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- nicotinic mechanisms

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CONDITIONAL AND NON-CONDITIONAL REWARD-RELATED RESPONSES TO ALCOHOL − NICOTINIC MECHANISMS

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ABSTRACT

The mesolimbic dopamine system is believed to mediate the positive reinforcing and rewarding effects of addictive drugs by increasing dopamine levels in its terminal area, the nucleus accumbens (nAc). Nicotinic acetylcholine receptors (nAChRs) within this system appear important for the pharmacological actions of both alcohol and nicotine, which may explain the frequent co-abuse of these two drugs. Despite available pharmacological and psychological therapies, most addicts relapse to smoking and alcoholism, often due to the impact of drug-associated stimuli (cues) on craving and compulsive drug-seeking.

The first part of this thesis investigated the role of nAChRs in the effects of alcohol-associated as well as sucrose-associated cues on mesolimbic dopamine activity and/or behaviors related to drug-seeking (responding with conditioned reinforcement) in the rat. In the second part, *in vivo* microdialysis was utilized to characterize the ethanol-induced dopamine elevation in the rat nAc and the consequences thereon by subchronic pre-treatment with nicotinic drugs.

The data demonstrate that antagonism of ventral tegmental area (VTA) nAChRs abolishes the ethanol cue-induced dopamine elevations in the nAc and the conditioned reinforcing properties of ethanol cues. Moreover, nAChRs appear to mediate responding with conditioned reinforcement to sucrose. The results also indicate that the most important site of interference for ethanol-induced dopamine elevations is in the nAc, but that once the ethanol action is present in this brain region, ethanol may act also in the VTA to produce add-on effects. Furthermore, the decline in dopamine that is observed following the initial elevation after ethanol administration may be due to recruitment of dopamine inhibitory $GABA_A$ receptors in the nAc, as demonstrated by the ability of a $GABA_A$ antagonist to attenuate this effect. Pre-treatment with a nicotinic drug abolished the dopamine declining phase.

We hypothesize a novel mechanism by which alcohol-associated cues stimulate mesolimbic dopamine activity and promote drug-seeking behavior by activation of VTA $\alpha3\beta2^*$ and/or $\alpha6^*$ nAChRs. Interestingly, the same nAChR subtypes were previously demonstrated to mediate the pharmacological effects of ethanol. This coincidence may play a critical role in the well known phenomenon of "loss of control" of drinking, a hallmark of alcoholism. Pharmacological manipulations of specific nAChR subtypes may thus be possible treatment strategies to prevent cue-induced relapse to alcoholism. The demonstration that nAChRs mediate responding with conditioned reinforcement also to sucrose, may explain the enhanced sugar intake associated with smoking cessation and alcohol abstinence.

The second part of the thesis suggests that recruitment of $GABA_A$-receptor activity is responsible for the second, declining phase with respect to nAc dopamine levels following ethanol administration and that pre-treatment with nicotinic drugs produces tolerance to this effect in the nAc and other brain regions. This phenomenon could be part of the explanation to why the sedative effects of ethanol are reduced in some nicotine users.

These results contribute with novel explanations for the common co-abuse of nicotine and alcohol and suggest specific nAChRs as potential targets for novel pharmacological interventions aimed at reducing cue-induced craving and relapse in alcoholism.

Key words: ethanol, nicotine, ventral tegmental area, nucleus accumbens, dopamine, nicotinic acetylcholine receptor, γ-amino-butyric acid receptor A, conditioned reinforcement, *in vivo* microdialysis, rat

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IV. Löf E., Chau P., Stomberg R., Söderpalm B. Ethanol-induced dopamine elevation in the rat - modulatory effects by subchronic treatment with nicotinic drugs. In press, Eur. J. Pharmacol.

Till mina föräldrar

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LIST OF ABBREVIATIONS

α-CtxMII	α-conotoxin MII
ANOVA	Analysis of variance
CR	Conditioned reinforced (lever)
CS	Conditioned stimulus/stimuli
CS+	Paired Conditioned stimulus
CS-	Unpaired stimulus
DA	Dopamine
DHβE	Dihydro-β-erythroidine
PLSD	Protected least-significant difference
GABA	γ-amino-butyric acid
HEX	hexamethonium
LDTg	Laterodorsal tegmental grey
MEC	Mecamylamine
MLA	Methyllycaconitine
nAc	Nucleus accumbens
nAChRs	Nicotinic acetylcholine receptor
NCR	Non-reinforced (lever)
NIC	Nicotine
NMDA	*N*-methyl-*D*-aspartate
PBS	Phosphate buffered saline
PPTg	Pedunculopontine tegmental grey
SEM	standard error of the mean
US	Unconditioned stimulus/stimuli
VTA	Ventral tegmental area

BACKGROUND

ALCOHOLISM AND SMOKING

Alcoholism is a world-wide problem causing considerable suffering to the individual and enormous costs to society. In Sweden alone, the annual cost for alcohol abuse is estimated to 150 billion SEK (Johnson, 2000). Alcoholism not only has direct medical and social consequences for the afflicted, but is often a cause of traffic accidents, violent crime and fetal alcohol syndrome. A high alcohol consumption is also associated with a high consumption of nicotine, and vice versa (*e.g.* Walton, 1972; Craig and Van Natta, 1977; Mello et al., 1987; DiFranza and Gurrera, 1990; Zacny, 1990). Alcoholism is estimated to be over 10 times more common among smokers than non-smokers, and nicotine dependent subjects have a greater severity of alcohol dependence (Daeppen et al., 2000). Moreover, alcoholics fail to a significantly greater extent than non-alcoholics when trying to quit smoking (DiFranza and Gurrera, 1990). It appears unlikely that these relationships are explained just by environmental or psychosocial factors, and the interaction between the two drugs can be observed also pre-clinically. Animal studies demonstrated that nicotine treatment can increase ethanol intake and preference in the rat (Potthoff et al., 1983; Blomqvist et al., 1996; Ericson et al., 2000a; Le et al., 2000; Clark et al., 2001; Olausson et al., 2001), indicating that the association between smoking and drinking may be due to neurochemical effects produced by nicotine, secondarily influencing the propensity for ethanol intake (Potthoff et al., 1983; Blomqvist et al., 1996; for review, see Soderpalm et al., 2000). Indeed, a common point of action for nicotine and alcohol is the nicotinic acetylcholine receptors (nAChRs) in the so called "brain reward system" (Blomqvist et al., 1993; for reviews, see Soderpalm et al., 2000; Larsson and Engel, 2004).

Relapse to smoking and alcoholism is unfortunately common despite available pharmacological and psychological treatment strategies and may occur after extensive drug-free periods. Under these circumstances, relapse may be induced by the impact of drug-associated stimuli (cues) on motivation, craving and drug-seeking behavior (Pomerleau et al., 1983; Niaura et al., 1988; Drummond et al., 1990; Carter and Tiffany, 1999; Mucha et al., 1999; Caggiula et al., 2001; Grusser et al., 2004; for review, see Spanagel and Zieglgansberger, 1997). Studying the behavioral and neurochemical consequences of these drugs and their associated cues is important, as it provides knowledge to serve as a basis for development of new treatment strategies for alcohol and nicotine addiction.

ADDICTION

The neurobiological effects of drugs (repeated/chronic pharmacological actions) and also the psychological and behavioral consequences (associative learning processes involving cues and motor actions) together mediate the transition from voluntary drug use

to compulsive drug taking and addiction. An important diagnostic criterion for addiction is the loss of control, which can be described as unsuccessful efforts to stop, cut down, or control use of the drug despite harm (ICD-10: International Classification of Disease – 10th edition, 1990, World Health Organization; DSM-IV: the Diagnostic and Statistical Manual of Mental Disorders – 4th edition, American Psychiatric Association, 1994). Loss of control is especially pertinent to alcohol abuse, where the alcoholic continues to consume alcohol, in spite of high levels of intoxication. Moreover, there may be common mechanisms behind the development and expression of drug addiction in general. It is therefore possible that the use of one drug, such as nicotine, increases the vulnerability to become dependent to another drug, such as alcohol, or vice versa.

DRUGS OF ABUSE AND DOPAMINE

Most addictive drugs stimulate the mesolimbic dopamine system which originates in the ventral tegmental area (VTA) and terminates in the ventral part of the striatum, the nucleus accumbens (nAc) (Fig. 1) (Dahlström and Fuxe, 1964; Koob, 1992). Activation of this system results in elevated levels of extracellular dopamine in the nAc (Di Chiara and Imperato, 1988; for review, see Wise, 1996). The dopamine increase is believed to mediate the subjective feelings of pleasure from natural rewards as well as from drugs of abuse including nicotine and alcohol (Engel and Carlsson, 1977; Gessa et al., 1985a; Koob and Bloom, 1988; Grenhoff and Svensson, 1989; Wise and Rompre, 1989; Di Chiara and North, 1992), and to stimulate appropriate motor responses and motivation that support reward-seeking and consumption (Hodge et al., 1994; for review, see Le Moal and Simon, 1991). Consequently, the mesolimbic dopamine system is a central part of what is often referred to as the "brain reward system". Furthermore, it is hypothesized that the increase in extracellular dopamine in the nAc associated with reward consumption behavior moreover mediates facilitation of learning of association between cues and reward (Richardson and Gratton, 1996; Schultz, 1998a; Balfour et al., 2000). Thus, this system appears to be involved in the effects on behavior by reinforcers that are primary (the actual reward) as well as conditioned (the cues).

THE VENTRAL AND DORSAL STRIATUM IN REWARD

The rat nAc consists of two anatomically and functionally different subdivisions (Graybiel and Ragsdale, 1978; Jones et al., 1996; Bassareo and Di Chiara, 1999a), the core and the surrounding shell (Voorn et al., 1989; Heimer et al., 1991; Zahm and Brog, 1992). The dopaminergic neurons that project from the VTA and terminate in the nAc shell, respond to acute administration of drugs of abuse, such as nicotine (Cadoni and Di Chiara, 2000; Iyaniwura et al., 2001) and ethanol (Bassareo et al., 2003) as well as highly palatable food (Bassareo and Di Chiara, 1999a). The consequent release of dopamine in the nAc shell is believed to facilitate learning about reinforcing stimuli (Di Chiara, 1999). It is speculated that, in the case of normal eating behavior, there is an adaptation to the

dopamine stimulating effects of food in the nAc shell (Bassareo and Di Chiara, 1997, 1999b; Bassareo et al., 2003). This adaptation may not occur to drugs of abuse (Di Chiara, 1998, 2000; Bassareo et al., 2003) or in the case of disturbed eating behaviors (Di Chiara, 2005; Rada et al., 2005). In the nAc core, which anatomically is an extension of the striatum (Heimer et al., 1991), sensitization of the dopamine response to repeated administration of psychostimulant drugs of abuse (Cadoni et al., 2000) and nicotine (Cadoni and Di Chiara, 2000; Iyaniwura et al., 2001), has been observed. This phenomenon is implicated in the formation of cue-conditioned responses (*e.g.* Parkinson et al., 1999; Di Ciano and Everitt, 2001), that may be central in the development of dependence (Balfour et al., 2000; Di Chiara, 2000). Thus, the nAc may be a key substrate for the learning of cue-reward relationships (Cador et al., 1989; Parkinson et al., 1999; Parkinson et al., 2000; Hutcheson et al., 2001), involving dopamine receptors in this brain region (Kelley et al., 1997; Smith-Roe and Kelley, 2000; Di Ciano et al., 2001; Parkinson et al., 2002). Since both the core and the shell of the nAc appear to be important for the drug effects that lead to addiction, a dialysis probe with an active space that covers both the core and the shell areas were used in the *in vivo* microdialysis experiments of the present thesis.

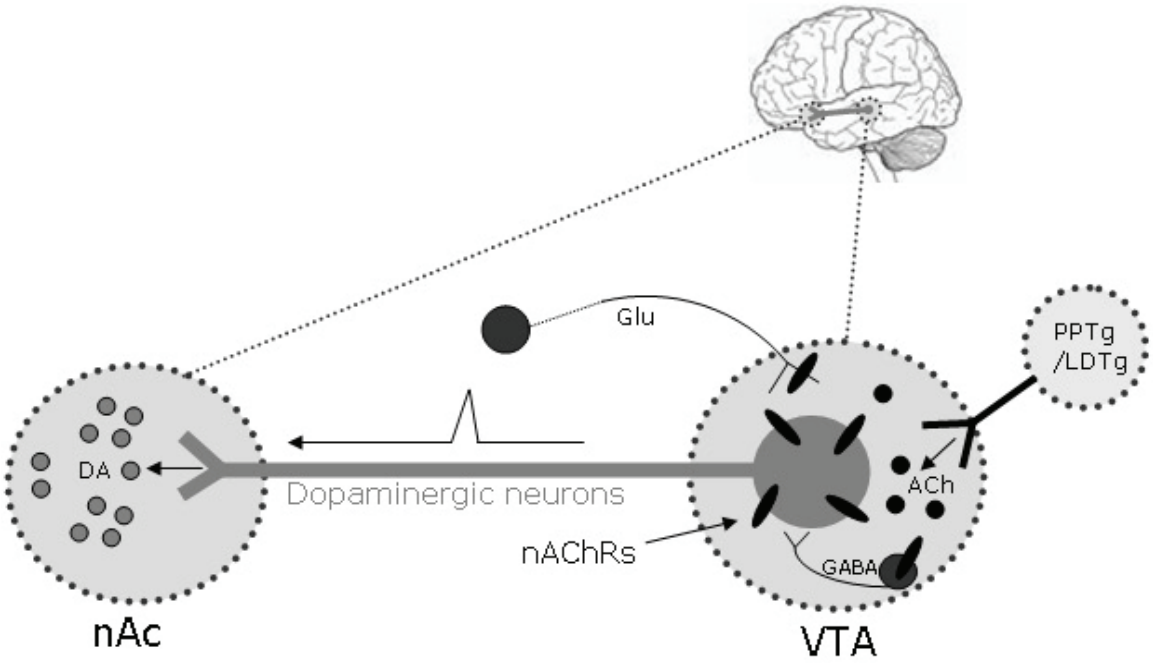


Fig. 1. The mesolimbic dopamine system.
The dopaminergic cell bodies are located in the VTA and send axonal projections to *i.a.* the nAc, where dopamine (DA) is released following neuronal stimulation. The activity of these neurons is stimulated by glutamatergic (Glu) input from the prefrontal cortex and inhibited by GABA released by interneurons and afferents. Cholinergic input to the VTA from mesopontine nuclei (PPTg/LDTg) modulates the dopaminergic activity by acting directly on nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) on dopaminergic neurons and indirectly by acting on nAChRs on GABAergic and glutamatergic neurons. ACh = acetylcholine

Whereas dopamine activity in the ventral striatum is believed to be important in the early stages of drug use (for reviews, see Koob, 1992; Wise, 1998; Koob and Le Moal, 2001), dopamine in the dorsal part of the striatum (caudate and putamen) has recently received increasing attention as a mediator of the development of more persistent habitual, compulsive consequences of drug abuse in both rodents (Jog et al., 1999; Faure et al., 2005), primates (Letchworth et al., 2001; Porrino et al., 2004) and humans (Lehericy et al., 2005; Volkow et al., 2006; for reviews, see Robbins and Everitt, 1999; Everitt and Wolf, 2002; Gerdeman et al., 2003). The dorsal striatum receives dopaminergic projections predominantly from the substantia nigra (Anden et al., 1964; Jimenez-Castellanos and Graybiel, 1987;. for reviews, see Koob, 1992; Haber and Fudge, 1997). Studies demonstrate that behavioral sensitization to morphine in rats is associated with increased dopamine transmission in the caudate-putamen in addition to the nAc core (Cadoni and Di Chiara, 1999). Drug taking also in nicotine and alcohol dependent individuals appears strongly habitual and compulsive and a habitual component of ethanol seeking in rats was recently demonstrated (Dickinson et al., 2002). Thus, Paper IV investigated whether the dopaminergic responses to alcohol also in the dorsal striatum may be altered by subchronic pre-treatment with nicotinic drugs that increase alcohol consumption in the rat.

INCENTIVE MOTIVATION AND CONDITIONED REINFORCEMENT

Relapse to drug abuse can occur following extensive drug-free periods. In this case, craving and relapse may be induced by stimuli (cues) that during previous drug-taking have become associated with the behavior that results in consumption and the subjective rewarding effects of the drug. Thus, these cues have acquired incentive motivational values to become a prediction of reward and can thereby exert powerful behavioral effects by acting as conditioned reinforcers (for review, see Wise, 2004). This hypothesis is in line with the well known observations of Pavlov (1927) that environmental signals for food become conditioned stimuli for the unconditioned effects of eating.

The dopamine hypothesis of incentive motivation and conditioned reinforcement

The incentive motivational theories of cue conditioning state that drug-associated cues have the ability to activate reinforcement circuits in the brain (Stewart et al., 1984; Robinson and Berridge, 1993). Indeed, the nAc and its dopamine innervations are implicated in mediation of the effects that drug-associated cues exert over motivational behavior (Taylor and Robbins, 1984, 1986; Cador et al., 1991; Wolterink et al., 1993; Hodge et al., 1994; Parkinson et al., 1999; Parkinson et al., 2000; Wyvell and Berridge, 2000; Hall et al., 2001; Cardinal et al., 2002b; Parkinson et al., 2002; for reviews, see Robinson and Berridge, 1993; Spanagel and Weiss, 1999; Everitt et al., 2001). Dopamine neurons respond to unexpected rewards and to cues that predict reward (*e.g.* Schultz,

1998a) and this dopamine release mediates the selection of appropriate behaviors in order to acquire the predicted reward (for review, see Wise, 2004). Moreover, it contributes to reinforcement by associating predictive cues with specific outcomes of these behaviors (McClure et al., 2003). The specific mechanism by which drug-associated cues increase nAc dopamine and enhance incentive motivation is, however, unknown. The nAc core has been attributed a role in processes involved in conditioned reinforcement (Burns et al., 1993; Parkinson et al., 1999; Ito et al., 2004). Psychostimulant drugs can amplify the control over behavior by a conditioned reinforcer via the dopaminergic innervation of the nAc (Taylor and Robbins, 1984, 1986; Wolterink et al., 1993). This process, on the other hand is suggested to depend on the nAc shell (Parkinson et al., 1999). Whereas the present thesis investigated dopaminergic and cholinergic mechanisms involved in conditioned reinforcement to ethanol, data suggest additional mechanisms involving several other brain systems in the mediation of cue-induced alcohol seeking, such as the opioid (Ciccocioppo et al., 2002; Ciccocioppo et al., 2004; Bechtholt and Cunningham, 2005), GABAergic (Bechtholt and Cunningham, 2005), nitric oxide (Liu and Weiss, 2004), endocannabinoid (Cippitelli et al., 2005) and glutamatergic systems (Bachteler et al., 2005; for review, see Weiss et al., 2001).

Conditioned reinforcement and alcoholism

Conditioned reinforcement is the process whereby a previously conditioned stimuli act as the reinforcer for a new instrumental action, such as lever pressing in the rat (Mackintosh, 1974; Robbins, 1978; Taylor and Robbins, 1984). The cues acquire motivational values by acting as conditioned reinforcers to addicts making them search for the cues, or through Pavlovian Instrumental Transfer where the cues trigger or enhance drug-seeking. Conditioned reinforcement may be viewed as a memory of addiction, where drug-associated behavioral conditioning is a reflection of inappropriate learning mechanisms in the brain (cf. Schroeder et al., 2001). The development of Pavlovian conditioning can be assessed by measuring the approach to the conditioned stimuli. The acquired motivational valance of such conditioned stimuli to serve as a conditioned reinforcer is therefore assessed by its ability to reinforce the acquisition of a novel and arbitrary response (for review, see Everitt et al., 1999). In the present thesis, the novel response consisted of lever responding to achieve alcohol- or sucrose-associated conditioned stimuli (see "Methods" section).

In alcohol-dependent subjects, alcohol-associated cues can produce urges to drink and smoke (Rohsenow et al., 1997; Cooney et al., 2003), elicit craving (Pomerleau et al., 1983; Spanagel and Zieglgansberger, 1997; Carter and Tiffany, 1999) and precipitate relapse (Niaura et al., 1988; Drummond et al., 1990; Grusser et al., 2004). In line with the dopamine hypothesis of incentive motivation and conditioned reinforcement, these cues may have the ability to activate the ventral striatum in abstinent high-risk drinkers and alcoholics (Braus et al., 2001; Kareken et al., 2004) and to elevate extracellular dopamine levels in the rat nAc (Weiss et al., 1993; Katner et al., 1996; Gonzales and Weiss, 1998; Katner and Weiss, 1999; Melendez et al., 2002; Doyon et al., 2003), suggesting dopamine as a neurobiological substrate for the ability of cues to promote alcohol-seeking and -taking behavior (Liu and Weiss, 2002). This is further supported by data

demonstrating that activation of dopaminergic transmission in the nAc by local amphetamine administration can enhance the conditioned reinforcing effects of an ethanol-paired cue (Slawecki et al., 1997).

THE ACTIVITY OF THE MESOLIMBIC DOPAMINE SYSTEM

The dopamine releasing neurons of the mesolimbic system can fire as single spikes or in clustered bursts (Grace and Bunney, 1984b, 1984a). Bursting patterns evoke a relatively brief (seconds) phasic increase in "synaptic" dopamine levels explicitly signaling rewarding and alerting stimuli (Schultz, 1998b), whereas increases in spontaneous (random) firing promote slow (minutes) tonic elevations in "extrasynaptic" dopamine levels proposed to result in the expression of motor, cognitive and motivational behaviors (Schultz, 1998a). Increases in spikes produce less dopamine release than bursts, but nevertheless results in a dopamine overflow into the extrasynaptic space that is detected by *in vivo* microdialysis (Gonon, 1988; Manley et al., 1992; Nissbrandt et al., 1994), the neurochemical method applied to measure dopamine in the present thesis. Additionally, there can be a selective increase in a population of spontaneously active neurons resulting in an increased tonic activity, without concomitant changes in burst firing (Floresco et al., 2001; Floresco et al., 2003).

In addition to classical release from the axon terminals, midbrain dopaminergic neurons can release dopamine from their soma and dendrites (Bjorklund and Lindvall, 1975; Geffen et al., 1976; Kalivas and Duffy, 1991; Elverfors et al., 1992; Rice et al., 1994). In rodents, this allows an intrinsic regulatory component of dopamine activity via somatodendritic D_2 autoreceptors (tonic inhibition) (White and Wang, 1984; Lacey, 1993) and via D_1 receptors (excitation) on GABA- and excitatory amino acids-containing terminals in the VTA (Cameron and Williams, 1993; Yung et al., 1995; Lu et al., 1997; Koga and Momiyama, 2000). Extensive research has provided evidence of modulation of dopamine cell firing patterns by afferents from *e.g.* the prefrontal cortex, where many studies have focused on the *N*-methyl-*D*-aspartate (NMDA)-dependent tonic excitatory influence (Karreman et al., 1996; for review, see Kitai et al., 1999). However, several other systems possess modulatory roles on the mesolimbic dopaminergic activity by their actions preferentially within the VTA, such as gamma-amino-butyric acid (GABA) interneurons (Sesack and Pickel, 1995), projecting GABA-fibers from other brain areas such as the nAc (Walaas and Fonnum, 1980; Kalivas et al., 1993; Steffensen et al., 1998), as well as cholinergic neurons originating in the pedunculopontine tegmental (PPTg) and laterodorsal tegmental (LDTg) nuclei (Futami et al., 1995; for review, see Kitai et al., 1999; Omelchenko and Sesack, 2005, 2006) (Fig. 1).

Cholinergic regulation of the VTA

The majority of the cholinergic drive onto the VTA is believed to involve inputs to GABAergic, rather than dopaminergic, neurons (Garzon et al., 1999; Fiorillo and Williams, 2000), although this idea was recently challenged by a study demonstrating the reversed (Omelchenko and Sesack, 2006). The PPTg cells project mainly to the substantia nigra (Beninato and Spencer, 1987; Futami et al., 1995), but there are also PPTg projections to the VTA (for recent review, see Laviolette and van der Kooy, 2004b). The LDTg cells project mainly to the VTA (Oakman et al., 1995; Blaha et al., 1996). Electrical stimulation of the PPTg elicits striatal dopamine efflux (as measured by *in vivo* microdialysis) (Forster and Blaha, 2003), whereas LDTg stimulation produces a similar dopamine overflow in the nAc through the activation of cholinergic (muscarinic and nicotinic) and glutamatergic receptors in the VTA (Blaha et al., 1996; Forster and Blaha, 2000). Moreover, the PPTg was recently demonstrated to selectively control the bursting of dopamine cells rather than their tonic resting activity (Floresco et al., 2003). This is in agreement with the notion that the mesolimbic dopamine system is phasically rather than tonically regulated by cholinergic input into the VTA (Nisell et al., 1994b; Westerink et al., 1996).

