectively (59).

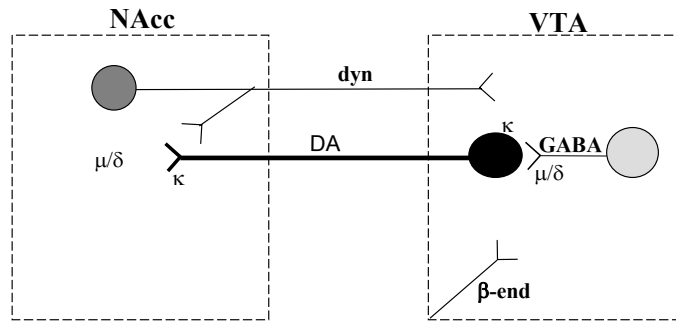


Figure 2. Diagrammatic representation of possible interactions between the endogenous opioid system and the mesolimbic dopamine system. Ventral tegmental area (VTA) dopamine cellbodies are under tonic GABAergic inhibition. This inhibition may be removed after stimulation of μ - (and δ -) opioid receptors located on GABA interneurons in the VTA. In addition, dopamine release might be decreased through stimulation of pre-synaptic κ -opioid receptors in the nucleus accumbens (NAcc). DA, dopamine; dyn, dynorphin; β -end, β -endorphin.

Similarly, D2 receptor stimulation has been shown to produce an increased expression of μ -opioid receptors (25), while D2 receptor blockade decreases the expression of μ -opioid receptors in the striatum (24).

The endogenous opioid systems have been suggested to be tonically active, stimulating basal dopamine release, with a balance between the stimulating μ - and δ -opioid systems and the opposing κ -opioid system. This is supported by studies using mice that are lacking an opioid receptor subtype. For example, mice lacking μ - or δ -receptors have been suggested to have a decreased basal dopamine release based on the observations that these mice have a decreased basal dopamine up-take in the nucleus accumbens yet no changes in basal extracellular levels (23) and mice lacking the κ -opioid receptor show increased basal dopamine levels in the nucleus accumbens (22).

1.4.4 Nociceptin and ORL1

Based on sequence homology (65%) with the known opioid receptors, a new G-protein coupled receptor was identified and named ORL1 (67, 177). A dynorphin-like peptide, nociceptin was later shown to be the endogenous ligand of the ORL1 receptor (169, 222). None of the known opiate ligands bind to the ORL1 receptor with high affinity, nor does nociceptin bind to μ -, δ - or κ -receptors (221). ORL1 receptor messenger ribonucleic acid (mRNA), protein levels and the nociceptin precursor mRNA are widely distributed in the central nervous system (67, 177, 6, 99, 108).

1.4.5 Nociceptin and dopamine

Nociceptin has been shown to reduce dopamine levels in the nucleus accumbens after intracerebroventricular (180), or intra ventral tegmental area (181) administration of the peptide suggesting that the ventral tegmental area may be the site of action for this effect possibly mediated through a direct activation of ORL1 receptors located on dopaminergic neurons (155, 188, 297, 182). It has also been demonstrated that nociceptin inhibits the activity of β -endorphinergic neurons of the arcuate nucleus (271) and could thereby affect the mesolimbic dopamine system.

Taken together, the modulatory action of the endogenous opioid peptides and nociceptin on the dopamine system suggests that these systems could play a part in neurobiological mechanisms that might be underlying reward and dependence of drugs of abuse, such as cocaine and ethanol.

1.5 COCAINE

Cocaine is one of the most powerful and reinforcing central nervous system stimulants in humans. Animal experiments clearly demonstrate the rewarding effects of cocaine. For example, rats learn to self-administer cocaine easily (48, 13) and if given unlimited access, they will self-administer up to the point of severe weight loss and death (13). Cocaine is also behaviorally activating which in rats is seen as locomotor hyperactivity at low and intermediate doses of cocaine and as stereotyped behavior at high doses (15).

1.5.1 Cocaine, dopamine and reward

Cocaine binds to and inhibits the dopamine, norepinephrine and serotonin transporters which increases and prolongs the transmitter concentration in the synaptic cleft (223). The increase of dopamine concentration in the terminal region of the mesolimbic dopamine system has been suggested to initiate the rewarding effects and the psychomotor activation effects of cocaine (282, 142). The importance of the nucleus accumbens in mediating cocaine reward and reinforcement has been demonstrated in several studies. For instance, animals self-administer cocaine directly into the nucleus accumbens (166) and toxic lesions of the dopamine fibers in the nucleus accumbens reduced cocaine self-administration (202).

Nevertheless, mice lacking the dopamine transporter continue to self-administer cocaine (227), implicating other neurotransmitter systems to be involved in cocaine reward and reinforcement. Besides serotonin and norepinephrine cocaine interacts with many other neurotransmitter systems including glutamate (123), GABA (143) and opioids (136) which directly or indirectly could play a role in the reinforcing properties of these drugs.

1.5.2 Cocaine and the endogenous opioid system

The interaction of cocaine with endogenous opioid systems has been recognized for several years. Evidence suggests that cocaine alters the activity of the opioid peptides in the brain and these alterations may in part modulate some of the behavioral effect of cocaine, including cocaine reinforcement. Increased levels of endorphin peptides were observed in the nucleus accumbens after a single (193, 232), and after chronic (232), cocaine administration and dynorphin peptide levels were increased in the striatum after multiple doses of cocaine (248, 249). At least some of these effects are mediated through activation of dopamine receptors, since dopamine agonists have been shown to increase the release of dynorphin (256) which might cause further changes at the opioid receptor levels. Cocaine has been shown to alter the density (267, 113, 268, 115, 298, 255, 264, 162, 265, 36) and activity (115, 242) of specific opioid receptors in discrete regions of the brain reward circuit. Such changes are likely to alter the interaction of opioid receptors with their endogenous ligand and perhaps further influence the response of a drug.

Opioid agents in turn affect dopamine levels and other effects caused by cocaine. For example, the non-selective opioid antagonist naltrexone has been shown to reduce cocaine self-administration in experimental animals (41, 213) as well as relapse to cocaine abuse in human cocaine addicts (241). When using more specific agents it was shown that suppression of the μ - and δ -opioid systems reduces the rewarding effects of cocaine (258, 220, 42, 209, 231). In addition, mice lacking the μ -opioid receptor have been reported to show reduced cocaine-induced place-preference (9, 86). Several investigations have provided evidence that stimulation of κ -opioid receptors suppresses several pharmacological and behavioral effects induced by cocaine. For instance, κ -receptor stimulation attenuates cocaine-induced reward in self-administration studies (74, 145, 184, 167, 239).

1.5.3 Cocaine and the nociceptin system

Neurochemical studies lend support for a role of nociceptin in the modulation of reward related behaviors. First, intracerebroventricular injection of nociceptin reduced cocaine-elevated dopamine concentrations in the nucleus accumbens (151). The rewarding effects of cocaine are reduced after treatment with nociceptin as measured by conditioned place preference (134, 236). Nociceptin has also been reported to decrease cocaine-induced locomotion (151, 134) and cocaine-induced sensitization (152) after intracerebroventricular administration possibly due to nociceptins effects in the ventral tegmental area (152).

1.6 ETHANOL

Ethanol is a substance that does not bind to specific sites as many other drugs of abuse, instead ethanol is interacting non-specifically with several different neurotransmitter systems occurring at widespread anatomical sites in the brain.

1.6.1 Ethanol, dopamine and reward

A considerable amount of publications has reported that the reinforcing and behavioral effects of ethanol are mediated by an increase in the dopamine concentration in the nucleus accumbens (110, 52). Ethanol affects many properties of the dopaminergic neurons. Firstly, ethanol has been shown to directly excite dopaminergic cell bodies in the ventral tegmental area (71, 17, 16). Secondly, ethanol might indirectly affect dopamine release through inhibition of GABAergic neurons in the substantia nigra pars reticulata and ventral tegmental area leading to a disinhibition of dopamine neurons, possibly through activation of opioid receptors (43). Pharmacological treatment with both dopamine receptor antagonists (217) and dopamine agonist (218, 187) have been shown to decrease ethanol intake and supports a role for dopamine in ethanol reward and reinforcement. However, neurotoxic lesions of dopamine neurons did not interfere with ethanol consumption or operant responding for ethanol (219, 109) showing a complex situation suggesting that other systems besides dopamine influence the intake of ethanol.

Indeed, ethanol has been shown to facilitate GABA transmission by increasing chloride conductance through GABA_A receptors (46) and to increase serotonin concentrations in the nucleus accumbens (294). Moreover, acute ethanol has also been associated with decreased glutamate activity (19), while chronic ethanol administration up-regulates NMDA glutamate receptors (263). A recent study has proposed that glycine receptors may be involved in controlling ethanol consumption, since stimulation of glycine

receptors in the nucleus accumbens decreases ethanol intake while increasing accumbal dopamine concentrations (176). Several studies also suggest important interactions between ethanol and opioid systems that might contribute to the initiation, maintenance and relapse to alcohol dependence (95).

