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Glutamatergic Modulation of Cue-Induced Drug-Seeking Behavior in the Rat

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National Public Health Institute, Helsinki, Finland
and
Department of Biological and Environmental Sciences
University of Helsinki, Finland

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A C A D E M I C D I S S E R T A T I O N

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ABSTRACT

The characteristics of drug addiction include compulsive drug use despite negative consequences and re-occurring relapses, returns to drug use after a period of abstinence. Therefore, relapse prevention is one of the major challenges for the treatment of drug addiction. There are three main factors capable of inducing craving for drugs and triggering relapse long after cessation of drug use and dissipation of physical withdrawal signs: stress, re-exposure to the drug, and environmental stimuli (cues) that have been previously associated with drug use.

The neurotransmitters dopamine and glutamate have been implicated in the modulation of drug-seeking behavior. The aim of this project was to examine the role of glutamatergic neurotransmission in relapse triggered by conditioned drug-associated stimuli. The focus was on clarifying whether relapse to drug seeking can be attenuated by blockade of glutamate receptors. In addition, as the nucleus accumbens has been proposed to participate in the modulation of drug-seeking behavior, the effects of glutamate receptor blockade in this brain structure on cue-induced relapse were investigated.

The studies employed animals models in which rats were trained to press a lever in a test cage to obtain alcohol or intravenous cocaine. Drug availability was paired with distinct olfactory, auditory, or visual stimuli. This phase was followed by extinction training, during which lever presses did not result in the presentation of the drug or the drug-associated stimuli. Extinction training led to a gradual decrease in the number of lever presses during test sessions. Relapse was triggered by presenting the rats with the drug-associated stimuli in the absence of alcohol or cocaine. The drug-associated stimuli were alone capable of inducing resumption of lever pressing and maintaining this behavior during repeated testing. The number of lever presses during a session represented the intensity of drug-seeking and relapse behavior.

The results suggest that glutamatergic neurotransmission is involved in the modulation of drug-seeking behavior. Both alcohol and cocaine relapse were attenuated by systemic pretreatment with glutamate receptor antagonists. However, differences were found in the ability of ionotropic AMPA/kainate and NMDA receptor antagonists to regulate drug-seeking behavior. The AMPA/kainate antagonists CNQX and NBQX, and L-701,324, an antagonist with affinity for the glycine site of the NMDA receptor, attenuated cue-induced drug seeking, whereas the competitive NMDA antagonist CGP39551 and the NMDA channel blocker MK-801 were without effect. MPEP, an

antagonist at metabotropic mGlu5 glutamate receptors, also decreased drug seeking, but its administration was found to lead to conditioned suppression of behavior during subsequent treatment sessions, suggesting that MPEP may have undesirable side effects. The mGluR2/3 agonist LY379268 and the mGluR8 agonist (S)-3,4-DCPG decreased both cue-induced relapse to alcohol drinking and alcohol consumption. Control experiments showed however that administration of the agonists was accompanied by motor suppression limiting their usefulness.

Administration of the AMPA/kainate antagonist CNQX, the NMDA antagonist D-AP5, and the mGluR5 antagonist MPEP into the nucleus accumbens resulted also in a decrease in drug-seeking behavior, suggesting that the nucleus accumbens is at least one of the anatomical sites regulating drug seeking and mediating the effects of glutamate receptor antagonists on this behavior.

Keywords: relapse, drug seeking, glutamate, cues, alcohol, cocaine, rats

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TIIVISTELMÄ

Alkoholi- ja huumeriippuvuudelle on tyypillistä pakonomainen tarve käyttää päihdettä ja toistuvat retkahtamiset raittiiden kausien jälkeen. Stressi, päihteelle altistuminen ja päihteen käyttöön liittyneet ehdollistuneet ärsykkeet voivat aiheuttaa voimakkaan ja hallitsemattoman päihteen himon ja retkahtamisen kauankin päihteen käytön ja fyysisten vieroitusoireiden loputtua. Näin ollen tehokas päihderiippuvuuden hoito edellyttää paitsi päihteiden akuuttien vaikutusmekanismien selvittämistä myös retkahtamisen neurobiologian tuntemista.

Dopamiini ja glutamaatti ovat pääasialliset päihdehakuista käyttäytymistä säätelevät hermovälittäjäaineet. Tämän tutkimuksen tarkoituksena oli selvittää glutamatergisen hermovälityksen merkitystä aistiärsykkeiden laukaisemassa retkahtamisessa. Erityisesti pyrittiin selvittämään, voidaanko retkahtamisalttiutta vähentää glutamaattireseptoreiden salpauksella. Lisäksi tutkittiin retkahtamiskäyttäytymisen kannalta tärkeänä pidetyn accumbens-aivotumakkeen merkitystä aistiärsykkeiden laukaisemassa päihdehakuisessa käyttäytymisessä.

Tutkimuksissa käytettiin koe-eläinmalleja, joissa rotat opetettiin annostelemaan itselleen alkoholia tai kokaiinia koehäkissä olevaa vipua painamalla. Päihteiden saatavuuteen yhdistettiin valo-, ääni- tai tuoksuärsykkeitä. Opetusta seuranneen ekstinktiovaiheen aikana päihdettä ei ollut saatavilla eikä koehäkissä ollut aistiärsykkeitä. Tällöin vivun painaminen vähitellen lakkasi palkinnon puuttuessa. Retkahtaminen laukaistiin altistamalla rotat päihteiden vaikutuksiin yhdistyneille aistiärsykkeille. Tällöin vivun painaminen alkoi uudelleen, vaikka alkoholia tai kokaiinia ei ollut testitilanteessa saatavilla. Vivunpainallusten määrä retkahtamistestissä mittasi retkahtamisalttiutta ja päihdehakuisuutta.

Tulokset osoittavat, että glutamaterginen hermovälitys säätelee päihdehakuista käyttäytymistä. Sekä alkoholi- että kokaiiniretkahtamista voitiin vähentää systeemisesti annostelluilla glutamaattireseptoriantagonisteilla. Ionotrooppisten glutamaattiantagonistien vaikutus riippui kuitenkin reseptorityypistä sekä sitoutumiskohdasta reseptorissa. AMPA/kainaattiantagonistit CNQX ja NBQX sekä NMDA-reseptorin glysiinikohtaan sitoutuva antagonisti L-701,324 vähensivät retkahtamista. Sen sijaan kompetitiivisella NMDA-antagonisti CGP39551:llä ja NMDA-reseptorin kanavasalpaaja MK-801:llä ei ollut vaikutusta retkahtamisalttiuteen. mGluR5-tyypin metabotrooppinen glutamaattireseptoriantagonisti MPEP vähensi päihdehakuista käyttäytymistä, mutta lääkeaineen annostelulla oli myös ehdollistuneita, rottien vivunpainamista alentavia pitkäaikais-

vaikutuksia. mGluR2/3-agonisti LY379268 ja mGluR8-agonisti (S)-3,4-DCPG vähensivät sekä alkoholin juomista että aistiärsykkeiden laukaisemaa retkahtamista, mutta molemmat agonistit heikensivät myös motorista suorituskykyä. Ei-toivotut sivuvaikutukset tulevat todennäköisesti estämään lääkeaineiden hyötykäytön.

AMPA/kainaattiantagonisti CNQX, NMDA-antagonisti D-AP5 ja mGluR5-antagonisti MPEP vähensivät kokaiiniretkahtamista myös aivojen accumbens-tumakkeeseen annosteltuna. Tämä viittaa siihen, että accumbens-tumake on yksi aivoalueista, joiden kautta glutamaattireseptoriantagonistien retkahtamista hillitsevät vaikutukset välittyvät.

Avainsanat: retkahtaminen, päihteet, glutamaatti, alkoholi, kokaiini, rotta

CONTENTS

ABBREVIATIONS

ADE	alcohol deprivation effect
AGS3	activator of G protein signaling 3
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CGP39551	DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid carboxyethylester
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione disodium
CRF	corticotropin-releasing factor
$CS^{+/-}$	conditioned (discrete) stimulus
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
DRL	differential reinforcement of low rates of responding
FI	fixed interval
FR	fixed ratio
GABA	γ-aminobutyric acid
ip	intraperitoneally
iv	intravenously
LAAM	L-α-acetylmethadol
LY37926	(-)-2-oxa-4-aminobicyclo hexane-4,6-dicarboxylic acid
mGlu	metabotropic glutamate
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
MPEP	2-Methyl-6-(phenylethynyl)pyridine hydrochloride
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetra-hydrobenzo[f]quinoxaline-7-sulfonamide
NMDA	N-methyl-D-aspartate
(S)-3,4-DCPG	(S)-3,4-dicarboxyphenylglycine
S	stimulus (in reinforcement schedules)
$S^{+/-}$	discriminative stimulus
w/v	weight/volume
v/v	volume/volume
VI	variable interval
VR	variable ratio
VTA	ventral tegmental area

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Bäckström P., Hyytiä P. Attenuation of cocaine-seeking behaviour by the AMPA/kainate receptor antagonist CNQX in rats. Psychopharmacology 2003;166:69-76.[a]

II Bäckström P., Bachteler D., Koch S., Hyytiä P., Spanagel R. mGluR5 antagonist MPEP reduces ethanol seeking and relapse behavior. Neuropsychopharmacology 2004;29(5):921-928.[b]

III Bäckström P., Hyytiä P. Ionotropic glutamate receptor antagonists modulate cue-induced reinstatement of ethanol-seeking behavior. Alcoholism: Clinical and Experimental Research 2004;28(4):558-565.[c]

IV Bäckström P., Hyytiä P. Suppression of alcohol self-administration and cue-induced reinstatement of alcohol seeking by the mGlu2/3 receptor agonist LY379268 and the mGlu8 receptor agonist (S)-3,4-DCPG. European Journal of Pharmacology 2005;528(1-3):110-118.[d]

V Bäckström P., Hyytiä P. Ionotropic and metabotropic glutamate receptor antagonism attenuates cue-induced cocaine seeking. Neuropsychopharmacology 2006;31(4):778-786.[b]

VI Bäckström P., Hyytiä P. AMPA/kainate, NMDA, and mGlu5 receptor antagonism in the nucleus accumbens core attenuates cue-induced reinstatement of cocaine seeking in rats. Psychopharmacology (submitted).

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1 INTRODUCTION

The characteristics of drug addiction include impaired ability to control drug taking, compulsive drug use, continued use despite obvious psychological and social harm, and drug craving (O'Brien and Gardner, 2005). Addiction is also characterized by relapses, returns to drug use after a period of abstinence. Stress, re-exposure to drugs, and drug-associated environmental stimuli have been shown to trigger craving in abstinent drug and alcohol users, but also in non-addicted social drinkers (Cooney et al., 1997; de Wit and Chutuape, 1993; Greeley et al., 1993). The susceptibility to relapse can be predicted by the intensity of craving (Cooney et al., 1997; but see Tiffany and Carter, 1998).

Drugs that are abused by humans are in most cases also self-administered by experimental animals. However, it is likely that the mechanisms that mediate the acute effects of drugs differ from those controlling drug-seeking and relapse behavior. As the subjective feeling of craving is difficult to measure in laboratory animals, several experimental paradigms modeling different aspects of relapse and drug-seeking behavior have been developed (Markou et al., 1993). These models concentrate on measuring the motivation of the animal to obtain drugs.

Evidence from animal experiments suggests that dopamine, which is clearly involved in the acute reinforcing effects of most drugs of abuse, modulates also drug-seeking behavior. In addition, glutamate and several other transmitter have been found to be involved. It seems that drugs, drug-associated cues, and, to a lesser extent, stress are all capable of activating dopaminergic and glutamatergic neurotransmission which leads to an increased probability of relapse.

So far, there are few effective pharmacological treatments for drug and alcohol addiction (Heidbreder, 2005). Successful pharmacological relapse prevention requires insight into the neurobiology of the phenomenon. Therefore, the present experiments were designed to specifically examine the involvement of different glutamate receptor subtypes in modulation of drug-seeking behavior induced by drug-associated cues. The hypothesis was that cue-induced drug seeking can be decreased by counteracting the effects of cue-induced glutamate release by (presumably) postsynaptically acting glutamate receptor antagonists or by decreasing glutamate release by presynaptic modulation. To better assess the extent to which glutamate receptor antagonists and agonists are able to modulate drug seeking, experiments were conducted with both alcohol and the psychostimulant cocaine.

2 REVIEW OF THE LITERATURE

2.1 Addiction and relapse

Relapse, return to drug use after a period of abstinence, is one of the major challenges in the treatment of drug and alcohol addiction. Escape from physical withdrawal symptoms that emerge following discontinuation of drug use can sometimes be a factor leading to relapse. However, there are several drugs of abuse, such as cocaine and amphetamine, that do not produce severe somatic withdrawal signs (Lago and Kosten, 1994). Despite this, abusers of these drugs frequently relapse. Relapse can also, and often does, occur long after the signs of physical withdrawal have dissipated (Hyman, 2005). This indicates that drug seeking and subsequent relapse are induced by other processes than physical discomfort during withdrawal.

Initially, drug taking is driven by the acute reinforcing effects of the drug. With prolonged drug use, additional factors start regulating drug seeking and taking. One consequence of repeated drug taking is an adaptation to the presence of the drug and a change in the functioning of the brain (Tzschentke and Schmidt, 2003). Thus, stopping drug use upsets the balance of brain systems, which can manifest itself as physiological and psychological withdrawal signs. Repeated use of drugs also leads to the association of different discrete and environmental cues with the act of drug taking and the effects of the drug. These cues can acquire conditioned reinforcing properties of their own and later on trigger craving and drug-seeking behavior in the absence of the drug (Di Ciano and Everitt, 2004; Grüsser et al., 2004; See, 2005). It has been proposed that in an addicted person, drug-associated cues acquire more and more significance while natural reinforcers start to be ignored. As a result, behavior becomes pathologically oriented towards drug-associated stimuli and drug seeking at the expense of other behaviors (Hyman, 2005).

In addition to drug-associated cues, negative mood and re-exposure to the drug can trigger relapse (Self, 1998). Studies in abstinent alcohol and drug users have shown that all of these factors – cues, stress, and re-exposure to the drug – increase the urge and probability of consuming drugs or alcohol. Presentation of an alcoholic beverage to abstinent alcoholics (Cooney et al., 1997) or the smell of alcohol alone (Schneider et al., 2001) increases subjective ratings of craving and desire to drink, as does negative mood (Cooney et al., 1997). In subjects with a history of cocaine use, videos showing the drug taking act, imagery of autobiographical memories of drug use, or other cocaine-related cues increase self-reported cocaine craving (Ehrman et al., 1992; Foltin and Haney, 2000; Kilts et al., 2001; Kilts et al., 2004). In addition to subjective feelings of craving, drug-associated cues can lead to changes in physiological measures such as skin resistance, temperature, and heart rate (Ehrman et al., 1992).

Brain imaging studies have shown that drug imagery or the presentation of drug-associated cues to abstinent drug users leads also to activation of brain areas including

the prefrontal cortex, nucleus accumbens, and amygdala (Kilts et al., 2001; Schneider et al., 2001). Activation of these brain regions has been found to correlate with the probability of future relapse (Kosten et al., 2006), showing that craving and relapse have a neuroanatomical basis.

As a result of repeated and prolonged drug use the mechanisms mediating craving and relapse behavior probably differ from those mediating the acute reinforcing effects of drugs. Therefore, understanding the neurobiology of relapse is essential for the development of new pharmacological treatments for drug addiction.

2.2 Animal models of drug seeking

Many aspects of drug and alcohol addiction can be modelled and studied in laboratory animals. Animal experiments allow studies that for ethical reasons cannot be conducted in human drug addicts. Also, in animal studies the background of the subjects can be strictly controlled. In human subjects this is often a difficult task, as polydrug use is common among addicts, and so are differences in mental, nutritional and socioeconomic status. There is consistency among the findings from animal experiments and clinical trials. For example, buprenorphine and L-α-acetylmethadol (LAAM), which are used as opioid replacement pharmacotherapy, decrease opiate use in laboratory animals (Grabowski et al., 2004). The suppressive effects on alcohol drinking by naltrexone that is used in the treatment of alcoholism were also discovered in animal studies (O'Brien and Gardner, 2005).

Behavioral animal models of addiction often involve measures of drug taking, drug seeking, or evaluation of the reinforcement obtained from the drug. The term positive reinforcement is sometimes used to describe reward, but, by definition, a positive reinforcer is a stimulus that increases the frequency of behavior that leads to its presentation (Stolerman, 1992). 'Reward' implies a hedonic or positive affective state, i.e. suggests pleasure, whereas reinforcement is not necessarily perceived as pleasurable, or perceived at all. A negative reinforcer, correspondingly, increases the frequency of behavior that prevents or terminates its presentation (Stolerman, 1992). For example, a laboratory rat may be willing to perform a task if this results in the delivery of a positive reinforcer (such as a sucrose pellet) but also to perform a task if this prevents a negative reinforcer (e.g. electric shock) from being delivered. In other words, a positive reinforcer reinforces when it is presented and a negative reinforcer reinforces when it is withdrawn.

2.2.1 Operant models

Animals can be trained to perform an operant task, e.g. press a lever, in a test cage to obtain a reinforcer. The cages are called operant chambers or Skinner boxes after B.F. Skinner, the discoverer of operant behavior (see www.bfskinner.org). Animals are either living in the operant chambers or transferred to them for the duration of a test session. The chamber typically contains a manipulandum, often a lever, turning wheel, or nose-poke hole that the animal can "operate" to control the delivery of the reinforcer. An

inactive manipulandum is usually present to provide better measurement of response specificity. If a stimulus is reinforcing, the animal should respond significantly more on the active manipulandum. In addition, the chamber can contain lights, loudspeakers etc. for delivering visual or auditory stimuli during the session or the chamber may be equipped to deliver food pellets to a food tray or small electrical currents, foot shocks, through the floor. A computer is used to control the contingencies of the sessions and record the response data of the animals.

The task that the animal is required to perform in the chamber can be chosen so that it allows the measurement of different aspects of behaviour. Operant chambers can be used to measure drug or food reinforcement (i.e. drug/food taking, self-administration), drug seeking, relapse, or to compare the subjective effects of drugs. In addition, the effects of different manipulations on these behaviours, once established, can be examined.

Models of drug taking

One of the simplest operant tasks, or schedules of reinforcement, is the continuous reinforcement or fixed ratio 1 (abbreviated FR1) schedule. Under this schedule, every operant response (e.g. lever press) on the active manipulandum is reinforced, usually with a drug dose or food pellet. Increasing the number of responses required for reinforcement delivery allows better measurement of the selectivity and motivation of responding. Therefore, schedules such as FR5, under which five responses are required for reinforcement delivery, are frequently used.

The progressive ratio schedule measures especially well the motivation to obtain the reinforcer. Under these schedules, very few responses are required for the delivery of the first reinforcer, but for each subsequent reinforcer the response requirement is increased (sometimes even doubled) according to a predetermined schedule (Richardson and Roberts, 1996; Stafford et al., 1998). A session is considered completed when the animal fails to receive a reinforcer within a specified time period, often one hour. The final response ratio reached is called the breaking point and represents the maximal effort the animal is willing to make to receive the reinforcer. The motivation to obtain the reinforcer (drug) is considered higher with higher breaking points.