Thus, the dopaminergic cell bodies in the VTA express both groups of receptor types for the cholinergic transmittor acetylcholine, *i.e.* muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs) (Weiner et al., 1990; Charpantier et al., 1998), and stimulation of these receptors, by acetylcholine or other agonists, results in enhanced *in vivo* dopamine output in the terminal areas such as the striatum and the nAc (Blaha et al., 1996; Westerink et al., 1996; Forster and Blaha, 2000; Tzschentke, 2001). Studies implicate fast-activating ionotrophic nAChRs in the rapid excitation of dopamine neurons, whereas slow-activating metabotrophic mAChRs, probably of M5 subtypes (Yeomans et al., 2001; Miller and Blaha, 2005), mediate the sustained tonic excitation. These data are in agreement with the suggestion that VTA nAChRs do not tonically regulate the dopaminergic neurons (Blomqvist et al., 1993; Nisell et al. 1994b; Westerink et al., 1996; Westerink et al., 1998; Grillner and Svensson, 2000). Moreover, both nAChRs and nAChRs in the VTA contribute to brain-stimulation reward (Yeomans et al., 1993; Yeomans and Baptista, 1997). These effects are consistent with the facilitation of dopamine-related behaviors following administration of cholinergic agonists, systemically or locally into the VTA or the substantia nigra pars compacta (Yeomans, 1995; Winn et al., 1997)(*vide infra*).

GABAergic regulation of the VTA

Inhibitory GABAergic inputs to the dopamine neurons in the VTA arise from local interneurons or descending projections from the nAc and the ventral pallidum (Walaas and Fonnum, 1980; Kalivas et al., 1993; Yoshida et al., 1997; Steffensen et al., 1998) activating mainly $GABA_A$ and $GABA_B$ receptors (Johnson and North, 1992; Sugita et al., 1992; Kalivas et al., 1993; Xi and Stein, 1998). In the VTA, the $GABA_A$ receptors are located mainly on GABAergic interneurons, but can additionally be found on dopaminergic cell bodies (Churchill et al., 1992; Westerink et al., 1996; Xi and Stein,

1998), whereas the $GABA_B$ receptors are primarily localized to the dopamine neurons (Xi and Stein, 1998; Margeta-Mitrovic et al., 1999). GABA acting on $GABA_A$ receptors in the VTA tonically inhibits dopamine release in the nAc (Westerink et al., 1996) and in the prefrontal rat cortex (Westerink et al., 1998). $GABA_B$ receptor stimulation was demonstrated to inhibit spontaneous pacemaker-like activity of VTA dopamine neurons in slice preparations (Johnson and North, 1992; Seutin et al., 1994; Wu et al., 1999) and to decrease firing rate and burst firing of these cells *in vivo* (Olpe et al., 1977; Erhardt et al., 2002). While it remains unclear whether $GABA_B$ receptors in the VTA tonically inhibit terminal dopamine release, the somatodendritic dopamine activity in the VTA appears to be tonically regulated by $GABA_B$ receptors (for review, see Adell and Artigas, 2004). Moreover, $GABA_A$-receptors within the VTA have also been proposed to send bidirectional reward information between dopaminergic VTA neurons and cholinergic neurons in the PPTg (Laviolette and van der Kooy, 2001, 2004a).

VTA acetylcholine and reward-related behaviors

At the behavioral level, acetylcholine injected into the VTA enhances hypothalamic self-stimulation (Redgrave and Horrell, 1976), whereas this behavior is attenuated by cholinergic muscarinic antagonists (Yeomans et al., 1985; Yeomans et al., 1993; Rada et al., 2000). Moreover, hypothalamic self-stimulation as well as food and water consumption cause acetylcholine to be released in the rat VTA (Rada et al., 2000), as does electrical self-stimulation of the medial forebrain bundle (Nakahara et al., 2001). With regards to aspects of addictive drugs, the PPTg neurons are involved in mediation of *e.g.* nicotine self-administration (Lanca et al., 2000) and voluntary ethanol consumption increases acetylcholine levels in the rat VTA and concomitantly, almost time-locked, increases dopamine in the nAc (Larsson et al., 2005). Moreover, ethanol application into the nAc is proposed to result in endogenous acetylcholine release in the VTA (Ericson et al., 2003) (*vide infra*).

The firing of nAc dopamine neurons during consummatory events is not strongly modulated by detection of reward, but rather elicited by reward-predictive cues. Consequently, it has been proposed that these neurons are activated in response to the incentive properties of rewards (Richardson and Gratton, 1996; Schultz, 1998a). An alternative suggestion is that they encode information about the motor activity of reward consumption, or both (Nicola et al., 2004b). PPTg neurons respond with brief bursts of activity at the onset of salient sensory cues (Pan and Hyland, 2005). This is the same type of activity recorded in dopaminergic neurons after appetitive cue presentation (Schultz, 1998b). Lesions of PPTg affect conditioning of tasks with specific sensory cues (Inglis et al., 2000) and impairs the acquisition of responding to such cues (Inglis et al., 1994; Inglis et al., 2000), but do not impair the learning of tasks such as place preference to opiates, stimulants or food (*e.g.* Bechara and van der Kooy, 1989; Bechara et al., 1992; Olmstead and Franklin, 1993, 1994). Neither do PPTg lesions appear to disrupt primary motivation or the ability to respond to changes in the reward strength (of sucrose) (Olmstead et al., 1999). Taken together, these results suggest that cholinergic neurotransmission within the VTA regulates the activity of dopamine neurons in response to salient cues promoting reward-seeking behaviors (Inglis et al., 1994; Schultz, 1998a;

Brown et al., 1999; Inglis et al., 2000; Miller et al., 2002; Roitman et al., 2004; Yun et al., 2004a; Pan and Hyland, 2005), a hypothesis that was investigated in Paper I of the present thesis.

ALCOHOL ADDICTION

Dopamine and alcohol consumption

In humans, a dopaminergic dysfunction has been suggested to underlie alcohol addiction (Balldin et al., 1985; for review, see Heinz, 2002). This notion was earlier implied by results obtained in animal studies, showing that ethanol enhances catecholamine activity in the brain and that this activity may underlie the stimulatory properties of the drug (Carlsson et al., 1972; Engel and Carlsson, 1976). Also in humans evidence for catecholamine involvement in ethanol-induced stimulation and euphoria was presented early on (Ahlenius et al., 1973). Accordingly, an innate abnormal functioning of the VTA dopamine system may facilitate the rewarding actions of ethanol in ethanol high-preferring rats (McBride et al., 1993). Chronic alcohol ingestion may itself reduce dorsal and ventral striatal dopamine, potentially contributing to alcohol's addictive properties by dampening the basal activity of this system and necessitating alcohol intake to maintain dopamine levels (Rothblat et al., 2001). Indeed, withdrawal from chronic alcohol consumption progressively suppresses the release of dopamine in the nAc and ethanol intake in dependent rats greatly exceeds that in non-dependent rats (Weiss et al., 1996). This increased self-administration restores the nAc dopamine levels to normal, suggesting that decreased dopamine levels trigger ethanol-seeking behavior (Weiss et al., 1996). Moreover, a high ethanol preference in the rat may be determined by a greater dopaminergic response to ethanol within the nAc (Weiss et al., 1993; Smith and Weiss, 1999; Katner and Weiss, 2001).

An important role has been attributed to the dopamine D_2 receptors in processing the effects of dopamine on alcohol consumption. For instance, alcohol preferring rats (Stefanini et al., 1992) as well as human alcohol-dependent individuals (Jarmo et al., 1994; Volkow et al., 1996; Tupala et al., 2001; Heinz et al., 2004) display fewer dopamine D2 receptors in the striatum, and overexpression of D_2 receptor in nAc reduces alcohol intake in non-preferring and alcohol preferring rats (Thanos et al., 2001; Thanos et al., 2004). However, local nAc administration of dopamine agonists increase (*e.g.* Hodge et al., 1992; Samson et al., 1993), whereas disruption of nAc dopamine activity and D_2 receptor antagonists decrease responding for ethanol (Pfeffer and Samson, 1986; Rassnick et al., 1992; Samson et al., 1993; Hodge et al., 1997; Czachowski et al., 2001). More specifically, dopamine activity in the nAc may primarily regulate ethanol seeking, as compared to ethanol consumption (Samson et al., 1993; Samson and Chappell, 2004), and this is proposed to be related to the stimulus processing function of the nAc core of rodents (for review, see Czachowski et al., 2001). Altogether, these data suggest that dopamine in the ventral striatum plays an important but complex (Czachowski et al., 2001; Samson and Chappell, 2004) role in alcohol seeking and consumption both in laboratory animals and in humans. Thus, the ability of nicotine to increase alcohol

consumption could involve its pharmacodynamic impact on nAChRs that may alter the dopaminergic response to ethanol in the nAc and possibly other parts of the brain reward system such as the dorsal striatum. This hypothesis was tested in paper IV.

Not only is the involvement of dopamine in alcohol consumption complex. The effect of ethanol *per se* on the mesolimbic dopamine system is also an issue of debate. Thus, there is some controversy regarding the point of action for the stimulatory effect of ethanol on nAc dopamine. Although rodents self-administer ethanol into the posterior VTA (Gatto et al., 1994; Rodd-Henricks et al., 2000; Rodd et al., 2004; Rodd et al., 2005) and nAChRs in the VTA clearly are involved in the dopamine activating effect of ethanol (Blomqvist et al., 1996; Ericson et al., 1998), *in vivo* microdialysis studies demonstrate that ethanol application in the VTA fails to affect nAc dopamine levels (Ericson et al., 2003). Rather, data imply the nAc as the primary point of action for the effects of ethanol on rat mesolimbic dopamine activity (Ericson et al., 2000b; Soderpalm et al., 2000; Ericson et al., 2003; Molander and Soderpalm, 2005), a hypothesis that was challenged in Paper III.

Alcohol and GABA receptors

The $GABA_A$ receptors have long been implicated in several effects of ethanol. This conclusion was based on observations of pharmacological similarities between ethanol and GABAergic drugs in humans (Cole and Davis, 1975) and that the sedative/hypnotic actions and anxiolytic effects of ethanol in laboratory animals could be blocked by GABA antagonists (Cott et al., 1976; Liljequist and Engel, 1982). Moreover, benzodiazepines which are positive modulators of the $GABA_A$ receptors, are used routinely during alcohol detoxication in the clinic and commonly as self-medication of alcohol withdrawal symptoms (cf. SBU, 2001; Johansson et al., 2003). $GABA_A$ receptors mediate the majority of the fast inhibitory synaptic transmission in the mammalian central nervous system (Krnjevic, 1991; Thompson, 1994). Some of the behavioral and cognitive consequences of ethanol consumption are suggested to be due to potentiation of this inhibition, especially the acute sedative effects (Cott et al., 1976; Liljequist and Engel, 1982; Palmer et al., 1987; Poelchen et al., 2000; for reviews, see Grobin et al., 1998; Harris, 1999; Ueno et al., 2001).

Being ligand-gated pentamers, the $GABA_A$ receptors have several features in common with nAChRs (*vide supra*), such as a variety of functional subunit combinations, the susceptibility to be modulated by ethanol as well as the propensity to desensitize (for review, see *e.g.* Grobin et al., 1998). Important factors for the transition to alcohol dependence appear to be the development of cross-tolerance between sedative effects of ethanol and other GABAergic drugs such as the benzodiazepines, as well as the reduced responsiveness of the $GABA_A$ receptors to the endogenous agonist GABA (Ticku and Burch, 1980; Allan and Harris, 1987; Morrow et al., 1988; Sanna et al., 1993; Devaud et al., 1996). Alterations of the $GABA_A$ receptor subunit composition is one proposed mechanism behind development of tolerance to effects of ethanol and GABAergic compounds after chronic intermittent ethanol treatment (Mahmoudi et al., 1997; Cagetti et al., 2003; Liang et al., 2004). It has been suggested that the molecular mechanisms behind these changes involve the effects of ethanol on receptor density (Ticku and Burch,

1980), posttranslational modification (Kumar et al., 2002), receptor trafficking (Grobin et al., 1998), and subunit expression (Mhatre et al., 1993; Devaud et al., 1995; Devaud et al., 1997; Follesa et al., 2003; Sanna et al., 2003; for review, see Grobin et al., 2000).

The extrasynaptically located α6β2/3δ* and α4β3δ* $GABA_A$ receptors that are particularly sensitive to low concentrations of ethanol (in the millimolar range) (Nusser et al., 1998), have been suggested to be the primary targets of ethanol at concentrations reached during moderate social drinking (Wallner et al., 2003). In the hippocampus, chronic intermittent ethanol administration results in a reduction of extrasynaptically located $GABA_A$ receptors in favor of intrasynaptic less alcohol-sensitive (Cagetti et al., 2003; Liang et al., 2006). These changes may correlate with the development of tolerance to the sleep inducing effects and the lack of tolerance to anxiolytic effects, observed in both animals and humans (Allen et al., 1977; Brower, 2001; Roehrs and Roth, 2001) after chronic intermittent ethanol administration (Liang et al., 2006). The persistent switch from extrasynaptic to intrasynaptic $GABA_A$ receptors in chronic intermittent ethanol treated rats may be one mechanism by which ethanol dependence is maintained (Liang et al., 2006). This could also explain why multiple withdrawal episodes seem to produce more severe dependence than continuous alcohol administration (Brown et al., 1988; Olsen et al., 2005; Liang et al., 2006). In addition to its direct effects on postsynaptic $GABA_A$ receptors, alcohol also appears to produce an indirect activation of presynaptic $GABA_B$ receptors and a consequent reduction in the release of endogenous GABA, *i.e.* ethanol also possesses an inhibitory regulatory property of its own GABAergic actions (Ariwodola and Weiner, 2004; for review, see Follesa et al., 2006).

The benzodiazepine diazepam binds to a specific site at the $GABA_A$ receptor (Stephenson, 1995). Although addictive (Woods et al., 1992; Martin et al., 1993b), benzodiazepines, like other $GABA_A$ agonists, do not acutely activate the mesolimbic dopamine system, rather they reduce nAc dopamine (Zetterstrom and Fillenz, 1990; Ferraro et al., 1996; Yan, 1999). $GABA_A$ receptor agonists also appear to inhibit cholinergic interneurons in the nAc (Rada et al., 1993; DeBoer and Westerink, 1994; Rada and Hoebel, 2005), and reduce nAc acetylcholine levels (Rada and Hoebel, 2005). These observations have lead to the recent hypothesis that the ability of drugs of abuse to increase the ratio between nAc levels of dopamine and acetylcholine is of more importance for their addictive properties than their dopamine elevating effects alone (Rada and Hoebel, 2005). Thus, GABA and acetylcholine may interact in the nAc, an assumption supported by data demonstrating that GABAergic neuronal activity in the nAc is modulated by cholinergic afferents acting on nAChRs and mAChRs (Pickel et al., 1988; de Rover et al., 2002). This could indicate that chronic nicotine use may produce alterations of the neuronal regulation of the nAc and possibly other parts of the striatum which may also affect the response of these brain regions to *e.g.* ethanol or benzodiazepines, a hypothesis that was challenged in the present thesis.

NICOTINE

Approximately one third of the world's adults smoke tobacco. Most of them start as adolescents, and half of those who continue smoking die from smoking-related diseases (WHO 1997). In the developing countries, the use of tobacco is increasing and smoking is estimated to be the largest single cause of premature death (Peto et al., 1996).

Nicotine is the main addictive component of tobacco (Jaffe, 1990; Karan et al., 2003). It acts by affecting nAChRs, which are expressed in brain areas associated with reward, including dopaminergic, glutamatergic and GABAergic neurons in the VTA and the nAc (*e.g.* Clarke and Pert, 1985; Klink et al., 2001; Picciotto and Corrigall, 2002) (Fig. 2). Extensive data from *in vivo* microdialysis studies have revealed that nicotine increases extracellular dopamine levels in the nAc by activation of nAChRs in the VTA (Imperato et al., 1986; Di Chiara and Imperato, 1988; Nisell et al., 1994a, 1994b; Benwell and Balfour, 1998; Schilstrom et al., 1998a; Sziraki et al., 1998; Sziraki et al., 2002; Tizabi et al., 2002). These receptors are crucial in mediating the reinforcing effects of nicotine in rodents (Corrigall et al., 1994; Nisell et al., 1994a; Picciotto et al., 1998), since blockade of nAChRs in the VTA but not in the nAc abolishes nicotine self-administration (Corrigall et al., 1994). Because the postsynaptic nAChRs on the dopamine neurons in the VTA desensitize within seconds to minutes in the presence of nicotine (Pidoplichko et al., 1997; Wooltorton et al., 2003)(*vide infra*), the main synaptic mechanisms by which nicotine is suggested to stimulate the dopaminergic cell bodies in the VTA are the following two: (1) long-term potentiation of the glutamatergic excitatory drive by activation of pre-synaptic nAChRs on glutamatergic terminals (Schilstrom et al., 1998a; Mansvelder and McGehee, 2000) and (2) depression of GABAergic inhibitory input by desensitization (*vide infra*) of post-synaptic nAChRs on GABAergic interneurons, reducing the impact of endogenous acetylcholine on these receptors (Mansvelder et al., 2002). Also in the nAc, nicotine briefly stimulates nAChRs on GABAergic interneurons that promote inhibition of the output neurons (de Rover et al., 2002), followed by desensitization (for review, see Mansvelder et al., 2003).

NICOTINIC ACETYLCHOLINE RECEPTORS

In 1907, Langley described the neuromuscular nAChR as the "receptive substance" (Langley, 1907), but it took many years before the existence of central nAChRs was experimentally confirmed (Caulfield and Higgins, 1983; Clarke et al., 1984; Clarke and Pert, 1985; Clarke et al., 1985; Collins et al., 1986; Sargent et al., 1989; Sargent, 1993; McGehee and Role, 1995)

Neuronal nAChRs are pentamers composed of two α- and three β-subunits, α-heteromers (*e.g.* α9/α10) or α-homomers composed of five α-subunits (*e.g.* α7, α9) (Couturier et al., 1990; Elgoyhen et al., 2001; Le Novere et al., 2002; Sgard et al., 2002). Nicotine stimulates nAChRs by binding at the acetylcholine binding site of the α-subunits (Arias, 2000), while the β subunits are merely structural components. Until recently, 17 nAChR subunits have been identified (for review, see Lukas et al., 1999), of which the α1

and β1 subunits are found exclusively at the motor endplate, whereas the neuronal nAChR type contain α2–α10 and β2–β4 subunits (McGehee and Role, 1995; Lindstrom, 1996; Elgoyhen et al., 2001; Lustig et al., 2001; Khiroug et al., 2002).

The rat midbrain dopamine neurons generally express the α3-α7 and β2-β3 subunits (Klink et al., 2001). In the rat VTA, mRNA for the α2-α7 and the β2-β4 subunits have been found (Charpantier et al., 1998). Here, the homopentameric α7 nAChR is suggested to reside on dopaminergic (Klink et al., 2001; Wooltorton et al., 2003) and GABAergic neurons (Klink et al., 2001), as well as presynaptically on glutamatergic terminals (Nomikos et al., 2000) (Fig. 2). The main heteromeric nAChR subtypes in this brain region may be of α6β2β3* and α4α5β2* subunit compositions on the dopamine neurons and of the $(\alpha4)_2(\beta2)_3$ composition on the GABAergic neurons (Le Novere et al., 1996; Klink et al., 2001; Azam et al., 2002; Champtiaux et al., 2003; for recent review, see Olsen et al., 2005). However, additional functional nAChR subtypes such as the α3β2* nAChR may be present in the rat VTA (Jerlhag et al., 2006).

nAChR subtypes and their specific antagonists

The α4β2* and α7* nAChRs are the predominant nAChR subtypes in the brain (for review, see Lindstrom et al., 1995). Still, the function of the α7* nAChRs in the actions of nicotine is unclear. In the VTA, α7* nAChRs may mediate the nicotine-induced increase in extracellular levels of excitatory amino acids in the VTA (Schilstrom et al., 2000) and dopamine in the nAc (Schilstrom et al., 1998b). Previous studies propose that these receptors contribute to the development of long-term adaptations to nicotine exposure (Mansvelder and McGehee, 2000), to the rewarding effects of nicotine and cocaine (Panagis et al., 2000) and are involved in the nicotine withdrawal syndrome (Nomikos et al., 1999). However, a recent study suggested no involvement of the α7* nAChRs in nicotine dependence, by demonstrating the lack of precipitation of the nicotine withdrawal syndrome when blocking this receptor at a dose that reduce nicotine self-administration (Markou and Paterson, 2001). Experimentally, the plant alkaloid methyllycaconitine citrate (MLA) is a common tool to investigate the α7 nAChR. MLA was long considered a selective antagonist for this receptor subtype (Macallan et al., 1988; Ward et al., 1990; Alkondon et al., 1992; Davies et al., 1999), but was recently demonstrated to block also heteromeric α3 and/or α6β2β3* nAChRs (Klink et al., 2001; Mogg et al., 2002; Salminen et al., 2004), which may explain some of the inconsistencies in the conclusions of the abovementioned studies on the involvement of the α7* nAChR in nicotine dependence.

Studies utilizing animals genetically modified with respect to nAChRs suggest that the α4 and the β2 nAChR subunits are critically involved in nicotine addiction (Picciotto et al., 1998; Tapper et al., 2004). In the VTA, the α4β2* nAChR subtype is important for the dopamine-enhancing properties of nicotine as well as crucial for nicotine self-administration, as demonstrated by the ability of local VTA administration of dihydro-ß-erythroidine (DHßE, a relatively selective competitive α4β2* nAChR antagonist) to antagonize these effects (*e.g.* Alkondon and Albuquerque, 1993; Corrigall et al., 1994; Grillner and Svensson, 2000; Dwoskin and Crooks, 2001; Khiroug et al.,

2004). Moreover, in rats, systemic DHβE blocks several behavioral effects by nicotine such as locomotor activation (Stolerman et al., 1997).

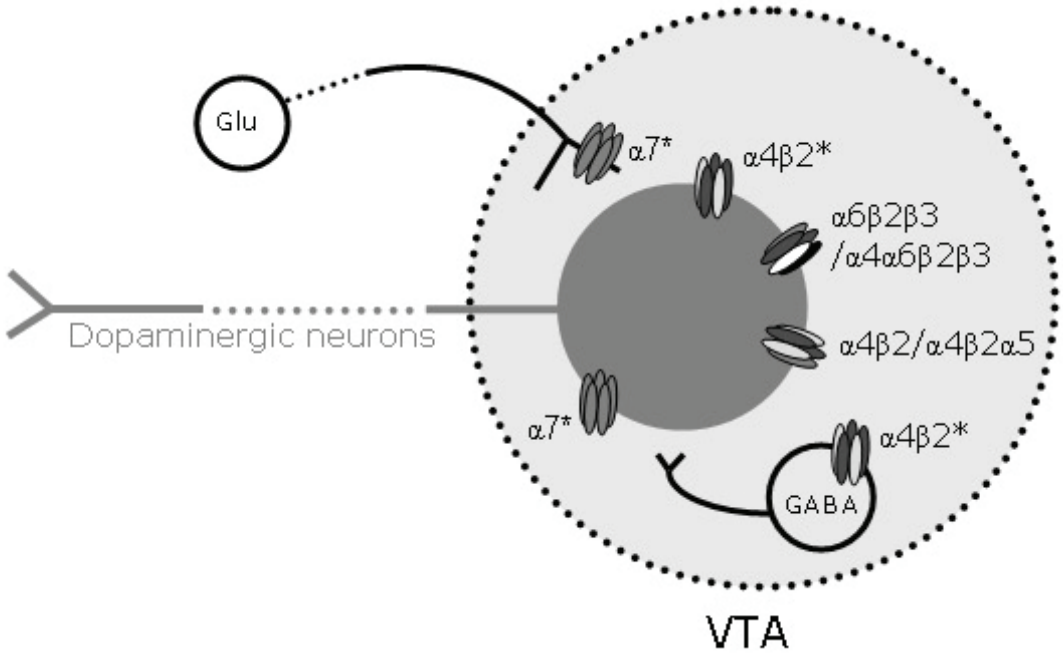
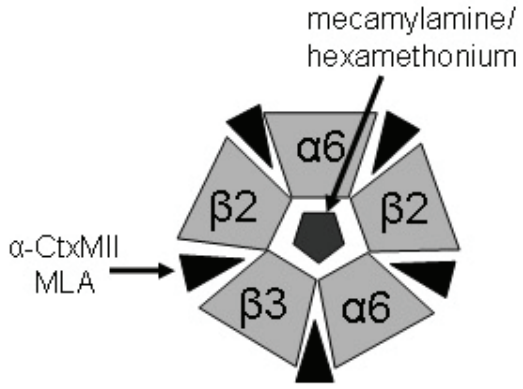


Fig. 2. **Neuronal nAChR subtype compositions suggested to be functional on dopaminergic, glutamatergic (Glu) and GABAergic neurons in the VTA.**
The relative distribution of different functional nAChR subtypes within the VTA, is suggested to be α4β2: 62%, α7: 22% and other: 16% (Champtiaux et al., 2003; for recent review, see Jensen et al., 2005). α-CtxMII sensitive nAChRs modulate the function of dopaminergic projections from the SN and VTA (Grady et al., 1997; Kulak et al., 1997).