1.6.2 Ethanol and the endogenous opioid system

Several reports indicate that ethanol alters the activity of the opioid peptides and these changes may play an important role in the reinforcing properties of ethanol. For example, an acute challenge of ethanol increases the extracellular levels of endorphins in the nucleus accumbens (216, 193, 159) and in the ventral tegmental area (216) and a correlation between the risk of developing alcoholism and an increased ethanol-induced release of β -endorphin in humans has been observed (73). Substantial evidence shows that ethanol does not only affect the opioid peptide levels but also the opioid receptors. The ethanol-induced changes in opioid peptides and in opioid receptors vary with the brain region investigated as well as the strains of animals used. Acute and chronic ethanol administration increases μ -opioid receptor binding (72, 168) and δ -receptor binding is increased in rat brain following chronic ethanol treatment (72).

Non-selective opioid receptor antagonists administered both systemically (157, 238) and locally in the nucleus accumbens (96) reduce ethanol self-administration and ethanol-induced stimulation of dopamine release (78). On the basis of findings in animal models, naltrexone, a non-selective opioid antagonist was tested clinically and is now used for treatment of alcohol dependence in humans (190, 287). Evidence for participation of μ -opioid system in modulation of ethanol-related behaviors comes from studies where rodents treated with μ -opioid receptor antagonists (106, 141, 107) and mice lacking μ -opioid receptors (224, 87) display reduced ethanol intake. In addition, a low basal activity of the dynorphinergic/ κ -receptor system is associated with high ethanol intake in rodents with an alcohol-preference (189, 116) and treatment with a κ -opioid agonist reduced voluntary ethanol intake in rats (149).

1.6.3 Ethanol and the nociceptin system

Centrally administered nociceptin has been shown to reduce ethanol self-administration (31, 30). Nociceptin or an ORL1 receptor agonist abolished ethanol-induced place preference (144) and ethanol reinstatement either caused by stress (160), ethanol paired cues (30) or a stimulating dose of ethanol (144).

Taken together, these data provide evidence for shared biochemical processes of cocaine and ethanol as shown by the participation of dopamine and opioid systems in modulation of both cocaine and ethanol-related behaviors.

1.7 CONCURRENT COCAINE AND ETHANOL

The combined use of alcohol and cocaine has become a significant social and public health concern worldwide, with evidence that sudden death and incidence of medical care are increased when this drug combination is used (229). Despite this concern, there are currently few efficacious medications for the treatment of each kind of drug dependence or a general approach treating the co-abuse of these drugs.

The high prevalence of cocaine and alcohol co-abuse in humans might be explained at genetic and pharmacokinetic levels as well as by additive properties when both drugs are taken in combination. Alcohol preferring rats consume more cocaine than non-preferring rats (105) and they respond with an increased dopamine release after cocaine administration (171), supporting a hypothesis of common genes that control cocaine and ethanol intake. Ethanol can modify cocaine responses through alterations in cocaine pharmacokinetics, where co-administration of ethanol increases the plasma and brain extracellular fluid concentrations of cocaine (270, 90, 194). Ethanol pre-treatment has also been demonstrated to potentiate cocaine's rewarding properties in conditioning place preference (18) and self-administration (170) paradigms suggesting that the combination of cocaine and ethanol results in a greater cocaine reward. Concurrent cocaine and ethanol also results in an enhanced locomotor response (163, 198) than what is produced by either drug alone and cross-sensitization between cocaine and ethanol has been observed (114, 146), suggesting that these two drugs act on a common neural circuit to mediate these behavioral effects.

Understanding possible common neurochemical mechanisms of these two drugs in drug reward and dependence might help explain the high frequency of cocaine and alcohol abuse and further lead to suitable pharmacological treatment for a concurrent cocaine and alcohol dependence.

2 AIMS OF THE STUDY

The general aim of the present thesis was to analyze separate and combined effects of cocaine and ethanol given acutely and chronically on the endogenous opioid and ORL1 systems as well as on dopamine release in the rat mesolimbic dopamine pathway.

In particular, the experiments were designed to:

Examine the acute effects of cocaine and ethanol on dynorphin and κ -opioid receptor mRNA levels in the mesolimbic and nigrostriatal dopamine systems by using *in situ* hybridization technique.

Study the acute effects of cocaine and ethanol on opioid and ORL1 receptors in the central nervous system by using autoradiography.

Examine the effects of subacute cocaine administration on κ - and μ -opioid receptor mRNA levels in the nucleus accumbens.

Analyze the chronic effects of cocaine and ethanol on κ -opioid receptor mRNA levels in the mesolimbic dopamine pathway by using quantitative reverse transcriptase polymerase chain reaction (RT-PCR). In addition, study the effects of κ -opioid receptor ligands on dopamine release following repeated ethanol administration.

Investigate the chronic effects of cocaine and ethanol on dopamine output in the nucleus accumbens by using microdialysis.

3 MATERIALS AND METHODS

3.1 ANIMALS

Male Sprague-Dawley rats (BK Universal, Sollentuna, Sweden) weighing from 200-350 grams at the beginning of the experiments were used. Before the experiments the animals were housed for one week in a modern animal care facility in a temperature and humidity controlled environment with *ad libitum* access to food and water under a 12 hour light/dark cycle. All experiments were performed according to the guidelines in our applications with permit numbers N302/97, N183/98, N289/99 approved by the Animal Ethics Committee of Northern Stockholm, Sweden.

3.2 DRUGS AND CHEMICALS

Ethanol (AB Svensk sprit) and cocaine hydrochlorid (Apoteket AB, Sweden) were dissolved in saline (0.9%) and administered by intraperitoneal (i.p.) injections in all experiments. In paper VI, U50, 488H and nor-binaltorphimine (nor-BNI) (Bio-nuclear AB, Stockholm, Sweden) were dissolved in saline and diluted to 20 μ M and 10 μ M respectively, in artificial cerebrospinal fluid (aCSF; 148 mM NaCl, 2.7 mM KCl, 0.85 mM MgCl₂, 1.2 mM CaCl₂, pH 7.1 to 7.4, Apoteket AB, Sweden) and administered into the nucleus accumbens by reverse microdialysis. [³H] CI-977 ((-)-N-Methyl-N-[7-(1-pyrroindinyl)-1-oxospiro [4, 5]dec-8-yl]-4-benzofuranacetamide), [³H] D-Ala², Asp⁴-deltorphin I (DELTA I), [³H] D-Ala²-Methyl-Phe⁴-Gly-ol⁵ enkephalin (DAMGO) and naloxone (Nycomed Amersham plc, Buckinghamshire, England) were dissolved in a 50 mM Tris-HCl buffer, pH 7.4 to 2.5 nM, 7nM, 4nM and 10 μ M, respectively and used in receptor binding studies (Paper IV). *leucyl* [³H] Nociceptin and Nociceptin were dissolved in 50 mM Tris-HCl, pH 7.4 containing 3 mM MgCl₂, 2 mM EGTA, 1.26 x 10³ U/L Bacitracin and 0.1% BSA to a concentration of 0.4 nM and 1 μ M, respectively.

3.3 EXPERIMENTAL DESIGN

3.3.1 Acute cocaine and ethanol administration (Paper IV and V)

Rats received i.p. injections of saline (0.9% w/v) twice daily for thirteen days, followed by one day of cocaine and/or ethanol injections, see Table 2. Ethanol was given at 09.00 (2 g/kg, 18% v/v in saline) followed by a cocaine “binge” paradigm (156). Thus, cocaine (45 mg/kg/day) or saline was administered by i.p. injections three times with one hour intervals, starting at 09.30. Rats were killed by decapitation thirty minutes after the final injection. Brains were rapidly removed and immediately frozen in isopentane at -20°C and subsequently stored in -80°C.

3.3.2 Chronic ethanol and/or subacute cocaine administration (Paper I-III, VI)

Ethanol (2 g/kg, 18% v/v in saline) or saline was given twice daily for seven (Paper VI), eight (Paper III) or fourteen days (Paper I, II). On the last two days of ethanol treatment, rats were challenged with cocaine (45 mg/kg/day) or saline “binges” (Paper I-III), see Table 2. In Paper I and II, rats were killed by decapitation 30 minutes after the final injection, brains were removed, dissected on ice and brain regions were immediately frozen on dry ice and stored in -80°C. In Paper VI the rats were treated with ethanol for seven days and on day 8, κ -receptor ligands were infused by reverse microdialysis into the nucleus accumbens.

TABLE 2
Drug treatment schedules

	Group	Day 1-13	Day 14
Paper IV, V	Saline control	Saline 1ml /kg (0.9% w/v) 2 x day	Saline 1 ml/kg / “Binge” saline 1 ml/kg x 3
	Acute cocaine	Saline 1ml/kg 2 x day	Saline 1 ml/kg / “Binge” cocaine 15 mg/kg x 3
	Acute ethanol	Saline 1ml/kg 2 x day	Ethanol 2 g/kg / “Binge” saline 1 ml/kg x 3
	Acute cocaine and ethanol	Saline 1ml/kg 2 x day	Ethanol 2 g/kg / “Binge” cocaine 15 mg/kg x 3
	Group	Day 1-6 (Paper III) Day 1-12 (Paper I, II)	Day 7-8 (Paper III) Day 13-14 (Paper I, II)
Paper I-III	Subacute cocaine	Saline 1ml /kg (0.9% w/v) 2 x day	Saline 1 ml/kg / “Binge” cocaine 15 mg/kg x 3
	Chronic ethanol	Ethanol 2 g/kg 2 x day	Ethanol 2 g/kg / “Binge” saline 1 ml/kg x 3
	Chronic ethanol and cocaine	Ethanol 2 g/kg 2 x day	Ethanol 2 g/kg / “Binge” cocaine 15 mg/kg x 3
	Group	Day 1-7	Day 8
Paper VI	Chronic ethanol	Ethanol 2 g/kg 2 x day	U50, 488 H (20 μ M) Nor-BNI (10 μ M)

In Paper III and VI microdialysis was performed on the last day of experiment. At the end of the microdialysis experiment rats were killed by decapitation, the brains rapidly removed and frozen in ice-cold acetone and thereafter stored in -80°C.