Self-administration schedules can be complicated further. To mention a few examples, under variable ratio (VR) schedules the number of responses per reinforcer is varied, resulting in very high rates of responding. Differential reinforcement of low rates of responding (DRL) schedules result in very low response rates as a response is reinforced only if it is performed following a defined period of non-responding. Premature responses "reset the clock" and are not rewarded (Monterosso and Ainslie, 1999). DRL schedules can be used to measure impulsivity of behavior. Under interval schedules the first response after a defined period of time is reinforced, but premature responding does not lead to punishment or postponement of the reinforcer. Variable interval (VI) schedules take his further by varying the time to the reinforced response. Both of these interval schedules usually lead to a scalloped pattern of responding when the animal first responds very little or not at all, but increases its response rates towards the end of the interval.

Second-order schedules

Second-order schedules are often used to incorporate stimulus presentations (e.g. light or tone presentations) into the schedule (Schindler et al., 2002). During repeated training sessions, these stimuli acquire conditioned reinforcing properties and the ability to control behavior on their own. The conditioned reinforcing properties of the stimuli can be demonstrated by experiments in which they support learning of a new response that leads to their presentation (Di Ciano and Everitt, 2004).

Second-order schedules contain two components, usually a fixed ratio schedule and a fixed-interval schedule or two fixed ratio schedules. For example, under the second-order schedule FR4(FR5:S) the animal is required to press a lever (or perform some other response such as nose-poke) five times to receive a stimulus presentation (FR5:S component). Simultaneously with the fourth stimulus presentation, the animal receives the reinforcer (FR4 component). Under a FI15min(FR5:S) schedule every fifth response leads to a stimulus presentation, and the first stimulus presentation after fifteen minutes has elapsed leads to reinforcer delivery.

Fixed-interval second-order schedules of self-administration are usually employed to measure drug seeking after short, over-night abstinence periods. Responding under these schedules shows often a scalloping pattern with very little initial responding that increases towards the end of the interval. A session contains several intervals, typically 4-5. Responding during the first interval measures drug seeking in the absence of the drug, whereas during subsequent intervals the animal has received at least one drug dose and is thus under its influence. Response rate as well as the latency to iniate responding is influenced by three factors: the conditioned reinforcing properties of the stimulus, the anticipation of obtaining the reinforcer available at the end of the interval, and the incentive value (strength) of the reinforcer. During the second and subsequent intervals, the drug itself contributes to responding by its reinforcing and response-invigorating or suppressing effects.

In contrast to fixed ratio schedules, fixed-interval schedules have been shown to promote the development of habitual responding, as there is no linear relationship between response rate and reinforcer delivery (Coutureau and Killcross, 2003; Yin et al., 2006). This may sometimes confound the interpretation of results, if one wants to dissociate motivation from habit as the driving force of drug seeking.

Reinstatement models

In drug seeking studies, reinstatement refers to the resumption of extinguished behavior such as lever pressing following exposure to drugs, drug cues, or stress (Shalev et al., 2002). The reinstatement paradigm can be divided into three phases: self-administration, extinction, and reinstatement. During the self-administration phase, the animal is trained to self-administer the drug. Studies examining the effects of cues on reinstatement utilize this phase to associate stimuli with the delivery of the drug. Therefore the self-administration phase is often also called the conditioning phase. Different schedules of reinforcement, including fixed ratio and second-order schedules, can be used. When the

animals have reached stable levels of self-administration and maintained this behaviour for some time, extinction training is begun. During extinction the drug is withheld and responding has either no programmed consequences or, as often during intravenous self-administration, results only in the delivery of a neutral solution such as saline. In the absence of the reinforcer, response rates may increase during the first session, but during subsequent training rapidly decline to very low levels. When extinction responding has stabilized, reinstatement testing can begin. Reinstatement can be induced either by administering the animal a small dose of the drug, called priming, exposing the animal to stimuli (cues) previously associated with drug delivery, or by exposing the animal to a stressor like foot shock or food deprivation. Regardless of the reinstatement-inducing factor, the drug is not available. However, re-exposure to the priming dose of the drug, the drug-associated stimulus, or stress alone is capable of reinstating extinguished responding. The number of responses performed by the animal measures the strength of reinstatement. As in human addicts, the propensity to relapse is long lasting. Thus, the same animals can be tested repeatedly for reinstatement. Reinstatement sessions are typically separated by a few days during which the animals either remain in their home cages or receive extinction training. In addition to these between-sessions reinstatement models, within-session models exist. In these models, animals go through the self-administration, extinction and reinstatement phases during a single session.

2.2.2 Alcohol deprivation effect

Alcohol-seeking behavior can be studied using the reinstatement paradigm described above, but in addition, the so-called deprivation model is often used. The alcohol deprivation effect (ADE) refers to an increase in alcohol consumption that animals show after a period of withdrawal from alcohol access (Sinclair and Senter, 1967). The length of the deprivation period is usually between 3 and 14 days, but can range to as long as over a year (Lê and Shaham, 2002). The subsequent increase in alcohol consumption is temporary and can be seen for some days after which animals return to their pre-deprivation consumption levels. Factors contributing to increased alcohol consumption may include decreased aversive effects (mostly taste) of alcohol (Pinel and Huang, 1976), an increase in the novelty of the alcohol solution (Sinclair, 1979; Lê and Shaham, 2002), changes (probably an increase) in the reinforcing properties of alcohol (Heyser et al., 1997; Spanagel and Hölter, 2000), or even anticipation of and preparation for future deprivation periods (Heyser et al., 1997). The alcohol deprivation effect has sometimes been described as a model of relapse in contrast to reinstatement models, as the alcohol deprivation model measures actual alcohol consumption after abstinence whereas reinstatement paradigms measure drug seeking after extinction without the possibility to consume alcohol (Spanagel, 2005).

2.2.3 Reinstatement of conditioned place preference

The conditioned place preference method is based on the ability of contextual cues to become conditioned to drug effects (see Tzschentke, 1998 for review). The basic

procedure consists of a conditioning phase and an expression phase. When the method is used to measure reinstatement, an extinction and a reinstatement phase are added.

The place preference apparatus consists most often of two compartments differing in e.g. color, texture, odor, and floor material. During the conditioning phase, animals are repeatedly confined to one side of the apparatus after a drug injection and to the other side after vehicle injection. During the expression phase, animals are allowed to freely explore both compartments in the drug-free state. If the drug effects have been perceived as pleasurable during conditioning, animals spend more time in the drug-paired compartment., i.e. they show place preference. If the drug has aversive subjective effects, animals show an aversion towards the drug compartment (termed conditioned place aversion). After the expression test animals can be subjected to an extinction phase. Animals are either allowed to freely explore both compartments for several sessions or both compartments are repeatedly paired with saline or another neutral compound until the preference for the drug-paired compartment has subsided (Mueller and Stewart, 2000; Sanchez et al., 2003). Reinstatement of extinguished place preference is then induced by injecting the animals with a priming dose of the drug or exposing them to a stressor after which the compartments can be explored freely (Mueller and Stewart, 2000; Wang et al., 2001; Sanchez et al., 2003). The measure of reinstatement is the amount of time spent in the initially drug-paired compartment compared to the initially vehicle-paired compartment.

2.3 Relapse-inducing factors and brain areas involved

There are three main factors capable of inducing relapse: stress, drug-associated cues, and re-exposure to the drug (Self, 1998). Dopamine and glutamate are considered the major neurotransmitters mediating drug-seeking behavior, but in addition, several other transmitters participate in the regulation of this behavior. Several brain regions are also involved (Figure 2.1). The key dopaminergic circuit consists of the dopaminergic projections from the ventral tegmental area to the nucleus accumbens, prefrontal cortex, and amygdala (McLaughlin and See, 2003; Schmidt et al., 2005). The glutamate system regulating drug seeking consists of the prefrontal cortex, hippocampus, and amygdala, all of which send glutamatergic projections to the accumbens (Tzschentke and Schmidt, 2003; Kalivas, 2004). In addition, a glutamatergic connection from the amygdala to the prefrontal cortex is involved. Alhough the relative strength of these brain areas varies in modulating drug seeking induced by different factors – cues, stress, and drug re-exposure – the circuitries largely overlap.

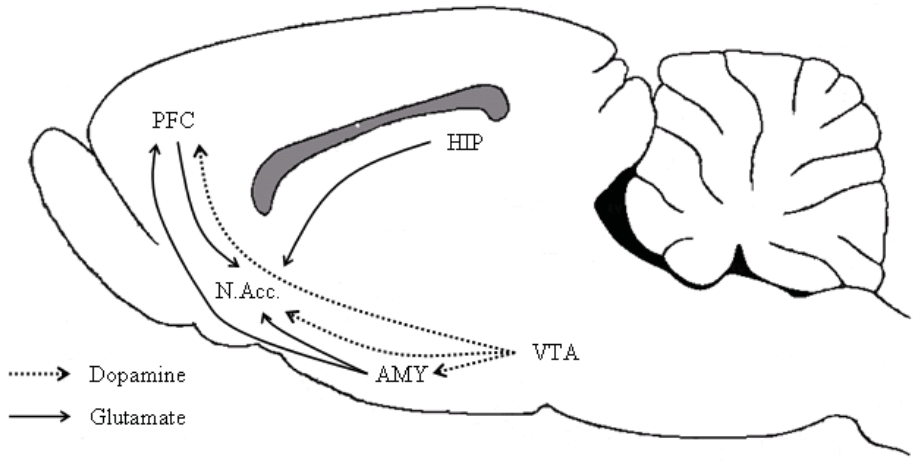

Figure 2.1. A sagittal rat brain section showing a simplified presentation of the dopaminergic and glutamatergic pathways regulating drug-seeking behavior. AMY, amygdala; HIP, hippocampus; N.Acc., nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

2.3.1 Drug re-exposure

In animal studies, reinstatement is often triggered by administering a small priming dose of the drug. Usually, priming is conducted with the training drug but also other drugs can induce reinstatement. This is called cross-reinstatement and, from a clinical point of view, means that re-exposure to other drugs than the one initially abused may trigger relapse. For example, in laboratory animals heroin seeking can be reinstated by heroin, but also by morphine, cocaine, and amphetamine (de Wit and Stewart, 1983; De Vries et al., 1998), and cocaine seeking can be reinstated by cannabinoid agonists and caffeine (Schenk and Partridge, 1999; De Vries et al., 2001).

Compounds mimicking the subjective or reinforcing effects of the training drug are often found to reinstate drug seeking. This is however not always the case (Spealman et al., 1999). The priming effects of cocaine, which is an indirect dopamine, norepinephrine and serotonin agonist, can be mimicked by dopamine agonists of the D2 type, whereas administration of D1 type dopamine agonists does not result in reinstatement (Khroyan et al., 2000). The subjective effects of both D1 and D2 agonists are, however, similar to cocaine, at least as measured by the drug discrimination paradigm, where animals are trained to make a certain response if the test drug (in this case dopamine agonist) resembles the training drug (cocaine) (Spealman et al., 1991). This indicates that in addition to the subjective effects of the priming drug, other properties can regulate drug-seeking behavior.

Reinstatement induced by drug priming is mediated mainly by the nucleus accumbens and the prefrontal cortex. A priming injection of cocaine elevates extracellular dopamine and glutamate levels in the nucleus accumbens and dopamine levels in the prefrontal cortex (McFarland et al., 2003). The ventral tegmental area, which projects to the nucleus accumbens and prefrontal cortex, also contributes to drug-seeking behavior as inactivation of this brain region attenuates cocaine-induced reinstatement (McFarland and Kalivas, 2001). Reinstatement induced by drug priming has been hypothesized to activate the VTA-prefrontal cortex dopamine projection, leading to elevated prefrontal dopamine levels, after which the glutamatergic projection from the prefrontal cortex to the nucleus accumbens is critical (McFarland and Kalivas, 2001; McFarland et al., 2003). Priming dose –induced reinstatement can be blocked by inactivation of any of the brain regions in this circuitry (McFarland and Kalivas, 2001) and also by attenuation of dopamine transmission in the prefrontal cortex or glutamate transmission in the nucleus accumbens (Cornish and Kalivas, 2000; McFarland and Kalivas, 2001; Park et al., 2002; Sanchez et al., 2003; Sun and Rebec, 2004; Peters and Kalivas, 2006). However, dopamine transmission in the accumbens is probably also involved, as accumbal dopamine antagonism attenuates cocaine-induced reinstatement (Anderson et al., 2003; but see Cornish and Kalivas, 2000; McFarland and Kalivas, 2001).

2.3.2 Stress

The most common stressor used in relapse studies is footshock, a small electrical current delivered through the grid floor of the operant box. Footshock stress has been found to reinstate alcohol, cocaine, heroin, and nicotine seeking (Erb et al., 1996; Shaham et al., 1996; Lê et al., 1998; Buczek et al., 1999). Other stressors that have been used include acute 1-day food deprivation (Shalev et al., 2000), forced swimming (Kreibich and Blendy, 2004), and administration of the stress hormone corticotropin-releasing factor (CRF) (Shaham et al., 1997; Shaham et al., 2003), the levels of which have been shown to be elevated following footshock stress (Wang et al., 2005). Selective CRF_1 or nonselective CRF receptor antagonists attenuate footshock-induced reinstatement of heroin, cocaine, and alcohol seeking (Shaham et al., 1997; Erb et al., 1998; Shaham et al., 1998; Lê et al., 2000). The neurotransmitter noradrenaline is also implicated in stress-induced relapse. α_2-adrenergic antagonists, which increase noradrenergic cell firing, induce reinstatement (Lee et al., 2004; Shepard et al., 2004), whereas α_2-adrenergic agonists attenuate footshock-induced reinstatement of heroin, cocaine, as well as speedball (a combination of heroin and cocaine) seeking (Erb et al., 2000; Shaham et al., 2000; Highfield et al., 2001). The brain stuctures critically involved in stress-induced reinstatement include the bed nucleus of the stria terminalis and the central nucleus of the amygdala (Shaham et al., 2000; Erb et al., 2001), both of which receive noradrenergic connections from the noradrenergic lateral tegmental cell groups and also contain CRF receptors (Shaham et al., 2000; Shaham et al., 2003). The central nucleus of the amygdala has been hypothesized to activate the ventral tegmental area and its dopaminergic projections, thus increasing dopamine levels in the prefrontal cortex (McFarland et al., 2004). The dopamine rise would induce reinstatement by activating the glutamatergic projection from the prefrontal cortex to the nucleus accumbens (McFarland et al., 2004).

Food-deprivation –induced reinstatement is thought to involve the adrenal hormone corticosterone and leptin, a hormone secreted by adipose tissue and involved in energy balance and body weight regulation (Shalev et al., 2001; Shalev et al., 2003). Intracerebroventricular administration of leptin decreases food intake and increases energy use (Ahima and Flier, 2000), but is also able to attenuate heroin seeking induced by a 1-day food deprivation (Shalev et al., 2001). Leptin has however no effect on heroin seeking induced by footshock or a priming injection (Shalev et al., 2001).

Orexin (hypocretin), a neuropeptide that controls sleep, arousal, and feeding, also mediates reinstatement of drug seeking (Carr and Kalivas, 2006). Activation of orexin neurons in the lateral hypothalamus or orexin receptors in the terminal field of this projection, the ventral tegmental area, reinstates morphine seeking (Harris et al., 2005). Intrecerebroventicular administration of orexin also reinstates cocaine seeking, an effect that can be prevented by blockade of noradrenergic or CRF systems, suggesting that the effects of orexin are at least partly mediated via activation of stress pathways (Boutrel et al., 2005).

2.3.3 Cues

Reinstatement induced by cues, i.e. drug-associated stimuli, involves the process of classical conditioning. As a result of repeated association with drug exposure, the stimuli acquire the ability to trigger drug-seeking behavior when presented in the absence of the drug (Self, 1998). Cues can be either discrete, discriminative, or contextual. Discrete cues are stimuli (e.g. tones or lights) presented simultaneously with drug delivery, whereas discriminative cues signal drug availability. For example, in the presence of a discriminative light stimulus responding on a lever in the test chamber is reinforced with drug delivery, whereas in the absence of the light responding has no consequences. Contextual cues are part of the self-administration environment. For example, two chambers differing in terms of size, color, material, and odor can be used for conditioning, extinction, and reinstatement sessions. Self-administration training and conditioning is conducted in one chamber, after which extinction training is carried out in the other chamber. Extinguished responding can then be reinstated, or renewed, by exposing the animals to the first chamber during reinstatement sessions. In addition to physical differences, the contexts may deviate also in interoceptive or circadian elements such as the time of testing.

Reinstatement induced by different kinds of cues is in most cases similarly modulated by pharmacological manipulations (Bossert et al., 2005). Dopamine antagonism attenuates reinstatement induced by discrete, discriminative, and contextual cues in a similar manner (Weiss et al., 2001; Alleweireldt et al., 2002; Crombag et al., 2002). Dopamine antagonists are effective also when injected directly into the amygdala, more precisely the basolateral region, indicating that the amygdala is involved in cue-induced drug seeking (See et al., 2001; Alleweireldt et al., 2006). This is further supported by findings that inactivation and lesions of the amygdala attenuate reinstatement induced by discrete, discriminative, and contextual cues (Meil and See, 1997; Fuchs and See, 2002; Yun and

Fields, 2003; Fuchs et al., 2005). In addition, the nucleus accumbens and glutamate transmission therein are critically involved. Accumbal glutamate receptor blockade decreases cue-controlled drug seeking under a second-order schedule of self-administration and dampening glutamate release attenuates cue-induced reinstatement (Di Ciano and Everitt, 2001; Baptista et al., 2004). Inactivation studies have shown that the hippocampus, ventral tegmental area, and prefrontal cortex also modulate cue-induced drug seeking (McLaughlin and See, 2003; Sun and Rebec, 2003; Di Ciano and Everitt, 2004; Fuchs et al., 2004). The hippocampus may however be more strongly involved in drug seeking induced by drug-associated contexts than discrete cues, as hippocampal inactivation was found to inhibit reinstatement induced by contextual but not discrete cues (Fuchs et al., 2005).

Human addicts are exposed to a vast array of stimuli during the acquisition and consumption of drugs. Although reinstatement can be induced by a single stimulus, compound stimuli have been shown to be more effective in animal studies (See et al., 1999). The constituents of the compound stimulus used during testing can also be conditioned separately as single stimuli. In a study where rats had been trained to associate a light and a tone separately as discriminative stimuli signalling food availability (i.e. food was available during presentation of either the light or the tone but not in their absence) responding was significantly higher in the presence of a light+tone compound stimulus than either the light or tone alone (Kearns and Weiss, 2005). Depending on the compound stimulus and the sensory modalities of its components, one component may dominate and thus induce stronger reinstatement than the others if presented alone. Reinstatement may also be completely absent, if the constituents are presented alone. In a study by See et al. (1999) this was examined by training rats to associate different kinds of discrete stimuli with cocaine delivery. Thereafter, responding was extinguished in the absence of the stimuli and the effects of different combinations of the stimulus components on reinstatement responding were examined. Rats that had been trained with a light and tone compound stimulus showed reinstatement of responding following presentation of the compound stimulus. However, neither of the stimulus constituents (light or tone) alone reinstated responding in these animals. When rats were trained with a single stimulus (either light or tone), the light stimulus was found to induce modest reinstatement, whereas a similarly conditioned and presented tone was ineffective.

Another important issue in animal reinstatement studies is the manner in which the stimulus is presented. Discrete cues can be presented either response-contingently, i.e. as a consequence of behavior or noncontingently, without any effort from the animal. Reinstatement induced by discrete cues has been found to be stronger following contingent than noncontingent stimulus presentation (Meil and See, 1996; Grimm et al., 2000; Di Ciano and Everitt, 2003). This may be related to stimulus compounding, as a stimulus that has been presented simultaneously with drug delivery during conditioning has usually also been presented in close temporal relationship with the response (e.g. lever press). Thus, the response (lever press) may have been incorporated in the compound stimulus (Grimm et al., 2000). A similar phenomenon has been observed in

addicts: those that were allowed to touch and manually examine cues (drug-related paraphernalia) showed greater craving and changes in physiological measures such as pulse, skin conductance, and temperature (Johnson et al., 1998).