Also the α6 subunit has been implicated in nicotinic reward mechanisms (Le Novere et al., 1996). Although sharing high sequence homology with the α3 subunit (Le Novere and Changeux, 1995), the α6* nAChRs (Whiteaker et al., 2000; McIntosh et al., 2004), but not α3* nAChRs (Whiteaker et al., 2002), appear to be functional in dopaminergic areas in the rodent striatum. α-conotoxin MII (α-CtxMII, a small polypeptide isolated from the venom of cone snails (McIntosh et al., 1999)) is a selective competitive antagonist for nAChR subtypes containing α3β2* and/or α6* subunits (Cartier et al., 1996; Kuryatov et al., 2000; McIntosh et al., 2004). α-CtxMII-sensitive sites also contain β2 (Grady et al., 2001), β3 (Cui et al., 2003) and α4 (Marubio et al., 2003) subunits, and recent data suggest that the α-CtxMII-sensitive α6β3β2* and α4α6β3β2* nAChR subtypes are functional by means of dopamine release assays from mice synaptosomes (Zoli et al., 2002; Champtiaux et al., 2003; Cui et al., 2003; Salminen et al., 2004) as well as membrane binding (Salminen et al., 2005). [125I]-α-CtxMII binding is absent in α6, but not in α3, null mutant mice (Whiteaker et al., 2002). This indicates that *in vivo*, the effects of α-CtxMII administration on dopaminergic activity are

mainly mediated via blockade of α6* nAChRs. This conclusion further supports the idea that the dopaminergic brain areas in mice lack functional α3* nAChRs and suggests a role for α6β3β2* nAChRs in nicotine-induced dopamine release (Champtiaux et al., 2002).



nAChR ANTAGONIST	SPECIFICITY
Mecamylamine	neuronal non-selective
α-CtxMII	α3β2* and α6*
MLA	α7 and α3/α6*
DHβE	α4β2
Hexamethonium	non-selective, peripherally acting

Fig. 3. Schematic drawing of a nAChR pentamer and the access points for some of its antagonists. Table insert accounts for the nicotinic antagonists that were utilized in the experiments of this thesis.

ACUTE AND CHRONIC EFFECTS OF NICOTINE;

DESENSITIZATION AND RECEPTOR UP-REGULATION

nAChRs can undergo desensitization, a state of reversible reduction in response during sustained agonist application (Katz and Thesleff, 1957; for review, see Quick and Lester, 2002). As mentioned above, both stimulation and desensitization of nAChRs may be important for the effects of nicotine in the VTA (Mansvelder and McGehee, 2000; Mansvelder et al., 2002; Wooltorton et al., 2003) and the nAc (de Rover et al., 2002; de Rover et al., 2004). Although α7* nAChRs can desensitize rapidly by very high agonist concentrations (Couturier et al., 1990; Zhang et al., 1994), they have a lower affinity for nicotine than the α4β2* nAChRs and are consequently not significantly desensitized by the nicotine levels achieved from smoking (Mansvelder et al., 2002; Quick and Lester, 2002; Wooltorton et al., 2003). The α4β2* nAChRs on the other hand, which are the predominant subtypes on the GABAergic neurons, desensitize after some exposure to nicotine concentrations relevant to smoking (Benwell et al., 1995; Pidoplichko et al., 1997; Dani et al., 2000). Thus, the consequence of these synaptic events is prolonged firing of dopamine neurons in response to nicotine. It is also possible that additional dopamine stimulating effects of smoking, independent of nicotine, are maintained by sensory cues or other chemicals present in the smoke (Balfour et al., 2000; Balfour, 2002).

One important aspect of nicotine addiction is the phenomenon of nicotine-induced up-regulation of nAChRs (Schwartz and Kellar, 1985). Chronic nicotine, as well as chronic mecamylamine, treatment increases the number of [3H]-nicotine binding sites in the mouse brain (Collins et al., 1994; Pauly et al., 1996). Moreover, additive increases in the number of binding sites were observed after co-treatment with the two drugs (Collins et al., 1994). In adult human brains, post-mortem studies have revealed an increased number of nicotine-binding sites in smokers (Benwell et al., 1988; Perry et al., 1999). There are several different theories on the mechanism(s) behind the enhanced receptor activity following chronic nicotine, although most of them can be explained by post-translational mechanisms (*e.g.* Mugnaini et al., 2002; Sokolova et al., 2005). Nicotine was recently suggested to act as a pharmacological chaperone to up-regulate human α4β2* nAChRs (Kuryatov et al., 2005). Results from studies of the effects of chronic nicotine on specific nAChR subtypes suggest that different subtypes in different brain areas are differently affected by chronic nicotine. Increases in the α4β2* nAChRs (Marks et al., 2004) and α6* nAChR (Lai et al., 2005; Mugnaini et al., 2006), have been observed following nicotine treatment, whereas α7* (Pauly et al., 1991; Collins et al., 1994; Pauly et al., 1996) and α3* (Davila-Garcia et al., 2003; Nguyen et al., 2003, 2004) nAChRs are reported to remain unaltered in the rodent striatum.

NICOTINE AND ETHANOL INTERACTIONS

Ethanol consumption in rats is increased by subchronic pre-treatment with nicotine, mecamylamine, or the peripheral nAChR antagonist hexamethonium, as well as the combined treatment of nicotine and either of these two nAChR antagonists (Blomqvist et al., 1996; Ericson et al., 2000a; Olausson et al., 2001). As mentioned above, chronic treatment with nicotine or the non-selective nAChR antagonist mecamylamine, results in up-regulation of at least some nAChR subtypes. Although controversial, this has *i.a.* been suggested to be a consequence of nicotine-induced desensitization of these receptors (Wonnacott, 1990; Paterson and Nordberg, 2000; Buisson and Bertrand, 2002; Gentry and Lukas, 2002). Consequently, the enhanced ethanol intake observed following subchronic nicotine treatment may involve subchronic consequences of a functional blockade of nAChRs by nicotine rather than stimulation of these receptors. This may explain why also antagonists of the nAChRs such as hexamethonium increase ethanol self-administration.

A combination of many factors may underlie the often combined use and abuse of alcohol and nicotine. Apart from psychosocial factors, one suggestion is the common pharmacological actions of both these drugs on nAChRs (for reviews, see Soderpalm et al., 2000 and Balogh et al., 2002; Larsson et al., 2002). For instance, nAChR binding is altered in specific brain regions in ethanol dependent rats (Yoshida et al., 1982; Penland et al., 2001), and treatment with chronic nicotine as well as chronic ethanol can increase the number of rat brain nAChRs (Rezvani and Levin, 2002). However, other studies demonstrate no alterations in ^{3}H-nicotine binding in mice (Burch et al., 1988; Collins et al., 1988a) or in rats (Nordberg et al., 1985) following chronic ethanol. It was therefore

suggested that the effect of alcohol on brain nAChR binding may be influenced by the length of ethanol treatment and genetic factors and may also be limited to certain brain regions (Booker and Collins, 1997). Despite these inconsistencies, substantial data demonstrate indirect as well as direct effects of ethanol on nAChRs (for review, see Narahashi et al., 1999).

The first evidence for ethanol interactions with nAChRs was obtained in preparations from the frog neuromuscular junction demonstrating stimulatory activity of ethanol on muscle nAChRs (Bradley et al., 1980). Shortly, this data was followed by illustrations of ethanol interactions with *Torpedo* neuronal nAChRs (Ei-Fakahany et al., 1983; Forman et al., 1989). *In vitro* studies demonstrate that, at behaviorally relevant concentrations, ethanol can act as an allosteric modulator stimulating recombinant human (Cardoso et al., 1999) and rat (Covernton and Connolly, 1997) α4β2* nAChR subtypes, and ethanol may moreover enhance the electrophysiological response to nicotine in certain rat brain regions *in vivo* (Criswell et al., 1993). Ethanol is suggested to act as a positive modulator of the α-bungarotoxin insensitive (Wu et al., 1994; Aistrup et al., 1999) or human α4β2 nAChRs, potentiating acetylcholine-induced currents (Zuo et al., 2001). Ethanol may also interact directly with peripheral nAChRs at physiologically relevant concentrations (Aracava et al., 1991). The α3β4* subunit combination may be especially sensitive to modulation by low ethanol concentrations and the α7* nAChRs are modulated by higher relevant levels of ethanol, where ethanol inhibits these receptors' activation by agonists (Yu et al., 1996; Covernton and Connolly, 1997; Aistrup et al., 1999). Also, *in vitro* studies suggest specific interaction sites for alcohols on the transmembrane 2 segment of the α2 subunit of the neuronal $(\alpha2)_2(\beta4)_3$ nAChR (Borghese et al., 2003b), where alcohol binding at Leucine 263 enhances receptor function (Borghese et al., 2003a).

Both ethanol- and nicotine-induced dopamine overflow in the nAc involve activation of nAChRs in the VTA (for reviews, see Svensson et al., 1990; Soderpalm et al., 2000). Several studies support the idea that the α4β2* nAChRs (Butt et al., 2003; Owens et al., 2003; Butt et al., 2004), as well as the α7* nAChR (Wehner et al., 2004), are modulated by ethanol. Still, neither MLA, nor DHβE had any effect on ethanol-induced dopamine elevations in rats or locomotor activity in mice, suggesting that the stimulatory and dopamine enhancing effects of ethanol involve nAChRs composed of other subunits (Ericson, 2000; Larsson et al., 2002; Ericson et al., 2003; Larsson et al., 2004; for review, see Larsson and Engel, 2004). This notion is supported by the lack of impact of systemic DHβE on alcohol consumption in rats (Le et al., 2000), and is further supported by data demonstrating that local VTA administration of α-CtxMII can reduce the ethanol-induced dopamine overflow in the nAc, locomotor stimulation as well as ethanol intake in rodents (Larsson et al., 2004). This indicates that α-CtxMII sensitive receptors (α3β2* and/or α6* nAChRs) may be important in mediating the stimulatory, dopamine-enhancing, and rewarding effects of ethanol. Interestingly, a recent paper specifically dedicated these effects to α3β2* nAChRs in the mouse VTA (Jerlhag et al., 2006), a hypothesis that is supported by the demonstration that 18-methoxycoronaridine, a selective α3β4* nAChR antagonist (Pace et al., 2004), reduces both nicotine (Glick et al., 2000) and ethanol (Rezvani et al., 1997; Maisonneuve and Glick, 2003) intake in rats. Apart from the diverse effects upon nAChR activity and dopaminergic neurons, ethanol modulates numerous other transmittor systems/receptors, including strychnine-sensitive

glycine receptors, NMDA receptors, serotonin and GABA receptors (Engel et al., 1992; Molander et al., 2005; Molander and Soderpalm, 2005; for review, see Koob et al., 1998).

Studies also propose several common genetic vulnerabilities to nicotine and alcohol dependence (*e.g.* Swan et al., 1996, 1997; True et al., 1999; Bierut et al., 2004). In synaptosomes from the mouse brain, α4β2* nAChRs are stimulated by physiologically relevant doses of ethanol (Butt et al., 2003), an effect that was modulated by a naturally occurring alanine-threonine switch polymorphism. Behavioral studies indicate that the strains of rodents bred for differences in sensitivity to ethanol may share a similar genetic constitution for nicotine sensitivity (De Fiebre et al., 1987; de Fiebre and Collins, 1989; de Fiebre et al., 1991) and it was recently demonstrated that rats selectively bred for high alcohol intake display increased nicotine self-administration (Le et al., 2006). Moreover, nicotine and ethanol demonstrate cross-tolerance to some effects in animals (Burch et al., 1988; Collins et al., 1988b; Collins et al., 1993; Collins et al., 1996), a phenomenon that appears to be genetically dependent (Luo et al., 1994; Madden et al., 1995; Madden et al., 1997). In null mutant α7-/- nAChR knock outs, there was an increase in sensitivity to the sedative-hypnotic effects of ethanol, as measured by ethanol-induced loss of righting reflex (Bowers et al., 2005). Indeed, a low sedative response to acute alcohol is considered a risk factor of alcohol dependence (Schuckit and Smith, 1996; Schuckit, 1998; Heath et al., 1999). Clinical studies suggest that nicotine has the ability to attenuate subjective sedative effects of alcohol intoxication (Zacny, 1990; Perkins et al., 1995; Perkins et al., 2000). However, these data are inconsistent with some results of other human studies demonstrating that nicotine can increase sedative-like effects of acute ethanol (Acheson et al., 2006). This and other discrepancies between results from clinical studies on nicotine and alcohol interactions may be due to methodological differences such as doses, administration regimens or the sex of the tested individuals (*e.g.* Perkins et al., 2002).

Nicotinic drugs for the treatment of alcoholism

Acute mecamylamine reduces alcohol intake in alcohol-preferring Wistar rats (Blomqvist et al., 1996; Le et al., 2000). Interestingly, clinical studies demonstrate that mecamylamine reduces the euphoric and stimulant subjective effects of acute alcohol intoxication in social drinkers, and decreases the subjects´ desire to drink more (Blomqvist et al., 2002; Chi and de Wit, 2003; Young et al., 2005). The higher the stimulatory effects that drinkers experience from alcohol, the more alcohol they choose to drink (Holdstock and de Wit, 2001; King et al., 2002; Thomas et al., 2004; Young et al., 2005). The ability of mecamylamine to reduce the stimulatory effect of acute alcohol consumption may thus have important clinical implications. Unfortunately, the use of this compound is limited by its many peripheral side effects (Young et al., 2001) such as dizziness, lightheadedness, fainting, tremors, chorieform convulsions, in addition to mental aberrations and dysphoria. Not to forget, chronic mecamylamine administration increases ethanol consumption in the rat (*vide supra*) (Ericson et al., 2000a). Thus, the availability of more selective nAChR modulators for treatment of alcohol dependence is warranted. The present thesis therefore investigated the effect of selective nAChR antagonists on responding with conditioned reinforcement.

Nicotinic drugs in conditioned reinforcement to natural rewards

If the impact of drug-associated cues on craving, drug-seeking and relapse can be reduced by nicotinic modulators, it is important to know how these modulators affect also basic motivation and seeking for natural rewards. Preventing alcohol relapse with a pharmacological molecule that reduces the conditioned reinforcing properties also of food cues may not be an optimal treatment. On the other hand, a compound that blocks cue-induced compulsive feeding behavior may be a promising candidate for the treatment of eating disorders. Moreover, during smoking cessation, weight gain may be a consequence of increased value of food reward (Lerman et al., 2004). Indeed, at least in women, fear of weight gain inhibits attempts to quit smoking (for review, see Perkins et al., 1997). Not to forget, alcohol consumption is often accompanied by sugar intake and studies suggest a genetic association between high sweet preference and alcohol dependence (Kampov-Polevoy et al., 1997; Kampov-Polevoy et al., 1999). Therefore, by studying the effects of various nAChR antagonists on responding with conditioned reinforcement to sucrose, the second paper of the present thesis studied the role of nAChRs in behavioral effect of cues associated with natural reward.

AIMS

The comprehensive aim of the present thesis was to investigate the neurobiological mechanisms by which nAChRs and nicotinic drugs can influence the conditioned reinforcing, as well as the primary reinforcing, effects of alcohol in the rat. Increased knowledge in nicotinic mechanisms behind facilitation of ethanol drinking and relapse to ethanol abuse has important clinical implications and may provide novel explanations to why nicotine and alcohol commonly are co-abused.

Specific aims of the present thesis

Nicotinic mechanisms in the conditioned reinforcing effects of alcohol

1) To investigate the involvement of specific nAChRs in the VTA in the mediation of nAc dopamine overflow induced by alcohol-cues.
2) To investigate the involvement of specific nAChRs in the VTA in the mediation of reward-seeking behavior induced by alcohol-cues.
3) To investigate the involvement of nAChRs in the mediation of reward-seeking behavior induced by cues associated with natural reward.

Nicotinic mechanisms in the primary reinforcing effects of alcohol

4) To elucidate the primary brain site of interference for ethanol-induced dopamine elevations in the nAc.
5) To investigate the tentative involvement of GABAergic effects behind the lack of association between the ethanol-induced elevations in nAc dopamine and the concomitant ethanol concentrations in the same brain region.
6) To investigate the impact of chronic administrations of nicotinic drugs on the ethanol-induced dopamine elevations in the nAc and the dorsal striatum.

METHODS AND METHODOLOGICAL CONSIDERATIONS

Experimental design

Paper I

Rats were trained during six weeks to associate a discriminative cue (a tone) with the access to a bottle of 10% ethanol in their home cages. The training period was followed by implantation of microdialysis probes into the nAc and the VTA. The nAc dopamine response to the presentation of the alcohol cue was measure by means of *in vivo* microdialysis. The possible involvement of VTA nAChRs in the dopaminergic response to the alcohol cue was investigated by local perfusion of mecamylamine or DHβE into the VTA.

Another set of animals underwent 9 weeks of ethanol pre-exposure, followed by implantation of guide cannulae in the VTA (Fig. 4). The rats were trained to associate a tone+light conditioned stimulus (CS) with the presentation of 10% ethanol in a magazine, and were subsequently tested on the acquisition of a new instrumental response (lever pressing) with conditioned reinforcement. The testing was preceded by an acute systemic injection of mecamylamine or DHβE or a local VTA infusion of mecamylamine or α-CtxMII.

The latter experimental design was used in an additional experiment (the last experiment of the present thesis), with the addition of a 15 day period of daily systemic injections of nicotine, hexamethonium or saline. The effect of an acute systemic injection of DHβE on responding with conditioned reinforcement was tested.

Paper II

Naïve rats were trained to associate a tone+light CS with the presentation of 0.1 M sucrose in a magazine, and were subsequently tested on the acquisition of a new instrumental response (lever pressing) with conditioned reinforcement (Fig. 4). The testing was preceded by an acute systemic injection of mecamylamine or MLA.

Paper III

Naïve rats were implanted with microdialysis probes in the nAc and the VTA. The nAc dopamine response to ethanol perfusion of either or both probes was analyzed by means of *in vivo* microdialysis. An additional group of naïve rats was implanted with microdialysis probes in the nAc. The accumbal dopamine response to perfusion of ethanol alone or co-perfused with picrotoxin was investigated by means of *in vivo* microdialysis.

Paper IV

In Paper IV, rats were pre-treated with systemic injections of nicotine, hexamethonium or saline once daily for 15 days. A microdialysis probe was implanted in the nAc or the dorsal striatum. By means of *in vivo* microdialysis, dopamine in the nAc was measured in response to a single systemic ethanol injection or during nAc perfusion of ethanol or diazepam. Dopamine in the dorsal striatum was measured during ethanol

perfusion into the same brain area. Samples from rats systemically injected with ethanol were analyzed also with respect to nAc ethanol concentrations.

Animals

All animals were allowed to adapt to the novel environment for one week following arrival. They were housed in humidity and temperature controlled environments on a 12/12 hour controlled light-dark cycle, and had free access to water and standard rat chow, unless stated otherwise.

For the microdialysis experiments in Paper I, male Wistar rats (220-250 g; B&K Universal AB, Stockholm, Sweden) were singly housed on a reversed artificial light-dark cycle (light off at 9 a.m. and on at 9 p.m.). All training sessions and microdialysis experiments took place in the animals´ home cages in the colony room. For the conditioned reinforcement experiments, Male Sprague-Dawley rats (225-275 g, Charles River, USA) housed in pairs. Male Wistar rats (260-280 g B&K Universal AB, Sweden) housed 5 per cage were used in Papers III and IV.

All experiments were approved as appropriate by the Ethics Committee for Animal Experiments, Göteborg, Sweden or the Yale Animal Care and Use Committee (YACUC) and were conducted in a manner complying with local and international guidelines for animal welfare.

Experimental techniques

Pavlovian training (Paper I)

Naïve rats (n = 77) were subjected to a limited access paradigm in their home cages where the daily presentation of a bottle of ethanol (in addition to a continuously available water bottle), was preceded by a CS, a 1 sec tone (paired; CS+). Following the CS+ presentation, the ethanol bottles were available for one hour/day. The concentration of the alcohol solution was gradually increased during 3 weeks (2% - 4% - 6% - 8% - 10% v/v) to a final concentration of 10% during the rest of the limited access experiment, lasting approximately six weeks in total, after which the alcohol high-preferring animals were selected for microdialysis experiment. Due to the restricted number of animals that could be tested in the microdialysis experiment setup simultaneously, all rats were not subjected to microdialysis at the same day. Thus, since both training and testing took place in the animal colony room, the total number of pairings varies. There are however no differences in the results from the rats trained the shortest period of time compared to those trained the longest. The rats received 34-43 days of one pairing/day in the mecamylamine experiment, 42-49 days of one pairing/day in the DHβE experiment and 29-41 days of one pairing/day in the control experiment. Only rats consuming ≥0.8 g ethanol/kg each limited access hour while displaying a stable high ethanol preference (≥60%) were selected for *in vivo* microdialysis experiments (n=30). These criteria were based on a recent study demonstrating that rats consuming more than 0.8 g/kg, but not

those consuming less than 0.5 g/kg, per drinking session displayed a significant increase in nAc dopamine levels during ethanol self-administration (Doyon et al., 2005). An ethanol intake of 0.8 g/kg produces blood alcohol levels of about 70 mg/dl (Gonzales and Weiss, 1998), or 8 mM (Nurmi et al., 1999), in male Wistar rats.

A control experiment was also performed where a group of 32 rats was trained daily to associate a tone with ethanol availability (CS+, exactly as described above). Another group of 12 rats was simultaneously housed in the same animal room and were thus exposed to the daily CS, however not paired with the access to ethanol, *i.e.* the CS was presented but alcohol was not made available (*i.e.* unpaired: CS-). Seventeen rats from the CS+ group (selected based on their ethanol preference and intake, see above) and 12 rats from the CS- group were subsequently implanted with a dialysis probe in the nAc and tested in the *in vivo* dopamine microdialysis experiment described below.

Surgeries (Papers I, III, IV and additional experiments)

The animals were anaesthetized with isoflurane (~3.5-4.0 % in air, microdialysis experiments) or Equithesin (conditioned reinforcement experiments) and prepared for stereotaxic surgery. Microdialysis probes were implanted monolaterally into the VTA and/or the nAc or the dorsal striatum. The coordinates used were, relative to the bregma (flat skull) and dura as appropriate, A/P +1.85, L/M -1.4, V/D - 7.8 for the nAc and A/P –5.4, L/M –0.7, V/D – 8.4 (Paper I) or A/P –5.2, L/M –0.7, V/D – 8.4 (Paper III) for the VTA and A/P +1.0, L/M –3.1 and V/D – 5.6 for the dorsal striatum (cf. (Paxinos and Watson, 2005). The microdialysis probes were kept in place with Phosphatine dental cement (Swedia or Dental AB, Sweden) and anchored to the skull by two stainless steel screws. The animals were injected with 2.0 ml 0.9% NaCl (s.c.) to prevent dehydration, and allowed to recover for 48 hours prior to initiation of the microdialysis experiment. During the recovery period they had limited access to ethanol as usual. In Paper I, bilateral infusion cannulae (PlasticsOne, USA) were implanted in the VTA using the following coordinates A/P –5.4, L/M ±0.7 (relative to the bregma) and V/D – 7.0 (relative to the dura). The cannula was attached to the skull using screws and dental cement (Ortho-Jet, Lang Dental, USA). Obturators were placed into the guide cannulae to prevent blocking. After the surgery, animals were allowed to recover for one week, while being on continuous 10% ethanol intake, before starting the experiment.

The microdialysis probes

All microdialysis experiments were performed using a modified version of the I-shaped probe, produced in our laboratory (cf. (Waters et al., 1993)). The inlet and outlet of the probes were made of 20 gauge PolyEtylene tubing (VWR, Sweden). During manufacturing and implantation of the probe a glass rod was used as a holder. The dialysis membrane was prepared from a copolymer of polyacrylonitrile and sodium methallyl sulfonate (Hospal-Gambro, Sweden) with an o.d./i.d. of 130/220 µm. The length of the exposed tip (the active space) was 2.0 mm, and the remaining area was covered with silicone glue (CAF 3; Rhodorsil Silicones, Saint-Fons Cedex, France). Before implantation the probes were perfused (2.0 µl/min) with 40 µl of ethanol (70 %) followed by approximately 120 µl Ringer's solution. The inlet and outlet tubes were

sealed with heating and the probes were stored at +4 ºC for a maximal number of four days before implantation.

In vivo microdialysis and biochemical assay (Papers I, III and IV)

In vivo brain microdialysis was performed in awake, freely moving Wistar rats in order to measure extracellular concentrations of dopamine as well as to locally administer experimental compounds, *i.e.* reversed microdialysis. The *in vivo* microdialysis method primarily measures overflow of neurotransmitters into the extrasynaptic space. The typical *in vitro* recovery of dopamine is estimated to be approximately 10 % in our laboratory (unpublished data). The data presented here are not corrected for recovery.