3.4 DISSECTION

After decapitation, the pituitary gland was removed and the brain was placed in a cooled brain blocker and sliced manually with razor blades in coronal sections. The nucleus accumbens (Paper I and II) and ventral tegmental area (Paper II) were dissected using a scalpel with guidance from the rat brain atlas of Paxinos and Watson (195, 196).

3.5 *IN SITU* HYBRIDIZATION

Coronal sections (15 μ m) were prepared in a cryostat (Zeiss Microm 505E). Sections were dried by using anhydrous CaSO₄ for one week at -20 °C. Prior to hybridization, the brain sections were warmed to room temperature and allowed to dry. Subsequently, the sections were fixed in 4% paraformaldehyde/ 1 x Phosphate buffered saline (0.9% PBS) for 5 minutes, rinsed twice in PBS and treated with 0.25% acetic anhydride/ 0.1 M triethanolamine/ 0.9% sodium chloride for 10 minutes. The sections were then rinsed in 2 x standard saline citrate (SSC; 1 x SSC = sodium chloride 0.15 M, sodium citrate 0.015 M), dehydrated in a graded series of ethanol (70%, 80%, 95%, 100%), delipidated with chloroform and air-dried before the hybridization procedure. All solutions were pretreated with 0.1% diethylpyrocarbonate before use. The hybridization buffer consisted of 0.5 mg/ml sheared single stranded DNA, 250 μ g/ml Yeast tRNA

(transfer RNA), 1 x Denhardt's solution (solution of 0.2% each, bovine serum albumin, ficoll, polyvinylpyrrolidone), 10% (w/v) dextran sulfate, 4 x SSC, and 50% formamide. Before hybridization, the labeled probe (prodynorphin RNA probe, bp 466-1101 (33); κ -opioid receptor probe, bp 628-1129 (Accession number NM 017167)) was added to the hybridization cocktail in a concentration of 20×10^3 cpm per μl , and 0.21 ml of this hybridization mixture was applied to the brain sections. The sections were coverslipped to prevent evaporation and the hybridization was carried out in a humidified chamber overnight at 55°C. Incubation was followed by RNase A treatment (40 $\mu\text{g/ml}$) for 30 min at 37°C and subsequent washes in a graded series of SSC solutions containing 1 mM dithiothreitol (2X SSC, 2 x 5 minutes; 1 x SSC and 0.5 x SSC, 10 min; 0.1 x SSC, 1 hour) all at room temperature except for the 0.1 x SSC (53°C). Dehydration was carried out with graded ethanol solutions containing 300 mM ammonium acetate. The slides were then air dried and exposed to β -max Hyperfilm (Amersham, Buckinghamshire, UK) along with ^{14}C standards (American Radiolabeled Chemicals, St Louis, MO, USA) for 14 days. Optical density values were measured from digitalized images with a resolution of 200 dpi (scanned by ScanMaker III; Microtek Electronics, Düsseldorf, Germany) using a Macintosh-based image analysis software system (IMAGE; Wayne Rasband, NIMH, MD, USA). The optical density values were converted to dpm/mg by reference to the co-exposed standards. The following anatomical sites were identified by using a rat brain atlas (197): nucleus accumbens core and shell, ventromedial, dorsomedial and dorsolateral striatum, ventral tegmental area, substantia nigra pars compacta and pars reticulata. The statistical significance of differences in prodynorphin and κ -opioid receptor mRNA expression levels between the groups was calculated using a one-way ANOVA, followed by the protected Fisher's LSD post-hoc test. Statistical calculations were performed using CSS Statistica software (v. 5.0 Stat. Soft Inc.).

3.6 AUTORADIOGRAPHY

For autoradiographic mapping 20 μm frozen coronal sections were cut (400 μm apart) in a cryostat (Zeiss Microm 505E). Adjacent sections were cut for determination of specific and non-specific binding for μ -, κ -, δ -, and ORL1 receptor binding using [^3H] DAMGO (4 nM), [^3H] Deltorphin-I (7 nM), [^3H] CI-977 (2.5 nM) and [^3H] Nociceptin (0.4 nM), respectively. Naloxone was used for determination of non-specific binding for all ligands (1 μM for [^3H] DAMGO, [^3H] CI-977, and 10 μM for [^3H] Deltorphin-I), except for [^3H] Nociceptin, where cold nociceptin (1 μM) was used. Binding and incubation conditions were performed as previously described (129, 34). Quantitative analysis of receptor binding on film autoradiograms was carried out by video-based computerised densitometry using a MCID image analyzer (Imaging Research, St. Catharines, ON, Canada). Fmol/mg tissue equivalents for receptor binding were derived from [^3H]-microscale (Amersham, UK) based calibrations laid down with each film after subtraction of non-specific binding images. Quantification from both the left and the right side of each brain section was carried out. All brain structures were identified by reference to the rat brain atlas of Paxinos and Watson (196). Overall comparison of quantitative measures between treatment groups across all regions was made using one-way ANOVA followed by Fisher's LSD post-hoc test. When significant main effects were observed a two-way ANOVA (treatment x region) was carried out followed by Fisher's post-hoc test to determine individual regions with significant changes. The statistical analysis were performed using GB Stat. software (Dynamics Microsystem, Inc., Silver Spring, MD, U.S.A)

3.7 QUANTITATIVE RT-PCR

Total RNA from each region was extracted using 1 ml of RNazol B (Biotech Laboratories, Huston, USA). The final RNA pellet was dissolved in 40 μ l of diethyl-pyrocabonate-treated water, followed by spectrophotometric measurement at 260 nm of the total RNA concentration. First strand complementary deoxyribonucleic acid (cDNA) synthesis of the total RNA was made using random hexamer primers, pd(N)₆ (Pharmacia Biotech, Uppsala, Sweden) using standard procedures (204). Primers and internal standards for the rat κ - and μ -opioid receptors and for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were added to the PCR amplifications. The competitive PCR reaction was performed in a total volume of 25 μ l, including 2 μ l cDNA as well as 1 μ l of each internal standard at six different concentrations (0.03125-1 attomoles/ μ l for the κ -receptor, 0.03125-1 x 10⁻² attomoles for the μ -receptor and 0.3125-10 attomoles for G3PDH). The PCR reaction was conducted under 35 cycles under the following conditions; denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, elongation at 72°C for 1 minute, followed by an extension step at 72°C for 5 or 10 minutes. The PCR products was separated on an ethidium bromide stained 2% agarose gel and the molar ratio between each specific gene product and the house-keeping gene was calculated for each brain region. Data were analyzed for overall treatment effect using Kruskal-Wallis ANOVA of median rating scores, followed by the Mann-Whitney U-test using the CSS Statistica software.

3.8 MICRODIALYSIS

Under pentobarbital anesthesia (60 mg/kg i.p.), rats were stereotaxically implanted with a shortened microdialysis guide cannula (CMA Microdialysis AB, Sweden) in the nucleus accumbens. The coordinates according to the atlas of Paxinos and Watson (195) with reference to the bregma were AP +1.6, ML +1.4 and DV -2.1 (from dura). The shortened guide cannula was fixed to the skull by stainless-steel screws and dental cement (AgnTho's, Sweden). Following surgery, the rats were housed individually and allowed to recover for two days before drug treatment began under the above described drug treatment schedule.

On the day before the microdialysis experiment, a probe (CMA/12, CMA Microdialysis AB, Sweden) was inserted via the guide cannula. On the day of microdialysis experiment the probe was connected to a microperfusion pump (Univentor syringe pump 801, AgnTho's, Sweden) and perfused at a rate of 2.5 μ l/min with artificial cerebrospinal fluid (aCSF) via a swivel. After equilibration, dialysate from the nucleus accumbens was collected over 20 minute intervals (Univentor microsample 810, AgnTho's, Sweden), starting 60 minutes before drug injection (ethanol or cocaine (Paper III), U50, 488H or nor-BNI (Paper VI)). The κ -receptor agonist U50, 488H and the κ -receptor antagonist nor-BNI were dissolved in aCSF and pH was adjusted before dialysis (Paper VI). The microdialysis probes were perfused for 20 minutes with aCSF containing either U50, 488H (20 μ M) or nor-BNI (10 μ M). Perfusate samples were loaded into the sample loop of the injector (MIDAS) and automatically injected into a high performance liquid chromatography (HPLC) system with electrochemical detection for extracellular concentrations of dopamine as well as the metabolites DOPAC (3, 4-dihydroxyphenylalanine) and HVA (Homovanillic acid). The mobile phase that consisted of 55 mM sodium acetate, 0.5 mM octanesulfonic acid, 0.01 mM Na₂EDTA and 10% methanol (pH was adjusted to 4.1 with acetic acid) was delivered by a HPLC pump (580, ESA Biosciences, Inc., Chelmsford, MA, USA) through a Reprosil pur C18 reversed phase column (150x4 mm; 3 μ m) at a constant flow rate of 0.7 ml/min. After separation, the analyte was passed

through a guard cell (5021, ESA Biosciences, Inc., Chelmsford, MA, USA) with an applied oxidizing potential of 150 mV. The electrochemical detection was thereafter accomplished using a coulometric detector (Coulochem II, 5200 A, ESA) connected with an oxidation and reduction of the microdialysis sample (coulometric electrode: 400 mV; amperometric electrode: -200mV). Chromatograms were both printed on a two-pen chartrecorder (Kipp&Zonnen) and recorded using the Chromatography Station for Windows (CSW) software. Dialysate concentrations were determined by comparison with the standard peaks resulting from injection of known concentrations of dopamine and the metabolites DOPAC and HVA. Statistical analysis of the results was performed on dopamine and metabolite levels expressed as percent of baseline levels. Data were analyzed by one- and two-way (treatment x time) ANOVA with or without repeated measures, followed by the Least Significant Difference (LSD) test for multiple comparisons when appropriate. Statistical calculations were performed by using the CSS Statistical software. The frozen brains were later analyzed for localization of correct probe placement.