2.4 Neuropharmacology of drug seeking

2.4.1 Dopamine

The mesocorticolimbic dopamine system

Most addictive drugs increase dopamine levels in the terminal fields of the mesocorticolimbic dopamine system (Di Chiara and Imperato, 1988). The cell bodies of dopamine neurons reside in the ventral tegmental area (VTA), from where projections extend to the nucleus accumbens, prefrontal cortex, and amygdala (Koob, 1992). Drug taking in laboratory animals can be modified by dopamine receptor agonist and antagonist injections administered either systemically or into the terminal fields of the dopamine system. Dopamine antagonists tend to attenuate, whereas agonists enhance, the reinforcing properties of e.g. cocaine (Hubner and Moreton, 1991; Caine and Koob, 1994; Caine et al., 1995; Caine and Koob, 1995). Drug-seeking behavior can be attenuated by inactivation of the VTA or any of the terminal regions of the mesolimbic dopamine system (McLaughlin and See, 2003; Di Ciano and Everitt, 2004; McFarland et al., 2004).

Dopamine receptors

Dopamine receptors are divided into two major groups, D1-like and D2-like receptors, based on their effects on the activity of adenylate cyclase, an enzyme responsible for the transformation of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), an intracellular second messenger. The D1-like receptors, which include D_1 and D_5 receptors, stimulate adenylate cyclase, whereas the D_2-like receptors D_2, D_3, and D_4 inhibit it (Nestler et al., 2001).

Dopamine and drug seeking

Drug priming increases accumbal dopamine levels (Di Ciano et al., 2001; McFarland et al., 2003) and drug-seeking behavior can be induced by injecting dopamine or dopamine agonists into the nucleus accumbens (Cornish and Kalivas, 2000; Park et al., 2002; Bachtell et al., 2005). Conversely, inactivation of the nucleus accumbens or intra-accumbal infusions of dopamine receptor antagonists attenuate reinstatement induced by drug priming or footshock stress (Di Ciano and Everitt, 2004; McFarland et al., 2004; Xi et al., 2004; Bachtell et al., 2005; Anderson et al., 2006; but see McFarland and Kalivas, 2001). Similar to drug priming, the presentation of drug-associated cues leads to elevations, although less pronounced, in accumbal dopamine levels (Katner and Weiss, 1999; Weiss et al., 2000). Although there are no direct studies examining the effects of accumbal dopamine receptor blockade on cue-induced drug seeking, the ability of a discriminative stimulus to guide behavior has been shown to be impaired following dopamine antagonist injections into the nucleus accumbens (Yun et al., 2004).

In addition to the accumbens, drugs of abuse elevate dopamine levels also in the prefrontal cortex (Sorg and Kalivas, 1993) and reinstatement can be induced by dopamine agonist administration into this brain region (McFarland and Kalivas, 2001; Park et al., 2002). In fact, elevated prefrontal dopamine levels have been suggested to be one of the initiators of drug-seeking behavior (McFarland and Kalivas, 2001). Therefore, it is not surprising that D1 and D2 dopamine receptor antagonism in the prefrontal cortex attenuates reinstatement induced by a priming dose of cocaine (McFarland and Kalivas, 2001; Park et al., 2002; Sanchez et al., 2003; Sun and Rebec, 2004) or footshock stress (Sanchez et al., 2003; McFarland et al., 2004).

Dopamine transmission in the amygdala is an important mediator of cue-induced reinstatement. Cocaine-associated discriminative stimuli, which reinstate drug seeking, increase dopamine levels also in the amygdala (Weiss et al., 2000) and infusions of d-amphetamine, an indirect dopamine agonist, into the basolateral amygdala enhance reinstatement induced by cocaine-associated cues (Ledford et al., 2003). In line with this, reinstatement induced by cocaine-associated stimuli can be attenuated by D1 or combined D1/D2 antagonism in the amygdala, whereas glutamate receptor antagonism is ineffective (See et al., 2001).

D1- and D2-type dopamine antagonists attenuate drug seeking also when injected systemically. For example, heroin, cocaine, and nicotine seeking induced by drug priming (Shaham and Stewart, 1996; Khroyan et al., 2000; Andreoli et al., 2003) as well as cue-induced cocaine and alcohol seeking (Alleweireldt et al., 2002; Liu and Weiss, 2002) and stress-induced cocaine seeking (Xi et al., 2004) can be attenuated by dopamine receptor antagonists. Surprisingly, a similar result has been reported with D1 agonists. Systemically administered D1-like agonists do not reinstate drug seeking like dopamine, nonselective dopamine agonists, and D2-like agonists do, but actually attenuate priming and cue-induced reinstatement (Self et al., 1996; Khroyan et al., 2000; Alleweireldt et al., 2002). The reason for this is largely unclear. In a study by Alleweireldt et al. (2003) a decrease in cue- or priming-induced cocaine seeking could be accomplished by either D1 antagonism or D1 agonism. When co-administered, the antagonist and agonist were found to be ineffective in modulating reinstatement induced by a cocaine priming dose. With cue reinstatement, no such interaction was found and reinstatement was attenuated similarly as following D1 agonism alone. A similar effect was discovered by Khroyan et al. (2000) on drug seeking induced by cocaine priming together with cocaine-associated cues: the reinstatement-attenuating effects of a D1 agonist or a D1 antagonist administered alone were partially reversed when the compounds were co-administered. This suggests that cocaine-induced reinstatement is dependent on an optimal level of dopamine receptor stimulation. Therefore, both overstimulation with agonists and understimulation with antagonists can abolish reinstatement. As for the different results between cue- and priming-induced reinstatement, several explanations are possible including higher extracellular dopamine levels following a priming dose of cocaine as compared to cue presentation and different populations or subtypes of D1 dopamine receptors involved. Inactivation studies have

shown that although the amygdala is involved in both cue and priming dose induced reinstatement (Fuchs and See, 2002; Yun and Fields, 2003), it may be more important in modulating cue-induced drug seeking (Grimm and See, 2000; McFarland and Kalivas, 2001). It has been hypothesized that in the amygdala most D1 receptors are probably not coupled to adenylate cyclase (Kilts et al., 1988; Alleweireldt et al., 2003), but possibly to phospholipase C (Alleweireldt et al., 2003). If so, this may be important as the effects of dopaminergic compounds often differ between adenylate cyclase and phospholipase C – coupled receptors, even to such an extent that no correlation has been found between the potencies or efficacies in stimulating these two receptors subtypes (Undie et al., 1994). For example, one of the D1 agonists used in the above studies, SKF81297, is classified as a full agonist at the adenylate cyclase -linked receptor but only as a partial agonist at the phospholipase C -linked receptor (Undie et al., 1994). Thus, partial agonism at amygdalar D1 receptors may not have been enough to counteract the effects of D1 antagonism on drug seeking.

2.4.2 Glutamate

The corticolimbic glutamate system

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system. With regard to drug addiction and drug-seeking behavior, glutamate transmission in the nucleus accumbens and its afferent projections plays a central role. The nucleus accumbens as well as the the ventral tegmental area (VTA) receive glutamatergic projections from the amygdala, prefrontal cortex, and hippocampus (Tzschentke and Schmidt, 2003). The VTA is the cell body region of the mesolimbic dopamine system, and therefore the nucleus accumbens and the VTA are sites of glutamate-dopamine interactions. In the VTA, glutamate increases the activity of dopaminergic neurons, which results in increased dopamine release in the accumbens (Kretschmer, 1999). At the level of the nucleus accumbens, research on glutamatergic modulation of dopamine neurotransmission has produced complex and controversial data, but it seems that glutamate receptors can differentially modulate impulse-dependent and impulse-independent dopamine release (see David et al., 2005 for a review).

Glutamate receptors

Glutamate receptors are divided into ionotropic receptors, which are intrinsic to an ion channel, and metabotropic receptors, which are G-protein coupled (Figure 2.2.). Ionotropic receptors are further subdivided into NMDA, AMPA, and kainate receptors, whereas metabotropic glutamate receptors (mGluR) are comprised of 8 subtypes (mGluR1-8) belonging to three groups (I-III) (Witkin et al., 2002; David et al., 2005).

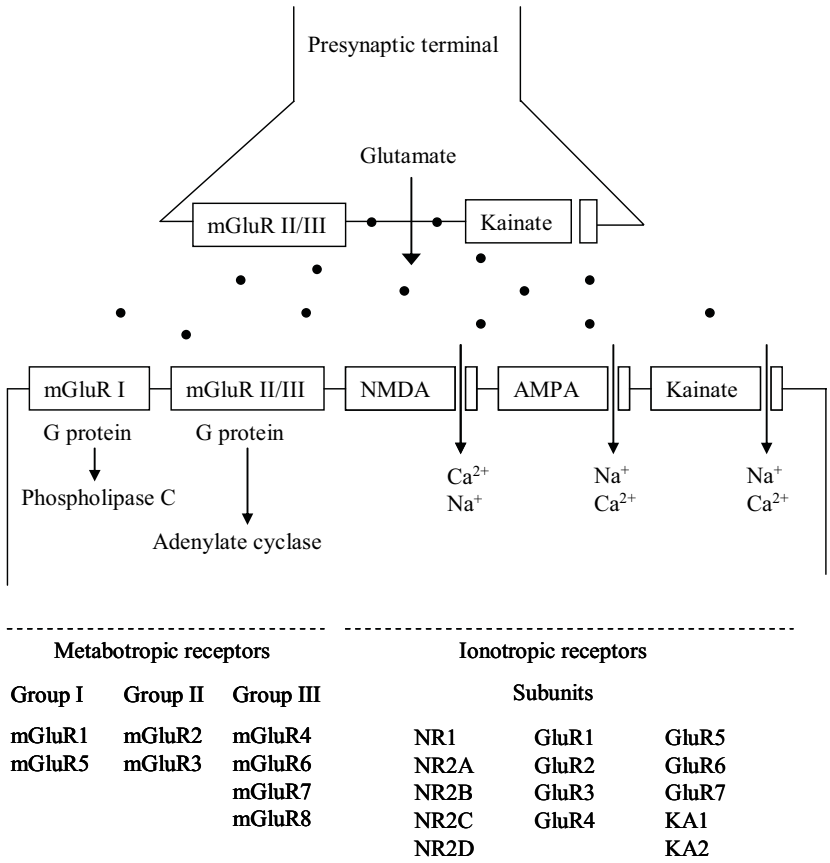

Figure 2.2. The major glutamate receptor subtypes in a hypothetical synapse. Shown are the G-protein coupled metabotropic glutamate receptors (mGluR) with their second messengers phospolipase C and adenylate cyclase and the ionotropic receptor channels with the ions to which they are mainly permeable. In the lower part, the receptor subtypes comprising each group (I-III) of metabotropic receptors are listed. For ionotropic receptors, the subunits from which the different receptors are composed are shown. Adapted from Bleakman and Lodge, Neuropharmacology (1998) 37:1187-1204.

NMDA receptors are composed of NR1 and NR2 subunits, of which NR1 has eight different splice variants and NR2 four different subtypes ($NR2_{A-D}$). In the brain NMDA receptors are believed to exist as NR1-NR2 heteromeric complexes (Nestler et al., 2001; Waxman and Lynch, 2005). The pharmacological properties of NMDA receptors are influenced by their subunit composition in terms of agonist affinity, antagonist sensitivity, desensitization, and sensitivity to Mg^{2+} blockade (see Yamakura and Shimoji, 1999 for review).

NMDA receptor function can be modulated through several binding sites: glutamate, $glycine_B$, polyamine, and phencyclidine sites. Competitive agonists and antagonists bind at the glutamate site whereas noncompetitive ligands bind at one of the other binding sites. Glycine is required as a co-agonist with glutamate for receptor activation. Under physiological conditions, however, the glycine concentration is believed to always be sufficient. The polyamine site potentiates receptor function in a pH-dependent fashion. The phencyclidine site is located within the receptor ion channel and its blockers are called uncompetitive antagonists. In addition to both glutamate and glycine, proper functioning of NMDA receptors requires a depolarization to release a Mg^{2+} block from the channel. One consequence of the Mg^{2+} block is that channel blockers (uncompetitive antagonists) are able to access their binding site only following receptor activation (Nestler et al., 2001). Compared to AMPA receptors, the affinity of NMDA receptors for glutamate is about 500 times higher, but because of the voltage-dependent Mg^{2+} block NMDA receptors are thought to contribute less to basal synaptic glutamate transmission (Simeone et al., 2004). However, after the NMDA receptor -containing neurons have been depolarized by activation of other receptors, the contribution of NMDA receptors to glutamatergic transmission can be substantial.

AMPA receptor are comprised of four subunits, GluR1-4, which most likely can assemble in all combinations as homomers or heteromers (Nestler et al., 2001). As with NMDA receptors, the subunit composition influences e.g. receptor desensitization (Dingledine et al., 1999). Compared to NMDA receptors, AMPA receptors have fewer binding sites. Competitive ligands bind at the glutamate recognition site whereas noncompetitive modulators bind at a distinct allosteric regulatory site. In addition, those AMPA receptors that lack the GluR2 subunit and are therefore permeable to Ca^{2+} can be antagonized by cationic channel blockers such as certain spider and wasp toxins. The function of kainate receptors, comprised of GluR5-7 and KA1-2 subunits, was until recently poorly understood because of the lack of selective agonists and antagonists (Witkin et al., 2002). Kainate receptors may however be future targets for the treatment for drug addiction as they have been found in the amygdala, one of the brain regions modulating drug-seeking behavior (Braga et al., 2004).

Metabotropic glutamate receptors are divided into three groups. Group I is comprised of mGlu1 and mGlu5 receptors, group II of mGlu2 and 3 receptors, and group III of mGlu4 and mGlu6-8 receptors. Group I mGlu1/5 receptors are predominantly postsynaptic, whereas group II and III agonists can negatively modulate glutamate transmission via a presynaptic mechanism (Schoepp, 2001; Thomas et al., 2001; Pothecary et al., 2002). Therefore, group I antagonists and group II/III agonists can have similar effects: group I antagonists by decreasing glutamate input to postsynaptic cells and group II/III agonists by decreasing the amount of glutamate released from presynaptic terminals.

Effects of repeated drug treatment on glutamatergic neurotransmission

Repeated drug treatment has been shown to change glutamatergic transmission in a way that increases the ability of drug-associated cues, drug priming, and stressors to trigger

relapse. The majority of studies have measured extracellular glutamate levels using the *in vivo* microdialysis technique. However, glutamate differs from e.g. dopamine in that its synaptic pool can rarely be sampled under physiological conditions as glutamate is rapidly cleared from the synapse after its release (Drew et al., 2004). Because of this the physiological origin of glutamate detected by microdialysis is uncertain and basal levels may mostly be of extrasynaptic or non-neuronal origin (Oldenziel et al., 2006). The role of glutamate released independently from neuronal activity should however not be belittled, as it may still contribute to the control of neuronal excitability under normal and pathological conditions (Nyitrai et al., 2006).

When animals have been repeatedly treated with a drug, so far all studies have used cocaine, decreased basal levels of extracellular glutamate are observed in the nucleus accumbens (Bell et al., 2000; Hotsenpiller et al., 2001; Baker et al., 2003; McFarland et al., 2003). This decrease in glutamate levels probably requires the pairing of the drug with environmental stimuli or cues, as so called "unpaired" rats, i.e. animals treated with cocaine in their home cage, mostly fail to show decreased glutamate levels when compared to rats for which the treatment has been paired with a distinct environment (thus called "paired" rats) (Bell et al., 2000; Hotsenpiller et al., 2001).

Upon re-exposure to either cocaine or cocaine-paired stimuli, a second change in glutamate transmission is observed. Compared to controls, repeatedly cocaine-treated animals show an enhanced glutamate release in the nucleus accumbens (Bell et al., 2000; Hotsenpiller et al., 2001; Baker et al., 2003; McFarland et al., 2003). As shown by McFarland et al. (2003), this increase is dependent on neuronal activation (i.e., sensitive to Na^+-channel blockade by tetrodotoxin). As with decreased basal glutamate levels, the enhanced glutamate release is observed only in animals in which cocaine has previously been paired with environmental stimuli, or in animals that have been self-administering cocaine. McFarland et al. (2003) showed also that a priming injection of cocaine to reinstate drug seeking increased glutamate release in rats that had been self-administering cocaine, but not in their yoked counterparts. The yoked and self-administering rats had received an equal amount of cocaine during the self-administration phase and similar treatment during cocaine withdrawal. In fact, the yoked rats even showed decreased basal glutamate levels, probably as a result of pairing cocaine with the self-administration chamber, but the enhanced glutamate release following cocaine priming was absent. This demonstrates that although passive exposure to cocaine can result in changes in glutamatergic neurotransmission, additional changes take place when experimenter-administered cocaine is paired with environmental stimuli or the drug is contingent upon the behavior of the animal. At the same time, the above experiments show that both cocaine priming and exposure to cocaine-associated stimuli elevate glutamate levels in the nucleus accumbens.

Repeated drug treatment has also been shown to induce changes in the glutamatergic responses to stress. Footshock stress has been found to increase glutamate levels in the ventral tegmental area of cocaine-treated, but not naïve, rats. Further examination

revealed that glutamate release was under the control of CRF and induced drug seeking indirectly, through activation of the mesolimbic dopamine system (Wang et al., 2005).

The increased glutamate release observed following cocaine priming and also reinstatement of cocaine seeking can be blocked by administration of the amino acid cystine (Baker et al., 2003). Cystine is the substrate for a carrier that exchanges extracellular cystine for intracellular glutamate (see Baker et al., 2002). The decreased basal glutamate levels following repeated cocaine treatment may result from decreases in this exchange process. In cocaine-naïve animals, blockade of the cystine/glutamate exchange in the nucleus accumbens was shown to result in decreased extracellular glutamate levels similar to cocaine withdrawal. When repeatedly cocaine-treated rats, which showed the characteristic decreased glutamate levels, were treated with exogenous cystine, normal glutamate levels were restored (Baker et al., 2002). The administration of cystine also abolished the ability of a cocaine priming injection to reinstate cocaine seeking. Microdialysis data showed that in cystine-treated rats the cocaine priming injection was no longer able to elevate glutamate levels above normal baseline levels (Baker et al., 2003) suggesting that the elevated accumbal glutamate levels mediate reinstament. It has been suggested that glutamate derived from the cystine/glutamate exchanger provides endogenous tone at extrasynaptic group II metabotropic glutamate receptors, thus decreasing the (cocaine-induced) release of glutamate and possibly also other transmitters such as dopamine, depending on whether the receptors function as auto- or heteroreceptors (Baker et al., 2002).

Glutamate and drug seeking

Evidence for the involvement of accumbal glutamate transmission in drug-seeking behavior comes from studies in which drug seeking was induced by injections of either AMPA or NMDA type glutamate agonists into the nucleus accumbens (Cornish et al., 1999; Cornish and Kalivas, 2000; Suto et al., 2004). Further, reinstatement induced by either systemically administered cocaine, intra-accumbal dopamine, or an intra-accumbal AMPA receptor agonist can be attenuated by blockade of accumbal AMPA receptors (Cornish and Kalivas, 2000). Interestingly, in this study an AMPA antagonist blocked reinstatement by all compounds, whereas a dopamine antagonist blocked only dopamine-induced reinstatement. This finding suggests that accumbal glutamate may be more important than dopamine in regulating drug seeking.