On the day of the microdialysis experiment, the rats were connected to the microdialysis apparatus and a microperfusion pump (U-864 Syringe Pump, AgnTho's, Sweden) via a swivel allowing the animal to move around freely. The dialysis probes were perfused with Ringer's solution at a rate of 2.0 µl/minute. Sample collection started approximately one hour after the animals had been connected to the pump. The dialysate was then collected from the nAc or the dorsal striatum every 15 (Paper I) or 20 minutes. After obtaining a stable dopamine baseline (±10%; usually requiring 5 samples), drug administration was initiated. The dopamine content of the collected samples was determined by means of HPLC with electrochemical detection, as previously described (Ericson, 2000). No food or liquid was available during testing. In Paper I, the inlet and outlet of the microdialysis probe were resealed at the end of the first experimental day and reopened the second day. Following completion of the *in vivo* microdialysis experiments, rats were sacrificed and the brains sectioned with a vibroslicer (Campden Instruments, UK) in order to verify the placements of the microdialysis probes. Only data from rats with correctly placed probes and no signs of tissue damage near the probes during placement evaluations were included in the results. Additional subjects were excluded from the data analysis due to technical issues such as clogged dialysis probes or failure to achieve stable dopamine baselines.

Drug administration and CS presentation during the in vivo microdialysis experiments

In Paper I, during acquisition of a stable baseline of dopamine, both the nAc and the VTA were perfused with Ringer's. Thereafter, the Ringer's perfusion of the VTA was switched to perfusions with mecamylamine (100 µM) or DHβE (1 mM), or the Ringer's perfusion continued throughout the experiment in both brain areas. Animals that were perfused with Ringer's in both brain areas on test-day 1, received drug (mecamylamine or DHβE) perfusion of the VTA on test-day 2, or vice versa, in a counterbalanced manner. The CS+ was presented after 45 minutes of drug perfusion, after which four additional samples were collected. These animals were presented with the CS+ alone. The rest of the animals in the colony room simultaneously went through the daily limited access paradigm, where they received the one hour access to the alcohol solution immediately after the presentation of the CS+. In the control experiment, the CS+ or the CS- was presented directly after obtaining a stable baseline.

Previous ethanol-microdialysis concentration-response studies performed at our laboratory demonstrate that ethanol (10-1000 mM) perfused into the rat VTA does not

influence dopamine output in the nAc. However, ethanol (300 mM) perfused into the nAc increases the nAc dopamine output levels to ~30%, *i.e.* to the same extent as observed after a systemic injection of ethanol (2.5 g/kg .i.p.), whereas ethanol (1000 mM perfused in the nAc) decreases the nAc dopamine output by ~50% (Ericson et al., 2003). The ethanol concentrations perfused in Papers III (100 mM, 200 mM or 300 mM) and IV (100 mM or 200 mM) were based on these previous studies. In Paper III, we hypothesized that chronic nicotine pre-treatment may increase the sensitivity to the dopamine enhancing properties of ethanol (Paper III). In order to investigate this hypothesis, relatively low ethanol perfusion concentrations (100 mM and 200 mM) and a low injection dose (1.0 g/kg, i.p.) were used in paper IV. Considering the limited excovery of the probe, flow rate and diffusion phenomena, the ethanol perfusion concentrations of 100 mM, 200 mM or 300 mM are expected to produce local extracellular ethanol levels of approximately 15-20 mM, 30-40 mM and 45-60 mM respectively, immediately outside the probe (Ericson et al., 2003; cf. Robinson et al., 2000).

In Paper III/Experiment 1, naïve rats were thus perfused with ethanol (100 mM, 200 mM or 300 mM) into the nAc and Ringer's into the VTA, ethanol (100 mM, 200 mM or 300 mM) into the VTA and Ringer's into the nAc, or ethanol (100 mM, 200 mM or 300 mM) into both brain regions. In Paper III/Experiment 2, the nAc of naïve rats was perfused with ethanol (300 mM) or with a picrotoxin (0.2 μM) and ethanol (300 mM) co-perfusion. Perfusion with this concentration of picrotoxin is estimated to produce extracellular levels of picrotoxin of ~0.02-0.04 μM immediately outside the dialysis probe (Molander and Soderpalm, 2005).

In Paper IV, dopamine in the nAc in response to a single systemic injection of ethanol (1.0 g/kg i.p.) or during nAc perfusion with ethanol (100 mM) or diazepam (10 μM) was measured. Dopamine in the dorsal striatum was collected during an ethanol perfusion concentration of 200 mM, since a pilot study in our laboratory demonstrated no dopamine response to perfusion with lower ethanol concentrations than 200 mM in this brain area. The diazepam concentration of 10 μM was chosen since it proved optimal in a concentration-response pilot study in our laboratory. Samples from rats systemically injected with ethanol were also analyzed with respect to brain ethanol concentrations with an ANALOX Alcohol Analyzer (DIFA, Stockholm, Sweden).

The conditioned reinforcement model (Papers I and II)

Ethanol pre-exposure (Paper I and additional experiments)

Rats were continuously presented with ethanol solutions of increasing concentrations during 3 weeks (2% - 4% - 6% - 8% - 10% v/v) and to 10% ethanol for 4 additional weeks (Fig. 4). *Limited access:* Animals had limited access to alcohol for 1 week to allow for individual assessment of ethanol preference. Here, all rats were housed separately for 1 hour/day during which they were allowed to choose between 10% ethanol and water. Surgeries were performed immediately following the limited access period. During behavioral testing, 10% ethanol was intermittently available in the operant chambers according to the behavioral task schedule (*vide infra*) as well as in the home cage for 60 min, beginning 30 min after the daily testing session.

Following the limited access period, an additional set of animals was subjected to 15 daily intermittent injections with nicotinic drugs (*vide infra*) ending 5 days before testing on responding with conditioned reinforcement.

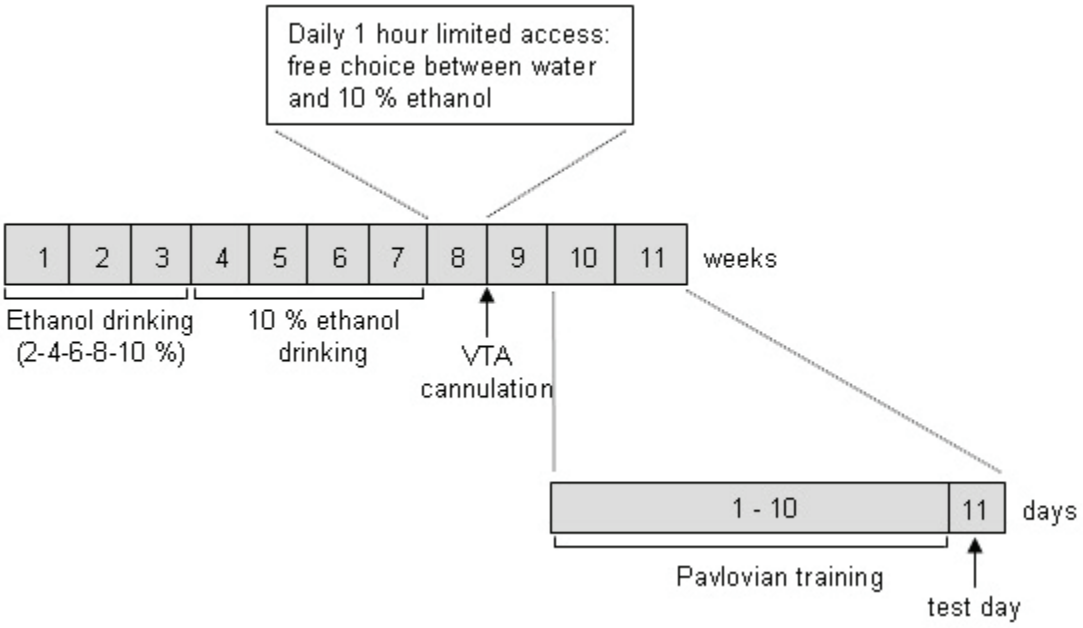


Fig. 4. Schematic illustration of the conditioned reinforcement model used in Paper I.
In paper II, the rats were exposed only to 10 days of Pavlovian training and one or two test days.

Pavlovian discriminative approach behavior (Papers I and II)

Standard operant chambers (30x20x25 cm) with grid floors (Med Associates, USA) were used for behavioral testing. Each chamber was housed in a sound attenuating outer box equipped with a white noise generator and a fan to reduce external noise. A liquid dipper (60 µl) delivered the reinforcer (Paper I: a 10% ethanol solution; Paper II: a 0.1 M sucrose solution) into the magazine. This sucrose concentration was selected based on a pilot study performed in our laboratory where free choice between tap water and 0.1 M sucrose in a 1 hour limited access paradigm, resulted in a close to 100% preference for the sucrose solution in all rats (unpublished data). Head entries were detected by a photocell mounted above the reinforcer receptacle. Above the magazine was a 2.5 W stimulus light, and the chamber was illuminated by a house light mounted on the back wall. A SonAlert tone (10 kHz) generator was mounted above the magazine. The operant chambers were controlled by a PC with interface and MedPC software (Med Associates, USA).

On the first day, a 5 s access to 60 µl of alcohol or sucrose (the unconditioned stimulus (US)) was available in the dipper on a fixed time 15 s (FT15) schedule; the session ended after delivery of 100 reinforcers. Beginning on the second day, the subjects received 30 pairings of a 5 s compound conditioned stimulus (CS; light+tone) followed immediately by 5 s access to 60 µl of the US solution; the CS+US pairings were

delivered on a random time 30 s (RT30) schedule. Head entries during the RT30 interval resulted in a 3 s delay during which time no reinforcement was given, and the schedule was restarted. Training on this schedule over a period of 10 days results in a discriminated pattern of approach of the magazine during CS and US, but not during inter-CS+US, periods (Taylor and Robbins, 1984; Burns et al., 1994; Taylor and Horger, 1999).

It should be noted that, each training session can result in a maximal total consumption of 1.8 ml of 10% ethanol. This produces an ethanol dose of no more than 0.35 g/kg per training session, which is likely below the threshold for the ethanol dose (0.5 g/kg in the rat) required to enhance nAc dopamine (Doyon et al., 2005). Thus, it could be questioned whether the training sessions were accompanied with any dopamine elevations. However, given that the CS+ acquired conditioned reinforcing properties (see Results), the dopaminergic response to the presentation of ethanol in this setting was clearly sufficient to support new associative learning between the alcohol solution and the cues. It is possible that a 2nd order conditioning between the sensory properties of alcohol (such as smell and taste previously associated with its pharmacodynamic effects during drinking in the home cages) and the CS+ contributed to the dopaminergic responses during conditioning. Since the elements of a reward that subserve appetitive Pavlovian conditioning may involve a complex array of sensory gustatory stimuli, it is likely that such processes also are important in establishing the reinforcing properties of alcohol cues in humans.

Conditioned reinforcement (Papers I, II and additional experiments)

After Pavlovian conditioning, all animals were tested in a conditioned reinforcement paradigm. Testing utilized the behaviorally stringent acquisition of a new response with conditioned reinforcement (Taylor and Robbins, 1984) and was performed in the absence of primary reinforcement. Here, two novel levers were introduced in the operant chambers. Responding on one lever (active or "CR lever") resulted in the presentation of a 5 s CS and elevation of the liquid dipper (without any reinforcer). Responding on the other (inactive or "NCR lever") had no programmed consequences and controlled for non-specific alterations in responding. The first three responses on the active lever elicited presentation of the CS, following which the CS was presented on a variable ratio (VR2) schedule. The session lasted for 30 min following the first response on the CR lever. The position of the CR lever (left/right) was balanced for all treatment groups, but remained the same for each rat in all testing sessions.

Subchronic intermittent drug pre-treatments (Paper IV and additional experiment)

Rats were divided into three groups and subjected to daily injections with 1) saline (s.c.) + saline (i.p.), 2) nicotine (0.35 mg/kg; s.c.) + saline (i.p.) or 3) saline (s.c.) + hexamethonium (10 mg/kg; i.p.) for 15 consecutive days. Previous studies have shown that such pre-treatment enhances ethanol consumption in male Wistar rats (Ericson et al., 2000b).

In Paper IV, the rats were subjected to surgery on day 14 of the subchronic drug pre-treatment period. Microdialysis was performed on day 16, *i.e.* the day following the last drug injection.

Another set of animals was first pre-exposed to ethanol for 8 weeks (see The conditioned reinforcement model). Subsequently, based on their ethanol preference, they were divided into three groups and injected with the drugs above for 15 consecutive evenings. Daily Pavlovian training session started on the 10^{th} pre-treatment day. To circumvent interference of the nicotinic drugs with the associative learning processes (Olausson et al., 2003; Davis and Gould, 2005), the rats were trained 6 hours before receiving nicotinic drug injections. Moreover, to avoid testing with conditioned reinforcement during withdrawal from the nicotinic drugs, the 15 day treatment period ended 5 days before testing of responding with conditioned reinforcement.

Acute systemic or local VTA antagonist administration (Papers I, II and additional experiments).

The doses for all experiments were selected based on consensus from previous studies and results demonstrating the lack of effect of administration of these antagonists at the doses used here, or higher, on basal dopamine activity in the nAc (Blomqvist et al., 1993; Blomqvist et al., 1997; Ericson et al., 1998; Seppa et al., 2000; Ericson et al., 2003; Larsson et al., 2004). Systemic administrations of PBS, mecamylamine (0.3 mg/kg or 1.0 mg/kg, i.p.), DHβE (3.0 mg/kg i.p.) or MLA (3.0 mg/kg or 6.0 mg/kg, i.p.) were performed 10 minutes prior to testing. In Paper I, the final set of experiments specifically tested the hypothesis that nAChRs in the VTA are required for the conditioned reinforcing properties of alcohol-associated cues using direct antagonist administrations into the VTA. Thus, intracerebral bilateral infusions of PBS (1μl/side), mecamylamine (10 pmol/side) or α-CtxMII (10 pmol/side) were made immediately prior to the conditioned reinforcement test. Infusion syringes (31 gauge), extending 1 mm below the tip of the guide cannula, were simultaneously lowered into the left and right VTA and infused 1.0 μl/side during a 4-min period at an infusion rate of 0.25 μl/min using a microinfusion pump (Harvard Apparatus, USA). The infusion syringes were kept in place for 2 additional minutes, the dummy cannnulae were then replaced and the animal was placed in the test box. Cannulae placements were histologically verified after completion of the behavioral experiments and only animals with correctly placed cannulae were included in the statistical analysis of the experimental data. Subjects that failed to press the levers were excluded from the data analysis.

Drugs

Isoflurane (Baxter, 3.5-4.0 % in air) or Equithesin (a mixture containing pentobarbital 25 mg/kg and chloral hydrate 183.6 mg/kg; administered 4.32 ml/kg i.p.) was used as an anesthetic. Carprofen (Rimadyl®, Pfizer, USA) was administered (5.0 mg/kg, 1.0 ml/kg s.c.) 30 minutes prior to surgery as an analgesic. Ringer's solution: 140 mM NaCl, 1.2 mM $CaCl_2$, 3.0 mM KCl and 1.0 mM $MgCl_2$ was perfused into the nAc,

dorsal striatum or VTA. Ethanol (Kemetyl AB, Sweden) 95% or 99% (Pharmco, USA) was diluted in 0.9% NaCl for i.p. injection (6.7 ml/kg), in tap water (2-10% v/v) and presented in 250 ml or 400 ml plastic bottles for the drinking experiments and in Ringer's solution for brain perfusion (100 mM and 200 mM). Picrotoxin (Sigma Chemical Co, US), a channel blocker at the $GABA_A$ receptor chloride channel was dissolved in Ringer's solution for nAc perfusion (0.2 μM). Diazepam (Sigma Aldrich Co, US) was dissolved in a few drops of concentrated acetic acid, diluted with Ringer's and neutralized with NaOH (1M) for nAc perfusion. Nicotine hydrogen tartrate salt (Sigma-Aldrich, Sweden or Sigma, USA) was dissolved in saline, neutralized with a few grains of sodium bicarbonate and injected s.c. 2.0 ml/kg. Nicotine doses are expressed as free base. Hexamethonium chloride (Sigma Chemical Co, US) was dissolved in saline and administered i.p. 2.0 ml/kg. Sucrose (J.T. Baker, USA) was dissolved in tap water (0.1 M) and presented in plastic 250 ml bottles. Mecamylamine HCl (purchased from Sigma, USA, Sigma Aldrich, Germany and generously provided by the NIDA research substance supply program), a non-selective non-competitive nAChR-antagonist, was dissolved in phosphate buffered saline (PBS; Invitrogen, USA) for systemic administration (2.0 ml/kg, i.p.) and local bilateral infusion (1.0 μl/side), or was dissolved in Ringer's solution for reverse microdialysis into the VTA. The plant alkaloid methyllycaconitine citrate (generously provided by the NIDA research substance supply program), a selective antagonist at the α7 and α6/α3β2β3* nAChRs, was dissolved in PBS for systemic administration (2.0 ml/kg i.p.). Dihydro-β-erythroidine HBr (Sigma Aldrich, Germany or Sigma, USA) was dissolved in PBS for systemic administration (2.0 ml/kg, i.p.) or in Ringer's solution for reverse microdialysis into the VTA. DHBE, an alkaloid from *Erythrina Americana,* is a tertiary amine that can penetrate the blood-brain barrier (Bowman and Rand, 1980; Decker et al., 1995), and is considered as a selective competitive antagonist of high-affinity α4β2* central nAChRs (Alkondon and Albuquerque, 1993; Dwoskin and Crooks, 2001; Khiroug et al., 2004), although it has also been demonstrated to have affinity for α4β4, α3β2 (Dwoskin and Crooks, 2001) and α2β4 nAChRs (Harvey et al., 1996). α-Conotoxin MII, a selective α3β2* and α6* nAChR antagonist, was dissolved in PBS for local bilateral infusion (1.0 μl/side).

Statistics

The data obtained in all experiments were analyzed using analysis of variance (ANOVA) with treatment as the independent factor. Paired t-tests or Fisher's protected least-significant difference (PLSD) were used for post-hoc comparisons of main effects. Multiple t-test comparisons were corrected for by Holm's sequential rejection procedure, a weighted improvement of the Bonferroni correction (Holm, 1979). Due to the variation in the lever responses recorded in Papers I and II, a statistical test of variance homogeneity recommended the use of square root transformation of the data to fulfill the requirements for ANOVA analysis, as is commonly used for analyses of conditioned reinforcement data. A probability level (p) of less than 0.05 was considered significant. The results are presented as means ± standard error of the mean (SEM).

Non-α4β2* nAChRs in the VTA are required for the stimulation of dopamine release in the nAc by ethanol-associated cues (Paper I)

First we confirmed that presentation of an alcohol-associated auditory CS has the ability to increase dopamine levels within the nAc using the present training parameters (Fig. 5), as has been demonstrated previously during anticipation for ethanol (Weiss et al., 1993; Katner et al., 1996; Gonzales and Weiss, 1998; Katner and Weiss, 1999; Melendez et al., 2002). These previous studies do not distinguish dopamine activity during incentive aspects of reinforcement from that associated with response activating effects of alcohol-associated stimuli, motor- or consummatory actions. We demonstrate here that the associative properties of an alcohol-associated tone (CS+) are sufficient to increase nAc extracellular dopamine levels in ethanol high-preferring rats. However, the same tone never paired with alcohol (CS-) had no effect on nAc dopamine levels. These results suggest that the increase in nAc dopamine is specifically related to the associative relationship between the CS+ and alcohol. The magnitude of the cue-induced elevation of nAc dopamine found here was in line with that observed in the abovementioned studies, but was as expected of a lower magnitude than following voluntary ethanol drinking in alcohol high-preferring rats (Ericson et al., 1998; Molander et al., 2005). These results demonstrate that an alcohol cue increases nAc dopamine levels and are consistent with the view that midbrain dopamine neurons are activated by the incentive properties of rewards (see Introduction).

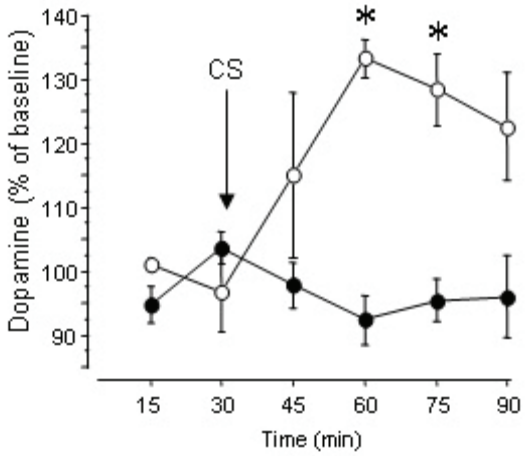


Fig. 5. Effects of presentation of an ethanol-associated (CS+, open circles) or a non-reinforced (CS-, filled circles) auditory stimulus on extracellular dopamine levels in the nAc.
Dopamine was measured by means of *in vivo* microdialysis in awake, freely moving rat during perfusion with Ringer's. * indicate significant increases in dopamine after presentation of the CS+ ($p < 0.05$, paired t-tests, n = 5-8). Shown are the means ± SEM.

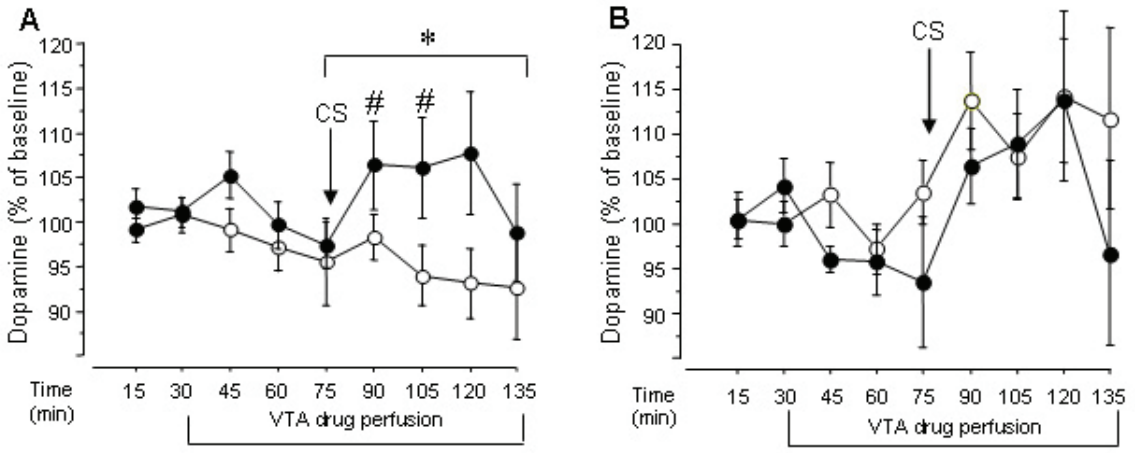


Fig. 6. Effects of presentation of an ethanol-associated cue (CS) on extracellular dopamine levels within the nAc.
A: Cue-induced nAc dopamine release during local perfusion of mecamylamine (100 μM, open circles) or Ringer's (filled circles) into the VTA. * and # indicate significant differences in nAc dopamine between perfusion with Ringer's and mecamylamine and significant increases in dopamine during Ringer's perfusion, respectively ($p < 0.05$; $n = 7$). **B:** Cue-induced nAc dopamine release during local perfusion of DHβE (1 mM, open circles) or Ringer's (filled circles) into the VTA ($n = 4$). Shown are the means ± SEM.

We next tested the hypothesis that nAChR stimulation in the VTA is required for this effect. Again, we confirmed that presentation of an alcohol-associated auditory CS increases dopamine levels within the nAc (Fig. 6A). Perfusion of the non-selective nAChR antagonist mecamylamine (100 μM) into the VTA prior to presentation of the ethanol-associated CS, antagonized the cue-induced elevation of nAc dopamine observed in Ringer's perfused control animals, without producing any significant changes in extracellular dopamine levels *per se*. This indicates that VTA nAChRs are required for cue-induced activation of mesolimbic dopamine neurons. These data further support the idea that blockade of VTA nAChRs not only inhibits ethanol-induced dopamine overflow in the nAc, but also blocks conditioned dopamine release that may provide an incentive signal initiating ethanol consumption (Ericson et al., 1998).

This experiment demonstrates that stimulation of VTA nAChRs is required for cue-induced increases in nAc dopamine output. However, the specific receptor subtype(s) involved in this effect was not determined. We therefore used DHβE, a specific antagonist at the α4β2* nAChRs, to determine the role of these receptors. Although presentation of the alcohol-associated CS increased dopamine output measured in the nAc, this effect was not modified by DHβE in the VTA (Fig. 6B). We have previously demonstrated that VTA perfusion with DHβE (1 mM) counteracts nicotine-induced, but not ethanol-induced, nAc dopamine release, suggesting that α4β2* nAChRs are not involved in the ethanol-induced nAc dopamine release (Ericson et al., 2003). Similarly, DHβE (1 mM) perfusion into the VTA did not affect the nAc dopaminergic response to an ethanol-associated cue in the present study. The same concentrations of mecamylamine or DHβE into the VTA do not affect basal extracellular dopamine levels

in the rat nAc (Blomqvist et al., 1997; Ericson et al., 1998; Ericson et al., 2003), supporting the notion that VTA nAChRs do not tonically regulate the dopaminergic neuronal activity (see Introduction). Consequently, in the present study, mecamylamine prevented cue-induced dopamine release that is likely to be mediated by stimulation of non-α4β2* nAChRs in the VTA.