4 RESULTS AND DISCUSSION

Ethanol has been reported to potentiate the euphoric effects of cocaine. Given that both cocaine and ethanol affect the mesolimbic dopamine system and the endogenous opioid system, we hypothesized that this drug combination would act synergistically. Thus, we examined the effects of acute as well as chronic cocaine and ethanol in combination on the opioid and dopamine system in the nucleus accumbens.

4.1 ACUTE EFFECTS OF COCAINE AND ETHANOL

4.1.1 Effects of cocaine and ethanol on the prodynorphin/ κ -system (Paper V)

By using *in situ* hybridization we studied the acute effects of combined as well as separately administered ethanol and cocaine “binge” on κ -opioid receptor mRNA and prodynorphin mRNA expression in discrete brain regions. Cocaine and ethanol in combination increased prodynorphin mRNA in all regions analyzed, Figure 3A. The combination of cocaine and ethanol increased prodynorphin mRNA in the striatum and nucleus accumbens, with a potentiated effect on prodynorphin mRNA levels in the dorsolateral striatum (212%, $p < 0.0001$). The dorsal striatum is relevant for motor functions that might be altered by cocaine intake, such as increased locomotor activity and stereotypic behaviors but also in drug-seeking behavior (112). We have previously not observed any changes in stereotypic behaviors after acute co-administration of cocaine and ethanol (unpublished data), whereas another study reported increased locomotor activity after acute concurrent cocaine and ethanol in mice (163). On the other hand, concurrent cocaine and ethanol down-regulated κ -opioid receptor mRNA levels in the striatum, the nucleus accumbens, the ventral tegmental area and the substantia nigra compacta with an additive effect on κ -opioid receptor mRNA levels in the nucleus accumbens core (74%, $p = 0.0012$), Figure 3B. The prolonged dopamine release following cocaine and ethanol leads to excessive stimulation of dopamine receptors which in turn could induce dynorphin release and biosynthesis (147, 256). Acute administration of cocaine increases the expression of dynorphin mRNA in the striatum (102, 253, 45, 264) and in the nucleus accumbens (264) suggesting that cocaine exerts a modulatory influence on the dynorphin system. Dynorphin peptide levels have been reported to be increased after chronic but not after a single cocaine administration (248), although Turchan and coworkers reported increased release of α -neoendorphin (another prodynorphin gene product) following acute cocaine administration (264) and a different study showed increased levels of dynorphin peptides after multiple cocaine injections during one day (249). Since the actual release of dynorphin peptides is troublesome to measure, it can only be suggested that an elevation of dynorphin mRNA and peptide levels gives a higher dynorphinergic tone. This elevated dynorphin tone might act as a feed-back inhibition to counteract the excessive dopamine transmission from the substantia nigra and ventral tegmental area as seen by decreased concentrations of dopamine in the nucleus accumbens (53, 252), and in the striatum (295) following κ -opioid receptor stimulation. Acute administration of ethanol has not been shown to influence the dynorphin system (244). Thus, the elevation of dynorphin mRNA levels seen after co-administration of cocaine and ethanol in this study might mainly depend on the effect that cocaine plays on the dynorphin system, except in dorsolateral striatum, where the combined drug treatment increased dynorphin mRNA levels, while cocaine and ethanol administered

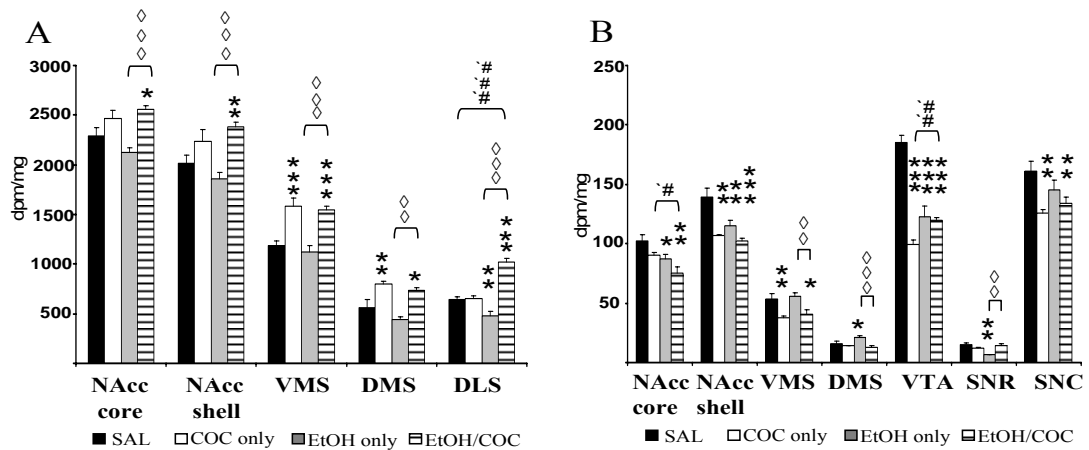


Figure 3. Histograms showing A. prodynorphin mRNA B. κ -opioid receptor mRNA expression after acute treatment with cocaine and/or ethanol in different brain areas. NAcc core, nucleus accumbens core; NAcc shell, nucleus accumbens shell; VMS, ventromedial striatum; DMS, dorsomedial striatum; DLS, dorsolateral striatum VTA, ventral tegmental area; SNR, substantia nigra reticulata; SNC, substantia nigra compacta. Values are means \pm S.E.M. (n=5). *P<0.05; **P<0.01; ***P<0.001 indicates significant difference when comparing control animals with drug-treated animals. #P<0.05, ##P<0.01, ###P<0.001 indicates significant difference when comparing cocaine-treated animals with cocaine/ethanol-treated animals. \diamond P<0.01, $\diamond\diamond$ P<0.001 indicated significant difference when comparing ethanol-treated animals with animals treated with cocaine/ethanol.

separately did not. The apparent lack of effect after single ethanol treatment might also be due to a rapid and transient elevation of dynorphin mRNA (228) that escapes detection in our study where ethanol was administered four hours before analysis, as compared to the last cocaine injection that was given thirty minutes before mRNA measurement. An increase in dynorphin release as a response to drug administration might be counteracted through changes in the synthesis of κ -opioid receptors as seen in this study by a decreased κ -opioid receptor mRNA expression.

4.1.2 Acute cocaine and ethanol, effects on opioid receptor levels (Paper IV)

Given that acute cocaine and ethanol, both separately and in combination, affected the levels of κ -opioid receptor mRNA, expression of the receptor protein might also be altered. Using the same drug-treatment schedule we analyzed the opioid receptor levels in rat central nervous system using autoradiography. Cocaine and ethanol in combination as well as acute ethanol administration increased μ -opioid and ORL1 receptor binding, while decreasing κ -opioid receptor levels, Figure 4. An acute cocaine “binge” did not cause any general changes in opioid and ORL1 receptor binding thus, the effects on receptor densities seen after concurrent cocaine and ethanol implicates a stronger influence of ethanol on the combined drug effect. However, in discrete regions there are alterations in receptor levels that seem to be due to the combination of ethanol and cocaine. For example, in the nucleus accumbens core there is an increase in μ -opioid receptor densities (P<0.05) that is not seen in any other treatment groups. The general increase in μ -opioid receptor densities after ethanol and ethanol and cocaine in combination might be explained by the fact that ethanol has a more wide-spread effect on the central nervous system than cocaine. Increased μ -opioid receptor densities after acute ethanol administration might change the sensitivity to the rewarding effects of ethanol. Rats selectively bred for alcohol preference showed a higher density of μ -opioid receptors than rats with no alcohol preference (47, 250) and mice lacking the μ -opioid receptor gene displayed reduced ethanol intake (224, 87). Our study reports no changes in δ -receptor densities in any treatment groups. Similarly, unaltered levels of δ -receptor in alcohol preferring rats has been reported (250), suggesting that direct changes in δ -receptor levels are unlikely to underlie behavioral consequences

of ethanol and/or cocaine administration. However, others have proposed a role for δ -receptors in both alcohol and cocaine dependency where blockade of δ -receptors attenuates the reinforcing effects of cocaine (258) and reduces volitional ethanol intake in rats (66, 139, 140, 64). A general decrease in κ -opioid receptor densities was observed after ethanol or concurrent ethanol and cocaine, which is in agreement with reports showing that mice with a preference for ethanol show reduced κ -opioid receptor levels in the nucleus accumbens and other limbic regions as compared to alcohol-avoiding mice (116),