Accumbal glutamate transmission modulates also drug seeking induced by drug-associated stimuli. Glutamate receptor blockade in the nucleus accumbens or more dorsal parts of the striatum has been fount to attenuate cocaine seeking under a second-order schedule of self-administration (Di Ciano and Everitt, 2001; Vanderschuren et al., 2005). Interestingly, these data suggest that antagonism at AMPA, but not NMDA glutamate receptors decreases drug seeking. A similar finding has been reported by Cornish and Kalivas (2000) with regard to priming-induced reinstatement. Also an anatomical dissociation has been found. It seems that within the nucleus accumbens, the core area may be more important than the shell in modulating both priming dose and

cue-induced drug seeking (Di Ciano and Everitt, 2001; McFarland and Kalivas, 2001; Fuchs et al., 2004; Ito et al., 2004; Yao et al., 2005; but see Bossert et al., 2005).

The nucleus accumbens receives glutamatergic projections from the amygdala, hippocampus, and prefrontal cortex. Inactivation of the prefrontal cortex with GABA agonists prevents cocaine-induced elevations in accumbal glutamate levels (Pierce et al., 1998; McFarland et al., 2003) as well as reinstatement induced by either cocaine priming (McFarland and Kalivas, 2001; Capriles et al., 2003) or drug-associated cues (McLaughlin and See, 2003). In addition, lesions of the prefrontal cortex disrupt the ability of drug-associated stimuli to guide behavior under a second-order schedule of reinforcement (Weissenborn et al., 1997). Park et al. (2002) provided futher support for the role of the glutamatergic projection from the prefrontal cortex to the accumbens by showing that reinstatement induced by cocaine injected into the prefrontal cortex can be blocked by accumbal AMPA antagonism.

The amygdala sends glutamatergic projections both to the nucleus accumbens and the prefrontal cortex. Within the basolateral amygdala glutamate receptor blockade is ineffective in modulating cue-induced reinstatement (See et al., 2001). This does not mean that the glutamatergic projections originating in the amygdala would not participate in the regulation of drug-seeking behaviour. Several inactivation and lesion studies have shown the involvement of the amygdala in both priming dose and cue-induced relapse (Grimm and See, 2000; Fuchs and See, 2002; Yun and Fields, 2003; but see Di Ciano and Everitt, 2004) and, underlining the importance of glutamate, a study by Di Ciano and Everitt (2004) showed that serial interactions between the amygdala and the nucleus accumbens are crucial for drug seeking guided by cues. Unilateral injections of a dopamine antagonist into the amygdala and an AMPA/kainate glutamate antagonist into the nucleus accumbens core, neither of which had any effects when injected alone, attenuated drug-seeking behavior under a second-order schedule of self-administration (Di Ciano and Everitt, 2004).

Although the nucleus accumbens and the VTA receive glutamatergic projections from the hippocampus, the role of these projections in drug-seeking behavior has not been systematically explored. Studies so far have however found that inactivation of the hippocampus attenuates priming dose and cue-induced drug seeking (Sun and Rebec, 2003; Fuchs et al., 2005) and, more importantly, that stimulation of the hippocampus induces cocaine seeking through activation of ionotropic glutamate receptors in the ventral tegmental area (Vorel et al., 2001). Within the VTA, cocaine-primed reinstatement can be attenuated by ionotropic glutamate receptor blockade (Sun et al., 2005). Also metabotropic glutamate receptors in the VTA modulate drug seeking as infusions of an mGluR2/3 agonist, which decreases presynaptical glutamate release, were reported to attenuate context-induced heroin seeking (Bossert et al., 2004).

The majority of studies examining the effects of systemically administered glutamate receptor agonists and antagonists on reinstatement of drug-seeking behavior have targeted metabotropic receptors. Most studies have employed either LY379268, an mGluR2/3

agonist, or MPEP, an mGluR5 antagonist. Presumably, both compounds dampen glutamatergic neurotransmission; LY379268 by a presynaptic, release-decreasing effect, and MPEP by a postsynaptic effect. MPEP has been found to attenuate priming-induced cocaine and nicotine seeking (Tessari et al., 2004; Lee et al., 2005) and cue-induced nicotine seeking (Bespalov et al., 2005). The mGluR2/3 agonist LY379268 has so far been reported to attenuate priming dose induced cocaine seeking (Adewale et al., 2006; Peters and Kalivas, 2006) and cue-induced heroin and cocaine seeking (Baptista et al., 2004; Bossert et al., 2004; Bossert et al., 2005), showing that drug seeking induced by both drug re-exposure and drug-associated stimuli is modulated by metabotropic glutamate receptors. This could be an exciting finding, as medications targeting metabotropic instead of ionotropic receptors might have fewer side effects (Spooren et al., 2000; Breysse et al., 2002). These hopes may, however, be somewhat premature as there are some recent studies showing that both LY379268 and MPEP may possess undesirable effects (Harrison et al., 2002; Kenny et al., 2005; Peters and Kalivas, 2006).

There are surprisingly few studies on the effects of systemically administered ionotropic glutamate receptor agonists and antagonists on drug-seeking behavior. A study by De Vries et al. (1998) showed reinstatement of cocaine-seeking behavior following systemic administration of the uncompetitive NMDA antagonist MK-801. This is interesting, as intracranially injected AMPA antagonists have been reported to decrease drug seeking (see above). However, systemic MK-801 has been shown to induce a delayed increase in accumbal glutamate levels (Ito et al., 2006) and another uncompetitive NMDA antagonist, phencyclidine, similarly increases both extracellular glutamate and dopamine levels in the nucleus accumbens and prefrontal cortex (Adams and Moghaddam, 1998). If systemic administration of uncompetitive NMDA antagonists increases accumbal and prefrontocortical transmitter levels similar to drug-associated cues and/or drug priming, this might explain the potentiating effect of MK-801 on drug seeking. However, memantine, which is also an uncompetitive antagonist, has been reported to attenuate morphine-induced reinstatement of conditioned place preference (Popik et al., 2006) whereas another uncompetitive antagonist, neramexane, did not reach significance in reducing cue-induced alcohol-seeking in a study by Bachteler et al. (2005). The functional NMDA antagonist acamprosate, in its turn, attenuates cue-induced alcohol reinstatement (Bachteler et al., 2005). This suggests that systemically administered NMDA antagonists, at least uncompetitive channel blockers, may differentially modulate reinstatement behavior.

In contrast, systemically administered competitive, uncompetitive, and NMDA/glycine antagonists have all been found to attenuate alcohol relapse as measured by the alcohol deprivation model (Hölter et al., 1996; Heyser et al., 1998; Hölter et al., 2000; Vengeliene et al., 2005). Administration of ethanol also decreases the alcohol deprivation effect, suggesting that alcohol drinking after deprivation is driven by the pharmacological effects of alcohol (Vengeliene et al., 2005). It has also been found that uncompetitive, competitive, and glycine site antagonists of the NMDA receptor are all capable of substituting for ethanol in drug-discrimination studies, i.e. they induce ethanol-like subjective effects (Kotlinska and Liljequist, 1997; Bienkowski et al., 1998;

Hölter et al., 2000). Ethanol itself inhibits NMDA receptor function via a noncompetitive mechanism (Krystal et al., 2003). Thus, NMDA antagonists probably substitute for ethanol during alcohol re-access and decrease deprivation-induced relapse-like drinking through this mechanism.

The role of glutamate in relapse induced by stressors has been less extensively studied. However, footshock stress has been found to increase dopamine and glutamate release in the prefrontal cortex and ventral tegmental area as well as glutamate, but not dopamine, release in the nucleus accumbens core (McFarland et al., 2004). Inactivation of the prefrontal cortex, which sends glutamatergic projections to the accumbens, blocked the stress-induced rise in accumbal glutamate levels and cocaine seeking (McFarland et al., 2004). In the VTA, footshock stress has been found to elevate levels of the stress hormone corticotrophin-releasing factor (CRF), glutamate, and dopamine in cocaine-experienced animals (Wang et al., 2005). Stress-induced increases in dopamine and glutamate levels as well as stress-induced reinstatement were blocked by intra-VTA infusions of a CRF antagonist. Similarly, blockade of ionotropic glutamate receptors blocked the increases in dopamine levels and reinstatement (Wang et al., 2005). Thus, stress probably acts to increase VTA CRF levels, which in turn increase local glutamate release. Glutamate activates dopaminergic projections to the accumbens and prefrontal cortex, brain regions where dopamine has been shown to induce drug-seeking behavior (McFarland et al., 2004).

Length of withdrawal period and drug seeking

The length of the withdrawal period that usually separates drug use and relapse also modulates the strength of drug-seeking behavior. Over the first two months of withdrawal, time-dependent changes in the intensity of craving and drug seeking accompanied by neurochemical findings have been demonstrated (see Lu et al., 2004 for a detailed review). Withdrawal-induced changes that are linked to glutamatergic neurotransmission include upregulation of AMPA and NMDA glutamate receptor subunits in the ventral tegmental area, amygdala, and nucleus accumbens (Carlezon and Nestler, 2002; Lu et al., 2003; Lu et al., 2005). Also increased expression levels of activator of G protein signaling 3 (AGS3) have been found in the prefrontal cortex and the nucleus accumbens core following 3 weeks, but not 1 or 7 days of withdrawal from cocaine (Bowers et al., 2004). AGS3 regulates the signaling of $G_{i/o}$-protein coupled receptors by inhibiting the reassociation of the two subunits ($G_{i\alpha}$ and $G_{\beta\gamma}$) of the G-protein after receptor activation. Thus, changes in AGS3 levels may alter the functioning of G-protein coupled dopamine and metabotropic glutamate receptors, thus affecting drug-seeking behavior. In drug-naïve animals, the phenotype of repeated cocaine treatment can be mimicked by injections of an AGS3-derived peptide. These animals show an enhanced locomotor response to an acute injection of cocaine (Bowers et al., 2004), much like animals that have been repeatedly treated with cocaine (Vanderschuren and Kalivas, 2000). More interestingly, these animals also show an enhanced glutamate release in the nucleus accumbens in response to an acute injection of cocaine, and cocaine-induced drug seeking is potentiated (Bowers et al., 2004). Decreasing levels of functional AGS3 by expression of AGS3 antisense was found to block heroin and cocaine seeking induced by priming injections (Bowers et al., 2004; Yao et al., 2005).

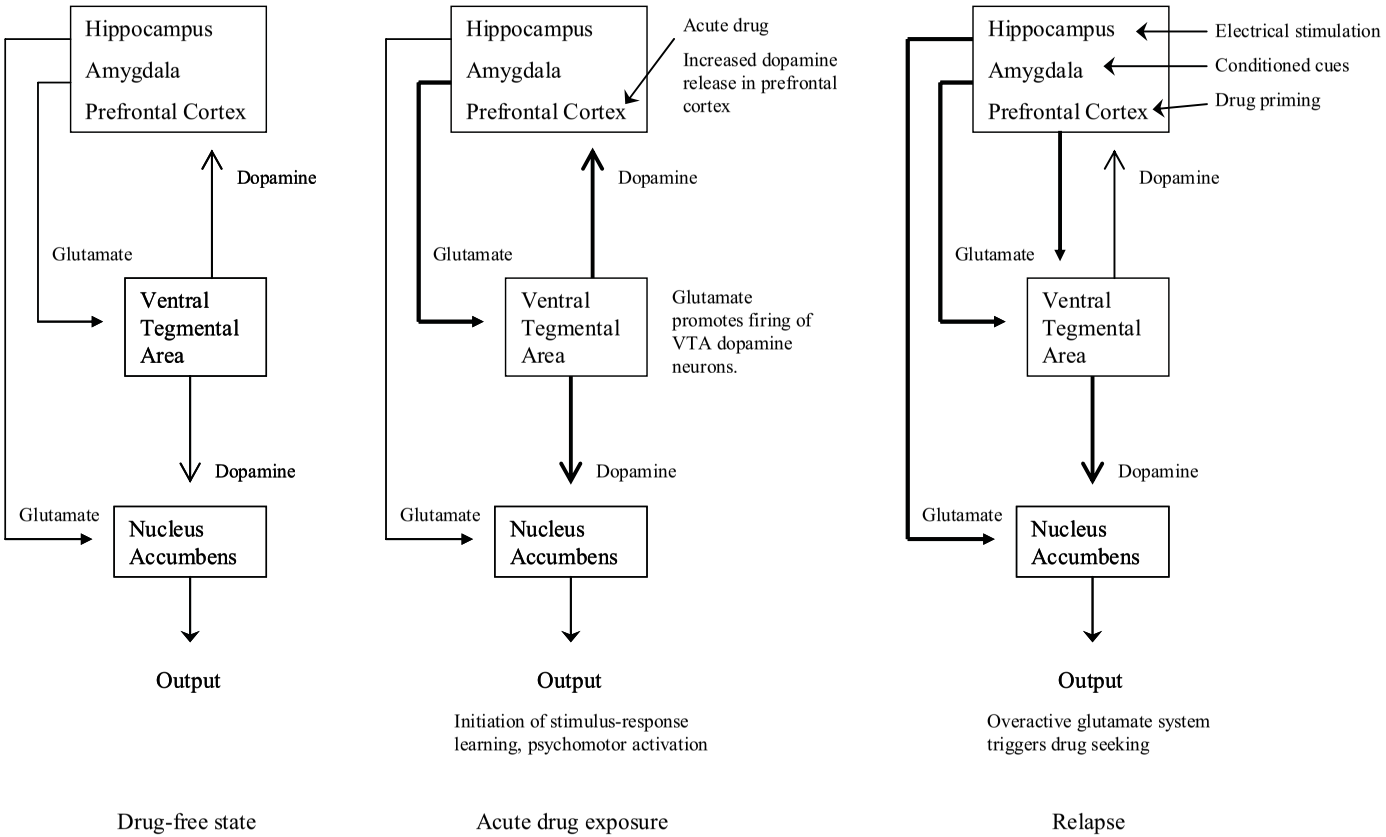

Figure 2.3. Summary of dopaminergic and glutamatergic neurotransmission in the drug-free state, during initial drug taking, and following transition to an addicted state where stress, drug-associated cues, and drug priming are capable of inducing relapse in drug-free subjects. Adapted from Tzschentke and Schmidt, Molecular Psychiatry (2003) 8:373-382.

Table 1. ***Effects of intracranial pharmacological manipulations on drug seeking induced by drug-associated cues or priming***

Prefrontal Cortex	Cues		Priming	
Dopamine agonists			Induction	McFarland and Kalivas, 2001 Park et al., 2002
Dopamine antagonists			Attenuation	McFarland and Kalivas, 2001 Park et al., 2002 Sanchez et al., 2003 Sun and Rebec, 2004
Nucleus Accumbens	**Cues**		**Priming**	
Dopamine agonists			Induction	Cornish and Kalivas, 2000 Park et al., 2002 Bachtell et al., 2005
Dopamine antagonists			Attenuation	Anderson et al., 2003 Anderson et al., 2005 Bachtell et al., 2005
			No effect	McFarland and Kalivas, 2001 Cornish and Kalivas, 2000
NMDA agonists			Induction	Cornish et al., 1999
NMDA antagonists	No Effect	Di Ciano and Everitt, 2001	Induction	Park et al., 2002
			No effect	Cornish and Kalivas, 2000
AMPA agonists			Induction	Cornish et al., 1999 Cornish and Kalivas, 2000

				Suto et al., 2004
AMPA antagonists	Attenuation	Di Ciano and Everitt, 2001	Attenuation	Cornish and Kalivas, 2000
mGluR2/3 agonists	Attenuation	Baptista et al., 2004 Bossert et al., 2005	Attenuation	Peters and Kalivas, 2006

Amygdala	Cues		Priming	
Dopamine agonists	Enhancement	Ledford et al., 2003		
Dopamine antagonists	Attenuation	See et al., 2001 Alleweireldt et al., 2006	No effect	See et al., 2001 Alleweireldt et al., 2006
NMDA agonists			Induction	Hayes et al., 2003
NMDA antagonists	No effect	See et al., 2001	No effect	See et al., 2001
AMPA agonists				
AMPA antagonists	No effect	See et al., 2001	No effect	See et al., 2001

2.4.3 Additional transmitters

In addition to dopamine and glutamate, the transmitters noradrenaline and gamma-aminobutyric acid (GABA) as well as the endocannabinoid system contribute to modulation of drug-seeking behavior. Modulators of the opioidergic system such as the nonselective opioid antagonist naltrexone have also been studied, especially with regard to attenuation of opiate- and alcohol-seeking behavior (see e.g. Lê and Shaham, 2002; O'Brien, 2005).

Noradrenaline participates extensively in the modulation of stress-induced relapse. Footshock stress has been shown to increase noradrenaline levels in the prefrontal cortex, amygdala, and bed nucleus of the stria terminalis (Erb et al., 2000; Morilak et al., 2005). Noradrenergic receptors, called adrenoceptors, are divided into α and β receptors with the subtypes α_1, α_2, and $\beta_{1\text{-}3}$. α_2 antagonists, which stimulate noradrenaline release, are capable of inducing relapse (Lee et al., 2004; Shepard et al., 2004), whereas α2 agonists (which decrease noradrenaline release) have been shown to attenuate stress-induced heroin and cocaine seeking (Erb et al., 2000; Shaham et al., 2000). Also β-adrenergic antagonists attenuate stress-induced drug seeking (Leri et al., 2002). In contrast, drug priming is seldom affected by treatment with noradrenergic agonists or antagonists (Erb et al., 2000; Leri et al., 2002; but see Zhang and Kosten, 2005).

The GABA agonists baclofen and muscimol have been widely used in reinstatement studies to inactivate brain regions (see e.g. McFarland and Kalivas, 2001; Fuchs et al., 2004). However, GABA agonists have been found to reduce drug-seeking behavior also after systemic administration (Johnson et al., 2005). Cue-induced nicotine, heroin, and cocaine seeking as well as priming dose -induced cocaine seeking were decreased by baclofen or another GABA agonist, CGP44532 (Campbell et al., 1999; Di Ciano and Everitt, 2003; Paterson et al., 2005). Baclofen has been tested also in clinical trials, where it has reduced craving for alcohol and cocaine as well as increased abstinence days (see O'Brien and Gardner, 2005), suggesting that GABA agonism may be effective in the treatment of drug addiction. GABA agonists of the benzodiazepine class are currently used for the treatment of acute alcohol withdrawal and symptoms secondary to alcohol dependence, including anxiety (Lejoyeux et al., 1998).

Endocannabinoids function as retrograde signaling messengers and modulators of synaptic transmission. Cannabinoid agonists are capable of inducing relapse of heroin, alcohol, cocaine, and cannabinoid seeking (De Vries et al., 2002; Fattore et al., 2003; De Vries et al., 2003; Spano et al., 2004; McGregor et al., 2005). Recently, cannabinoid antagonists have received a lot of attention in drug seeking studies. The CB_1 receptor antagonist rimonabant (SR-141716A) has been reported to decrease cue-induced alcohol, nicotine, methamphetamine, and heroin seeking (Anggadiredja et al., 2004; Cippitelli et al., 2005; De Vries et al., 2005; Economidou et al., 2006) and priming dose -induced cannabinoid, methamphetamine, and heroin seeking (De Vries et al., 2003; Spano et al., 2004; Anggadiredja et al., 2004; Fattore et al., 2005). Rimonabant has been tested in

clinical trials with promising results for smoking cessation and the treatment of obesity (Gadde, 2005; Pi-Sunyer et al., 2006; Maldonado et al., 2006).