Dopamine in the nAc has been given an important role in the reinforcing properties of cues associated with alcohol and other addictive substances (see Introduction). Since the ability of alcohol cues to increase dopamine output was blocked by the nAChR antagonist mecamylamine in the VTA, the functional correlate to this effect was tested next using a behaviorally stringent paradigm assessing the acquisition of a new response with conditioned reinforcement. This paradigm can be used to examine incentive motivational processes and the reinforcing effects of reward-associated cues (see Introduction).

Non-α4β2* nAChRs are required for the conditioned reinforcing effects of ethanol-associated stimuli (Paper I)

We first tested whether nAChRs were required for the reinforcing effects of alcohol cues using systemic administration of mecamylamine. The observation that control animals made significantly more responses on the CR lever (eliciting the presentation on the CS) than the NCR lever (which had no programmed consequence), demonstrates that the alcohol cues had acquired conditioned reinforcing properties (Fig. 7A). However, following acute systemic administration of mecamylamine (1.0 mg/kg i.p.), a dose that does not affect baseline nAc dopamine levels (Blomqvist et al., 1993) (Fig. 7A), responses on the CR and NCR lever were not different. These results indicate that nAChRs are required also for the reinforcing properties of alcohol-associated cues.

Again, since mecamylamine is a non-specific nAChR antagonist this experiment does not provide insight into the nAChR subtypes mediating the reinforcing effects of alcohol cues. Thus, the role for the high-affinity α4β2* subunit on responding with the ethanol-associated CS was then tested, using the selective competitive α4β2* nAChR antagonist DHβE (3.0 mg/kg i.p.). This dose blocks several behavioral effects of nicotine (Damaj et al., 1995; Stolerman et al., 1997) but has no effect on basal nAc dopamine (Seppa et al., 2000). Interestingly, there was no effect of systemic DHβE administration on responding with conditioned reinforcement to ethanol; both controls and DHβE-treated animals responded on the CR lever significantly more than the NCR lever (Fig. 7B). These results suggest that the α4β2* nAChRs are not required for the conditioned reinforcing properties of ethanol-associated cues and are consistent with the lack of involvement of this receptor subtype in cue-induced nAc dopamine release. The inability of DHβE to influence the conditioned reinforcing properties of alcohol cues may be unexpected, since this receptor subtype in the VTA is clearly involved in the reinforcing properties of nicotine (see Introduction). The present data are, however, in accordance with studies demonstrating that DHβE does not reduce the dopamine- or locomotor stimulating effects of ethanol in rodents (Larsson et al., 2002; Ericson et al., 2003), nor ethanol self-administration (Le et al., 2000). The results are moreover supported by data demonstrating preserved responding with conditioned reinforcement in β2-KO mice

(Brunzell et al., 2005). Interestingly, prior subchronic nicotine exposure enhances responding with conditioned reinforcement in rodents (Olausson et al., 2004a; Brunzell et al., 2005). This enhancement is, however, selectively prevented by genetic deletion of the β2 nAChR subunit is mice (Brunzell et al., 2005) (see also below and General Discussion).

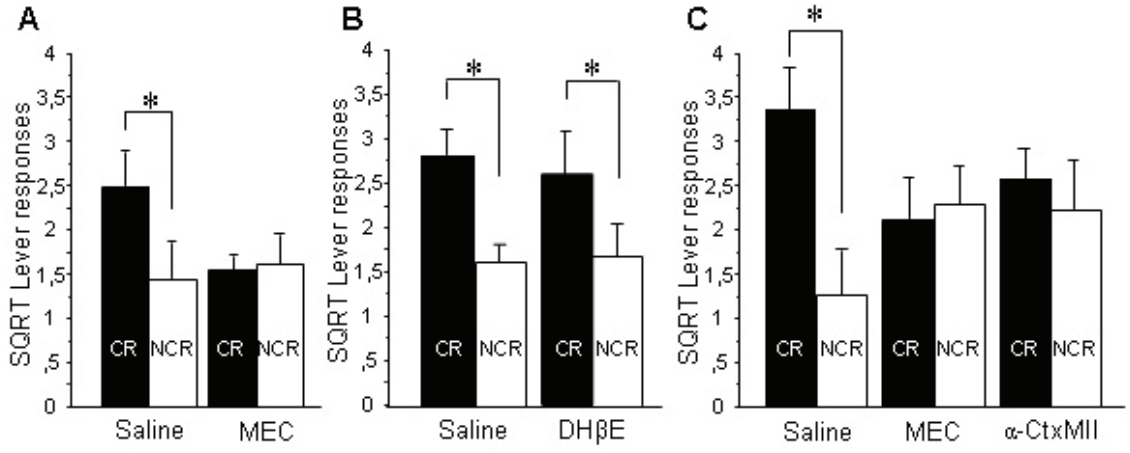


Fig. 7. Antagonists of nAChR decrease responding with conditioned reinforcement using an ethanol-associated cue.
A: Effects of systemic injection of mecamylamine (MEC, 1.0 mg/kg, i.p., -10 min) on responding with conditioned reinforcement (n = 8-9). **B**: Effects of systemic injection of DHβE (3.0 mg/kg, i.p., -10 min) on responding with conditioned reinforcement (n = 8). **C:** Effects of local ventral tegmental infusions of mecamylamine (MEC, 10 pmol/side/1µl) or α-CtxMII (10 pmol/side/1µl) on responding with conditioned reinforcement (n = 7-9). * indicate effect of lever ($p < 0.05$, paired t-test). CR = active lever, NCR = inactive lever. Data is presented as square root of mean lever responses + SEM.

Antagonism of α-CtxMII sensitive nAChRs in the VTA is sufficient to block the reinforcing effects of alcohol-associated stimuli (Paper I)

We next examined whether blockade of nAChRs specifically in the VTA was sufficient to attenuate the reinforcing effects of alcohol-associated stimuli. Here, bilateral infusion of mecamylamine (10 pmol/side) into the VTA abolished the reinforcing effects of the alcohol cue; the number of CR and NCR responses was not different (Fig. 7C). This observation is consistent with the ability of VTA mecamylamine to block the activation of nAc dopamine induced by alcohol cues in the first experiment and suggests that VTA nAChRs mediate the impact of cholinergic projections from the mesopintine nuclei on conditioned reinforcement processes (see Introduction and General Discussion). Although this experiment demonstrates that VTA nAChRs are required for the reinforcing effects of alcohol cues, it does not reveal which receptor subtype that is involved. The final experiment therefore tested the ability of VTA infusions of α-CtxMII (10 pmol/side), an antagonist selective for the α3β2* (Cartier et al., 1996) and α6* (Kuryatov et al., 2000) nAChRs. Here, infusions of α-CtxMII into the VTA, like mecamylamine, completely abolished the preference for the CR lever (Fig. 7C). This

suggests that α-CtxMII sensitive nAChR subtypes in the VTA are required for the conditioned reinforcing properties of alcohol cues.

With respect to these data, some important points should be considered. First, although the number of lever responses was consistently low, in all experiments the differences between CR and NCR levers were significant in control animals (Fig. 7A-C). The training procedure therefore established the alcohol-associated cues as a conditioned reinforcer, as measured by the stringent acquisition of a novel response procedure (lever pressing). This novelty is one critical requirement to demonstrate that cues have acquired conditioned reinforcing properties (Mackintosh, 1974). Accordingly, the low number of lever responses can be explained by the fact that these animals were not previously trained to press the levers since these were presented for the first time during the test day. Second, although it is possible that non-specific effects of the present treatments on locomotor activity could influence our behavioral findings, neither systemic mecamylamine (2.0 mg/kg or 4.0 mg/kg i.p.) nor local bilateral VTA infusion of α-CtxMII (5 nmol/side) affect locomotor activity in rats or mice (Blomqvist et al., 1992; Larsson et al., 2004). Moreover, and importantly, the doses of nAChR antagonists used here failed to change the total number of lever responses, strongly suggesting that the nAChR antagonists produced a selective reduction in responding for the alcohol-associated cue.

In summary, the data presented in this section demonstrate that antagonists of VTA nAChRs have the ability to block dopamine release associated with the presentation of an alcohol cue. Moreover, the conditioned reinforcing effects of alcohol-associated cues were selectively blocked by VTA administration of α-CtxMII, a selective α3β2* and/or α6* nAChRs antagonist. This suggests that modulators of nAChRs in the VTA may be used as medication for prevention of relapse to alcohol abuse.

nAChRs are required for the conditioned reinforcing effects of stimuli associated with natural reward (Paper II)

Paper II first tested the effects of systemic administrations of two different doses of the non-selective nAChR antagonist mecamylamine (0.3 mg/kg or 1.0 mg/kg, -10 min) on responding with conditioned reinforcement using a stimulus that had been associated with the delivery of a 0.1 M sucrose solution. Control-treated rats responded more on the lever resulting in presentation of the sucrose-associated CS, demonstrating that this compound stimulus had acquired conditioned reinforcing properties (Fig. 8A-B). However, following systemic administration of the higher dose of mecamylamine (1.0 mg/kg i.p.), responses on the CR lever and the NCR lever were not different (Fig. 8B). Interestingly, this is the same dose of systemic mecamylamine that blocked the conditioned reinforcing effects also of alcohol-associated stimuli (*vide supra*).

It should be noted that mecamylamine, like several other ganglion blockers, is not a competitive antagonist, but rather acts at the ion-channel of the nicotinic receptor (Ascher et al., 1979; Lingle, 1983; Varanda et al., 1985; Martin et al., 1989; Banerjee et al., 1990), and channel-blockers are generally considered less specific than competitive antagonists. Accordingly, *in vitro* studies suggest that mecamylamine also inhibits NMDA receptor complex-mediated currents (O'Dell and Christensen, 1988) and NMDA-

induced noradrenaline release from hippocampal rat slices (Snell and Johnson, 1989). Since the competitive nAChR antagonist α-CtxMII has no known affinity for NMDA receptors, the above data examining ethanol cues using α-CtxMII, are consistent with the idea that mecamylamine acts specifically through antagonism of nAChRs. Moreover, pharmacological studies indicate that, at doses equivalent to those used in the present experiment, mecamylamine does not interact with these NMDA receptors (Ericson et al., 2003). Thus, the present observations suggest that mecamylamine (1.0 mg/kg, i.p.) selectively reduced or blocked the conditioned reinforcing effects of the alcohol-associated, as well as the sucrose-associated, cues by antagonizing nAChRs. This assumption was supported by data from the final conditioned reinforcement experiment.

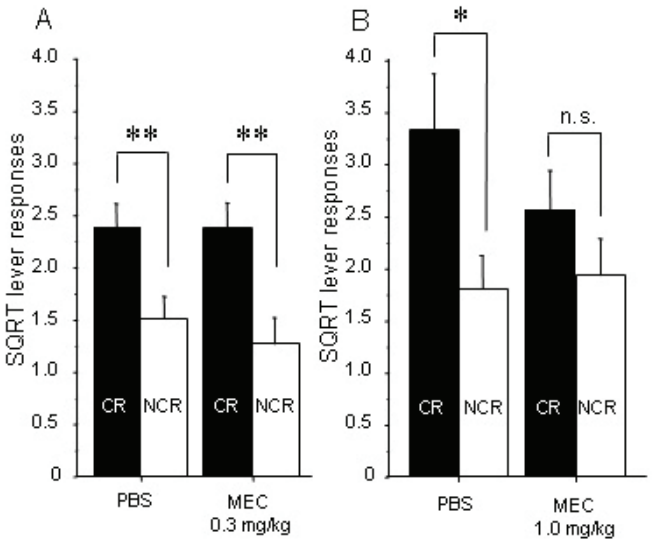


Fig. 8. Antagonists of nAChRs decrease responding with conditioned reinforcement using a sucrose-associated cue.
A: Effects of systemic injection of mecamylamine (MEC, 0.3 mg/kg, i.p., -10 min) on responding with conditioned reinforcement (n = 14). **B**: Effects of systemic injection of mecamylamine (MEC, 1.0 mg/kg, i.p., -10 min) on responding with conditioned reinforcement (n = 12). ** and * indicate effect of lever ($p < 0.001$ and $p < 0.05$, respectively, paired t-test). CR = active lever, NCR = inactive lever. Data is presented as square root of mean lever responses +SEM.

Antagonism of MLA sensitive nAChRs is sufficient to block the reinforcing effects of stimuli associated with natural reward (Paper II)

This experiment examined the effects of the selective competitive nAChR antagonist MLA on responding with conditioned reinforcement for sucrose-associated conditioned stimuli. Here, an acute systemic injection of 3.0 mg/kg (i.p.) MLA 10 minutes prior to testing, did not reduce the effect on behavior by the sucrose-associated stimuli, *i.e.* the animals pressed the CR lever significantly more than the NCR lever (Fig. 9A). However, the responses on the CR lever and NCR lever were not different following administration of the higher dose of MLA (6.0 mg/kg i.p.), suggesting that this dose of MLA blocks responding with conditioned reinforcement to sucrose (Fig. 9B). MLA has generally been regarded as an α7 nAChR antagonist, but was recently demonstrated to block α3/α6β2β3* nAChRs in a similar concentration range (Klink et al., 2001; Mogg et al., 2002; Salminen et al., 2004). The latter subtypes are also antagonized by α-CtxMII, which blocked the conditioned reinforcing effects of ethanol-associated cues (*vide supra*). Thus, the observations that responding with conditioned reinforcement to ethanol

as well as sucrose may be attenuated by blocking α3/α6β2β3* nAChRs support the suggestion of a common basis for the impact of reward-related cues on behavior (Kelley and Berridge, 2002; Nie and Janak, 2003).

Whereas the involvement of the α3/α6β2β3* nAChRs in mediating the reinforcing effects of alcohol-associated cues was localized to the VTA (Paper I), the present study investigated the effects of systemic antagonist administrations. While both antagonists tested here are expected to be specific for nAChRs at the present doses, MLA has been reported to be less selective at higher concentrations. Since the achieved brain concentrations of these drugs were not determined, we cannot exclude the possibility that MLA also interferes with the function of additional nAChR receptor configurations. Nevertheless, a pharmacokinetic study reported that an MLA dose of 5.4 mg/kg i.p. results in brain drug levels of approximately 50-100 nM (Turek et al., 1995), levels sufficient to displace α-CtxMII from α3/α6β2β3* nAChRs in binding studies (Mogg et al., 2002) and to inhibit α7 nAChR-mediated responses *in vitro* (Alkondon and Albuquerque, 1993; Yu and Role, 1998). Interestingly, nAChRs in separate brain regions appear to exhibit different affinities for MLA (Yum et al., 1996), where intermediate levels of MLA binding sites have been found in several of the brain areas involved in reward and motivation, such as the VTA, locus ceruleus and the basolateral/basomedial amygdala (Mugnaini et al., 2002).

Comparing the results from all experiments involving responding with conditioned reinforcement, there are some variations in the amount of lever presses performed by the different sets of rats. However, this variability is generally observed also within groups of rats that have been trained with the same unconditioned stimulus. Thus, the higher responding for the sucrose-associated CS in Fig. 9B compared to the alcohol-associated CS in Fig. 7A represents normal variation in response behavior rather than a higher conditioned value of the sucrose cues. This variability is the rationale for using of SQRT of the data in the present thesis.

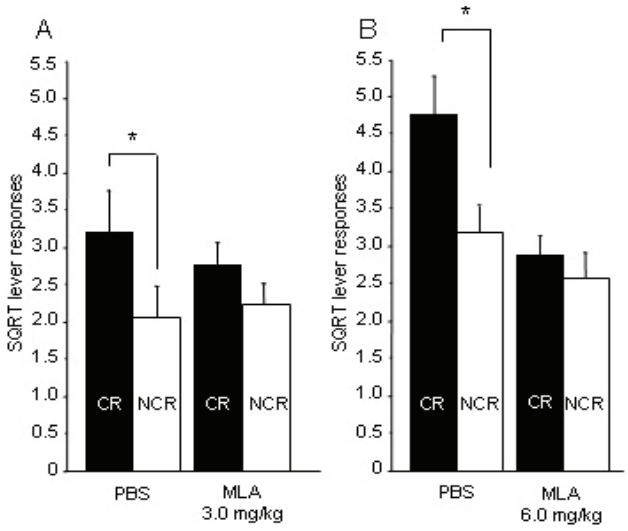


Fig. 9. Antagonists of nAChRs decrease responding with conditioned reinforcement using a sucrose-associated cue.
A: Effects of systemic injection of MLA (3.0 mg/kg, i.p., -10 min) on responding with conditioned reinforcement (n = 10). **B**: Effects of systemic injection of MLA (6.0 mg/kg, i.p., -10 min) on responding with conditioned reinforcement (n = 11). * indicate effect of lever ($p < 0.05$, paired t-test). CR = active lever, NCR = inactive lever. Data is presented as square root of mean lever responses + SEM.

In summary, the present section of this thesis suggests that antagonism of MLA-sensitive (α7* and/or α3β2* and/or α6*) nAChRs prevents the ability of sucrose-associated cues to act as a reinforcer in the stringent acquisition of a new response

paradigm. The α3β2* and/or α6* nAChRs were also suggested to be required for responding with conditioned reinforcement to ethanol in paper I. Consequently, these nAChR subtypes may be a common mediator of the incentive motivational properties of various conditioned reinforcers.

Ethanol perfused into the VTA can only increase extracellular dopamine levels in the nAc when the nAc is concomitantly co-perfused with ethanol (Paper III)

Whereas the first part of the present thesis investigated the conditioned reinforcing effects of ethanol, the second part moved on to study mechanisms behind the pharmacological dopamine responses to acute ethanol administration. In line with previously published data, Paper III demonstrate that local application of 200 mM ethanol into the nAc alone elevates extracellular nAc dopamine levels by approximately 40%, whereas no nAc dopamine changes were observed during perfusion of ethanol into the VTA alone (Fig. 10). These results argue against a direct dopamine stimulatory action of ethanol in VTA. Co-perfusion of ethanol into both the VTA and the nAc, on the other hand, produced significantly higher dopamine levels than ethanol perfusion into the nAc alone in the later part of the experiment (time-points 120-180 minutes, Fig. 10). Together these findings suggest that ethanol can stimulate dopaminergic neurons in the VTA only when it is concomitantly present in the nAc.

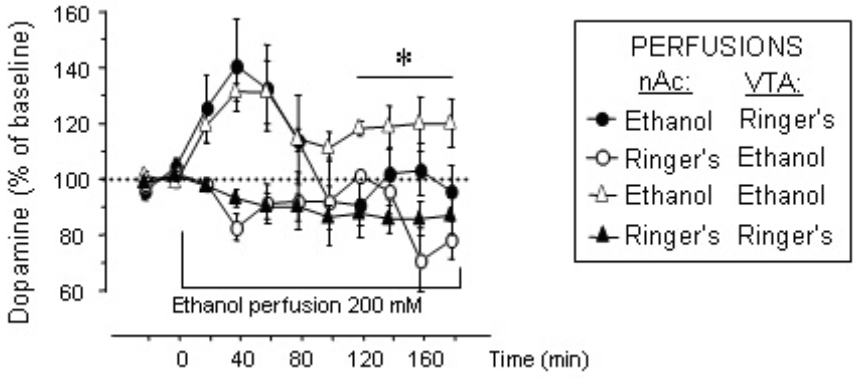


Fig. 10. nAc dopamine response to 200 mM ethanol or Ringer's perfused into the nAc, the VTA or the VTA + the nAc.
Dopamine was measured by *in vivo* microdialysis in awake, freely moving rats. n = 3-7. * indicates significant differences in nAc dopamine during ethanol perfusion concomitantly into the nAc + VTA compared to perfusion into the nAc only, into the VTA only, and compared to Ringer's controls ($p < 0.05$, Fisher´s PLSD). Shown are the means ± SEM.

It can only be speculated how the presence of ethanol in the nAc enables ethanol to act also in the VTA. Previous studies have demonstrated that the dopamine elevating effect of local nAc ethanol perfusion, systemic ethanol injections or voluntary ethanol drinking, are abolished by blockade of nAChRs in the VTA, but not in the nAc (Blomqvist et al., 1996; Ericson et al., 1998; Ericson et al., 2003). Taken together with

findings that ethanol drinking in the rat concomitantly increases acetylcholine levels in VTA and dopamine in the rat nAc (Larsson et al., 2005), it was hypothesized that ethanol interacts with a mechanism in the nAc, that, probably via a neuronal loop, enhances acetylcholine release in the VTA and thereby stimulates dopamine activating nAChRs (Soderpalm et al., 2000; Ericson et al., 2003; Molander and Soderpalm, 2005). Ethanol is suggested to act as a positive allosteric modulator of nAChRs, stabilizing the open state of the receptor and thereby enhancing the effects of acetylcholine (Wu et al., 1994; Aistrup et al., 1999). In the VTA, a general blockade of nAChRs by means of local mecamylamine perfusion alone does not alter basal dopamine levels in the nAc (Nisell et al., 1994a; Blomqvist et al., 1996; Ericson et al., 2003). Thus, under basic conditions, there is probably no ongoing endogenous cholinergic stimulation of dopamine activating nAChRs in the VTA (Nisell et al., 1994a; Westerink et al., 1996; Westerink et al., 1998; Grillner and Svensson, 2000). Therefore the prerequisite for an interaction of ethanol with VTA nAChRs may be lacking when ethanol is perfused into this brain region alone. After systemic administration on the other hand, actions of ethanol in the nAc may release acetylcholine in the VTA, allowing ethanol to act as a co-agonist with acetylcholine on VTA nAChRs. The data in Fig. 10 may support such a hypothesis, although cholinergic mechanisms were not specifically studied here.

The present findings should be related to previous studies in which ethanol was self-administered into the posterior VTA of alcohol-preferring rats (Gatto et al., 1994) and of female Wistar rats (Rodd-Henricks et al., 2000). Acute ethanol administration was moreover reported to excite the VTA dopamine neurons of rats *in vivo* (Gessa et al., 1985b) and *in vitro* (Brodie et al., 1990; Brodie et al., 1999). However, those studies utilized perfusion coordinates of the posterior VTA. Therefore it cannot be excluded that local ethanol application more posterior than in the present study would have activated the mesolimbic dopamine system. This assumption is supported by preliminary data demonstrating a slight increase in nAc dopamine (by ~ 20 %) during 300 mM ethanol perfusion (Ericson, oral communication) into the posterior VTA. It is also possible, however, that local self-administration into the posterior VTA is mediated via mechanisms unrelated to dopamine and/or via mechanisms that are irrelevant to those involved in oral self-administration of ethanol. The results of the present study also disagree with *in vitro* findings showing that ethanol directly activates VTA dopamine neurons (Brodie et al., 1990; Brodie et al., 1999). However, in those studies the neurons are isolated from their physiological context and the direct effect of ethanol observed could be cancelled out or prevented by other mechanisms *in vivo*.

Although not measured, the continuous local ethanol perfusion is expected to rapidly produce stable ethanol concentrations in the nAc. Thus, the accumbal dopamine response to ethanol did not correlate with the expected ethanol level in the same brain area. Rather, the dopamine response to continuous ethanol perfusion was transient, lasting for approximately 40 minutes, which is in line with previous studies demonstrating a time-wise dissociation between ethanol levels and dopamine in the nAc (see Introduction) (Yim et al., 1998; Yim et al., 2000; Doyon et al., 2003; Doyon et al., 2005) (Fig. 10). This dissociation could be due to acute tolerance to ethanol, as suggested in those studies. Alternatively, it may involve recruitment of dopamine release inhibitory $GABA_A$ receptors, a hypothesis that was tested in the next experiment.

Antagonism of $GABA_A$ receptors prevents the declining phase of the dopamine response in the nAc to ethanol (Paper III)

By reversed *in vivo* microdialysis, ethanol (300 mM) alone or in combination with the $GABA_A$ receptor channel blocker picrotoxin (0.2 μM – a concentration not affecting dopamine levels by itself; Fig. 11) was perfused into the nAc, while local dopamine levels in the same brain area were analyzed. Again, ethanol perfusion alone produced a transient 40 minute long increase in accumbal dopamine, whereas during co-perfusion of ethanol and picrotoxin the dopamine elevation lasted for 140 minutes (Fig. 11). These results suggest that recruitment of $GABA_A$ receptor activity in or near the nAc is responsible for the second, declining phase with respect to dopamine levels after ethanol administration. At the end of the present experiment, the levels of dopamine during co-perfusion of ethanol and picrotoxin appear to decline to the levels of dopamine during perfusion of ethanol alone. It is possible that this decrease in dopamine levels represents a general run-down of the system during prolonged *in vivo* microdialysis or that tolerance gradually develops to ethanol's dopamine activating effects.