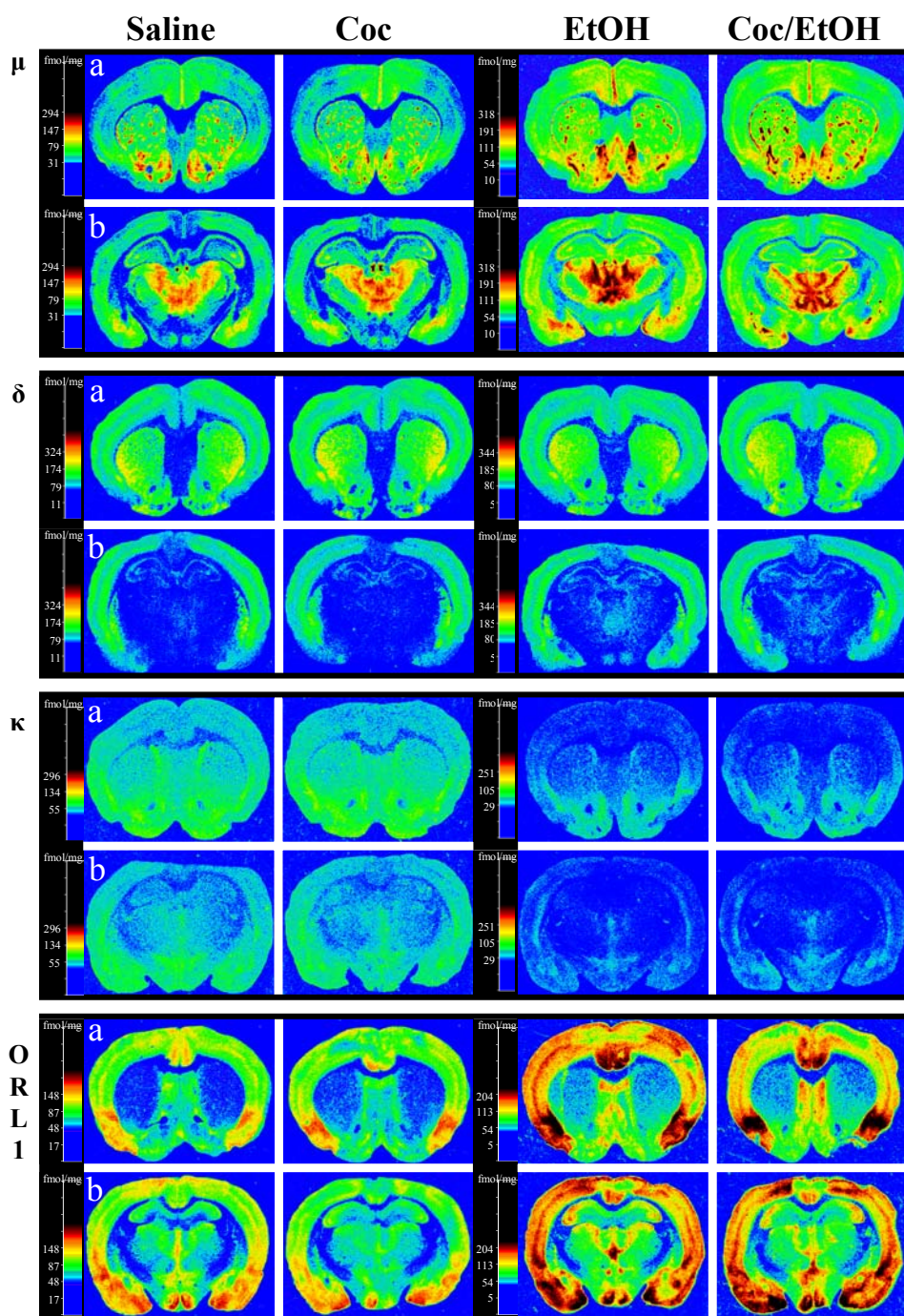


Figure 4. Computer enhanced colour autoradiograms of coronal brain section from saline, cocaine (Coc), ethanol (EtOH) and cocaine/ethanol (Coc/EtOH) treated rats showing μ -, δ -, κ - and ORL1 receptor binding. (a) Show sections cut at the level of the striatum (bregma 1.60). (b) Show sections cut at the level of the hippocampus (bregma -3.14). The colour bars show a pseudo-colour interpretation of relative binding density of black and white film images calibrated in fmol/mg tissue.

although contrasting data has been reported (250). The κ -agonists U-69593 or U50, 488 reduce cocaine self-administration, cocaine-induced locomotor activity as well as cocaine-induced sensitization (74, 245, 145, 35) and reduce ethanol consumption in rats (149). The general decrease of κ -opioid receptors might reflect an increase in dynorphin release and κ -opioid receptor occupation. ORL1 receptor levels were generally increased after ethanol and concurrent cocaine and ethanol in the present study. Nociceptin reduces morphine-induced reward (179) and has therefore been suggested to act as a negative feed-back for the μ -opioid system (28) supposedly through opposing the actions of morphine on the mesolimbic dopamine system (55). Nociceptin has also been shown to reduce ethanol self-administration (31, 30) and ethanol-induced place preference (144), possibly through acting as an anti-opioid peptide (28).

The mechanism behind the increase in μ - and ORL1-receptor binding could indicate an enhanced gene transcription, an increase in mRNA stability, stimulation of inactive receptors or decreased degradation of receptors due to ethanol or ethanol/cocaine treatment. On the other hand, the decreased κ -opioid receptor levels following ethanol or ethanol/cocaine treatment can reflect adaptations such as internalization or down-regulation that might occur through increased degradation or decreased synthesis of the receptor. Although, autoradiography gives no information about the actual activity of the receptors measured, the decrease of κ -opioid receptors seen in this study in combination with the increase in μ -opioid receptors might indicate that acute ethanol administration can lead to an imbalance in the opioid system that in turn might lead to a change in ethanol and cocaine preference (189).

Taken together, these studies show that the combination of acute cocaine and ethanol increases dynorphin mRNA while decreasing κ -opioid receptor mRNA and receptor protein levels.

4.2 CHRONIC EFFECTS OF ETHANOL AND COCAINE

The acute effects of cocaine and ethanol prove that this drug combination can cause initial changes on opioid peptide and opioid receptor mRNA levels important for drug reinforcement. These changes might be sustained or even enhanced after continued drug exposure and if so we were interested in exploring whether these changes are relevant for the dopamine response in the nucleus accumbens.

4.2.1 Ethanol and cocaine, effects on opioid receptor mRNA levels (Paper I and II)

By using a semi-quantitative competitive RT-PCR method we studied the effects of “binge” cocaine administration on κ - and μ -opioid receptor mRNA levels in the nucleus accumbens (Paper II). Two days of cocaine “binge” produced a down-regulation (88%, $P=0.006$) of κ -opioid receptor mRNA in the nucleus accumbens whereas μ -opioid receptor mRNA was not significantly changed, Figure 5 a. We further performed an experiment analyzing κ -opioid receptor mRNA in the nucleus accumbens and in the ventral tegmental area after a combined treatment with chronic intermittent ethanol and subacute cocaine “binge” administration (Paper I). Cocaine and ethanol in combination lead to reduced levels of κ -opioid receptor mRNA in the ventral tegmental area (88%, $P=0.00075$) and in the nucleus accumbens (76%, $P=0.0007$), Figure 5 b and c. There are several reports of increased prodynorphin mRNA levels in the nucleus accumbens after chronic cocaine (101, 264) and ethanol (148). Peptide levels in the striatum,