3 AIMS OF THE STUDY

Relapse is a major problem in the treatment of drug addiction. Dopamine and glutamate are suggested to be the main neurotransmitters involved in mediating the effects of drug re-exposure and drug-associated stimuli on drug-seeking behavior. Research has, however, largely concentrated on dopamine, and the role of glutamate has until recently received relatively little attention. Existing data suggests that drug-seeking behavior can be attenuated by dampening glutamatergic neurotransmission, but the role of different glutamate receptor subtypes in relapse induced by drug-associated cues has so far not been systematically explored. Also, it should be clarified whether the effects of glutamate transmission on drug seeking are universal or specific to a certain drug or drug group.

The specific aims of the present study were:

I To examine the role of glutamate receptor subtypes in cue-induced drug-seeking behavior using animal models

II To examine whether glutamate receptor antagonism similarly modulates alcohol- and cocaine-seeking behavior

III To examine, using glutamate receptor antagonists, whether glutamatergic neurotransmission in the nucleus accumbens is involved in cue-induced reinstatement of drug seeking

4 MATERIALS AND METHODS

4.1 Animals

Male Wistar rats (HsdCpb:Wu, Harlan, The Netherlands, or Martinsread, Germany) were used in the cocaine seeking and alcohol deprivation studies and male Long-Evans rats (HsdBlu:LE, Harlan Sprague Dawley, Indianapolis, USA) in the alcohol reinstatement studies. Rats weighed 160-180 g upon arrival to the animal facilities and were allowed an acclimatization period of at least 1 week prior to the experiments. The rats were housed in pairs in Eurostandard Type IV cages (transparent polycarbonate, dimensions 595 x 380 x 200 mm) in a temperature and humidity controlled room under a reversed 12-h light-dark cycle. Water and pellet food (RM1, SDS, Witham, UK) were available *ad libitum* in the home cage unless otherwise stated (see subsequent description). Behavioral testing was conducted during the dark phase of the light-dark cycle. All experimental procedures using animals were carried out under the National Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at the National Public Health Institute and the Chief Veterinarian of the County Administrative Board.

4.2 Drugs

Cocaine hydrochloride was obtained from the University Pharmacy (Helsinki, Finland) and dissolved in sterile 0.9% physiological saline. In the alcohol seeking studies, the 10% w/v alcohol solution was diluted from 94% w/v ethanol (Berner Oy, Helsinki, Finland) with tap water. MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine hydrogen maleate], CGP39551 [DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid carboxyethylester], D-AP5 [D-(-)-2-Amino-5-phosphonopentanoic acid], CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium), NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetra-hydrobenzo[f]quinoxaline-7-sulfonamide), (S)-3,4-DCPG [(S)-3,4-dicarboxyphenylglycine], and MPEP [2-methyl-6-(phenylethynyl) pyridine hydrochloride] were obtained from Tocris Cookson Ltd. (Bristol, UK). LY379268 [(-)-2-oxa-4-aminobicyclohexane-4,6-dicarboxylic acid] was a generous gift from Eli Lilly & Co. MK-801, CGP39551, (S)-3,4-DCPG, and LY379268 were dissolved in saline. CNQX and NBQX were dissolved in distilled water. L-701,324 and MPEP were suspended in propylene glycol, Tween 80, and saline (ratio 1:1:18). All systemically administered drugs were injected intraperitoneally (ip) in a volume of 1 ml/kg. For intracranial injections, CNQX, D-AP5, and MPEP were dissolved in sterile saline and administered in a volume of 0.5 µl/side.

4.3 Surgery and intracranial injections

Animals in the cocaine seeking studies (I, V, VI) were implanted with chronic jugular catheters under isoflurane anaesthesia. The catheter assembly was made in-house and

consisted of a 13-cm length of silastic tubing (inside diameter 0.31 mm; outside diameter 0.64 mm), attached to a guide cannula that was bent at a right angle. The cannula was embedded into a dental cement base and anchored with surgical polypropylene mesh. The catheters were implanted with the proximal end reaching the heart through the right jugular vein, continuing subcutaneously over the shoulder, and exiting between the scapulae. After surgery, catheters were flushed once a day for the next 10 days with a 0.05 ml infusion of an antibiotic (Oriprim, Orion Pharma, Espoo, Finland) to prevent infection and a 0.1 ml infusion of 0.9% saline containing heparin (30 IU/ml). Thereafter, the catheters were flushed daily with heparinized saline at the end of each self-administration session. If an abrupt increase or decrease was observed in the number of cocaine infusions earned by an individual animal during the session, catheter patency was tested by infusing the short-acting anesthetic methohexital (Brevimytal Natrium, Lilly Deutschland GmbH, Giessen, Germany) through the catheter. Animals with patent catheters exhibited a rapid loss of muscle tone within 2 s of the methohexital infusion.

Rats receiving intracranial injections (study VI) were implanted with bilateral 23 gauge stainless steel guide cannulas (10 mm in length) aimed at the nucleus accumbens core [anteroposterior +1.9 mm, mediolateral ±1.8 mm relative to bregma, dorsoventral –5.2 mm from the skull surface, coordinates according to Paxinos and Watson (1997)] under halothane anaesthesia. Cannulas were fastened to the skull with three stainless steel screws and dental cement and sealed with stylet wires (30 gauge) to prevent occlusion.

Rats were allowed at least one week of recovery from surgery before behavioral training and testing was continued.

Intra-accumbal injections were given through 30 gauge injector cannulas connected to 10 µl Hamilton syringes via polyethylene tubing. The injectors extended 2 mm below the guides. Injections were delivered in a volume of 0.5 µl/side over a period of 90 sec with a Razel infusion pump (Model A-99, Razel Scientific Instruments, Inc., Stamford, CT, USA) 15 min before behavioral testing. After the injections, the injectors were left in place for a 45-sec diffusion time. Animals were gently hand-held during the injections and were habituated to the procedure before testing. After completion of the experiments, rats were anesthetized, decapitated, and the brains were removed and stored in formaline. Coronal sections were sliced on a cryostat and stained with thionin for examination of injector tip locations.

4.4 Cocaine seeking studies (I, V, VI)

4.4.1 Apparatus

Experiments took place in operant chambers (Model ENV-112B, MED Associates, Georgia, VT, USA) enclosed in ventilated sound-attenuating cubicles. The chambers were equipped with a food hopper in the middle of the front panel. A food dispenser located behind the front panel delivered 45 mg Noyes pellets to the food hopper. Two

retractable levers were located on both sides of the food hopper. A white stimulus light was mounted above each lever and auditory stimuli were delivered from a speaker positioned on the front panel. Intravenous infusions were delivered at the volume of 0.1 ml by means of activating an infusion pump outside the sound-attenuating cubicle. The infusion pump was attached to a counterbalanced liquid swivel with Tygon tubing. From the swivel, Tygon tubing protected by a steel spring passed through a hole in the operant chamber and was connected to the catheter base at the midscapular region of the animal. Schedule contingencies and data collection were controlled by a computer using MED-PC behavioral software (MED Associates).

4.4.2 Food training

To facilitate acquisition of cocaine self-administration, rats were trained to lever press for food reinforcement before they were implanted with iv catheters. During training rats were restricted to 4 g of standard pellet food per day and trained under a fixed ratio 1 (FR1) schedule with a time-out (TO) duration of 1 s on both response levers. A lever press response resulted in the delivery of a 45 mg Noyes pellet (Formula "A/I", Research Diets Inc., New Brunswick, NJ, USA). Rats were trained during daily 60 min sessions until they earned 100 pellets during the session. Once rats had reached this criterion (2–4 sessions), they were returned to *ad libitum* food and implanted with iv catheters.

4.4.3 Cocaine seeking under a second-order schedule of self-administration (Study I)

One week after implantation of jugular catheters, rats were allowed to respond for a 0.1 ml intravenous infusion of cocaine (0.25 mg/infusion, dissolved in 0.9% physiological saline) under an FR1 TO 20-s schedule during daily 2-h sessions. At the beginning of each session, the house light was turned off and the two response levers were extended. Responding on the active lever resulted in an infusion that was signalled by a 20-s illumination of the stimulus light and a 3.5-s tone. Responses on the inactive lever were recorded but had no programmed consequences. Once animals had acquired reliable responding under the FR1 TO 20-s schedule, a second-order schedule of reinforcement of the type FR*x*(FR*y*:S) was introduced, where *x* was the number of conditioned stimulus (CS) presentations (1-s stimulus light and tone) required for the delivery of a cocaine infusion and *y* was the number of lever presses required for a CS presentation. Therefore, rats were presented a 1-s stimulus light and tone after *y* responses, and a 20-s stimulus light and a 3.5-s tone after completion of *x*, i.e., during each cocaine infusion. The schedule requirements were gradually increased as follows: FR1(FR2:S), FR1(FR3:S), FR1(FR5:S), FR1(FR7:S), FR2(FR5:S), FR3(FR5:S), FR4(FR5:S), FR5(FR7:S). When rats had reached the FR5(FR7:S) schedule or self-administered less than 10 infusions during the 2-h session, a second-order schedule consisting of two fixed intervals (FI) followed by a 2-hour period of self-administration under FR4(FR7:S) was introduced. The length of the fixed interval was gradually increased from 5 to 15 minutes. Thus, under the final reinforcement schedule, rats were allowed to respond for the first two cocaine infusions under an FI15min(FR7:S) schedule, and then take an

unlimited number of cocaine infusions under an FR4(FR7:S) schedule for 2 hours. The first interval measured drug seeking in the undrugged state whereas the second interval measured drug seeking under the influence of cocaine. The purpose of the latter schedule was to strengthen action-outcome associations and the importance of the CS in the maintenance of cocaine responding as well as measure cocaine taking. When rats had reached this schedule, the cocaine unit dose was increased to 0.5 mg/infusion as this dose results in higher response rates during the fixed interval component of the schedule.

The effects of the AMPA/kainate antagonist CNQX (0, 0.75, 1.5, 3 mg/kg ip, n = 11) administered in a within-subjects, Latin-square design 20 minutes before self-administration sessions on responding for cocaine were examined.

4.4.4 Cue-induced reinstatement of cocaine seeking (Studies V and VI)

The experiment consisted of three phases: conditioning, extinction, and reinstatement. As in study I, rats were allowed to respond for a 0.1 ml infusion of cocaine (0.25 mg/infusion, dissolved in 0.9% physiological saline) under an FR1 TO 20-s schedule during daily 2-h sessions. Once animals had acquired reliable responding under the FR1 TO 20-s schedule, a second-order schedule of reinforcement of the type FRx(FRy:S) was introduced, where x was the number of CS presentations (1-s stimulus light and tone) required for the delivery of a cocaine infusion and y was the number of lever presses required for a CS presentation. The schedule requirements were gradually increased as follows: FR1(FR2:S), FR1(FR3:S), FR1(FR5:S), FR1(FR7:S), FR2(FR5:S), FR3(FR5:S), FR4(FR5:S).

Extinction training began after 4 to 6 days of cocaine self-administration under the FR4(FR5:S) schedule. During the extinction phase, responding had no programmed consequences. Extinction sessions continued until no further trend for either increased or decreased responding was seen in the group mean for at least 5 consecutive sessions.

Reinstatement testing began on day 1 post-extinction. During the first minute of a reinstatement session the cocaine-associated light and auditory stimuli were presented 6 times non-contingently (once every 10 seconds). Then the response levers were extended into the self-administration chamber and the cocaine-associated stimulus complex was contingently available under the FR4(FR5:S) schedule that was used during the conditioning phase, but no cocaine was available. Reinstatement sessions were conducted twice a week with the rats remaining in their home cages on intervening days.

In order to exclude the possibility that responding during reinstatement sessions was induced by the intermittent testing schedule and not by presentations of the cocaine-associated stimulus, a group of rats (n = 7) with reliable reinstatement baselines was tested during seven consecutive twice-a-week reinstatement sessions under extinction conditions.

The effects of the competitive NMDA antagonist CGP 39551 (0, 2.5, 5, 10 mg/kg, n = 8), the NMDA/glycine antagonist L-701,324 (0, 0.63, 1.25, 2.5 mg/kg, n = 10), the AMPA/kainate antagonists CNQX (0, 0.75, 1.5, 3 mg/kg, n = 8) and NBQX (0, 1.25,

2.5, 5 mg/kg, n = 7), and the mGluR5 antagonist MPEP (0, 1.25, 2.5, 5 mg/kg, n = 10) on cue-induced reinstatement of cocaine seeking were examined. The drugs were administered intraperitoneally 20 minutes before behavioral testing in a within-subjects Latin-square design.

To examine the involvement of the nucleus accumbens in cue-induced cocaine seeking, the effects of the AMPA/kainate antagonist CNQX (0, 0.01, 0.03 µg/side, n = 6), the competitive NMDA antagonist D-AP5 (0, 1, 2 µg/side, n = 8), and the mGluR5 antagonist MPEP (0, 0.5, 1 µg/side, n = 9) administered into the accumbens core on reinstatement responding were examined. The antagonists were administered 15 minutes before testing in a within-subject, Latin-square design. Responding was allowed to return to baseline levels between injections.

4.5 Alcohol seeking studies (II, III, IV)

4.5.1 Apparatus

All operant sessions took place in operant chambers (Lafayette Instrument, Lafayette, IN) enclosed in ventilated sound-attenuating cubicles. The front panel of each chamber was equipped with two response levers and two drinking cups between the levers. A blue stimulus light was mounted above the right response lever. Auditory stimuli (2.9 kHz, 65 dB) were delivered from a loudspeaker positioned on top of the self-administration chamber. Responses at the appropriate lever activated a syringe pump that delivered a 0.1-ml drop of fluid to one of the two drinking cups. MED-PC behavioral software (MED Associates Inc., Georgia, VT) was used for controlling the operant chambers and collecting data.

4.5.2 Operant alcohol self-administration training

Rats were trained to orally self-administer ethanol by using a modification of a training protocol described previously by Samson (1986). Briefly, rats were deprived of water for 12 h prior to training sessions for three consecutive days and were trained to respond for a 0.1-ml drop of 0.2% (w/v) saccharin solution on both levers under a fixed ratio 1 (FR1) schedule of reinforcement. After this initial training, water deprivation was terminated, and animals had free access to food and water in their home cages throughout the subsequent training and testing. Non-deprived rats were given two additional saccharin sessions to confirm that they had acquired responding for saccharin before ethanol self-administration training started. Then, during the next three sessions, responses at the right lever resulted in the delivery of 0.1 ml of 5% (w/v) ethanol + 0.2% saccharin solution. Responses at the left lever were recorded but had no programmed consequences. Thereafter, the concentration of ethanol was increased first to 8% and then to 10% w/v and the concentration of saccharin was decreased until saccharin was eliminated completely from the drinking solution.

4.5.3 Operant alcohol self-administration (Study IV)

The final schedule of reinforcement for the 10% w/v ethanol concentration was similar to the training schedule except that a stimulus light was added. Thus, during the 30-min sessions responses on the active lever resulted in the delivery of 0.1 ml of ethanol and, in addition, in the illumination of the stimulus light for 3 s. The left lever remained inactive.

When rats had reached stable ethanol self-administration under these conditions, the effects of the mGlu2/3 receptor agonist LY379268 (0, 1, 3, 5 mg/kg ip, n = 10) and the mGlu8 receptor agonist (S)-3,4-DCPG (0, 5, 10, 15 mg/kg ip, n = 8) on ethanol self-administration were examined in a Latin-square, within-subjects design. The agonists were administered 30 min (LY379268) or 15 min [(S)-3,4-DCPG] before start of the self-administration session.

4.5.4 Cue-induced reinstatement of alcohol seeking (Studies II, III and IV)

During the conditioning phase, rats self-administered 10 % w/v alcohol and 80 µM quinine hydrochloride during 30-min sessions on semi-randomly alternating days. Olfactory discriminative stimuli (S^D) predicting alcohol or quinine availability were presented during the sessions. Alcohol availability (S^+) was signaled by anise odor (trans-anethole, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), whereas quinine availability (i.e. non-reward) was signaled by citrus odor [S^-, (R)-(+)-limonene, Sigma-Aldrich Chemie GmbH, Steinheim, Germany]. The olfactory stimuli were generated by placing a small piece of absorbent paper containing a drop of either anethole or limonene next to the self-administration chamber inside the sound-attenuation cubicle immediately before the start of the session. In addition to these discriminative stimuli, each alcohol delivery was accompanied by a 3-s light stimulus (CS^+), while a 3-s auditory stimulus (CS^-) was presented with quinine delivery. During the first week of the 7-week conditioning phase rats were given alcohol sessions only. Thereafter, alcohol and quinine sessions were given in a semi-random order until rats received a total of 18 alcohol and 17 quinine sessions.

The conditioning phase was followed by extinction training. During 30-min extinction sessions, responding had no programmed consequences, the olfactory stimuli signaling alcohol or quinine availability were withheld, and the liquid delivery lines remained empty. Extinction sessions continued until no further trend for either increased or decreased responding was seen in the group mean for at least five consecutive sessions.

Reinstatement sessions began on day 1 post-extinction. During the first session, rats were presented with the S^D predictive of alcohol non-availability (i.e. quinine availability, citrus odor), and responses on the previously active lever resulted in a 3-s presentation of the auditory stimulus (CS^-) and activation of the syringe pump motor, but not in the delivery of any drinking solution. During the next two sessions rats were tested for alcohol seeking under two conditions: with the alcohol-associated stimuli (S^+/CS^+) present and with the alcohol-associated stimuli accompanied by a response-

contingent 0.2 ml priming dose of 10% w/v alcohol (S^+/CS^+/priming). Both sessions started with the presentation of the alcohol-associated S^D (anise odor) and active lever responses turned on the syringe pump motor and the alcohol-associated CS^+, the 3-s light. In addition, during the alcohol priming sessions, the first two lever responses produced 0.1 ml of alcohol solution to the drinking cup. This provided rats with two additional stimuli, namely the taste and smell of ethanol that had been present during the conditioning, but not the extinction phase. Half of the rats were tested under the S^+/CS^+ condition on day 2 postextinction and the S^+/CS^+/priming condition on day 5 postextinction. On intervening days rats remained in their home cages. The conditions were reversed for the other half of the animals. Thereafter, reinstatement sessions were conducted twice a week (on Tuesdays and Fridays) under the S^+/CS^+/priming condition. The rats remained in their home cages on intervening days.

The effects of the uncompetitive NMDA receptor antagonist MK-801 (0, 0.5, 0.15 mg/kg i.p., $n = 10$), the competitive NMDA antagonist CGP39551 (0, 5, 10 mg/kg i.p., $n = 8$), the NMDA/glycine antagonist L-701,324 (0, 2, 4 mg/kg i.p., $n = 9$), the AMPA/kainate antagonist CNQX (0, 0.5, 1.5 mg/kg i.p., $n = 10$), the mGluR5 antagonist MPEP (0, 1, 3, 10 mg/kg i.p., $n = 10$), the mGluR2/3 agonist LY379268 (0, 1, 3, 5 mg/kg i.p., $n = 12$), and the mGluR8 agonist (S)-3,4-DCPG (0, 5, 10, 15 mg/kg i.p., $n = 9$) on alcohol-seeking behavior were examined in a Latin-square, within-subjects design under the S^+/CS^+/priming condition described above. The pretreatment times were 30 min for MPEP and LY379268, 20 min for MK-801, CGP39551, L-701,324 and CNQX, and 15 min for (S)-3,4-DCPG. On the reinstatement session preceding the first drug pretreatment session rats were administered a saline injection (1 ml/kg i.p.) 15-30 min before testing to habituate them to the injection procedure.