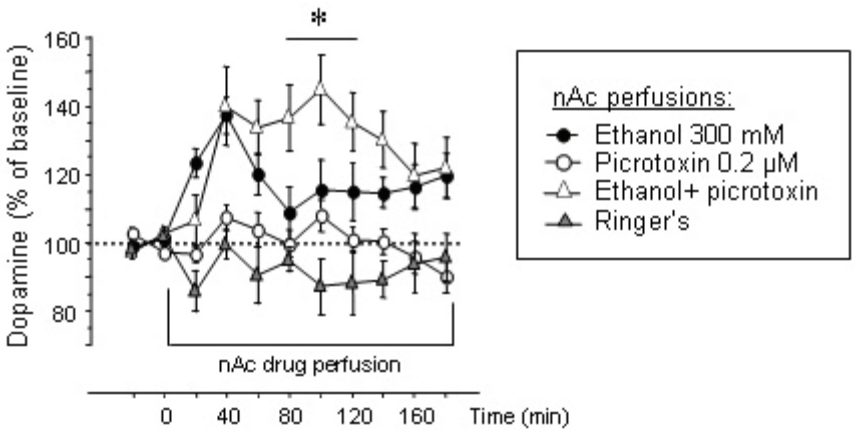


Fig. 11. Effect of picrotoxin on the nAc dopamine response to ethanol.
Dopamine in the nAc before and during nAc perfusion of Ringer's, 300 mM ethanol or 0.2 μM picrotoxin or 300 mM ethanol + 0.2 μM picrotoxin perfused simultaneously into the nAc, as measured by *in vivo* microdialysis in awake, freely moving rats. * indicate that the nAc dopamine response to co-perfusion of ethanol and picrotoxin was significantly different from that of ethanol alone as well as that of picrotoxin alone and Ringer's alone ($p < 0.05$, Fisher's PLSD). Shown are the means ± SEM. n = 6-10.

$GABA_A$ receptors are suggested to mediate the sedative hypnotic effects of acute ethanol intoxication (see Introduction). The subjective experience of ethanol intoxication may be the result of a balance between the stimulatory effects of catecholamines and the sedative properties of GABA, where the catecholaminergic effects appear first and the GABAergic prevail in later stages of the intoxication phase (Engel and Liljequist, 1983; Schechter et al., 1989) – a between systems theory. The present results support and extend this suggestion. In addition to the general sedative effects produced by ethanol elsewhere in the brain, the higher ethanol concentrations may produce a concomitant shut-down of the stimulatory effects on catecholamines, such as nAc dopamine (a within

system balance). Since the dopamine activating effect of ethanol appears to involve two other ligand-gated ion-channels (glycine receptors and nAChRs), it appears that the subjective experience of an ethanol intoxication may ultimately depend on in what order and to what degree these different ligand-gated ion-channels ($GABA_A$, glycine receptors and nAChRs), as well as others, respond to various concentrations of ethanol (see also Lovinger, 1997). A further complication is that ligand-gated ion-channels are prone to desensitization and tolerance is also developed to some of the effects of acute ethanol involving $GABA_A$ receptors (Allan and Harris, 1987; June et al., 1995; Liang et al., 2006; Marutha Ravindran and Ticku, 2006). These mechanisms are likely to influence the outcome of the ethanol intoxication. Indeed, although not statistically significant, in the graphs of Paper III, there are trends for a second increase in ethanol-induced dopamine elevations following the transient, initial dopamine increase. Thus, the development of functional tolerance in the $GABA_A$ receptor response to ethanol may enable a second increase in nAc dopamine during ethanol perfusion. Such phenomena may explain the fluctuations in stimulation and sedation observed over time during ethanol intoxication in social drinkers.

In this context it should also be noted that chronic ethanol exposure, as in alcohol dependent individuals, is known to produce pronounced tolerance to the sedative effects of ethanol. This phenomenon is at least in part explained by changes in the subunit compositions of $GABA_A$ receptors, which result in reduced coupling between benzodiazepine agonist sites and the chloride channel and a consequent cross-tolerance between ethanol and benzodiazepines (Buck and Harris, 1990) (see Introduction). Thus, chronic alcohol consumption may very well attenuate also the dopamine reducing effects of ethanol demonstrated to be mediated via the $GABA_A$ receptor here, consequently resulting in enhanced dopamine activation upon ethanol exposure. This gained dopamine activation may in turn promote further ethanol consumption, as suggested by animal experiments (Weiss et al., 1993; Katner and Weiss, 2001).

Intermittent pre-treatment with a nicotinic antagonist alters the accumbal dopamine response to a systemic ethanol injection (Paper IV)

Intermittent, subchronic pre-treatment with nicotine or the nAChR antagonist hexamethonium increases ethanol intake and preference in the rat (Ericson et al., 2000a). By means of *in vivo* microdialysis, the first part of Paper IV investigated the effect of these pre-treatments on the dopaminergic response in the rat nAc to systemic or local ethanol administration. This response was compared to local concentrations of ethanol in the same brain region. In all three experimental groups, a systemic ethanol injection (1.0 g/kg, i.p.) produced similar ethanol levels in the nAc, indicating no differences in ethanol pharmacokinetics between saline, nicotine or hexamethonium pre-treated animals (Fig. 12B). Moreover, significant concentrations of ethanol were present in the nAc during the complete experiment following the injection (Fig. 12A). In nicotine pre-treated animals and in controls, there was a significant ethanol-induced increase in accumbal dopamine that returned to baseline before the local levels of ethanol declined in the same brain area (cf. Fig. 12A and B). Thus, in the nAc of these animals there was a dissociation between ethanol concentrations and the dopamine response to ethanol, as previously demonstrated

in nicotine naïve animals (Paper III; Yim, 2000 #337; Yim, 1998 #288; Doyon, 2005 #568; Doyon, 2003 #181}. Interestingly, this dissociation was absent in hexamethonium pre-treated rats. In this experimental group, the nAc dopamine levels were instead time-locked to the ethanol levels in the same area following a single systemic ethanol injection (Fig. 12A-B).

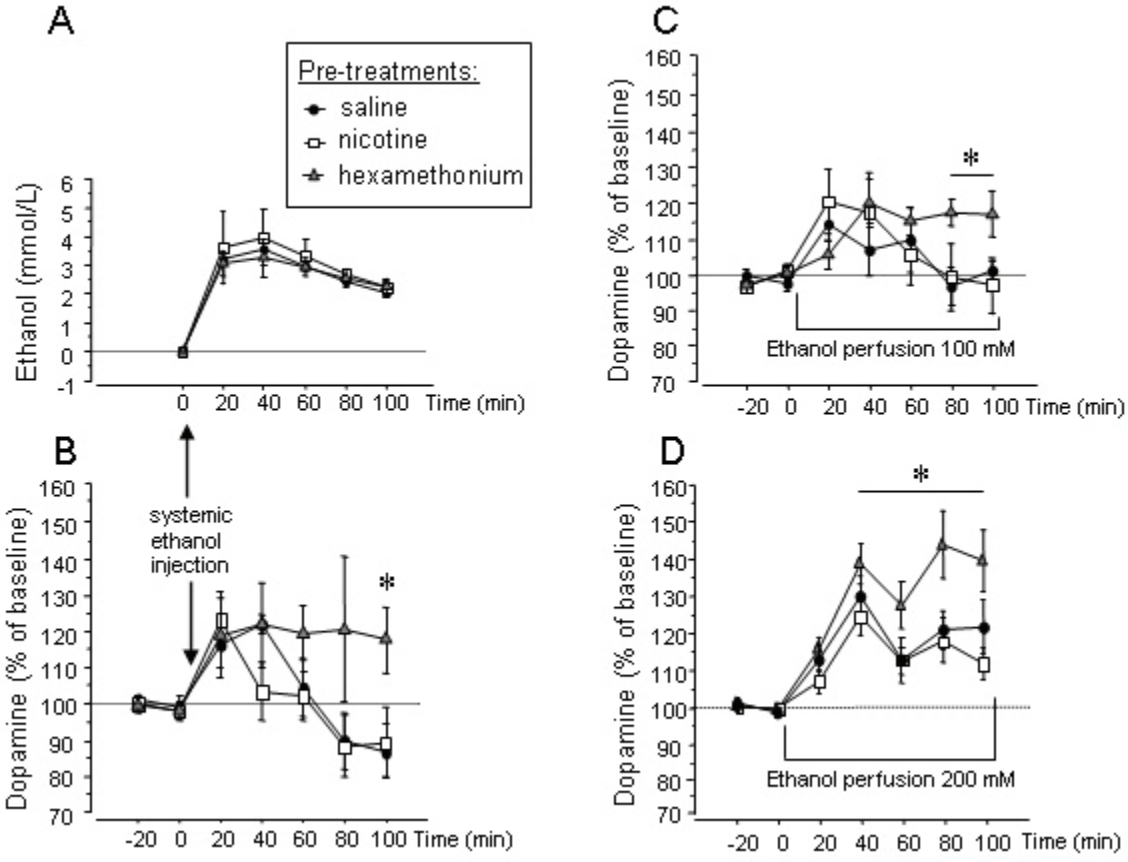


Fig. 12. The effect of acute ethanol administration as measured by *in vivo* microdialysis in awake, freely moving rats following 15 consecutive daily injections with saline s.c./saline i.p., nicotine (0.35 mg/kg) s.c./saline i.p. or saline s.c./hexamethonium (10 mg/kg) i.p.
A: nAc extracellular ethanol levels during the same experiment (n = 3-5). **B:** extracellular nAc dopamine levels before and after a systemic injection of ethanol (1.0 g/kg i.p.). * indicates significantly higher dopamine concentrations in hexamethonium pre-treated rats compared to controls or nicotine pre-treated animals ($p < 0.05$, Fisher's PLSD) (n = 6-9). **C:** extracellular nAc dopamine levels before and during local perfusion of ethanol (100 mM) into the same brain area. * indicates significantly higher dopamine levels in hexamethonium pre-treated rats compared to controls or nicotine pre-treated animals ($p < 0.05$, Fisher's PLSD) (n = 8-9). **D:** extracellular dopamine levels in the dorsal striatum before and during local perfusion of ethanol (200 mM) into the same brain area. * indicates significantly higher dopamine levels in hexamethonium pre-treated rats compared to nicotine pre-treated animals ($p < 0.05$, Fisher's PLSD) (n = 8-9). Shown are the means ± SEM.

Intermittent pre-treatment with a nicotinic antagonist alters the accumbal dopamine response to local ethanol perfusion into the nAc (Paper IV)

During local perfusion of ethanol (100 mM) into the nAc, extracellular dopamine levels were significantly increased in hexamethonium pre-treated rats only. Again, towards the end of the experiment, the dopamine levels in the nAc were significantly higher in these animals compared to the other two groups (Fig. 12C). These results indicate that subchronic antagonism of nAChRs modulates the pharmacodynamic properties of ethanol in the nAc and may explain why subchronic hexamethonium treatment increases alcohol consumption in the rat (Ericson et al., 2000a). It has been reported that ethanol perfusate concentrations of 170 mM are required to significantly stimulate nAc dopamine (Yim et al., 1998). Hence, the failure in the present study of the accumbal dopamine elevations to reach statistical significance after local ethanol perfusion in controls and nicotine pre-treated rats may be due to the low ethanol concentration applied (100 mM). This threshold concentration was chosen because in Paper III, it was hypothesized that the responsivity of midbrain dopamine neurons to local ethanol would be higher after pre-treatment with nicotinic drugs. The fact that local and systemic alcohol administration produced almost identical dopaminergic responses in the nAc, supports our previous suggestion that this brain area is the primary and most important site of action for ethanol with respect to mesolimbic dopamine activation (Ericson et al., 2003; Molander et al., 2005; Molander and Soderpalm, 2005; Paper III).

Although hexamethonium is regarded as a peripherally acting nAChR antagonist, it is possible that the dose used in the present experiments (10 mg/kg) may be high enough for some penetration through the blood-brain-barrier and a consequent blockade of centrally located nAChRs. Alternatively may a hexamethonium-induced blockade of peripheral ganglionic nAChRs result in compensatory mechanisms secondarily affecting central activity via *e.g.* hormonal or metabolic factors or afferent peripheral neuronal activity. Indeed, the same hexamethonium dose and administration regimen was unable to block the nicotine-induced increase in locomotor activity (that was blocked by mecamylamine) (Ericson et al., 2000b), suggesting that hexamethonium selectively blocked peripheral ganglionic nAChRs.

The hexamethonium-induced alterations of the dopaminergic responses to ethanol were restricted to the second, dopamine counteracting phase (Fig. 12B and C). This phase is most likely explained by ethanol stimulation of dopamine inhibiting $GABA_A$ receptors, since in Paper III local application of the $GABA_A$ antagonist picrotoxin into the nAc of naïve rats abolished this dopamine counteracting phase of local ethanol perfusion into the same brain area (Fig. 11). Indeed, ethanol is a positive allosteric modulator that enhances the stimulatory effect of GABA on the $GABA_A$ receptor (for review, see Grobin et al., 1998). Thus, it is possible that hexamethonium pre-treatment diminished the sensitivity of $GABA_A$ receptors to ligands in the nAc, or reduced the levels of endogenous GABA in the same area. This putative consequence of hexamethonium may reduce the ability of ethanol to lower the accumbal dopamine levels via the proposed $GABA_A$ receptor mediated mechanism. The third experiment of Paper IV was designed to test this hypothesis.

Intermittent pre-treatment with nicotine or a nicotinic antagonist alters the accumbal dopamine response to local diazepam perfusion into the nAc (Paper IV)

By means of *in vivo* microdialysis we demonstrate that perfusion of the positive allosteric $GABA_A$ receptor modulator diazepam (10 μM) into the nAc of control animals significantly reduces extracellular dopamine levels in the same brain region, which confirms the findings of others (see Introduction)(Fig. 13). Interestingly, prior subchronic treatment with hexamethonium totally abolished this effect. This result is congruent with the lack of the second, dopamine counteracting phase of acute ethanol administration observed following hexamethonium treatment in the first two experiments of paper IV. Thus, the data support our hypothesis that the dopamine counteracting action of ethanol is a consequence of ethanol stimulating dopamine inhibitory $GABA_A$ receptors in the nAc (Paper III). A partial attenuation of the dopamine reducing effect of accumbal diazepam was observed in nicotine pre-treated animals. This reduction in $GABA_A$ receptor sensitivity following subchronic nicotine treatment is not in complete agreement with the unaltered ethanol-induced dopamine release in this group of animals in the first experiments, but may nevertheless contribute to explaining why some smokers report less sedation from acute alcohol intoxication (Zacny, 1990; Perkins et al., 1995; Perkins et al., 2000). These observations are important, since a low sedative response to acute ethanol challenge is considered a risk factor of alcohol dependence (Schuckit and Smith, 1996; Schuckit, 1998; Heath et al., 1999).

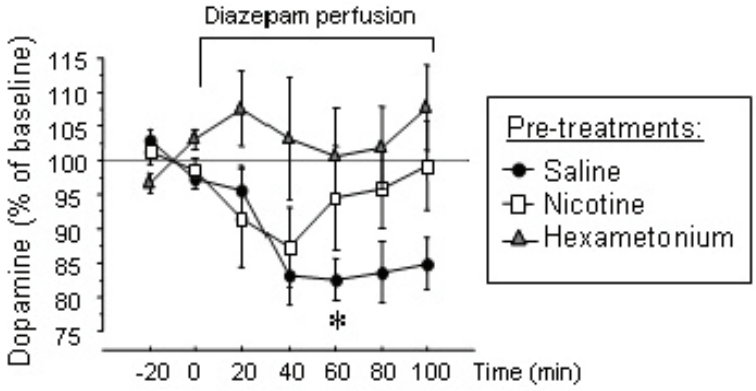


Fig. 13. Dopamine in the nAc as measured by *in vivo* microdialysis in awake, freely moving rats.
The effect of 15 consecutive daily injections with saline s.c./saline i.p., nicotine (0.35 mg/kg) s.c./saline i.p. or saline s.c./hexamethonium (10 mg/kg) i.p. on nAc dopamine levels before and during local perfusion of diazepam (10 μM) into the same brain area. Shown are the means ± SEM; n = 7-10. * indicates a significant reduction in nAc dopamine in controls.

Acute nicotine can increase GABAergic activity in several brain areas (Lena et al., 1993; McMahon et al., 1994a, 1994b; Lena and Changeux, 1997), although this effect of nicotine has not been demonstrated in the nAc. The nAc receives excitatory glutamatergic afferents from the prefrontal cortex, hippocampus and amygdala (Pennartz et al., 1994). These afferents also promote GABAergic feed forward inhibition onto medium spiny neurons both through inter-neurons and through axon collaterals of

neighboring medium spiny neurons (Chang and Kitai, 1985; Pennartz and Kitai, 1991; Kawaguchi et al., 1995; Taverna et al., 2004). GABA inter-neurons in the nAc express nAChRs of non-α7* subtypes (de Rover et al., 2002) which are desensitized by nicotine (Wooltorton et al., 2003). Accordingly, GABA inhibition of the output neurons are stimulated by spontaneously active cholinergic interneurons via these nAChR subtypes (for reviews, see Mansvelder et al., 2003; Pidoplichko et al., 2004). It is possible that such nAChRs possess a similar regulatory role also of GABAergic input neurons that can inhibit the release of dopamine in the nAc. This putative mechanism could be sensitive to chronic nicotine treatment. In the present study, nicotine-induced desensitization as well as hexamethonium-induced antagonism of nAChRs may result in a reduced stimulatory effect of acetylcholine on nAChRs residing on GABAergic input neurons. The consequence may be a reduced GABA release in the nAc which in turn may lower the impact of positive $GABA_A$ receptor modulators such as ethanol or diazepam on dopamine inhibiting $GABA_A$ receptors. This may explain why we observe a reduced sensitivity to a benzodiazepine - which requires GABA stimulation for being effective - after subchronic pre-treatment with nicotine as well as hexamethonium (Fig. 13). An alternative explanation may be that pre-treatment with nicotine or hexamethonium results in a compensatory up-regulation of nAChRs on GABAergic neurons, a subsequent increase in GABA release when the drugs are not present (*i.e.* during most of the time since the drugs are administered only once a day during the subchronic treatment) and a down-regulation of $GABA_A$ receptor function in response (Xi et al., 2003). Thus, it is possible that chronic nicotine treatment attenuates the $GABA_A$ receptor mediated reduction in accumbal dopamine elevation during acute alcohol administration, thereby reducing the sedative properties of ethanol as well as enhancing the incentive dopaminergic signal (Robinson and Berridge, 1993), two phenomena that both probably further promote alcohol consumption.

The observed effects of nicotinic drugs on GABAergic activity in the nAc may contribute to counteracting the sedative properties of ethanol by maintaining dopamine stimulation. However, it appears more likely that ethanol-induced sedation is mediated via a more general activation of central $GABA_A$ receptors. Some indication of this was obtained in the last experiment of Paper IV where the effect of local ethanol perfusion into the dorsal part of the striatum was investigated after subchronic pre-treatment with nicotinic drugs.

Intermittent pre-treatment with a nicotinic antagonist alters the dopamine response in the dorsal striatum to local ethanol perfusion into the same area (Paper IV)

Also in the dorsal striatum, ethanol perfusion (200 mM) resulted in elevated dopamine levels that were prolonged in hexamethonium pre-treated animals compared to saline or nicotine pre-treated rats (Fig. 12D). As judged from the shape of the ethanol-induced dopamine curves of the dorsal striatum, it appears likely that a $GABA_A$ mediated dopamine counteracting phase similar to that in the nAc is present also in the dorsal part of the striatum. This suggests that subchronic hexamethonium may produce $GABA_A$ receptor subsensitivity in several regions of the brain.

Subchronic, intermittent pre-treatment with nicotine or hexamethonium increases

both ethanol intake and preference in rats (Ericson et al., 2000a). High ethanol preference in rats has been related to a strong responsivity of nAc dopamine to ethanol (Weiss et al., 1993; Katner and Weiss, 2001). Thus, the enhanced ethanol consumption following hexamethonium pre-treatment observed by Ericson et al. (2000a) may be explained by the present results demonstrating that this nicotinic drug prolongs the ethanol-induced activation of the dopamine system (Fig. 12). With respect to nicotine no such effect was observed. However, subchronic intermittent nicotine treatment, markedly enhances dopamine receptor responsivity in the nAc (Fung and Lau, 1988; Suemaru et al., 1993; Molander and Soderpalm, 2003) and presumably also in the dorsal striatum. Thus, dopamine synaptic function is probably strengthened also in the nicotine pre-treated animals of the present study, which may explain why nicotine pre-treatment enhances ethanol consumption. In this context it should be recalled that in our previous behavioral studies, the increase in alcohol consumption was most pronounced in animals subjected to the combined treatment with hexamethonium and nicotine (Ericson et al., 2000a). This outcome could tentatively be explained by a simultaneous hexamethonium-induced prolongation of the dopamine release and a nicotine-induced enhancement of postsynaptic dopamine receptor sensitivity.

In conclusion, Papers I and II demonstrate that nAChRs are critically involved in the impact of reward-associated stimuli on the activity of the mesolimbic dopamine system as well as on reward-seeking behavior in the rat. The results from Paper III and IV demonstrate that pre-treatment with nicotine or hexamethonium, two nicotinic drugs that increase voluntary ethanol drinking in the rat (Ericson et al., 2000a), may attenuate $GABA_A$ receptor mediated brain effects of ethanol, *e.g.* the decline in nAc dopamine response observed approximately one hour after the ethanol challenge, thereby prolonging the stimulatory effects of ethanol. The final experiment of the present thesis was designed to investigate the effect of subchronic nicotine and hexamethonium pre-treatment on responding with conditioned reinforcement to ethanol.

α4β2* nAChRs in responding with conditioned reinforcement to alcohol following intermittent pre-treatment with nicotinic drugs

An additional experiment investigated the effect of intermittent subchronic pre-treatment with nicotine or the nAChR antagonist hexamethonium on responding with conditioned reinforcement to ethanol. The results show that in all animals, there was a significant effect of the CS on lever pressing behavior, demonstrating that the alcohol-cues had acquired conditioned reinforcing properties (Fig. 14). Pre-treatment with the nicotinic drugs did not modulate the number of lever responses for the alcohol cues. The present results were unexpected since previous studies demonstrate that intermittent subchronic pre-treatment with nicotine increases responding with conditioned reinforcement to natural reinforcers in rats (Olausson et al., 2004a) and in wild-type mice but not in β2-/- knock out mice (Brunzell et al., 2005). The present experiment moreover demonstrates that acute systemic administration of the selective α4β2* nAChR antagonist DHβE (3.0 mg/kg i.p.) had no effect on lever-pressing for an alcohol CS in controls or in hexamethonium pre-treated rats. These results support our suggestion that nAChRs of non-α4β2* subtypes mediate the conditioned reinforcing effects of alcohol cues (*vide*

supra). However, and interestingly, the nicotine pre-treated rats, did not respond with conditioned reinforcement following the acute injection of DHβE. This result is partly in line with the demonstration of Brunzell and colleagues (2005) that nicotine pre-treatment did not have the ability to increase responding in conditioned reinforcement to food in mice that lack the subunit that is selectively antagonized by DHβE.