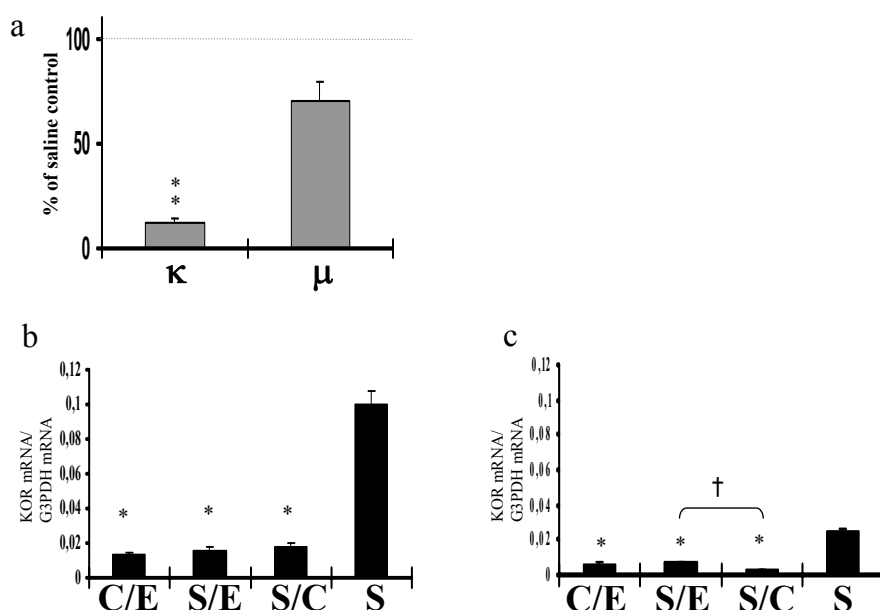


Figure 5. (a) κ - and μ -opioid receptor mRNA levels in the nucleus accumbens following 2 days of “binge” cocaine treatment. Data are expressed as % of the saline control group. Means \pm S.E.M. for six experiments are given. (b) and (c) Levels of κ -opioid receptor mRNA expression in rats treated with ethanol and “binge” cocaine, separately or in combination. Bars represent the means \pm S.E.M. of six rats for each group. Differences among treatments were estimated by Kruskal-Wallis ANOVA. (b) Ventral tegmental area; (c) Nucleus accumbens; C/E, cocaine/ethanol; E, ethanol; C, cocaine; S, saline. * $P=0.004$, ** $P=0.006$ vs. saline treated group. † $P=0.005$ between ethanol (E) and cocaine (C) treated animals by Mann-Whitney’s *U* test.

the nucleus accumbens and the substantia nigra are also increased after multiple cocaine treatments (248, 249). Although no direct measurements of dynorphin release has been reported, enhanced dynorphin activity have been proposed to act as a negative feed-back to the dopamine system in order to counter-act the drug-induced elevations of dopamine concentrations in the nucleus accumbens. The dramatic decrease of κ -opioid receptor mRNA levels seen in this study might be interpreted as a compensatory response to increased levels of dynorphin, which in turn could affect dopaminergic activity. Although mRNA levels can not be directly correlated to receptor levels there are studies reporting decreased levels of κ -opioid receptor binding following chronic cocaine administration (264).

4.2.2 Effects of ethanol on cocaine-induced dopamine concentrations (Paper III)

Activation of κ -opioid receptors in the nucleus accumbens has been shown to decrease dopamine concentrations in the nucleus accumbens, leading to the speculation that the down-regulation of κ -opioid receptor mRNA levels seen after both acute and chronic cocaine and/or ethanol treatment might change the response of dopaminergic cells. By using microdialysis, the combined effects of chronic intermittent ethanol administration and subacute cocaine “binge” on accumbal dopamine concentrations were measured. The combination of cocaine and ethanol produced a more pronounced increase in extracellular dopamine levels as compared to cocaine “binge” treatment ($p < 0.05-0.01$), Figure 6. Several factors might contribute to the enhanced cocaine-induced dopamine release in the nucleus accumbens after pre-treatment with ethanol. Cocaine and ethanol are both known to increase dopamine in the nucleus accumbens (52) via different mechanisms. The two drugs in combination could work synergistically, causing an elevated dopamine response. In this study, ethanol alone did not influence dopamine concentration in the nucleus accumbens, suggesting other mechanisms than a direct additive effect

underlying the enhanced dopamine response seen after concurrent cocaine and ethanol administration. Pre-treatment with ethanol may cause neuroadaptive changes influencing the cocaine-induced dopamine response. For example, the tissue concentration of dynorphin B was increased in the nucleus accumbens after repeated ethanol administration (148) that might lead to reduced κ -receptor mRNA levels as seen after chronic ethanol administration in Paper I, which in turn could affect the response of cocaine. Lower levels of κ -receptors might increase the dopamine response due to less inhibition of dopamine release in the nucleus accumbens. The observed effects could also originate from an increased plasma concentrations of cocaine after concurrent cocaine and ethanol intake, resulting in a higher brain extracellular fluid cocaine concentration (270, 90, 194), which could contribute to the increase in dopamine concentrations in the nucleus accumbens following cocaine and ethanol co-administration, since extracellular dopamine levels are highly correlated to brain cocaine levels (103).

A radically different possibility, where cocaine in the presence of ethanol is transformed into an active metabolite – cocaethylene (62), has been proposed to be responsible for the effects caused by cocaine and alcohol co-abuse. Cocaethylene binds to the dopamine transporter with similar affinity as cocaine (89, 288) that will increase the dopamine concentrations in the synaptic cleft. Cocaethylene produces reward as measured by self-administration in monkeys (117). The clinical relevance of cocaethylene in cocaine and ethanol reinforcement can be questioned since, in the actual co-abuse of alcohol and cocaine, the concentrations of cocaine most likely far exceed the concentrations of cocaethylene (199). In human studies cocaethylene is also less potent than cocaine at producing euphoria (200).

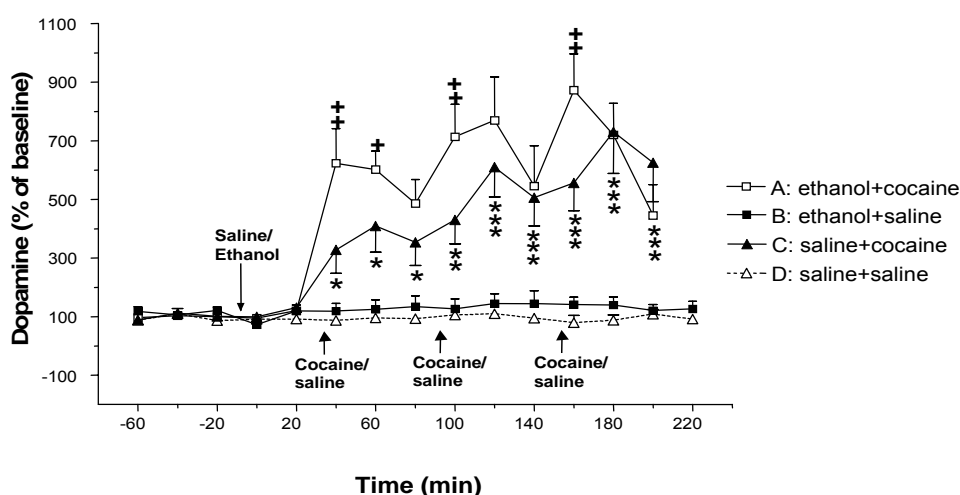


Figure 6. Effects of ethanol and/or cocaine administration on extracellular dopamine in the nucleus accumbens. The rats were pretreated with ethanol (2g/kg twice daily) or saline for 6 days. On treatment days 7 and 8, additional cocaine or saline injections were given in a “binge” pattern (15 mg/kg i.p. three times with 1 hour interval) starting 40 minutes after the morning ethanol/saline dose. Arrows indicate injections of ethanol, saline or cocaine. Stars indicate the difference in cocaine-induced dopamine release between rats pretreated with saline (C) and its saline pretreated control group receiving saline “binges” (D). Cocaine increased dopamine levels significantly in ethanol pretreated rats (A) as compared to ethanol pretreated rats receiving “binge” saline (B) (data not shown). Crosses indicate differences between the cocaine-induced dopamine release in ethanol pretreated rats (A) as compared to saline pretreated rats (C). No significant alterations on DOPAC and HVA levels were found. The data are expressed as mean±S.E.M. percent change of baseline values (i.e. three values preceding ethanol or saline injection) and analyzed by two-way ANOVA with repeated measures followed by one-way ANOVA and the least significant difference (LSD) test for multiple comparisons when appropriate (*+P<0.05, **,+P<0.01, ***,+P<0.001).

Taken together, cocaine and ethanol enhance dopamine release in the nucleus accumbens. Factors such as ethanol pre-treatment and an increased cocaine bio-availability might contribute to these effects. The increase in dopamine concentrations in the nucleus accumbens may be related to the enhanced euphoric experience produced by the combination of cocaine and ethanol seen in human subjects (199, 164, 61).

4.3 EFFECTS OF K-LIGANDS ON ACCUMBAL DOPAMINE RELEASE FOLLOWING CHRONIC ETHANOL ADMINISTRATION (PAPER VI)

Given that pre-treatment with ethanol induced a potentiated response on dopamine release in the nucleus accumbens, we further hypothesized that this response might partly be an effect of κ -opioid receptors, therefore we examined the effect of locally applied κ -ligands on the dopamine response after chronic ethanol administration by using microdialysis. Pre-treatment with ethanol for seven days did not change the baseline dopamine concentration or its metabolites in the nucleus accumbens as compared to saline treated animals (data not shown). Withdrawal from ethanol has previously been shown to decrease extracellular dopamine levels (230, 56) in the nucleus accumbens, an effect that has been suggested to contribute to continued drug-seeking behavior and relapse (277). Blockade of κ -receptors with nor-BNI (10 μ M) in the nucleus accumbens produced a significant increase in extracellular dopamine in ethanol pre-treated rats as compared to baseline dialysate levels ($p < 0.01$), Figure 7 a. The κ -receptor antagonist did not have an effect on dopamine concentrations in the nucleus accumbens in ethanol naïve rats. A previous study has shown elevated dopamine concentrations in the nucleus accumbens after intra-accumbal infusions of nor-BNI to ethanol-naïve rats, an effect that might be due to the higher dose used in that study (252). Perfusion with aCSF containing the κ -receptor agonist U50, 488H (20 μ M) for twenty minutes decreased dopamine levels in the ethanol group and in the control group, but in the ethanol pre-treated animals the decrease was only significant during the 40 minute post-infusion period ($p < 0.05$), while the dopamine concentration was reduced during the 120 minutes post-injection period in the