4.5.5 Alcohol relapse measured by the alcohol deprivation effect (Study II)

Rats in the alcohol deprivation experiments were given *ad libitum* access to tap water, 5%, 10%, and 20% (v/v) alcohol in their home cages. The positions of the water and alcohol bottles were changed weekly to avoid location preferences. After 8 weeks of continuous alcohol access, a schedule of 4 weeks of alcohol access followed by two weeks alcohol non-access (deprivation) was introduced. On the last three days of alcohol access, the bottles and rats were weighed daily for the calculation of alcohol and water consumption. At the beginning of the deprivation phase all alcohol bottles were removed from the cages leaving the animals with only water and food. After 14 days, the alcohol solutions were returned to the animals and the bottles and rats were again weighed daily for 4 days.

The effects of MPEP on alcohol drinking and deprivation were measured in rats with 12 months of experience of repeated cycles of alcohol access and deprivation. For evaluating the effects of MPEP on baseline drinking rats were divided into three groups ($n = 10$) and administered MPEP (0, 3 or 10 mg/kg ip) for three subsequent days before the alcohol deprivation phase. The effects of MPEP on the alcohol deprivation effect were examined in rats injected with MPEP on the last two days of the alcohol deprivation period as well as during the first day of alcohol re-access. To ensure that

previous MPEP treatment during baseline drinking did not influence measurements of the alcohol deprivation effect different groups received different treatments during baseline and alcohol deprivation drinking.

4.6 Sucrose self-administration (Study VI)

In order to assess the effects of intra-accumbally administered glutamate receptor antagonists on the ability to lever-press and also to determine whether the antagonists attenuate responding for a non-drug reinforcer, a group of rats was trained to self-administer 45-mg sucrose pellets (Noyes precision pellets PJFSC-0045, Research Diets Inc., New Brunswick, NJ, USA). Initially, rats were trained during 2-h sessions under an FR1 schedule on the right lever. Responding on the left lever had no programmed consequences. The response requirement was then increased to FR2, FR3, and FR5. Thereafter, a variable interval 30 s (VI30s) schedule followed by a VI60s schedule was introduced. No stimuli were associated with the delivery of pellets. The VI60s schedule was chosen because under it rats displayed similar sustained responding throughout the 2-hour session as seen during reinstatement sessions. When responding had stabilized under the VI60s schedule (i.e. the number of responses varied less than 20% and the number of pellets earned varied less than 10% over three days) rats were administered CNQX (0, 0.03 µg/side, $n = 8$), D-AP5 (0, 2 µg/side, $n = 7$), or MPEP (0, 0.5, 1 µg/side, $n = 6$) into the nucleus accumbens core 15 minutes before testing in a within-subjects, Latin square design. The effects of CNQX and D-AP5 were tested in the same group of rats ($n = 8$) in a within-subjects, Latin-square design, while MPEP was tested in a separate group ($n = 6$) because previous reinstatement tests had indicated possible conditioned aversive effects of MPEP. Responding was always allowed to return to baseline levels and stabilize between injections.

4.7 Locomotor activity (Studies I-VI)

As a separate control experiment, spontaneous locomotor activity was measured after pretreatment with glutamate receptor agonists and antagonists. This was to clarify whether the observed changes in operant responding were caused by effects on motor performance, e.g. drug-induced sedation.

Locomotor activity was measured in transparent Eurostandard Type III cages (transparent polycarbonate, dimensions 43 x 27 x 19 cm) that were placed inside photocell frames (Cage Rack Activity System, San Diego Instruments, CA, USA). The frames were equipped with seven pairs of photocells (5 cm off the cage floor) for measuring horizontal activity and eight pairs of photocells (12 cm off the floor) for measuring vertical activity. The number of photocell interruptions was recorded by a computer at 5-min intervals for 2 hours. During the first three sessions, rats were habituated to the test cages, and, on the third day, also administered with a habituation saline injection. Thereafter, rats were injected intraperitoneally with CGP39551 (0, 2.5, 5, 10 mg/kg), L-701,324 (0, 0.63, 1.25, 2.5 mg/kg), CNQX (0, 0.75, 1.5, 3 mg/kg),

NBQX (0, 1.25, 2.5, 5 mg/kg) or MPEP (0, 1.25, 2.5, 5 mg/kg) or intra-accumbally with CNQX (0, 0.03 µg/side), D-AP5 (0, 2 µg/side), or MPEP (0, 0.5, 1 µg/side) in a within-subjects Latin-square design 15-30 minutes before testing (n = 8 for each antagonist).

4.8 Statistical analysis

The effects of the glutamate receptor agonists and antagonists on alcohol and cocaine seeking, locomotor activity, and sucrose self-administration were analyzed with a within-subjects one-way analysis of variance (ANOVA) with repeated measures on dose. Following a significant main effect of dose, each individual drug dose was compared with the vehicle condition using a post hoc means comparison with Bonferroni correction. In the reinstatement experiments, paired t tests were used for demonstrating reinstatement of responding compared with the extinction baseline and for testing the stability of responding by comparing the vehicle injection with non-injection baseline sessions.

In the alcohol deprivation experiment, treatment effects on postabstinence days were analyzed with a two-way ANOVA with repeated measures (treatment x days). For *post hoc* comparisons, a Newman-Keuls test was applied when appropriate.

In all statistical analyses, criterion for significance was set at the 0.05 level.

5 RESULTS

5.1 Reinstatement of cocaine-seeking behavior by cocaine-associated cues

During the last three conditioning (cocaine self-administration) sessions of the experiment, the mean (±SEM) numbers of responses on the active and inactive lever, respectively, were 409.0±29.4 and 10.0±7.0 (Study IV). Following extinction training, responding decreased to a level of 14.8±1.3 on the previously active and 4.7±0.8 on the previously inactive lever. Re-introduction of the cocaine-associated stimulus complex reinstated responding on the active lever ($p<0.05$ compared to extinction baseline) and maintained responding reliably during repeated test sessions conducted twice a week (Figure 1). When the cocaine-associated stimulus complex was omitted in a separate group of rats, responding decreased rapidly towards extinction levels ($p<0.0001$) showing that responding was maintained by the cocaine-associated stimulus complex and not the intermittent testing pattern (Figure 2).

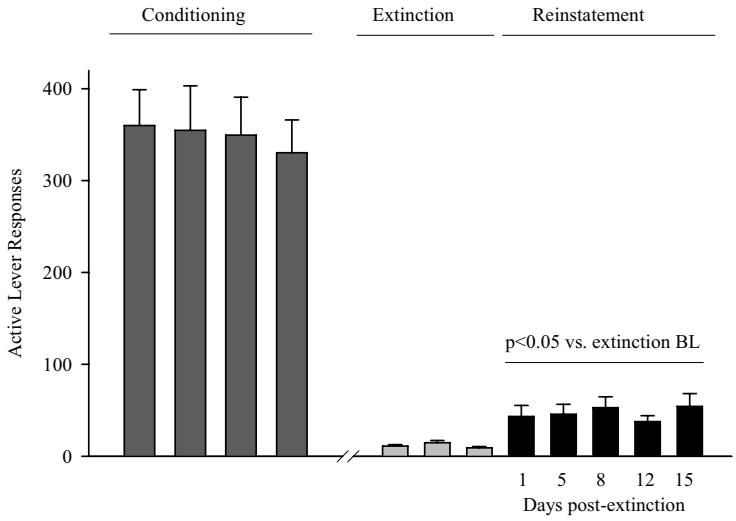

Figure 1. ***Conditioning, extinction, and reinstatement phases during the cocaine reinstatement experiment (n = 15). The mean (±SEM) number of responses on the active lever is shown. For the conditioning and extinction phases, the last four and three days of responding are shown, respectively. Re-introduction of the cocaine-associated stimulus complex reinstated and maintained responding above extinction levels throughout the 15-day testing period.***

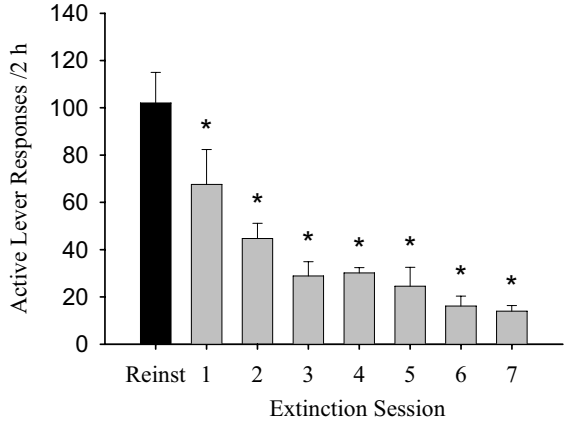

Figure 2. ***Effects of omission of the cocaine-associated conditioned stimulus on reinstatement of cocaine seeking (n =7) . Shown is the mean (±SEM) number of responses on the active lever during the reinstatement baseline ("Reinst"; stimulus complex available response-contingently) and seven consecutive extinction sessions (stimulus complex omitted) conducted every second or third day. * p < 0.05, significantly different from the reinstatement baseline.***

5.2 Reinstatement of alcohol-seeking behavior by alcohol-associated cues

During the conditioning phase of the experiment, rats developed stable responding for alcohol and quinine. The average number of alcohol responses was, depending on study, from 26.2±1.2 to 32.0±3.6 on the active lever and from 2.3±0.4 to 6.4±1.7 on the inactive lever, whereas quinine responses ranged from 9.6±0.6 to 11.9±2.0 responses on the active lever and from 4.5±0.7 to 9.9±2.2 on the inactive lever. Thus, rats discriminated well between alcohol and quinine. The average ethanol intake was 0.53-0.64 g/kg per session.

During the extinction phase responding decreased to a low level (from 4.8±0.5 to 6.3±0.7 responses on the previously active lever and from 2.5±0.3 to 4.6±0.6 responses on the previously inactive lever). During the alcohol reinstatement sessions, the alcohol-associated stimuli (S^+/CS^+) reinstated responding on the active lever ($p<0.05$ compared to last three extinction sessions), whereas the quinine-associated stimuli (S^-/CS^-) decreased responding below extinction levels (Figure 3). Responding during the reinstatement session that included the small (0.2 ml) response-contingent priming dose of 10% w/v alcohol was significantly higher compared to the session with alcohol-associated stimuli alone (Figure 3). The 0.2 ml alcohol priming resulted in an average alcohol dose of 0.03 g/kg, which all rats consumed during the session. Inactive lever responding did not differ from extinction levels under any of the reinstatement conditions.

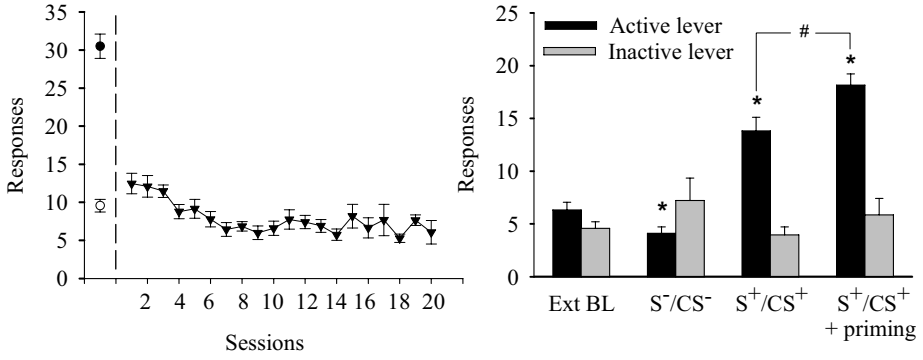

Figure 3. ***Left: Lever-press responses on the active lever during 30-min conditioning and extinction sessions (n = 24, Study II). For the conditioning sessions, the mean (±SEM) number of responses averaged over the last three self-administration sessions is shown. Extinction responses represent the mean (±SEM) number of responses during each successive extinction session. Inactive lever responses have been omitted for clarity. Right: Mean (±SEM) number of responses during the last three extinction sessions (Ext BL), the reinstatement session with stimuli previously associated with nonreward, i.e. quinine (S^-/CS^-), and the alcohol reinstatement sessions during which the rats were reintroduced to either the alcohol-associated stimuli alone (S^+/CS^+) or the alcohol-associated stimuli together with a priming dose of alcohol (S^+/CS^++priming). * p < 0.05, significantly different from extinction baseline.***

5.3 Effects of systemic ionotropic glutamate receptor antagonism on drug seeking

5.3.1 Reinstatement of cocaine seeking (Study V)

Cue-induced reinstatement of cocaine seeking was attenuated by systemic administration of the NMDA/glycine antagonist L-701,324 and the AMPA/kainate antagonists CNQX and NBQX (p´s<0.01). The competitive NMDA antagonist CGP39551 failed to alter reinstatement responding. Inactive lever responding was not affected by the pretreatments (Figure 4).

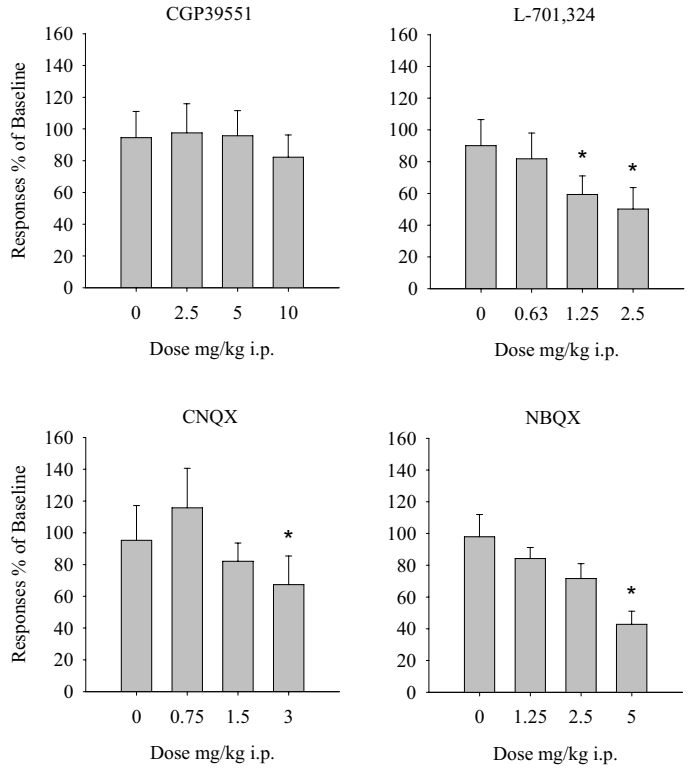

Figure 4. ***Effects of pretreatment with the ionotropic glutamate receptor antagonists CGP39551 (n = 8), L-701,324 (n = 10), CNQX (n = 8), and NBQX (n = 7) on cue-induced reinstatement of cocaine-seeking behavior during the 2-h session. Responses are expressed as the percentage (mean ± SEM) of the mean number of responses during three vehicle injection sessions that preceded the drug pretreatment sessions. * p < 0.05, significantly different from vehicle following a significant main effect of dose in ANOVA.***

5.3.2 Cocaine seeking under a second-order schedule of reinforcement (Study I)

The AMPA/kainate antagonist CNQX suppressed cocaine seeking during the first interval of the fixed interval, second-order schedule of self-administration ($p<0.001$), but did not alter responding during the second interval, or the number of cocaine infusions during the latter part of the session under the FR4(FR7:S) schedule. Inactive lever responding was not affected (Figure 5).

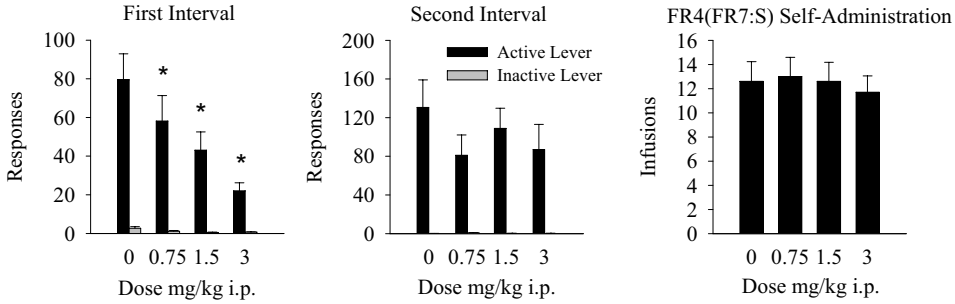

Figure 5. ***Effects of pretreatment with the AMPA/kainate antagonist CNQX (n = 10) on cocaine seeking under a second-order schedule of reinforcement. Shown is the mean (± SEM) number of responses during the first 15-minute interval measuring cocaine seeking in the undrugged state and the second 15-minute interval measuring cocaine seeking under the influence of the drug. For the 2-hour period, during which cocaine was freely available under an FR4(FR7:S) schedule, the mean (± SEM) number of cocaine infusions is shown. * p < 0.05, significantly different from vehicle following a significant main effect of dose in ANOVA.***

5.3.3 Reinstatement of alcohol seeking (Study III)

Administration of the NMDA/glycine antagonist L-701,324 ($p<0.05$) and the AMPA/kainate antagonist CNQX ($p<0.05$) decreased reinstatement induced by the alcohol-associated stimuli. The competitive NMDA antagonist CGP39551 and the noncompetitive NMDA antagonist MK-801 did not alter reinstatement responding (Figure 6). Inactive lever responding was not affected with the exception of L-701,324, which increased responding ($p<0.05$). This effect was however not detected by the post hoc test.

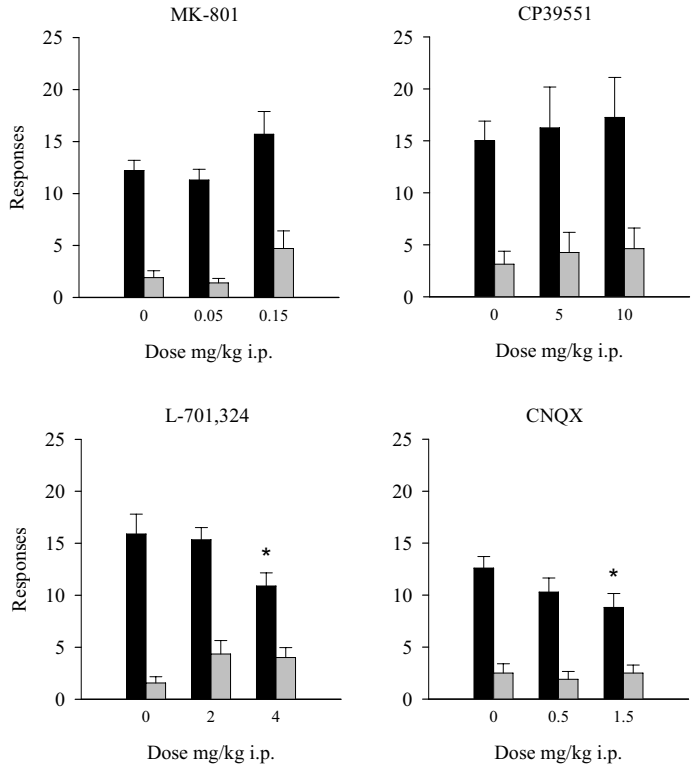

Figure 6. ***Effects of pretreatment with the ionotropic glutamate receptor antagonists MK-801 (n = 10), CGP39551 (n = 8), L-701,324 (n = 9), and CNQX (n = 10) on alcohol-seeking behavior induced by alcohol associated stimuli (S^+/CS^+) and a response-contingent 0.2-ml alcohol priming dose. Shown is the mean (± SEM) number of responses during the 30-min reinstatement sessions. * p < 0.05, significantly different from vehicle following a significant main effect of dose in ANOVA.***

5.4 Effects of systemic metabotropic glutamate receptor agonism and antagonism on drug seeking

5.4.1 Reinstatement of cocaine seeking (Study V)

Systemic administration of the mGluR5 antagonist MPEP decreased cue-induced reinstatement of cocaine seeking on the active lever ($p<0.001$) (Figure 7). Also inactive lever responding was decreased ($p<0.05$) although the effect did not reach significance in the post hoc test. In addition, a decrease in responding compared to reinstatement

baseline was observed after pretreatment with the saline vehicle included in the within-subjects Latin-square injection series ($p<0.05$).