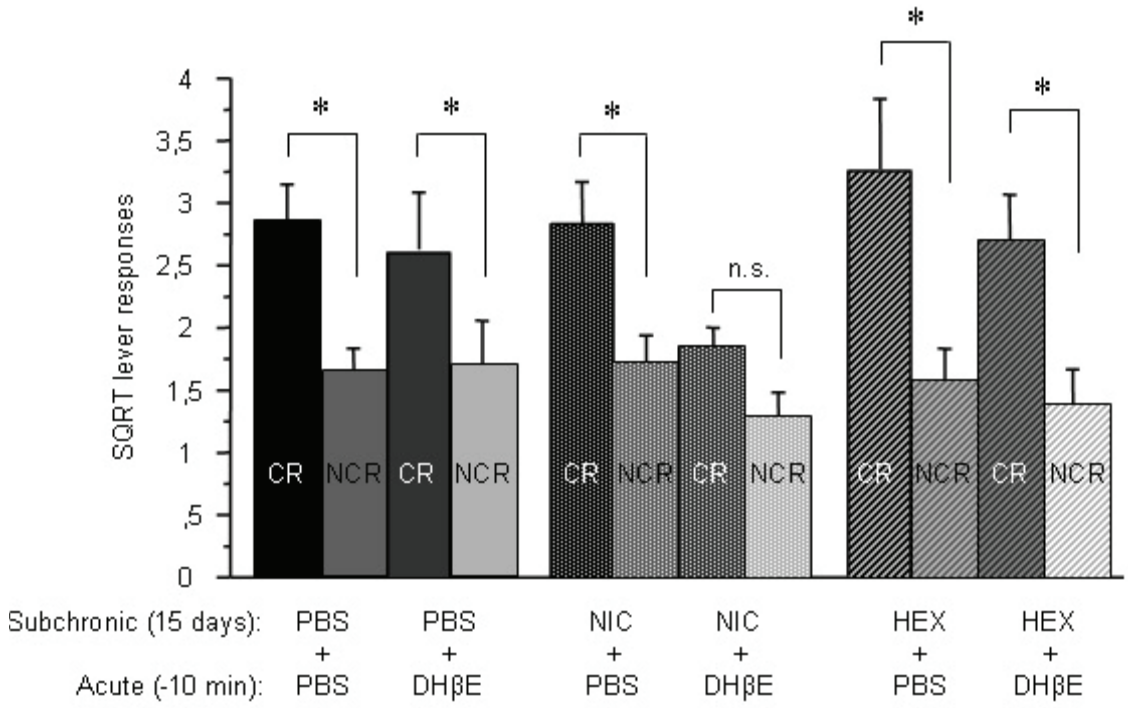


Fig. 14. Effect of subchronic pre-treatment with nicotinic drugs and acute DHβE on responding with conditioned reinforcement to 10 % ethanol.
Pre-treatment: 15 consecutive daily injections with saline s.c./saline i.p., nicotine (NIC; 0.35 mg/kg) s.c./saline i.p. or saline s.c./hexamethonium (HEX; 10 mg/kg) i.p. Acute treatment: PBS or the selective α4β2* nAChR antagonist DHβE (3.0 mg/kg i.p.) 10 minutes prior to testing. In nicotine pre-treated animals, the conditioned reinforcing properties of an alcohol associated compound stimulus was antagonized by an acute injection of DHβE (n = 7-8). * indicates effect of lever ($p < 0.05$, paired t-test). CR = active lever, NCR = inactive lever. Data is presented as square root of mean lever responses + SEM..

As mentioned in the Introduction, different neuronal nAChR subtypes respond differently to chronic nicotine administration, with an increase in the α4β2* nAChRs, and a down-regulation or no change in α3/α6β2* nAChRs. Here, we demonstrate that nAChRs located in the VTA mediate the conditioned reinforcing effects of alcohol cues. The main nAChR subtypes in this brain area are the α4β2* the α7 and the α6β2* nAChRs (see Introduction). It is possible that there are limited amounts of the β2 subunits in the VTA. If so, subchronic nicotine treatment may produce nAChR subtype changes in favor of the α4β2* composition at the expense of other β2* nAChRs, such as the α6β2*. The present thesis hypothesizes that cholinergic neurons in nicotine naïve rats synapse on VTA dopaminergic cell bodies to stimulate non-α4β2* nAChRs in response to cue

presentation. Following subchronic nicotine pre-treatment however, the dopamine stimulating nAChRs that are reached by these cholinergic projections may accordingly have switched from being of the α-CtxMII sensitive α3/α6β2* subunit composition to the DHβE sensitive α4β2* nAChRs. This hypothesis could explain why acute DHβE administration abolished responding with conditioned reinforcement to alcohol in nicotine pre-treated animals, but not in nicotine naïve animals in the present experiment.

The failure of nicotine pre-treatment in the present experiment to increase responding with conditioned reinforcement as opposed to other studies, has several possible explanations. It could be due to the different time-span between chronic nicotine pre-treatment and testing in the different studies, since time of withdrawal is crucial for nicotine induced changes in nAChR composition. For instance, α6β2*-nicotinic acetylcholine receptors in the rat VTA are down-regulated following 2 weeks of chronic nicotine treatment. However, this decrease recovered within one day of withdrawal (Mugnaini et al., 2006).

SUMMARY OF RESULTS

1) Non- α4β2* nAChRs in the VTA mediate the extracellular dopamine overflow in the nAc induced by alcohol-cues.
2) α-CtxMII sensitive (α3β2* and/or α6*) nAChRs in the VTA mediate responding with conditioned reinforcement to ethanol.
3) nAChRs suggestedly of (α3β2/α6*) mediate responding with conditioned reinforcement to a natural reward (sucrose).
4) The nAc is the primary brain site of interference for the ethanol-induced elevations in extracellular dopamine in the nAc. However, ethanol administered into the VTA can further stimulate nAc dopamine when ethanol is concomitantly available in the nAc, such as during drinking.
5) Recruitment of $GABA_A$ receptor mediated inhibitory activity appears to be responsible for the second, declining phase of this dopamine response to ethanol, which explains the lack of association between the ethanol-induced elevations in nAc dopamine and the concomitant ethanol concentrations in the same brain region.
6) Chronic administration of nicotinic drugs may reduce this $GABA_A$ receptor mediated activity, thereby possibly attenuating the sedative and prolonging the stimulatory, effects of ethanol. This is a novel explanation to why smokers report less sedation from alcohol consumption and implies that the use of nicotine may increase the reinforcing properties of ethanol and consequently also alcohol consumption.

GENERAL DISCUSSION

Using the *in vivo* microdialysis method and the conditioned reinforcement model, the first two papers of the present thesis investigated the tentative involvement of nAChRs in the neurochemical and behavioral effects of reward-associated cues, respectively. Paper I demonstrates that non-α4β2* nAChRs in the VTA are crucial for the nAc dopamine response to the presentation of an alcohol cue, as well as for the behavioral consequences thereof. Additionally, in the behavioral study this effect was mediated specifically via the α-CtxMII-sensitive α3β2* and/or α6* nAChRs in the VTA. In Paper II, MLA, a selective antagonist of the α7* and/or α3β2* and/or α6* nAChRs, blocked the conditioned reinforcing properties of sucrose-associated cues. It is tempting to suggest that the α-CtxMII-sensitive nAChRs, and not the α7* nAChRs, in the VTA, mediate the effects also of cues associated with natural reward, supporting the suggestion of a common basis for the impact of reward-related cues on behavior (Kelley and Berridge, 2002; Nie and Janak, 2003).

It should be noted that the 8 week long alcohol pre-exposure in Paper I is unlikely to produce neurochemical alterations similar to those underlying alcoholism in man. In humans, the process of transition to alcohol dependence often requires several years of high alcohol consumption. However, non-dependent light drinkers also show conditioned cue reactivity and mild craving in response to alcohol cue exposure (Greeley et al., 1993; Streeter et al., 2002), whereas in alcoholics the cue-induced craving is more severe and highly correlated with the history and degree of dependence (Laberg, 1986; Greeley et al., 1993; Streeter et al., 2002). In the present thesis, the degree of CR lever responding was rather low and similar to the alcohol cue and the sucrose cue, perhaps reflecting a relatively mild craving. Thus, additional studies may be necessary to conclude whether the nAChR mechanisms identified here prevails and/or are strengthened in alcohol-dependent animals.

The importance of nAChRs in modulating the conditioned effects of cues is supported by previous observations that acute and chronic nicotine enhance the reinforcing properties of primary and conditioned reinforcers (Popke et al., 2000; Olausson et al., 2004a, 2004b) as well as cue-induced cocaine-craving (Reid et al., 1998). Apart from nicotine's positive reinforcing effects on the brain reward system, its ability to desensitize nAChRs on dopaminergic terminals in the nAc is suggested to increase the responsivity of mesolimbic dopamine neurons to phasic bursts (Rice and Cragg, 2004), which is the type of neuronal activity that is promoted by salient cues (Schultz, 1998b). Additionally, nicotine enhances learning and memory (Levin and Simon, 1998; Newhouse et al., 2004), as exemplified by its ability to increase contextual and cue-induced fear conditioning, effects that can be modulated by MLA and DHBE (Davis and Gould, 2005). Moreover, the β2*, but not the β3* or β4* nAChRs are suggested to mediate the enhancing effects of nicotine on contextual fear conditioning (Wehner et al., 2004), as well as on responding with conditioned reinforcement to food (Brunzell et al., 2005). Thus, the use of nicotine during alcohol-consumption, may 1) facilitate the learning of cue-reward relationships, 2) up-regulate the VTA nAChRs demonstrated here to mediate the neurochemical and behavioral consequences of alcohol-associated cues,

and 3) up-regulate additional nAChRs such as the α4β2* subtype in the VTA. The α4β2* nAChRs had no role in cue-induced dopamine overflow or responding with conditioned reinforcement to alcohol in nicotine naïve rats. However, following chronic nicotine administration these receptors may become involved in conditioned reinforcement to alcohol if they are up-regulated in a fashion where they become targets for the VTA cholinergic activity that promotes responding for alcohol cues in Paper I. The results of the last experiment of this thesis, although preliminary, may support such as conclusion.

One consequence of drug withdrawal in humans alcoholics (Junghanns et al., 2000; for review, see Kampov-Polevoy et al., 1999) and smokers (Grunberg, 1982; Hatsukami et al., 1984; Hall et al., 1989; Hatsukami et al., 1993), as well as in nicotine-dependent laboratory animals (Grunberg et al., 1985; Grunberg et al., 1988a; Grunberg et al., 1988b), is the increased consumption of sweet-tasting and high-caloric food. This is an important aspect of withdrawal, since the fear of consequent weight gain may reduce the motivation to stay abstinent (for review, see Perkins et al., 1997). One hypothetical explanation to the increased consumption of high-caloric food during nicotine and alcohol withdrawal is the direct effect of drug consumption and withdrawal on appetite. Acetylcholine in the nAc is implicated in satiety (*e.g.* Rada et al., 2005), and acute administration of drugs that suppress appetite, such as nicotine, amphetamine and cocaine, releases acetylcholine in the nAc (Lindefors et al., 1992; Mark et al., 1999; Rada et al., 2001). This effect is suggested to mediate satiety when the dopaminergic activity in the same brain area is high, but not when it is low, such as during withdrawal (Rada et al., 2001). Only three studies have investigated the effect of ethanol on nAc acetylcholine. They have demonstrated that acetylcholine release in the nAc is increased *in vitro* in response to repeated alcohol administration (Nestby et al., 1997; Nestby et al., 1999), whereas no effects of acute or repeated ethanol injections were observed *in vivo* (Rada et al., 2004). Thus, at this point an accumbal cholinergic component in the increased appetite for sugars during alcohol withdrawal cannot be concluded.

Other studies suggest that elevated insulin plasma levels contribute to the increased sucrose consumption during nicotine as well as alcohol withdrawal (Grunberg et al., 1985; Grunberg et al., 1988a; Passilta et al., 1999). It is also possible that during withdrawal, alcohol and/or nicotine dependent individuals compensate for the lack of drug reward by increasing their consumption of other reinforcers that stimulate the mesolimbic dopamine system, such as sucrose (cf. Junghanns et al., 2000). Additionally, smoking cessation may increase the value of food reward in women (Lerman et al., 2004). The results of the present thesis provide an additional explanation. Here, the demonstration that common nAChRs mediate the impact of cues on reward-related behaviors, may suggest that drug-cues in abstinent alcoholics and/or smokers increase the motivation to consume natural rewards such as sucrose during the withdrawal phase. Alcohol dependent patients that are family history positive with respect to alcoholism report increased desire for sweets, cigarettes and coffee during alcohol detoxification (Junghanns et al., 2005). A pharmacological compound that reduces the conditioned reinforcing effects of rewards in general could be particularly beneficial to these individuals. The present results together with the suggestion that women are more reactive to smoking cues than men (Perkins et al., 2002), may moreover explain why weight gain is more commonly observed among women than men during smoking cessation (for review, see Perkins et al., 1997).

In humans, dysfunction of the cortical input to the nAc is hypothesized as the foundation of compulsive, repetitive pathological drug-seeking and taking behavior (London et al., 1999; Volkow and Fowler, 2000). Numerous studies propose that the sensitization of the mesoaccumbens dopamine response to a drug increases the motivational value also of stimuli associated with the drug (*e.g.* Robinson and Berridge, 1993; Schultz et al., 1997). In Paper I, a direct causal relationship between the cue-induced dopamine elevation in the nAc and responding with conditioned reinforcement was, however, not established. Apart from the projections to the nAc, dopaminergic cell bodies in the VTA also project to the amygdala (for reviews, see Alheid and Heimer, 1988; Heimer et al., 1997), another brain area implicated in conditioned reinforcement processes (Cador et al., 1989; Whitelaw et al., 1996; Grimm and See, 2000; See et al., 2001; for review, see Everitt and Robbins, 2000), as well as in the processing of fear conditioning (for review, see LeDoux, 2000). Presentation of cocaine-associated stimuli equally increases extracellular dopamine in the nAc and the basolateral amygdala (Weiss et al., 2000). Being parts of the forebrain continuum termed the extended amygdala (Alheid and Heimer, 1988; Heimer et al., 1991), these regions likely interact during cue-associated drug-seeking behavior (Burns et al., 1993; for reviews, see Mogenson et al., 1980; Everitt et al., 1999). Indeed, this whole circuitry is implicated in drug addiction as undergoing long-lasting functional changes following chronic drug exposure (Koob, 2000), and a recent study demonstrated a role of amygdala in the processing of alcohol cues (Zhao et al., 2006). It is therefore possible that the blocking of VTA nAChRs in Paper I, attenuates cue-induced dopamine overflow in the amygdala in addition to the nAc, and that this attenuation, in one or both of these brain regions, is responsible for the consequent reduction in lever-pressing for the cues.

Another brain area implicated in conditioned reinforcement processes is the dorsal striatum. A recent human study suggested that, in cocaine-dependent individuals, dopamine in the dorsal rather than the ventral striatum may be crucially involved in craving and addiction (Volkow et al., 2006). These data are supported by *in vivo* microdialysis experiments demonstrating increased extracellular levels of dopamine in the rat dorsal striatum during behavioral responding to cocaine cues (Ito et al., 2002). The dorsal striatum receives innervations mainly from the substantia nigra (Anden et al., 1964; Jimenez-Castellanos and Graybiel, 1987;. for reviews, see Koob, 1992; Haber and Fudge, 1997). Considering that α-CtxMII is a relatively large molecule it appears unlikely that α-CtxMII reached the substantia nigra by spreading in Paper I and that the observed effects of α-CtxMII involves nAChRs also in this brain area.

Pointing in the direction of the nAc as an important brain area for responding with conditioned reinforcement are data demonstrating that this behavior is modulated by local application of dopaminergic agents in the nAc (Taylor and Robbins, 1984, , 1986; Cador et al., 1991; Wolterink et al., 1993; Hodge et al., 1994; Parkinson et al., 1999; Wyvell and Berridge, 2000; Parkinson et al., 2002; Yun et al., 2004b). Rats with lesions of the nAc core failed to discriminate between the CS+ and the CS- (Cardinal et al., 2002a). Lesions of the central amygdala had no effect on performance in the same study, but were suggested to inhibit the associative learning between the cues and the conditioned approach response. Moreover, *in vivo* voltammetry studies have shown that dopamine is released in the nAc in response also to food predicting cues and the responding for the cue was time-locked to the sub-second dopamine increases (Roitman et al., 2004).

Indeed, a general function of the nAc may be to promote a specific behavior in response to the specific cues that indicate that the behavior will result in reward (Nicola et al., 2004b, 2004a).

It is difficult to determine which one of the mesopontine nuclei (PPTg or LDTg) that is critically involved in mediating the cholinergic input on VTA nAChRs in response to conditioned stimuli. On the one hand, anatomical studies suggest that the LDTg is the mesopontine nucleus that mainly projects to the VTA, whereas the PPTg preferentially projects to the substantia nigra pars compacta (Beninato and Spencer, 1987; Futami et al., 1995). Functional studies, on the other hand, generally attributes the main role in responding with conditioned reinforcement to the PPTg (with no effects on basic- or drug-enhanced motivation (for review, see Winn et al., 1997)). These studies propose that the PPTg cholinergic neurons projecting to the VTA relay sensory signals to regulate conditioned responses of dopamine cells (see Introduction), yet the mechanism is unknown. The PPTg neurons specifically fire to the presentation of context dependent tones (Reese et al., 1995; Dormont et al., 1998) in favor of light cues (Pan and Hyland, 2005). Conversely, the superior colliculus, which sends projections to dopaminergic neurons in the substantia nigra pars compacta (Comoli et al., 2003), responds with a bias towards visual stimuli, as compared to tones (Wallace and Fredens, 1988). Here, Paper I presents evidence that a compound stimulus of a tone + a light supports responding with conditioned reinforcement that was completely blocked by local VTA administration of nAChR antagonists. Paper I moreover suggests that the same nAChRs mediate the dopamine overflow in the nAc elicited by the presentation of an auditory cue. Together these results point towards the PPTg as the main source of cholinergic input to the VTA nAChRs involved in conditioned reinforcement processes. The LDTg, the PPTg and the superior colliculus were recently proposed to collaborate and fine-tune each others impact on the production of behavioral responses to conditioned stimuli (Pan and Hyland, 2005). A possibility that should be considered is that the balance between these inputs to the dopamine systems is shifted in drug dependent subjects.

Based on these facts and the results from Papers I and II, the following mechanism may be suggested (Fig. 15). Cue-induced release of acetylcholine in the VTA, possibly via the PPTg projections, stimulates VTA α-CtxMII-sensitive nAChRs that activate dopaminergic neurons resulting in a cue-induced release of nAc dopamine. This increase in dopamine neurotransmission within the nAc, and possibly other terminal regions of VTA dopamine projections, is likely to stimulate or enhance responding with ethanol-associated cues and probably other conditioned reinforcers. Indeed, sub-populations of nAc neurons that respond to sucrose cues require the dopaminergic projection from the VTA to promote reward-seeking behavior (Yun et al., 2004a). This proposed mechanism could be especially pertinent to the effects of ethanol cues since VTA α-CtxMII-sensitive nAChRs are suggested to mediate also the pharmacological dopamine-related effects of ethanol in rodents (Larsson et al., 2004; Jerlhag et al., 2006). The coincidence that the same receptor sub-population in the VTA mediates both the primary reinforcing and the conditioned reinforcing effects of ethanol may play a critical role in the well known phenomenon of “loss of control” of drinking, a hallmark of alcoholism.

In conclusion, these results suggest that drugs manipulating α-CtxMII-sensitive nAChRs in the VTA should be considered as candidates in the development of novel

pharmacological interventions aimed at reducing cue-induced craving and relapse in alcoholism. α-CtxMII-sensitive nAChRs are supposed, at least in rodents, to be rather concentrated to the central nervous system and especially to the brain reward system. Thus, a pharmacological modulator of these specific nAChR subtypes should produce significantly fewer side effects than mecamylamine (see Introduction). However, the α-CtxMII molecule is a polypeptide that probably is too large to pass the intestinal membranes and the blood brain barrier, and is likely also to be digested by circulating peptidases. Thus, new more selective nAChR antagonists of different molecular structure are warranted.

Paper III and IV demonstrate that the nAc most likely is the primary brain site of interference for the ethanol-induced elevations in extracellular dopamine in the nAc. However, ethanol administered into the VTA was able to stimulate nAc dopamine when ethanol was concomitantly applied in the nAc. These results may support the hypothesis that once acetylcholine is released in the VTA via ethanol's actions in the nAc, ethanol may act also as a co-agonist to acetylcholine on VTA nAChRs, thereby further promoting dopamine activation (Ericson et al., 2000b; Soderpalm et al., 2000; Ericson et al., 2003; Larsson et al., 2004; Molander and Soderpalm, 2005). If alcohol acts as a positive modulator of nAChRs of non-α7 subunit composition, there will be two additional consequences of the co-abuse of alcohol and nicotine. First, the tentative up-regulation of VTA nAChRs as a result of chronic nicotine use, may enhance the pharmacological effects of ethanol if it acts as a co-agonist to acetylcholine in this brain area (cf. Tizabi et al., 2002). This probably promotes further alcohol consumption, since human studies demonstrate that the higher the stimulatory effect that a drinker experiences from alcohol during drinking, the more alcohol is consumed (Holdstock and de Wit, 2001; King et al., 2002; Thomas et al., 2004; Young et al., 2005). Second, if ethanol is able to act together with acetylcholine on VTA nAChRs, it should enhance the effects of acetylcholine that is released into the VTA in response to the presentation of alcohol cues. *I.e.* drinking in an environment where established alcohol cues are present, may facilitate the conditioned reinforcing effects of the cues on behavior AND presentation of cues during drinking could facilitate the primary reinforcing effects of alcohol on the brain reward system via acetylcholine release in the VTA (Fig. 15). This should result in a continuously spiraling enhancement of compulsive drug-seeking and taking. These suggestions are merely hypothetical since we did not investigate the putative involvement of nAChRs in the VTA in Paper III. Thus, the action of ethanol in the VTA that required its presence in the nAc in Paper III may involve mechanisms unrelated to cholinergic activity.

Previous studies have demonstrated a lack of association between the concentrations of ethanol in the nAc and the ethanol-induced dopamine elevations in the same brain region. Rather, as illustrated in Paper III, the dopamine response to ethanol administration consists of an initial elevation in nAc dopamine levels when ethanol concentrations increase, followed by a reduction in nAc dopamine before the concentrations of ethanol have declined. Whereas the ascending limb of the blood alcohol curve may represent the stimulatory properties of alcohol, the sedative effects of alcohol are suggested to appear during the descending phase of the blood alcohol curve (*e.g.* Martin et al., 1993a). Paper III demonstrates that recruitment of $GABA_A$ receptor mediated activity appears to be responsible for the second, declining phase of the dopamine response to ethanol. In Paper IV, chronic administration of the nAChR

antagonist hexamethonium prevented this dissociation, thereby attenuating the inhibitory, and prolonging the stimulatory, effects of ethanol on nAc dopamine. Hexamethonium pre-treatment moreover abolished the nAc dopamine decrease in response to local administration of the sedative-hypnotic diazepam, a positive modulator of the $GABA_A$ receptor. Together with the fact that $GABA_A$ receptors mediate the sedative effects of ethanol (see Introduction), this may also suggest that the hexamethonium pre-treatment reduced the sedative effects of acute alcohol.

Also nicotine counteracted the inhibitory effect of locally applied diazepam on nAc dopamine. Human studies have reported that nicotine may attenuate the subjective sedative effects of acute alcohol intoxication (Zacny, 1990; Perkins et al., 1995; Perkins et al., 2000). This phenomenon could be related to nicotine-induced stimulation of noradrenergic arousal systems (Svensson and Engberg, 1980; Li et al., 1998; Saint-Mleux et al., 2004) but may also arise from attenuation of $GABA_A$ mediated effects of ethanol (Cott et al., 1976; Liljequist and Engel, 1982; Palmer et al., 1987; for recent review, see Ueno et al., 2001), as observed following chronic administration of nicotinic drugs in Paper IV. The accumbal $GABA_A$ receptors that were investigated in Papers III and IV may thus mediate some of the sedative properties of ethanol, although also other $GABA_A$ receptors than those located in the nAc most likely are involved in ethanol-induced sedation. This finding may be of great importance, since a low sedative response to acute ethanol challenge is suggested a risk factor of alcohol dependence (Schuckit and Smith, 1996; Schuckit, 1998; Heath et al., 1999).

It is possible that the subchronic treatments with nicotine and hexamethonium produced different alterations of $GABA_A$ receptor compositions (which is a common adaptive strategy of these receptors (see Introduction)). Such differential alterations taken together with the fact that ethanol and diazepam are believed to activate different $GABA_A$ receptor subunit configurations (for review, see Olsen et al., 2005), could explain why the consequences of both alcohol and diazepam on nAc dopamine were altered by hexamethonium pre-treatment while only the effect of diazepam was changed following nicotine. In support of nicotine-induced alterations of $GABA_A$ receptor activity is a recent human study suggesting that smoking influences some $GABA_A$ receptor adaptations associated with chronic alcohol consumption (Staley et al., 2005).

It remains to be resolved whether the reported consequences of subchronic pre-treatment with hexamethonium in the present thesis can be designated to its antagonistic effects on peripheral ganglionic nAChRs and/or to interference with central nAChRs. Like other quaternary ammonium compounds, hexamethonium is considered to poorly penetrate the blood brain barrier, although its selectivity for the periphery naturally decreases with increasing doses (Asghar and Roth, 1971). As previously mentioned, the hexamethonium dose and administration regimen used here increases voluntary ethanol intake (Ericson et al., 2000a) but fails to antagonize nicotine-induced neurochemical (*e.g.* Matta et al., 1995) as well as behavioral (*e.g.* Ericson et al., 2000b) consequences that are antagonized by mecamylamine. Moreover, an acute systemic hexamethonium injection at the dose used here, had no effect on voluntary ethanol intake in ethanol high-referring rats, unlike mecamylamine which significantly reduced ethanol intake in these animals (Blomqvist et al., 1996). Altogether, these results suggest that the effects observed following intermittent subchronic hexamethonium pre-treatment in the present thesis are

due to blockade of peripheral ganglionic nAChRs, although this assumption remains to be verified under our experimental conditions.