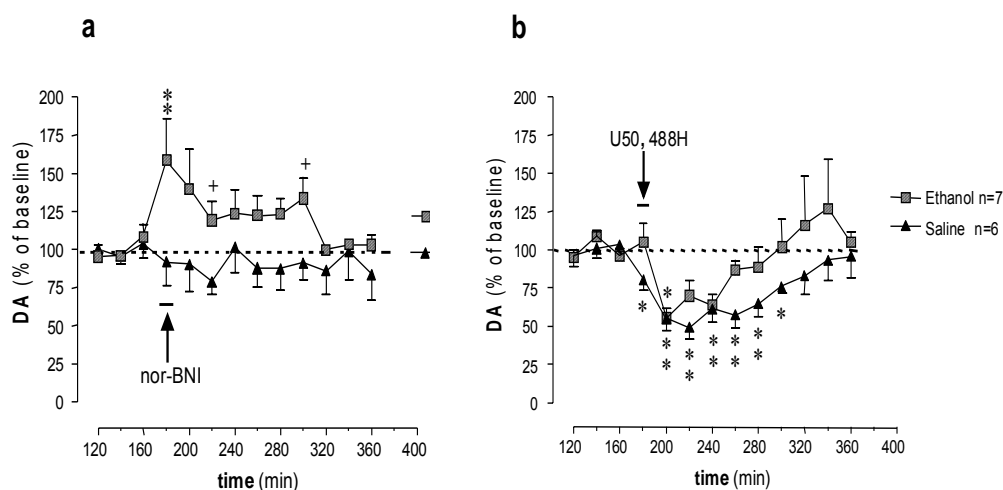


Figure 7. (a) Effects of the κ -antagonist nor-binaltorphimine (nor-BNI) or (b) the κ -agonist U50,488H on extracellular dopamine (DA) concentrations in the nucleus accumbens. The rats were pre-treated with ethanol (2g/kg twice daily) or saline for 7 days. On day 8, nor-BNI (10 μ M) or U50,488H (20 μ M) was administered by reverse microdialysis into the nucleus accumbens. Asterisks indicate within-group differences in DA release following κ -receptor blockade/stimulation. Crosses indicate differences between the DA release in ethanol as compared to saline pre-treated rats. The data are expressed as the mean \pm (S.E.M) percent change of baseline values (i.e. three values preceding ethanol or saline injection) and analyzed by one- and two-way ANOVA with repeated measures followed by the Least Significant Difference (LSD) test for multiple comparisons when appropriate. (*, + $p < 0.05$, **, ++ $p < 0.01$).

control group ($p < 0.05-0.01$), showing a slower onset and a more rapid return to baseline in the ethanol pre-treated animals as compared to the saline-treated animals, Figure 7 b. Ethanol exposure is believed to influence the endogenous opioid system (95), which in turn can regulate the dopamine activity (53, 252). It might be hypothesized that the increase in dynorphin activity after chronic ethanol administration (148) could in turn cause adaptive changes in the κ -receptor system, such as lower levels of κ -receptors and such an alteration could further influence the activity of the mesolimbic dopamine system as indicated in this study. By blocking lower levels of κ -receptors in the nucleus accumbens the effect of dynorphinergic inhibition of dopamine neurons will be reduced which might result in an increase in dopamine concentrations as seen in this study. In comparison, the responsiveness of dopaminergic neurons to cocaine has been shown to be enhanced following repeated treatment of κ -opioid receptor agonists (91), suggesting that an increased stimulation of κ -opioid receptors will lead to disinhibition of dopamine transmission. The smaller response from a κ -agonist on reducing dopamine output in rats chronically treated with ethanol might be an effect of an increased dynorphin tonus in these animals, leading to an altered response following κ -receptor stimulation.

Taken together, the results from this thesis show that cocaine and ethanol acutely affect the endogenous opioid system by producing drug-specific alterations in dynorphin mRNA, opioid receptor mRNA and opioid receptor binding in the rat brain, Table 3. Chronic studies on co-administration of cocaine and ethanol suggest that ethanol changes the sensitivity of the dynorphin/ κ -opioid system and consequently its ability to affect the dopamine system, Table 3. An imbalance in the opioid system has been suggested to influence the reinforcing properties of drugs of abuse and might therefore lead to an increased propensity to a continued drug intake.

TABLE 3
Summary of acute and chronic drug treatment on opioid and ORL1 systems in rat brain

	Opioid system	Cocaine	Ethanol	Cocaine/Ethanol
Acute	μ - receptor	\leftrightarrow	\uparrow	\uparrow ($\uparrow\uparrow$ NAcc core)
	δ - receptor	\leftrightarrow	\leftrightarrow	\leftrightarrow
	ORL1- receptor	\leftrightarrow	\uparrow	\uparrow
	κ - receptor	\leftrightarrow	\downarrow	\downarrow
	κ - receptor mRNA	\downarrow NAcc shell, VMS, VTA, SNC	\downarrow NAcc core, shell, VTA, SNR, \uparrow DMS	$\downarrow\downarrow$ NAcc core, \downarrow shell, VMS, VTA, SNC
Chronic	Prodynorphin mRNA	\uparrow VMS, DMS	\downarrow DLS	\uparrow NAcc core, shell, VMS, DMS, $\uparrow\uparrow$ DLS
	κ - receptor mRNA	\downarrow NAcc, VTA	\downarrow NAcc, VTA	\downarrow NAcc, VTA
	Dopamine	\uparrow NAcc	\leftrightarrow NAcc	$\uparrow\uparrow$ NAcc

\leftrightarrow ; no difference, \uparrow ; increased, \downarrow ; decreased as compared to control, $\uparrow\uparrow/\downarrow\downarrow$ as compared to separate cocaine or ethanol effects. NAcc; nucleus accumbens, VMS; ventromedial striatum, DMS; dorsomedial striatum, DLS; dorsolateral striatum, VTA; ventral tegmental area, SNC; substantia nigra compacta, SNR; substantia nigra reticulata.

5 GENERAL DISCUSSION

5.1 COCAINE AND ETHANOL PROFILES

The present thesis focused on the interactions between cocaine and ethanol, two drugs that show both similarities and differences. Pharmacologically they differ, where cocaine in contrast to ethanol acts as a powerful central nervous stimulant usually administered intranasally, intravenously or by smoking. Cocaine causes an instant euphoric “rush” in combination with increase in blood pressure, heart rate and motor activity. Ethanol belongs to the sedative-hypnotics group generally acting on the body and central nervous system as a depressant, although at lower blood alcohol concentrations drinkers report a feeling of joviality and increased self-confidence. However, the human experience of ethanol varies where some individuals experience relaxation at the same blood concentration where another person reports feeling elated. Both cocaine and ethanol are rapidly absorbed and widely distributed through body tissues, but the “high” caused by cocaine is relatively short acting (usually thirty minutes to one hour depending on the route of administration, frequency and amount used) due to a fast elimination rate. Similarly, the ethanol-experienced euphoria is short but it takes several hours to overcome the overall effects caused by ethanol as a result of the different elimination profile of ethanol at commonly used amounts (constant rate of metabolism due to saturation). Interestingly, the stimulating effects of ethanol occur when the blood alcohol concentrations is rising but disappear at higher levels and are substituted with a feeling of anger and fatigue when the blood alcohol levels are falling. When the stimulating effects of cocaine diminish they are also replaced with dysphoria which the abuser seeks to relieve by an additional cocaine dose in order to re-establish the initial euphoria. This pattern of frequent intake may be repeated several times until the cocaine user is completely exhausted and goes into a “crash”. The “crash” is characterized by symptoms such as depression, anxiety, and fatigue lasting for several days, which is followed by a later withdrawal syndrome resembling an episode of major depression where the user is most likely to fall into a new cycle of cocaine “binges” (68). These limited episodes of heavy drug use are mainly associated with cocaine, whereas excessive alcohol consumption is usually connected with drinking daily rather than intermittently, although heavy episodic drinking is also common. The term “binge” drinking has been linked to two main drinking patterns described either as one drinking episode leading to intoxication, often measured as having a large amount of drinks on one occasion or described as a pattern of heavy drinking that occurs over an extended period of time set aside for this purpose, and linked to more clinical definitions of abuse or dependence (291). Cocaine withdrawal is of more psychological character and does not require any medical support for the post-use period, whereas alcohol withdrawal after long-term use produces physical symptoms due to autonomic hyperactivity that in many cases might need medical attention. Interestingly, despite the many differences between cocaine and ethanol pharmacodynamics these two substances are among the most commonly used in combination today but it is unclear why. Therefore, the main objective of this thesis has been to search for possible interactions between cocaine and ethanol with the endogenous opioid system, the dopamine system in order to get a better understanding why these two substances are frequently co-abused.