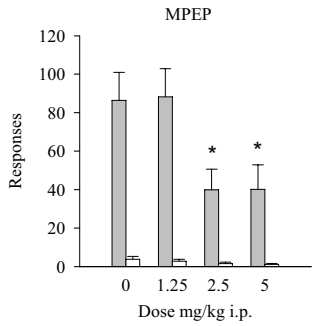

Figure 7. ***Effects of pretreatment with the mGluR5 antagonist MPEP (n = 10) on cue-induced reinstatement of cocaine-seeking behavior. Shown is the mean (± SEM) number of responses during the 2-h session. * p < 0.05, significantly different from vehicle following a significant main effect of dose in ANOVA.***

5.4.2 Reinstatement of alcohol seeking (Studies II and IV)

Administration of the mGluR2/3 agonist LY379268 ($p<0.05$) and the mGluR8 agonist (S)-3,4-DCPG ($p<0.0001$) as well as the mGluR5 antagonist MPEP ($p<0.0001$) decreased reinstatement induced by alcohol-associated stimuli (Figure 8). LY379268 and (S)-3,4-CDPG did not affect inactive lever responding, but with MPEP a decrease was observed ($p<0.05$).

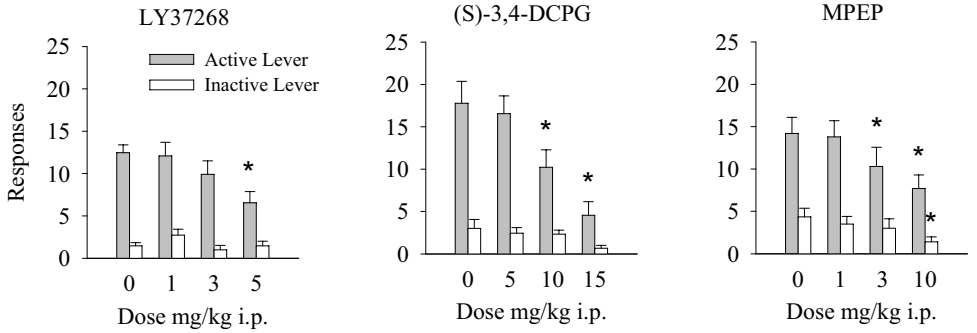

Figure 8. ***Effects of pretreatment with the mGluR2/3 agonist LY379268 (n = 12), the mGluR8 agonist (S)-3,4-DCPG (n = 9), and the mGluR5 antagonist MPEP (n = 10) on alcohol-seeking behavior induced by alcohol associated stimuli (S^+/CS^+) and a response-contingent 0.2-ml alcohol priming dose. Shown is the mean (± SEM) number of responses during the 30-min reinstatement sessions. * p < 0.05, significantly different from vehicle following a significant main effect of dose in ANOVA.***

5.4.3 Alcohol deprivation effect (Study II)

The basal alcohol intake of the rats in the alcohol deprivation experiment was 2.9±0.15 g/kg ethanol per day. MPEP decreased basal alcohol consumption compared to the saline control group ($p<0.05$). This was however mainly due to an increase in alcohol consumption of the control group.

After two weeks of abstinence (alcohol deprivation), an increase in alcohol consumption, characteristic to the alcohol deprivation effect, was observed ($p<0.001$). MPEP administration decreased alcohol intake ($p<0.05$) without any significant effect on body weight or water intake (Figure 9).

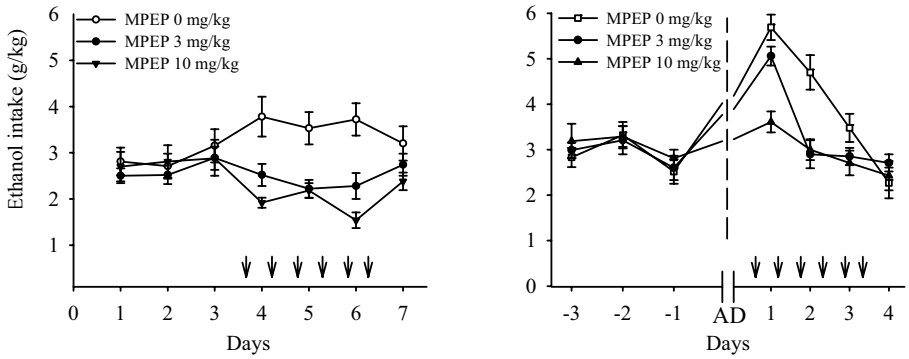

Figure 9. ***Effects of MPEP on baseline drinking (left) and the alcohol deprivation effect (right) (n = 10 per group). Shown is the mean daily alcohol intake in grams of ethanol/kg bodyweight (±SEM). Arrows indicate MPEP injections. Measurements were taken during the last 3 days before the 14-day alcohol deprivation (AD) period and the first 4 days after re-introduction of alcohol. Alcohol consumption on baseline days 4-6 and postdeprivation days 1-3 was significantly attenuated by MPEP compared to the saline control group.***

5.4.4 Alcohol self-administration (Study IV)

After 28 sessions of alcohol self-administration with 10% w/v ethanol rats showed an average (±SEM) number of 26.7±1.9 responses on the active lever and 4.2±1.5 responses on the inactive lever. This corresponded to an average ethanol intake of 0.59±0.04 g/kg. In another study with Long-Evans rats this alcohol amount was found to result in blood alcohol levels ranging from 3 to 43 mg/100 ml when measured immediately after alcohol access (Czachowski et al., 1999).

The mGluR2/3 agonist LY379268 and the mGluR8 agonist (S)-3,4-DCPG suppressed alcohol self-administration ($p<0.05$ and $p<0.01$, respectively). Inactive lever responding was not significantly affected by the agonists (Figure 10).

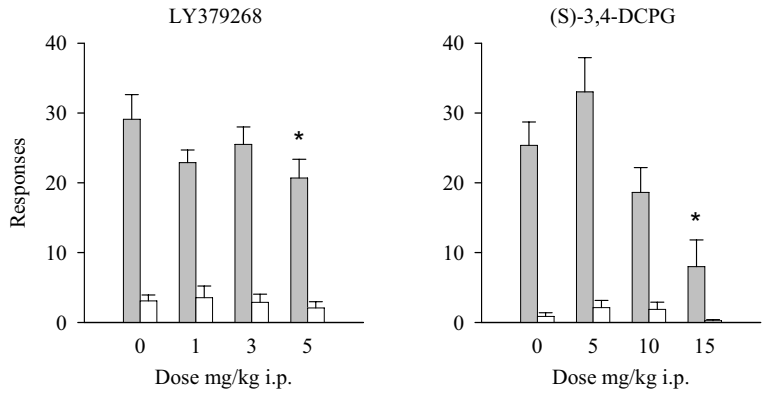

Figure 10. ***Effects of pretreatment with the mGluR2/3 agonist LY379268 (n = 10) and the mGluR8 agonist (S)-3,4-DCPG (n = 8) on alcohol self-administration. The mean (±SEM) number of responses during the 30-min self-administration sessions is shown. * p < 0.05, significantly different from the vehicle following a significant main effect of dose in ANOVA.***

5.5 Effects of intra-accumbal glutamate receptor antagonism on cocaine seeking (Study VI)

Following intra-accumbal administration of the NMDA antagonist D-AP5 and the AMPA/kainate antagonist CNQX, responding during reinstatement sessions remained stable during the period of testing as confirmed by paired *t* tests conducted separately for each antagonist between the vehicle dose and the reinstatement baseline ($p´s>0.05$). With the mGluR5 antagonist MPEP, however, responding during the vehicle session was decreased compared to baseline reinstatement sessions ($p<0.05$). D-AP5 and CNQX ($p´s<0.01$), but not MPEP ($p>0.05$), decreased responding compared to the vehicle condition (Figure 11). When compared to the reinstatement baseline, responding was suppressed following MPEP treatment ($p<0.001$). None of the antagonists affected inactive lever responding.

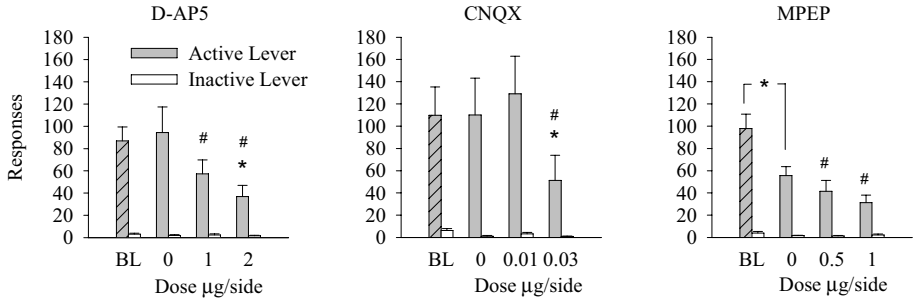

Figure 11. ***Effects of intra-accumbal pretreatment with the NMDA antagonist D-AP5 (n = 8), the AMPA/kainate antagonist CNQX (n = 6), and the mGluR5 antagonist MPEP (n = 9) on cue-induced reinstatement of cocaine-seeking behavior. Shown is the mean (± SEM) numbers of responses during the 2-h session. * p < 0.05, significantly different from the vehicle; # p < 0.05, significantly different from reinstatement baseline (BL, responding averaged over 3 sessions preceding any drug treatments).***

Compound	Ethanol	Cocaine	
	Systemic	Systemic	N. Acc.
Competitive NMDA antagonist CGP39551	No effect	No effect	-
Competitive NMDA antagonist D-AP5	-	-	↓
Noncompetitive NMDA antagonist MK-801	No effect	-	-
NMDA/glycine antagonist L-701,324	↓	↓	-
AMPA/kainate antagonist CNQX	↓	↓	↓
AMPA/kainate antagonist NBQX	-	↓	-
mGluR5 antagonist MPEP	↓	↓	↓
mGluR2/3 agonist LY379268	↓	-	-
mGluR8 agonist (S)-3,4-DCPG	↓	-	-

Table 2. ***Effects of glutamate receptor agonists and antagonists on alcohol and cocaine seeking following systemic or intra-accumbal (N.Acc.) administration. Significant decrease: ↓ , Not tested: - .***

5.6 Spontaneous locomotor activity

The mGluR2/3 agonist LY379268 and the mGluR8 agonist (S)-3,4-DCPG attenuated spontaneous locomotor activity (p´s<0.05). The NMDA/glycine antagonist L-701,324, the AMPA/kainate antagonists CNQX and NBQX, or the mGluR5 antagonist MPEP did not suppress locomotor activity after systemic administration. Similarly, the NMDA antagonist D-AP5, CNQX, and MPEP did not alter locomotor activity after intra-accumbal administration.

5.7 Sucrose self-administration

The mean (± SEM) number of responses during a habituation saline injection session was 2254.64 ± 510.10 on the active and 3.43 ± 1.41 on the inactive lever. This resulted in the delivery of 99.36 ± 4.61 sucrose pellets. D-AP5, CNQX, or MPEP did not affect either active lever responding or the number of pellets earned. Inactive lever responding was however increased by D-AP5.

6 DISCUSSION

6.1 Reinstatement of cocaine seeking by cocaine-associated cues

In the cocaine reinstatement model used in the present study, cocaine seeking was induced after extinction by reintroduction of a cocaine-associated stimulus complex. This is in agreement with previous studies showing that cocaine-paired discrete and discriminative cues are capable of reinstating cocaine-seeking behavior (Meil and See, 1996; Arroyo et al., 1998; Weiss et al., 2000; Alleweireldt et al., 2002).

The present experiments employed a second-order schedule of reinforcement to pair a stimulus complex consisting of a light and a tone with cocaine during the conditioning phase. Compared to simple ratio schedules, second-order schedules have been suggested to enhance the development of associations between actions and their outcomes and amplify the role of conditioned stimuli in exerting control over responding (Everitt and Robbins, 2000). Compound stimuli were chosen, because they have been shown to result in more robust reinstatement than single stimuli (See et al., 1999).

The conditioning phase was followed by extinction training, during which neither cocaine nor the stimulus complex were available. This resulted in a gradual decrease of responding to a very low level. During subsequent reinstatement sessions, noncontingent stimulus presentations followed by contingent access to the stimulus reinstated responding on the previously cocaine-paired lever. The cocaine-associated stimulus complex maintained responding above extinction levels during repeated reinstatement testing conducted twice a week.

One concern with the present reinstatement model is that the intermittent testing pattern could alone have resulted in reinstatement-like responding, as extinguished lever pressing has been shown to recover spontaneously with time after termination of extinction training (See et al., 1999; Bouton, 2002; Di Ciano and Everitt, 2002). However, when a group of rats was tested twice a week in the absence of the cocaine-associated stimulus complex, i.e. under extinction conditions, responding was significantly lower than during reinstatement sessions. This shows that responding in the present model was maintained by and under the control of the cocaine-associated stimulus complex.

6.2 Reinstatement of alcohol seeking by alcohol-associated cues

In the alcohol reinstatement studies a procedure with both discrete and discriminative stimuli, which is often utilized in alcohol seeking studies, was employed (Katner et al., 1999; Ciccocioppo et al., 2001). During the conditioning phase, one set of stimuli was paired with alcohol reward and another set of stimuli with alcohol non-reward. Following extinction, the alcohol-associated stimuli were found to selectively induce

reinstatement of responding. Responding was further enhanced when the animals were allowed to self-administer a small amount of response-contingent alcohol at the beginning of the reinstatement session. This finding is in line with previous studies showing that responding for alcohol can be reinstated by very small alcohol amounts (Chiamulera et al., 1995; Bienkowski et al., 1999)

Due to the small size of the priming dose it is unlikely that the pharmacological effects of alcohol contributed to reinstatement. There are, however, other reasons why the priming dose might have increased alcohol seeking. First, the priming dose allowed the animals to experience the taste and smell of alcohol. The extinction sessions had been carried out without alcohol access and with the fluid delivery lines of the apparatus empty, so the taste and smell were never extinguished. It is very likely that during conditioning sessions both the taste and smell of alcohol were incorporated in the compound stimulus signaling alcohol availability and reward. Thus, presenting the complete compound stimulus during reinstatement instead of only the S^+ (anise odor) and CS^+ (light) components might have produced stronger reinstatement (See et al., 1999). Second, it may be that simply the act of consuming a fluid contributed to reinstatement. It was shown by Bienkowski et al. (2000) that responding could be reinstated by presentations of a liquid dipper containing either alcohol or water, but not an empty dipper, suggesting that the orosensory properties of the fluid made available are alone capable of inducing reinstatement. In fact, we discovered later that the 0.2 ml alcohol priming dose used in the present study is capable of reinstating alcohol seeking to similar levels as the alcohol-associated stimuli (anise odor and light) presented alone (unpublished observation).

6.3 Effects of systemic ionotropic glutamate receptor antagonism

In the studies examining the effects of systemic ionotropic glutamate receptor antagonism on drug seeking, the NMDA/glycine antagonist L-701,324 and the AMPA/kainate antagonist CNQX decreased both cue-induced alcohol and cocaine seeking. In contrast, the competitive NMDA antagonist CGP39551 did not significantly affect reinstatement responding. In addition to these antagonists that were tested in both the cocaine and alcohol models, the AMPA/kainate antagonist NBQX was found to decrease cue-induced cocaine reinstatement whereas the uncompetitive NMDA antagonist MK-801 was ineffective in modulating alcohol reinstatement. In line with the findings from the reinstatement studies, cocaine seeking measured by a fixed-interval, second-order schedule of self-administration was also decreased by CNQX.

None of the glutamate receptor antagonists decreased spontaneous locomotor activity in control experiments, suggesting that the observed attenuation in reinstatement responding was not due to motor suppression. Also, examination of cumulative response patterns showed that rats responded at a stable level throughout the session, and the effects of antagonist pretreatments became visible slowly during the session without affecting initiation of responding. These findings are in agreement with previous data that L-701,324, CNQX, and NBQX do not show severe impairment of locomotion in the

dose range used in the present study (Danysz et al., 1994; Dalia and Wallace, 1995; Bristow et al., 1996; Mead and Stephens, 1999).

In the present study, alcohol- and cocaine-seeking behavior was found to be decreased by AMPA/kainate antagonism and NMDA antagonism of the glycine site, but not other binding sites of the NMDA receptor complex. Previously, systemically administered AMPA receptor antagonists have been found to attenuate the expression of conditioned and drug-related behaviors such as conditioned place preference and behavioral sensitization (Cervo and Samanin, 1995; Tzschentke and Schmidt, 1997; Jackson et al., 1998; Mead and Stephens, 1998; but see Li et al., 1997; Mead and Stephens, 1999), whereas NMDA antagonists seem to differ in their ability to modulate these behaviors (Bespalov, 1996; Kotlinska and Biala, 1999; Mead and Stephens, 1999; Kotlinska and Biala, 2000; Papp et al., 2002; Popik et al., 2003). When administered systemically in reinstatement studies, the AMPA antagonist GYKI52466 has been found to reduce cue-induced reinstatement of alcohol seeking (Sanchis-Segura et al., 2006). The uncompetitive NMDA antagonist memantine decreases responding for a cocaine-associated stimulus in monkeys (Newman and Beardsley, 2006), whereas another uncompetitive antagonist, neramexane, produces only a nonsignificant decrease in cue-induced alcohol seeking in rats (Bachteler et al., 2005). Further contradiction is added to these results by MK-801, which was found to increase responding nonselectively in an alcohol seeking reinstatement test (Vosler et al., 2001). Thus, the present results are in parallel with the above suggesting that AMPA antagonism mostly attenuates cue-conditioned behaviors whereas NMDA antagonism shows mixed results.

In contrast to cue-induced alcohol reinstatement, there is evidence that alcohol seeking measured by the alcohol deprivation effect is sensitive to NMDA antagonism regardless of binding site. The competitive NMDA antagonist CGP37849, the NMDA/glycine antagonist L-701,324, the polyamine site and NR2B subunit antagonist ifenprodil, and the NMDA channel blockers neramexane and memantine all suppress the alcohol deprivation effect (Hölter et al., 1996; Hölter et al., 2000; Vengeliene et al., 2005) suggesting that an inhibition of NMDA receptor function in general is sufficient for the reduction of alcohol seeking in the alcohol deprivation model. In addition to NMDA receptor antagonism, the AMPA antagonist GYKI52466 has also been found to attenuate the alcohol deprivation effect (Sanchis-Segura et al., 2006). The differences between the findings in the alcohol deprivation and reinstatement models are most likely accounted for by the fact that the alcohol deprivation paradigm, where alcohol consumption is a key factor, models different aspects of relapse behavior than the reinstatement model in which especially the conditioned drug responses promote relapse. Consistent with this, alcohol consumption can be attenuated by NMDA receptor antagonists (Hölter et al., 2000; McMillen et al., 2004).