Peripheral components in the modulation of alcohol consumption have previously been demonstrated. The unselective peripherally acting muscarinic antagonists methylatropine and methscopolamine acutely reduced ethanol consumption in rats (Rezvani et al., 1990; Sprague et al., 1994), and methscopolamine reversed the increase in ethanol intake and preference induced by subchronic pre-treatment with hexamethonium (Ericson et al., 2000a). Intermittent blockade of ganglionic nAChRs during subchronic hexamethonium pre-treatment should reduce the postganglionic activity innervating peripheral effector organs such as the stomach, the liver and/or the pancreas. It is possible that a reduction in parasympathetic acetylcholine stimulation of mAChRs on these organs following subchronic hexamethonium administration results in a compensatory mAChR up-regulation. This up-regulation may modify processes of these organs and their vagal afferent activity and/or increase the liberation of circulating hormones that target the central nervous system. The consequence may be alterations in central activity such as the GABAergic response to ethanol in the nAc and a consequent increase in alcohol consumption. Indeed, in support of such a hypothesis are findings showing that vagotomy, both subdiaphragmatic, gastric and hepatic, lower ethanol intake in the rat (Kulkosky et al., 1987; Toth et al., 1990). Several central as well as peripheral ganglionic nAChR subtypes readily desensitize (e.g. (Fenster et al., 1999), and during longer exposure to nicotine a greater fraction of these receptors becomes desensitized (Pidoplichko et al., 1997). In the case of nicotine treatment, it is therefore possible that a larger fraction of the nAChRs desensitize and/or are up-regulated following a longer nicotine treatment period than the 15 days used here, or during a more frequent injection schedule than once daily (cf. (Ulrich et al., 1997). Thus a more intense use of nicotine, such as that of heavy smokers or snuffers, may produce effects that resemble those of hexamethonium observed here.

Studying reward-related mechanisms in the rat, this thesis strongly suggests a role for nAChRs in conditioned reinforcement to ethanol. Although not clearly evidenced, the present data also suggest that nicotine may promote alcohol consumption due to reduction in GABAergic responses to ethanol in the brain. In spite of the fact that nicotine and alcohol are commonly co-abused, the extensive supply of clinical data investigating the subjective reward-related effects of co-consumption of these two drugs is inconclusive. A recent human study demonstrated that nicotine increases the motivation to consume alcohol among male, non-dependent smokers (Barrett et al., 2006). When the reversed was investigated, *i.e.* the effects of alcohol on nicotine reward and consumption, acute alcohol pre-treatment produced no significant alterations in discriminative stimulus, subjective or reinforcing effects of nicotine delivered by nasal spray in alcohol non-dependent young female smokers (Perkins et al., 2005). However, other recent clinical studies report that acute alcohol increases craving to smoke (Burton and Tiffany, 1997; Kouri et al., 2004; Rose et al., 2004; King and Epstein, 2005) and that nicotine is more reinforcing in smokers with a past history of alcoholism (Hughes et al., 2000). The present thesis may provide an explanation to the variation in the clinical data. If nAChRs, as suggested here, mediate conditioned reinforcement to reward in general, it may be the conditioned (rather than the pharmacological) consequences of nicotine administration that are increased by acute alcohol, and vice versa; conditioned stimuli

may be absent in clinical testing environments, especially when nicotine is administered i.v. to smokers. This conclusion is supported by data demonstrating that acute alcohol consumption increases the impact of smoking cues on nicotine craving in heavy smokers (Sayette et al., 2005) and that alcohol cues can increase the urge to smoke in alcoholic smokers when they are not nicotine deprived (Cooney et al., 2003). Moreover, cross reactivity to alcohol and nicotine cues was demonstrated among drinking alcoholics (Drobes, 2002).

One of the main purposes of the present studies was to provide a better comprehension of why nicotine and ethanol often are co-abused. Taken together, the results propose the following explanations (Fig. 15):

1) It is demonstrated that nAChRs mediate the dopamine activating and conditioned reinforcing effects of alcohol cues and cues associated with natural reward. Consequently, acute nicotine use may enhance the impact of reward-related cues on consumption of rewards and increase the vulnerability for drug-seeking and relapse to alcoholism.
2) Chronic nicotine use results in up-regulation of the activity of several nAChR subtypes and may thereby further enhance the alcohol consumption that is promoted by alcohol cues.
3) The present thesis suggests nAChRs as a common mediator for the impact of cues on drug-seeking behavior. Drug-related stimuli are important for self-administration and craving for alcohol as well as nicotine. Together, this may imply that nicotine-cues can enhance not only nicotine use, but also alcohol consumption, and vice versa. These results may also provide a novel mechanism underlying the common craving for sweets during alcohol and nicotine withdrawal.
4) It is suggested here, that chronic administration of nicotinic drugs may reduce the $GABA_A$ receptor mediated inhibitory action on ethanol-induced dopamine release. This effect should attenuate the sedative and prolong the stimulatory, effects of ethanol, and may explain why smokers report less sedation from alcohol consumption. It further implies that the use of nicotine may increase the dopamine stimulating properties of ethanol and consequently also alcohol consumption.
5) Since the nAChRs appear to be one common factor involved in nicotine and alcohol addiction, it is possible that genetic alterations in systems involved in nAChR function can further enhance the sensitivity to the impact of salient cues and increase the vulnerability for co-abuse of these two drugs.

The comprehensive conclusion is that nicotinic drugs can modulate the conditioned reinforcing, as well as the primary reinforcing, effects of alcohol in the rat. This coincidence may play a critical role in the well known phenomenon of “loss of control” of drinking, a hallmark of alcoholism. The findings moreover strongly suggest α-CtxMII-sensitive nAChRs as potential targets for pharmacological interventions aimed at reducing cue-induced craving and relapse in alcoholism.

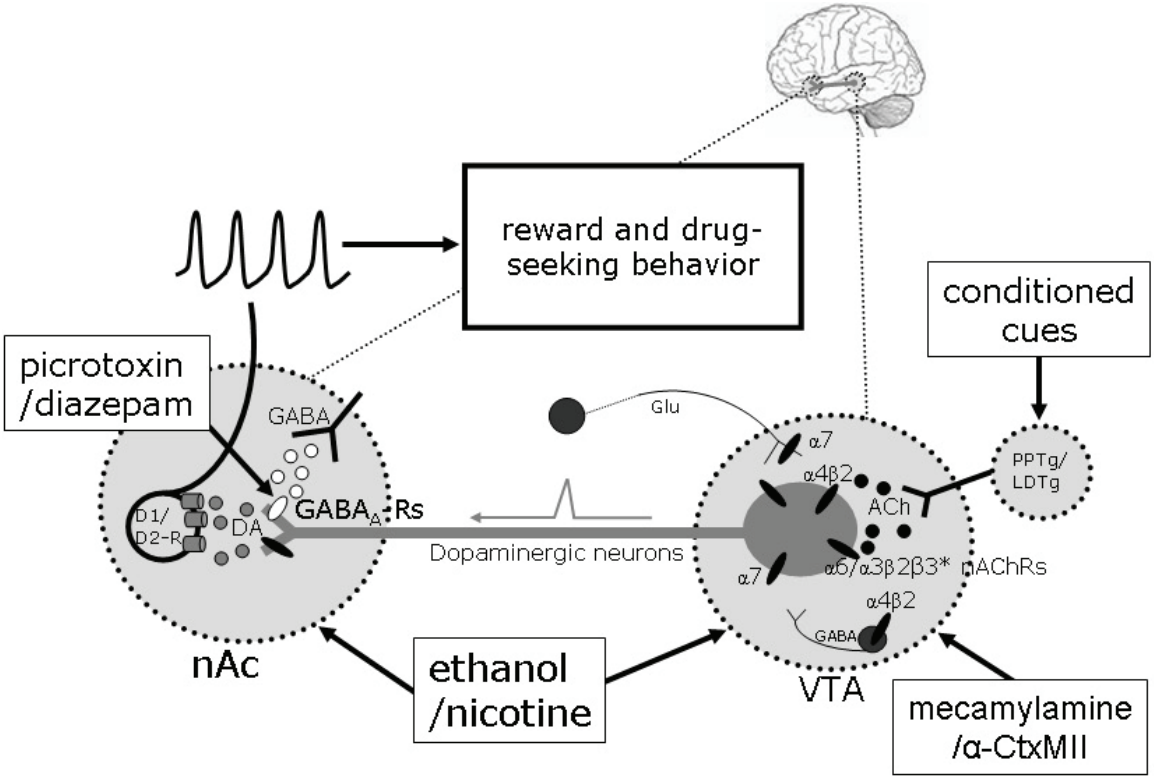


Fig. 15. Conditional and non-conditional reward-related responses of the mesolimbic dopamine system to alcohol – mechanisms studied in the present thesis.

Reward-associated conditioned cues may induce the release of acetylcholine (ACh) in the VTA, possibly via the PPTg projections. In the VTA, this acetylcholine stimulates α-CtxMII-sensitive nAChRs that activate the mesolimbic dopaminergic neurons, resulting in a cue-induced release of dopamine (DA) in the nAc. This increase in nAc dopamine neurotransmission likely stimulates reward-seeking behaviors and may also enhance the reinforcing properties of drugs that are consumed during cue presentation. These consequences can be antagonized by local administration of nAChR antagonists such as mecamylamine or α-CtxMII into the VTA. The concomitant use of nicotine, may facilitate learning about the cue-reward relationship through its general dopamine enhancing effects and via enhancement of dopamine firing in response to salient cues. Chronic use of nicotine may increase both the conditional and non-conditional reward-related responses to alcohol by up-regulation of VTA nAChRs. Chronic nicotine administration may also reduce the GABAergic activity in the nAc, as demonstrated by its ability to reduce the sensitivity of nAc $GABA_A$ receptors to the dopamine reducing effects of diazepam. Thus, smoking may prolong the stimulatory response to ethanol at the expense of the sedative properties of acute ethanol intoxication.

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SWEDISH SUMMARY FOR MY FAMILY AND FRIENDS

SVENSK SAMMANFATTNING

Denna avhandling studerar konsekvenser av alkohol- och nikotinkonsumtion på hjärnan, mekanismerna bakom återfall samt hur återfall kan framkallas av betingade faktorer hos råtta. Syftet är att generera ny kunskap om varför dessa två droger ofta missbrukas tillsammans för att kunna förbättra behandlingsmöjligheterna vid alkohol- och nikotinberoende.

Alkoholism är ett stort problem världen över. Utöver det lidande som drabbar den beroende individen och dess anhöriga, uppskattas alkoholrelaterade konsekvenser enbart i Sverige kosta det svenska samhället ca 150 miljarder kronor per år. Risken att drabbas av alkoholism påverkas av både arv och miljö. En rökare löper 10 gånger högre risk att bli alkoholist än en icke-rökare och 90 % av alla alkoholister röker, vilket inte enbart beror på sociala faktorer. Försöksråttor som vid fritt val mellan alkohol och vatten föredrar att dricka vatten, övergår till att välja alkohol om de behandlas med nikotin. Alltså tycks det finns biologiska orsaker till varför alkohol och nikotin ofta missbrukas tillsammans. Följande avhandling syftar till att utröna dessa orsaker med fokus på hjärnan.

De flesta beroendeframkallande droger stimulerar hjärnans belöningssystem (se figur A) så att signalämnet dopamin frisätts från dopaminnerverna; ju mer dopamin som utsöndras desto mer belönande och beroendeframkallande förefaller drogen vara. Både alkohol och nikotin stimulerar hjärnans belöningssystem via en gemensam nämnare, nikotinreceptorer (”mottagare”). Denna avhandling studerar två olika aspekter på hjärnans belöningssystem och nikotinreceptorerna.

Trots tillgång till både läkemedel och psykologisk terapi för behandling av alkoholberoende återfaller många. Generellt kan återfall i missbruk ske efter många års avhållsamhet, dvs. då drogen[1] sedan länge är ute ur kroppen och personen inte längre är drogberoende i dess rätta bemärkelse. I detta fall induceras återfallet ofta av miljöbetingade faktorer (Eng. *cues*) som tidigare associerats till drogen, såsom en speciell lokal, lukt eller ett musikstycke. Sådana faktorer framkallar känslor liknande dem som upplevts vid tidigare drogintag och ger åter ett drog-sug och ett drogsökande beteende. Fenomenet kallas betingning eller konditionering, och tycks spela en viktig roll vid intag av alkohol och nikotin. Även *cues* i sig kan öka dopaminet i hjärnans belöningssystem hos en beroende individ vilket anses framkalla drogsökande beteenden. På detta sätt kan alkoholister få en ökad aktivitet i delar av belöningssystemet av alkoholrelaterade objekt, såsom en bild av ett ölglas. Ju starkare hjärnområdet aktiverades av *cuen* i vissa studier, desto snabbare återföll senare personerna i alkoholmissbruket. Även när det gäller nikotinberoende, spelar betingning en stor roll för nikotinkonsumtionen. Hos försöksdjur tycks både akut och kronisk nikotinbehandling kunna förstärka effekten av *cues* på drogsökande beteenden.

Syftet med första delen av avhandlingen var att studera om de nikotinreceptorer som finns i hjärnans belöningssystem är inblandade då alkoholassocierade *cues* stimulerar hjärnans belöningssystem och framkallar drogsökande beteenden hos råtta. Ett

[1] Ordet drog syftar i denna text till beroendeframkallande ämnen i allmänhet, dvs. även alkohol och nikotin.

klarläggande av dessa mekanismer möjliggör utveckling av nya, mer selektiva läkemedel för förhindrande av det drog-suget och det reflexliknande, tvångsmässiga drogsökande beteende, som kan drabba en missbrukare som när han/hon hamnar i en miljö som påminner om tidigare drogkonsumtion.

Till försöken selekterades råttor som vid fritt val mellan alkohol (10%) och vatten föredrog att dricka alkohol (s.k. högprefererande råttor). Dessa djur tränades sedan i speciella burar, där de fick lära sig att tillgången till en 10 % alkohollösning under 5 sekunder alltid föregås av en signal i form av en ton och ett ljus (jmf. Pavlovs hundar). Efter en sådan träningsfas (30 min/dag i ca 2 veckor) kommer djuren att associera signalen med alkohol. Vid det efterföljande testtillfället fick råttorna under 30 minuter tillgång till två små pedaler i burarna (utan att få tillgång till alkohol). Tryckte råttan på den ena pedalen (CR-pedal), resulterade detta i att signalen presenterades (utan att råttan fick tillgång till alkohol). Tryckning på den andra pedalen (NCR-pedal) hade ingen konsekvens. Vi mätte hur mycket djuren tryckte på respektive pedaler. En preferens för CR-pedalen visar att signalen har fått betingade egenskaper, dvs. den har blivit en *cue*. Vi studerade om nikotinreceptorer är inblandade i betingningens effekt på drogsökande beteenden genom att injicera råttorna, antingen systemiskt eller direkt in i hjärnans belöningssystem, med olika ämnen som blockerar dessa nikotinreceptorer (s.k. nikotinreceptorblockerare). Lokala injektioner i hjärnan utförs via en tidigare inopererad kanyl. Råttorna förefaller inte alls störda av att denna kanyl finns närvarande, utan beter sig som vanligt efter en sådan operation. Resultaten visar att råttor som inte injicerats med nikotinreceptorblockerare främst tryckte på pedalen som levererade den *cue* råttorna tidigare hade lärt sig att associera med tillgången till alkohol. Alltså motiverar *cuen* dessa djur att söka belöningar. Råttor som å andra sidan injicerats med en nikotinreceptorblockerare innan testsessionen, tryckte lika mycket på båda pedalerna. Substanser som blockerar nikotinreceptorerna i hjärnans belöningssystem förefaller alltså kunna häva den motiverande effekten som *cuen* hade på råttans beteende.

Dopaminet i hjärnans belöningssystem mättes också samtidigt som råttan fick höra den inlärda *cue*-signalen (utan att ge dem alkohol vid testillfället). *Cuen* i sig kunde höja dopaminmängderna i hjärnan till nästan samma nivå som när en råtta dricker alkohol. Denna höjning, som tros stimulera till drogsökande beteenden, gick att motverka med en substans som blockerar nikotinreceptorer på dopaminnerverna.

Sammantaget visar resultaten att signaler som tidigare associerats med tillgången till alkohol stimulerar råttans belöningssystem, liksom bilden av ett glas öl kan stimulera hjärnan hos en alkoholist och framkalla drogsökande beteenden. Vidare visar resultaten att nikotinreceptorer i råttans belöningssystem måste aktiveras för att detta beteende ska framkallas.

Ett nytt försök visade att samma nikotinreceptorer också förmedlar liknande effekter av signaler som råttan lärt sig att associera med en sockerlösning istället för alkohol. Dessa data tyder på att nikotinreceptorerna är inblandade i effekten av *cues* på belöningssökande beteenden i allmänhet. Resultaten skulle kunna förklara varför suget efter och konsumtionen av sötsaker ofta ökar hos rökare och alkoholister under abstinensfasen. Det ökade socker-suget och påföljande viktökning är en vanlig orsak till att kvinnor återfaller i rökning. Nya framtida läkemedel som påverkar dessa receptorer skulle därför kanske kunna hindra att miljöbetingade faktorer inducerar återfall i alkoholism. Att beteendeeffekter av socker-associerade *cues* förmedlas via samma system

i hjärnan visar också att dessa hypotetiska läkemedel även skulle kunna användas för behandling av andra typer av beroende som t.ex. matmissbruk. Att nikotinreceptorerna verkar styra effekten av alkohol-*cues* på beteendet kan betyda att rökning förstärker denna effekt och kan vara en förklaring till varför rökning är en riskfaktor för alkoholism.

Som tidigare nämnts, aktiverar både alkohol och nikotin hjärnans belöningssystem. Hur nikotin aktiverar detta system är relativt väl utrönt medan alkoholens mekanismer är mer oklara. Syftet med andra delen av avhandlingen var därför att utröna på vilket sätt alkoholen frisätter det belönande dopaminet hos råttan. Dessutom, för att undersöka om rökning kan påverka belöningssystemet så att det reagerar annorlunda på alkohol, behandlades vissa råttor först med olika ämnen (t.ex. nikotin) som påverkar nikotinreceptorerna. Genom att samla upp vätska ur en prob som tidigare opererats in i råttans hjärna, mättes därför mängden dopamin i råtthjärnans belöningssystem före och efter en injektion av alkohol. Koncentrationerna av alkohol som fanns i hjärnans belöningssystem mättes också. Resultaten visar att alkoholens förmåga att öka det belönande dopaminet i hjärnan avtar efter en stund, trots att alkoholen fortfarande finns kvar i hjärnan. Avhandlingen visar också att en annan typ av receptorer som också finns i hjärnans belöningssystem ($GABA_A$, se figur A) ligger bakom denna avtagande effekt på dopaminet. Det är väl känt att dessa receptorer förmedlar den sövande egenskapen hos de flesta sömntabletter. Därav dras slutsatsen att den hämmande effekten alkoholen har på dopaminet (efter den initialt stimulerande effekten) bidrager till att framkalla den trötthetskänsla som mer eller mindre upplevs vid alkoholkonsumtion. Denna effekt uteblev hos råttor som hade förbehandlats kroniskt med olika substanser som påverkar nikotinreceptorerna. Detta kan vara en förklaring till varför vissa rökare inte får samma trötthetskänsla under alkoholintag som icke-rökare. Risken för alkoholism förefaller vara större hos personer som ej blir sömniga av alkohol. Således kan slutsatserna av den andra delen i denna avhandling ge ytterligare en förklaring till varför rökning ökar risken för alkoholism.

Den övergripande slutsatsen i denna avhandling är att både alkohol, nikotin och även miljöbetingade alkoholrelaterade signaler (*cues*) förefaller påverka aktiviteten i nikotinreceptorer hos råtta. Alltså tycks samma komponent i hjärnans belöningssystem vara viktig för både de farmakologiska och de psykologiska effekterna av alkohol och nikotin, som spelar en viktig roll vid abstinenssymptom och återfall. Detta kan vara en av orsakerna till att alkohol och nikotin så ofta konsumeras tillsammans samt en förklaring till den kontrollförlust över beteenden som kännetecknar återfall i alkoholmissbruk. Sammantaget tyder alltså resultaten på att framtida läkemedel som påverkar dessa nikotinreceptorer skulle kunna användas för behandling av både alkoholism och rökning.

Det kan tyckas långsökt att studera råttan för att få kunskap om en sjukdom som drabbar främst människan. Alkoholism är ju trots allt ingen vanlig sjukdom bland råttor. Dock har hjärnans belöningssystem bevarats väl under evolutionens gång och fyller en liknande funktion hos så skilda arter som människor och bananflugor. Anledningen till belöningssystemets bevarande är just dess huvudfunktion: att, oavsett art, frisätta dopamin och därigenom framkalla känslor av välbefinnande som motiverar till beteenden av betydelse för artens överlevnad, såsom sökandet av föda och fortplantning (även om dessa beteenden onekligen yttrar sig olika hos människor och bananflugor). Tyvärr har droger, liksom mat och sex under speciella omständigheter, förmågan att ge belöningssystemet en för stark genomslagskraft. Konsekvensen blir då att sökandet och

konsumtionen av belöningar dominerar det vardagliga beteendet hos den missbrukande individen. Det är därför en stor utmaning att utveckla nya läkemedel som kan manipulera dopaminaktiviteten så att de drog-inducerade sjukliga förändringarna motverkas utan att eliminera personens motivation att söka föda eller förmågan att uppleva känslor av tillfredställelse.

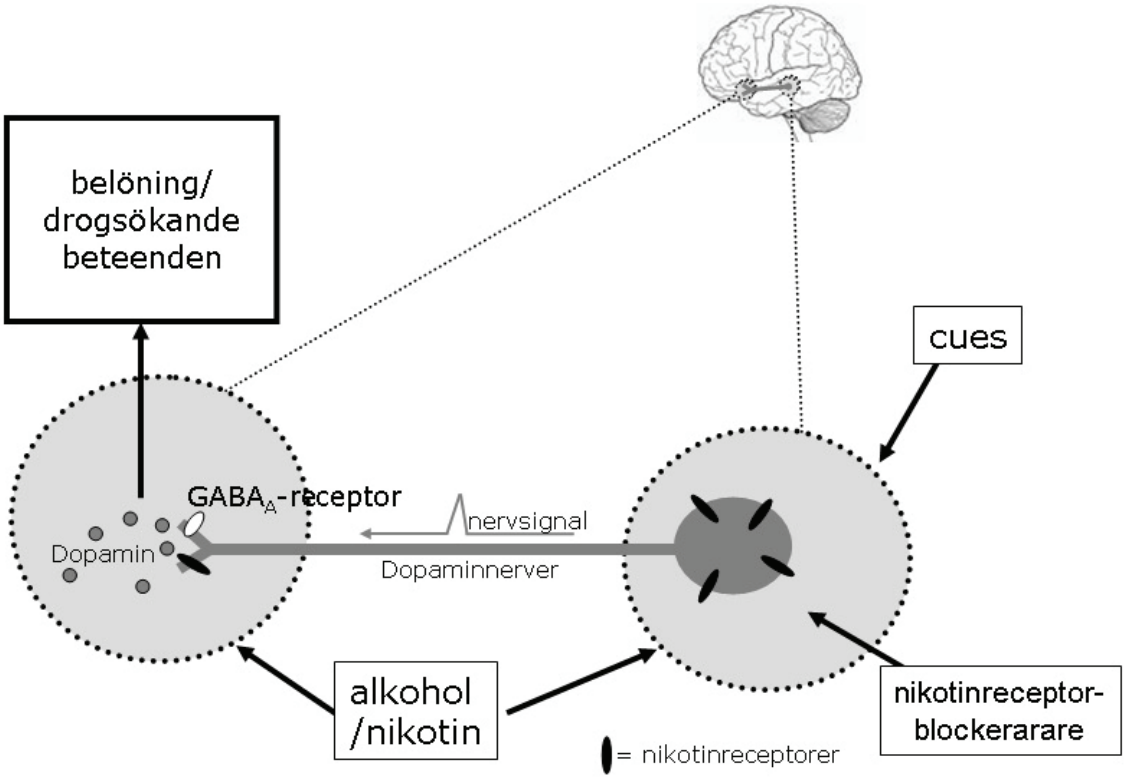

Fig. A. Dopaminnerverna i hjärnans belöningssystem.
Både nikotin och alkohol kan stimulera hjärnans belöningssystem via nikotinreceptorer. Resultatet blir att signalsubstansen dopamin frisätts, vilket framkallar känslor av belöning och motiverar till att söka mer drog. Avhandlingen visar att även miljöbetingade signaler (cues) som tidigare associerats med alkoholintag och dess effekter kan öka dopaminet via nikotinreceptorerna. Resultaten tyder på att framtida substanser liknande de nikotinreceptorblockerare som användes i försöken i denna avhandling, skulle kunna fungera som läkemedel mot återfall i alkoholism och rökning och troligtvis andra typer av beroende. Alkoholens förmåga att öka det belönande dopaminet i hjärnan avtar efter en stunds alkoholadministrering, trots att alkoholen fortfarande finns kvar i hjärnan. Avhandlingen visar att denna avtagande effekt på dopaminet tycks förmedlas via GABA$_A$-receptorer i hjärnans belöningssystem.

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