5.2 EXPERIMENTAL RESEARCH ON COCAINE AND ETHANOL

Experimental research on ethanol and cocaine are primarily using animal models in order to determine factors important for the development and maintenance of dependence. It is difficult to say whether the interactions of cocaine and ethanol seen in this study can be directly linked to human cocaine and alcohol co-abuse but the fact that animals generally self-administer these drugs in combination with several common characteristics in brain anatomy support a parallel between substance dependence in animals and in humans. However, it needs to be considered that in the present studies we used a forced administration model that might not be the optimal model for abuse in humans. On the other hand forced administration has the advantage of controlling the dose and the time of drug delivery. To mimic the human abuse, we administered cocaine in a “binge” pattern with three hourly injections of cocaine (156) and injected ethanol in a chronic intermittent pattern. There is no animal model that addresses all aspects of human alcoholism, since rodents are not motivated to drink or self-administer ethanol in amounts high enough to produce physiological dependence. The use of inbred rat or mice strains with preference for ethanol drinking has the advantage that they voluntarily choose an ethanol solution over water and they show signs of withdrawal during abstinence and might therefore be a more relevant animal model for alcohol dependence. However, these genetically predisposed animals seem to limit their drinking to amounts that are not producing intoxicating blood alcohol levels (178), one of seven important characteristics that have to be included in a representative animal model for alcoholism (32). Other criteria for such an animal model are the need for ethanol to be orally self-administered, to produce signs of physical dependence, to produce tolerance and that ethanol is consumed for its rewarding effects rather than its taste or smell. Considering that drinking in humans is a highly complex and individual experience, it is difficult to generalize from findings in animal experimental settings to humans but there is still much that can be learned about drug effects in humans from research in animals.

5.3 ACUTE EFFECTS OF COCAINE AND ETHANOL ADMINISTRATION

The acute effects of drugs of abuse might be important factors for the initiation, establishment as well as the maintenance of drug self-administration behavior. During the initial phase of drug dependence, the pharmacological properties of the drug of choice play an important part for a continued drug intake. Positive effects such as euphoria will make the drug of abuse more desirable and thus influence the drug-taker to re-experience that effect. In addition, a possible genetic predisposition or environmental factors might increase the initial “liking” of a drug in an individual. There are animal studies showing that prenatal exposure to drugs (212), early social isolation (206) as well as psychological stress (214) are factors contributing to an individual susceptibility for drug dependence. Apart from an increased dopamine transmission in the nucleus accumbens the initial positive effects of cocaine and ethanol have been suggested to depend on other neuroactive substances that are affected initially by cocaine and ethanol, including the endogenous opioid system (136, 95). The endogenous opioids have been suggested to have a modulatory role in drug reinforcement and might therefore also play an important part in the transition from drug use to drug dependence (69). We found that in the acute phase of cocaine and ethanol co-administration prodynorphin mRNA levels were increased in the nucleus accumbens and in the striatum. This acute increase in prodynorphin mRNA levels might be indicative of a counteradaptive process to dampen the excessive dopamine levels in the nucleus accumbens and striatum. Further, κ -opioid receptor mRNA levels and κ -opioid receptor levels were reduced that in turn have been suggested

to counteract the alterations of prodynorphin mRNA (264). In combination with the increase in μ - and ORL1 receptor binding, ethanol and cocaine administration causes changes in the opioid system that might be relevant for a continued drug intake.

5.4 CHRONIC EFFECTS OF COCAINE AND ETHANOL ADMINISTRATION

After the initial phase of drug dependence, continued use might change the initial “liking” of the drug to “wanting” the drug (226). Chronic drug administration produces long-lasting changes in the brain reward circuit which might contribute to the drug dependent state with a vulnerability to relapse and continued drug-use (132). These neuronal adaptations might underlie symptoms such as withdrawal and sensitization and therefore influence the development of drug dependence. There are studies indicating a correlation between alterations in opioid peptide content and an increased desire for the drug (70) which might be important for the daily craving and dysphoria present in dependent individuals prior to drug taking. It can be argued that in humans, chronic alcohol drinking can span over years and decades while experimental research focusing on chronic effects of ethanol are conducted over weeks or at best, months. In the present model, chronic intermittent ethanol administration preceded the cocaine “binge” paradigm, in view of the reports showing that ethanol and cocaine use alone antedates the use of the combination of ethanol and cocaine (21, 211). In addition, animals with a history of ethanol exposure have an altered sensitivity to cocaine (82), suggesting common neural substrates for cocaine and ethanol. We showed that pre-treatment with ethanol caused a potentiated cocaine-induced dopamine response in the nucleus accumbens that might contribute to the increased euphoria that has been reported by subjects given cocaine and ethanol in combination (199, 62, 164). Again, the increased dopamine concentrations might lead to an increase in dynorphin tonus acting as a negative-feedback mechanism to regulate the function of dopaminergic neurons and this increase in dynorphin might in turn lead to a compensatory down-regulation of κ -opioid receptors in the ventral tegmental area and nucleus accumbens. This down-regulation might further reduce the ability of κ -opioid receptor agonists to decrease dopamine release in the nucleus accumbens.

5.5 CLINICAL RELEVANCE

The definition of the neurobiological substrates mediating the reinforcing effects of cocaine and ethanol in combination may increase our knowledge of reinforcement mechanisms and provide useful new information for the development of pharmacotherapies. The treatment of cocaine and alcohol co-abuse is of great clinical concern since the combination of cocaine and ethanol increases the risk of cocaine-related morbidity (138, 1, 135) and mortality (135, 229). Results from this thesis show a substantial increase in dopamine concentration in the nucleus accumbens after a combined cocaine and ethanol administration, implicating dopamine as an important factor contributing to cocaine and alcohol co-abuse. However, clinical trials using dopaminergic agents for treating drug-dependence have not been successful, mainly due to severe side effects caused by these agents. The endogenous opioid system, with modulating actions on the dopamine system may serve as a better target for the treatment of drug-dependence which has been proven by the introduction of naltrexone in the treatment of relapse in individuals dependent on alcohol (190, 287). However, naltrexone did not produce any advantages over placebo in a population with a concurrent cocaine and alcohol dependency (94). The present results show that both acute and chronic cocaine and ethanol in combination cause a decrease in κ -opioid receptor

mRNA, whereas ethanol also suppresses κ -opioid receptor levels after an acute administration. A dysfunctional dynorphin/ κ -opioid receptor system may contribute to some of the mechanisms underlying dependence and this system could therefore be an interesting target for the treatment of drug addiction as previously suggested (137, 233). Accordingly, we found that chronic ethanol treatment changed the dopaminergic response to κ -opioid ligands which may be related to an increased dynorphinergic tone. Furthermore, it has been shown that the κ -opioid receptor agonist U50, 488H blocks the acquisition and maintenance of cocaine self-administration in animals (74, 145). However, a selective κ -opioid agonist has some disadvantages since they also have been shown to decrease food-intake in primates (167) and show other undesirable side-effects such as sedation and dysphoria (269). A recent more encouraging report has shown that a novel κ -opioid receptor agonist, TRK-820 suppressed the rewarding effects of cocaine without producing place aversion (88). A partial agonist or a metabolically stable peptide analogue acting on κ -opioid receptors might have pharmacotherapeutic potentials in drug dependence, if the side effects are less severe. Furthermore, agents affecting the ORL1 receptor may represent a promising treatment for relapse. Nociceptin and a synthetic ORL1 receptor agonist Ro64-6198 reduced ethanol reinforcement and reinstatement without having any motivational properties themselves (144, 30). In combination with these agents' anxiolytic and anti-stress actions (118, 29), ORL1 receptor activation might be beneficial in the treatment of drug dependence.

While the positive reinforcing effects of ethanol are essential to the initiation and early maintenance of intake, other studies suggest that ethanol-seeking behavior related to alleviation of symptoms during abstinence (negative reinforcement) is equally, if not more, effective in maintaining ethanol use (131). Therefore, when considering risk factors for alcohol and cocaine dependence, it is important to consider not only systems that are involved in ethanol and cocaine reward, but those activated during withdrawal and abstinence. Withdrawal from ethanol or cocaine is associated with reduced levels of dopamine in the nucleus accumbens (230, 56) and increased levels of dynorphin in this area have been associated with dysphoria (205, 186). Furthermore, an agonist acting at the κ -opioid receptor has been found to initiate withdrawal symptoms (210). The symptoms of withdrawal might lead to a subsequent relapse in order to reverse this negative state. To prevent relapse into drug-taking behavior is an important strategy for treating drug dependence, especially in cocaine and alcohol co-abuse considering that a single occasion of ethanol intake can increase the likelihood for a relapse into cocaine abuse in individuals that are long-term abstinent from a concurrent cocaine and alcohol dependency (165). I have not addressed the aspects of withdrawal or relapse in this study although this aspect of the drug addiction cycle is very significant and would therefore be an interesting study for the future.

In summary, the main finding in the present animal model of chronic concurrent cocaine and ethanol intake were a substantial increase of cocaine-induced extracellular dopamine concentrations in the nucleus accumbens. This effect might be explained by reduced κ -receptor mRNA levels observed after ethanol administration, since pre-treatment with ethanol changed the dopaminergic response following κ -receptor stimulation and blockade. Acutely, we found a potentiated effect of the drug combination on dynorphin mRNA levels and μ -opioid receptor levels in the dorsolateral striatum and nucleus accumbens core, respectively and an additive effect on κ -receptor mRNA levels in the nucleus accumbens core. Taken together these changes might be relevant for human cocaine and alcohol co-abuse and in the end lead to the development of new pharmacological targets.

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