6.4 Effects of systemic metabotropic glutamate receptor antagonism

Systemic administration of the mGluR5 antagonist MPEP decreased both cue-induced alcohol and cocaine seeking and also the alcohol deprivation effect. In line with previous

studies, spontaneous locomotor activity was not affected by pretreatment with MPEP (Ossowska et al., 2001; Henry et al., 2002) suggesting that motor performance of the animals was not severely impaired.

Examination of response patterns of the rats after MPEP pretreatment showed that the initial response rates were very low. In addition, the vehicle included in the within-subjects series of injections decreased responding compared to the reinstatement baseline. The same effect was seen later in the study (VI) with intra-accumbal MPEP administration. This observation led to the hypothesis that MPEP might have aversive effects that became associated with the injection procedure, leading to conditioned suppression by the vehicle. This seemed, at first, unlikely considering that MPEP has not been reported to produce either place preference or aversion when administered systemically to mice (Popik and Wróbel, 2002; McGeehan and Olive, 2003). However, two recent reports describe possible aversive effects of MPEP in rats. An elevation in intracranial self-stimulation thresholds was observed at MPEP doses only slightly higher than those used in the present study, suggesting that MPEP may induce a negative affective state (Harrison et al., 2002; Kenny et al., 2005). Our own experiments conducted after the completion of the MPEP reinstatement studies showed that systemically administered MPEP indeed has strong aversive effects as measured by the conditioned taste aversion paradigm (unpublished observation). Thus, aversive effects of MPEP may have contributed to decreased drug seeking.

MPEP has been the antagonist of choice in most studies examining the involvement of group I metabotropic receptors in drug self-administration and drug-seeking behavior. MPEP has been reported to reduce cocaine, nicotine, and ethanol self-administration as well as cocaine and nicotine seeking induced by priming (Sharko et al., 2002; Paterson et al., 2003; Tessari et al., 2004; Lee et al., 2005). In addition, and supporting the present findings with cocaine and alcohol, MPEP attenuates cue-induced nicotine seeking (Bespalov et al., 2005) as well as other conditioned drug effects, such as morphine-, amphetamine- and cocaine-induced place preference (Popik and Wróbel, 2002; McGeehan and Olive, 2003; Herzig et al., 2005). In the present study, MPEP decreased also relapse measured by increased alcohol consumption after a period of deprivation, an effect that was confirmed by a recent study (Schroeder et al., 2005), and baseline alcohol drinking. The latter finding is in agreement with several studies reporting a decrease in alcohol consumption by MPEP in either the choice or self-administration paradigm (McMillen et al., 2005; Olive et al., 2005; Schroeder et al., 2005).

6.5 Effects of systemic metabotropic glutamate receptor agonism

The mGluR2/3 agonist LY379268 and the mGluR8 agonist (S)-3,4-DCPG suppressed both alcohol self-administration and cue-induced alcohol seeking. The effects of the agonists were however found to be accompanied by motor suppression. This is in accordance with previous reports showing that LY379268 impairs motor performance in or near the dose range used in the present study (Cartmell et al., 1999; Cartmell et al., 2000), although the impairment may not be severe enough to fully account for the decreases in operant

responding (Baptista et al., 2004). Thus, although motor suppression may have contributed to the effects of LY379268 the possibility that the agonist could also alter the reinforcing properties of alcohol and the ability of alcohol-paired stimuli to reinstate alcohol-seeking behaviour cannot be excluded. In contrast, the effects of (S)-3,4-DCPG on locomotor activity were more pronounced and, in addition, cumulative response patterns of the rats revealed that the agonist decreased responding from the onset of the session, which can be interpreted as an indication of motor suppression. Intracerebroventricular administration of (S)-3,4-DCPG has previously been found to impair performance dose-dependently on the rotarod in mice (Moldrich et al., 2001), but the effects of systemic administration on motor functions in rats are not known. However, our observations from the present experiments suggest that motor suppressant effects by (S)-3,4-DCPG may have influenced responding in the operant tasks.

Drug self-administration reflects mainly the unconditioned, primary reinforcing effects of the drug, whereas the cue-induced reinstatement model measures the potency of alcohol-associated environmental cues to reinstate extinguished behavior and thus represents conditioned drug effects. LY379268 has previously been reported to attenuate both reinstatement of cue-induced cocaine seeking and cocaine self-administration (Baptista et al., 2004) as well as heroin seeking induced by a heroin-paired context (Bossert et al., 2004). The present results extend these findings to alcohol, although with some caution keeping the motor suppressive effects of LY379268 in mind.

In contrast to LY379268, very little is known about the effects of (S)-3,4-DCPG on drug-related behaviors. The racemic form (R,S)-3,4-DCPG has been reported to decrease amphetamine-induced hyperactivity in mice at doses that do not affect spontaneous locomotor activity (Ossowska et al., 2004). However, as the R-isomer has antagonistic effects at AMPA receptors (Thomas et al., 2001), it is unclear whether the effects were due to actions at mGlu or AMPA receptors, although a role for mGlu8 receptor agonism was proposed (Ossowska et al., 2004).

Group II/III agonists have been reported to decrease transmitter release presynaptically (Schoepp, 2001). Therefore, the effects of LY379268 and (S)-3,4-DCPG could be due to decreased presynaptical glutamate release in response to alcohol-associated stimuli. In line with this, LY379268 pretreatment has been reported to reduce enhanced glutamate overflow in the nucleus accumbens of amphetamine-sensitized rats (Kim et al., 2005). However, although re-exposure to drug-associated stimuli elevates accumbal glutamate levels (Hotsenpiller et al., 2001), also dopamine release has been shown to be enhanced following exposure to an alcohol-associated context (Katner and Weiss, 1999). As LY379268 attenuates also dopamine release (Kim et al., 2005) and cue-induced alcohol-seeking behavior can be reduced by dopamine receptor antagonists (Liu and Weiss, 2002), it is possible that LY379268 decreased alcohol seeking through both glutamatergic and dopaminergic mechanisms.

6.6 Effects of intra-accumbal glutamate receptor antagonism

To examine the possible anatomical site of action of the glutamate antagonists, the NMDA antagonist D-AP5, the AMPA/kainate antagonist CNQX and the mGluR5 antagonist MPEP were injected into the nucleus accumbens core, which has previously been suggested to be involved in mediating the effects of glutamate on drug seeking (Kalivas and McFarland, 2003). It was found that all three antagonists attenuated reinstatement induced by the cocaine-asociated stimulus complex without any significant effects on motor behavior. This is in accordance with previous findings on the enhancing rather than suppressing effects of intra-acumbal D-AP5 and CNQX on locomotor activity (David et al., 2004). Lever-pressing for sucrose pellets at response rates far higher than those in the reinstatement experiments was neither affected by intra-accumbal antagonist pre-treatment showing that the rats were capable of performing the lever-press task after antagonist administration.

A decrease in responding, compared to reinstatement baseline, was observed following MPEP administration at all doses, also the saline vehicle. The same effect was seen in study V after systemic administration. As responding was allowed to return to baseline levels between injections, residual effects of MPEP are an unlikely cause for the decrease, as is the composition of the vehicle (saline was used also with D-AP5 and CNQX). Therefore, it is possible that conditioned aversive effects of MPEP detected in a conditioned taste aversion study of ours (unpublished observation) contributed to the decrease in responding.

There are no previous reports on the effects of intracranially administered mGluR5 antagonists on drug-seeking behavior. With the ionotropic antagonists, the present finding that AMPA/kainate antagonism attenuates cue-induced drug seeking is in agreement with previous results (Di Ciano and Everitt, 2001). However, the attenuation by the NMDA antagonist D-AP5 is a new finding. Previously, NMDA antagonists have been ineffective at modulating drug seeking behavior when injected into the nucleus accumbens core or more dorsally into the striatum (Cornish and Kalivas, 2000; Di Ciano and Everitt, 2001; Vanderschuren et al., 2005). However, there are some differences between these studies and the present one making it possible that drug seeking in these models differs in sensitivity to NMDA antagonism. In the study by Cornish and Kalivas (2000), reinstatement was induced by a priming injection of cocaine, whereas in the present study cues were used. The study by Di Ciano and Everitt (2001) examined cue-induced drug seeking, but using the fixed-interval second-order schedule of self-administration after a very short withdrawal period, whereas the rats in the present study had been withdrawn from cocaine for several weeks before reinstatement testing. Following long-term withdrawal, drug-seeking behavior has been shown to be enhanced, and this effect is accompanied by neurochemical changes (see Lu et al., 2004 for review). For example, cocaine seeking has been found to increase over a 2-month withdrawal period (Grimm et al., 2001) and responding for an alcohol-associated cue in a reinstatement test is higher on day 56 of abstinence compared to days 1 and 28 (Bienkowski et al., 2004). One more difference between the present study and the one by

Di Ciano and Everitt (2001) is the schedule of reinforcement. Fixed-interval schedules, which were employed by Di Ciano and Everitt (2001), have been shown to promote the development of habitual responding, whereas ratio schedules, under which there is always a linear relationship between response and reward rates, do not (Coutureau and Killcross, 2003; Yin et al., 2006). The assumption that responding in the present study was not habitual is supported by the fact that it quickly decreased to extinction levels after omission of the cocaine-associated stimulus.

The decrease in cocaine seeking following intra-accumbal D-AP5 administration is also in contradiction with some of the findings in studies III and V, as the competitive NMDA antagonist CGP39551 was ineffective in modulating cue-induced cocaine or alcohol seeking after systemic administration. This is an interesting discrepancy, the reason of which is however unclear, and further adds to the complexity of findings with NMDA antagonist. As mentioned earlier, NMDA antagonists have either decreased or had no effect on drug-conditioned behaviors depending on antagonist and animal model employed.

6.7 Glutamatergic neurotransmission and drug seeking

Repeated treatment of laboratory animals with cocaine has been found to decrease basal extracellular glutamate levels in the nucleus accumbens (Bell et al., 2000; Hotsenpiller et al., 2001; McFarland et al., 2003). In addition, an enhanced glutamate release upon re-exposure to cocaine or cocaine-associated stimuli has been observed in repeatedly cocaine-treated, but not naive animals (Bell et al., 2000; Hotsenpiller et al., 2001). Against a decreased basal glutamate level this results in a very large proportional increase in accumbal glutamate. In contrast, re-exposure to a natural reinforcer does not lead to enhanced glutamate release. This was shown by McFarland et al. (2003) in a study where a cocaine priming injection induced cocaine seeking and elevated accumbal glutamate levels, whereas noncontingent delivery of food pellets reinstated food seeking but was without effect on glutamate levels. Glutamate levels have not been examined in cue-reinstatement tests, but based on the above it can be assumed that stimuli paired with drugs during self-administration are capable of inducing glutamate release in the nucleus accumbens when presented alone in reinstatement tests.

There is evidence that the changes in accumbal glutamate transmission following repeated drug treatment are connected to drug-seeking behavior. Normalizing the decreased basal glutamate levels was found to prevent the enhanced glutamate release induced by a cocaine priming injection and, more importantly, when accumbal glutamate levels were not markedly elevated by cocaine, drug-seeking behavior was abolished (Baker et al., 2003). This suggests that decreasing or counteracting glutamate release induced by drug priming or cues decreases drug seeking.

Supporting this view, cocaine-induced reinstatement can be blocked by accumbal AMPA/kainate antagonism or mGluR2/3 agonism (Cornish and Kalivas, 2000; Peters and Kalivas, 2006). Similarly, mGluR2/3 agonism suppresses context-induced heroin

seeking and cocaine seeking maintained by cues can be decreased by AMPA/kainate antagonism (Di Ciano and Everitt, 2001; Bossert et al., 2005). The present results add to these findings. Further support for the involvement of glutamate transmission in behavior guided by cues comes from a study in which accumbal AMPA/kainate antagonism attenuated the sensitized locomotor response to a challenge injection of cocaine. This effect was seen only in rats treated repeatedly with cocaine in a specific testing environment and not in rats that had received saline or cocaine in the home cage without stimulus pairing (Bell et al., 2000). The effects of ionotropic glutamate receptor antagonism seem to be specific to drug seeking, as food-seeking behavior is unaffected by systemic or intracranial treatments (Bespalov et al., 2005; Sun et al., 2005).

It is very likely that the rats in the present experiments developed changes in glutamate transmission similar to the ones described above following the 7-week drug conditioning and self-administration phase and subsequent withdrawal period. Therefore, re-exposure to cocaine- and alcohol-associated cues could have triggered drug-seeking behavior through increased glutamate release. The glutamate receptor agonists used in the experiments may have diminished glutamate release and the antagonists the effects of glutamate on postsynaptic cells, thus decreasing drug seeking. However, pretreatment with glutamate agonists and antagonists can also have had effects that are relatively independent of previous drug exposure. First, the pretreatments can have affected the motivation to seek drugs in general. Consistent with this, NMDA, AMPA, and mGluR5 antagonists have been reported to decrease the motivation to self-administer nicotine, cocaine, and alcohol under a progressive ratio schedule of reinforcement (Stephens and Brown, 1999; Allen et al., 2005; Paterson and Markou, 2005). Second, the pretreatments may have attenuated the ability of drug-associated stimuli to trigger drug-seeking behavior. There are several means to this including decreased perception of stimuli, decreased ability of stimuli to guide behavior despite intact perception, and decreased ability to retrieve the stimulus-reward association meaning that the rats were no longer able to associate the stimulus with its conditioned effects.

Memory retrieval, but not consolidation, is considered to require AMPA receptor activation (Izquierdo et al., 1997; Roullet et al., 2001). For example, AMPA/kainate antagonism decreases the ability of animals to detect a spatial re-arrangement of objects when administered before testing, but not if administered after training (Roullet et al., 2001). Di Ciano et al. (2001) showed that also discrimination is impaired as rats were unable to discriminate between stimuli paired with reward and nonreward after intra-accumbal infusions of an AMPA/kainate antagonist in a Pavlovian approach behavior study. NMDA antagonists, especially channel blockers, have also been shown to decrease discriminability and accuracy (Willmore et al., 2001; Gargiulo et al., 2005). Thus, decreased memory retrieval or stimulus recognition might explain the effects of the ionotropic antagonists used in the present study. For metabotropic agonists, decreased choice accuracy has been reported after administration of an mGluR2/3 agonist, LY354740 (Higgins et al., 2004). On the other hand, mGluR2/3 and mGluR4/6 agonists have been reported to enhance memory retrieval (Szapiro et al., 2001). A broad spectrum metabotropic glutamate receptor antagonist, MCPG, was found not to affect cue-guided

performance in the Morris water maze, where animals must to find a non-visible platform submerged in water with the help of external cues (Bordi et al., 1996). Also MPEP has been reported to leave object recognition memory intact in an object novelty preference task (Barker et al., 2006) suggesting that it does not affect memory retrieval.

6.8 Neural pathways mediating drug-seeking behavior

It has been shown that drug-seeking behavior can be induced by electrical or pharmacological stimulation of the prefrontal cortex, amygdala and hippocampus, all of which send glutamatergic projections to the nucleus accumbens (McFarland and Kalivas, 2001; Vorel et al., 2001; Hayes et al., 2003). Accordingly, inactivation or lesions of these structures attenuates cocaine seeking (Meil and See, 1997; McFarland and Kalivas, 2001; McLaughlin and See, 2003; Sun and Rebec, 2003). So far, at least the amygdala and the prefrontal cortex have been implicated as the source of accumbal glutamate released in response to drug-associated cues. Infusions of a dopamine antagonist into the basolateral amygdala and an AMPA/kainate antagonist into the contralateral nucleus accumbens core, neither of which had any effects on its own, abolished cue-induced drug seeking under a second-order schedule of self-administration showing that the connection from the amygdala to the nucleus accumbens is critically involved (Di Ciano and Everitt, 2004). Inactivation of the prefrontal cortex with the GABA agonists baclofen and muscimol, on the other hand, blocks cocaine-induced increases in nucleus accumbens glutamate levels (McFarland et al., 2003) and cue-induced reinstatement of cocaine seeking (Pierce et al., 1998; McLaughlin and See, 2003). In addition, lesions of the prefrontal cortex disrupt the ability of cocaine-associated stimuli to guide behavior under a second-order schedule of reinforcement (Weissenborn et al., 1997). This suggests that in addition to the nucleus accumbens, both the prefrontal cortex and the amygdala are involved in the guidance of drug-seeking behavior.

The core and shell subregions of the accumbens seem to differentially participate in the mediation of the effects of glutamate and cues on drug seeking. The injections in the present experiments (Study VI) were targeted at the accumbens core, but a possibility of diffusion into nearby shell structures cannot be totally ruled out. Manipulations targeting glutamate transmission in the accumbens shell have so far been mostly unsuccessful, whereas lesions or inactivation of the core impaired responding for cocaine and cocaine seeking under second-order schedules of reinforcement (Di Ciano and Everitt, 2004; Ito et al., 2004). Also cue-induced drug seeking can be blocked by GABA agonist-induced inactivation of the accumbens core (Fuchs et al., 2004) or by infusions of ionotropic glutamate receptor antagonists in the accumbens core, but not shell (Di Ciano and Everitt, 2001).

This view was recently challenged by Bossert et al. (2005) who found that the mGluR2/3 agonist LY379268, which acts to decrease presynaptic transmitter release, attenuated context-induced heroin seeking when administered into the shell but not core, except at very high doses. This may reflect a difference between contextual and discrete cues in inducing reinstatement, or, alternatively, the effects of LY379268 could have been mediated via decreased dopamine, and not glutamate, release (Kim et al., 2005).

Dopamine release has been shown to be enhanced following exposure to cocaine- or alcohol-associated discriminative stimuli (Katner and Weiss, 1999; Weiss et al., 2000), and cocaine and alcohol reinstatement can be attenuated by systemical dopamine antagonism (Crombag et al., 2002; Liu and Weiss, 2002). Therefore, the effects of LY379268 and possibly also (S)-3,4-DCPG seen in the alcohol reinstatement study (IV) could be due to either decreased glutamate or dopamine release. With regard to dopamine, the shell region of the accumbens may be more important than the core as reinstatement can be induced by infusing a dopamine agonist into the shell, but not core (Schmidt et al., 2006). Also, decreased reinstatement has been reported following dopamine antagonist administration into the accumbens shell, whereas administration into the core has been ineffective (Anderson et al., 2003; Anderson et al., 2006).

7 CONCLUSIONS

1. Drug-seeking behavior, as measured by fixed-interval second-order self-administration, the alcohol deprivation effect, or cue-induced reinstatement of responding, could be attenuated by antagonism at AMPA/kainate, NMDA, and metabotropic mGlu5 glutamate receptors. However, NMDA antagonists differentially modulated drug seeking with NMDA/glycine antagonism decreasing drug seeking and antagonism at the competitive glutamate binging site or the uncompetitive channel blocking site being ineffective. In addition, metabotropic glutamate receptor agonism at group II/III receptors, which presumably decreases postsynaptic glutamate release, attenuated alcohol seeking and self-administration. The results suggest that drug-associated cues activate glutamatergic neurotransmission and that counteracting the effects of glutamate can attenuate the ability of these cues to guide behavior.
2. Cocaine- and alcohol-seeking behavior induced by drug-associated cues was similarly affected by systemically administered glutamate receptor antagonists suggesting that glutamate transmission modulates drug seeking across drug classes.
3. Accumbal AMPA/kainate, NMDA, and mGluR5 antagonism attenuated cue-induced drug seeking suggesting that the nucleus accumbens is at least one of the anatomical sites where glutamate mediates the effects of cues on behavior.

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