THE CHOLINERGIC-DOPAMINERGIC REWARD LINK AND ADDICTIVE BEHAVIOURS

special emphasis on ethanol and ghrelin

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Abstract

The cholinergic-dopamienrgic reward link and addictive behaviours -special emphasis on ethanol and ghrelin

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An important part of the reward systems is the cholinergic-dopaminergic reward link. This reward link has been proposed to be involved in reward and motivated behaviours. It encompasses a cholinergic input from the laterodorsal tegmental area (LDTg) to the mesolimbic dopamine (DA) system that originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens.
Previous results demonstrate that nicotinic acetylcholine receptors (nAChRs), especially those located in the VTA, are involved in mediating the stimulatory, rewarding and DA enhancing properties of ethanol. One aim of the present thesis was therefore to investigate the functional role of different nAChR subtypes for the behavioural and neurochemical effects of ethanol. In Paper I a slightly modified method was used to synthesize α-conotoxins with various subunit selectivity; α-conotoxin MII (αCtxMII) and a α-conotoxin PIA-analogue. Furthermore, it was demonstrated that αCtxMII-sensitive (*i.e.* the $\alpha_3\beta_2$*, β_3* and/or α_6* subtypes), rather than αPIA-analogue-sensitive (the α_6* subtype), nAChRs in the VTA are involved in mediating the stimulatory and accumbal DA enhancing properties of ethanol. Given that ethanol concomitantly increases ventral tegmental ACh and accumbal DA levels and that some of the effects of ethanol are mediated via the $\alpha_3\beta_2$* and/or β_3*, rather than α_6* subtypes in the VTA, we hypothesize that ethanol activates the cholinergic-dopaminergic reward link.
There appears to be a neurochemical overlap between the reward systems and the systems regulating energy balance. Ghrelin is an orexigenic peptide, which *e.g.* via the hypothalamus increases food intake. Given that ghrelin is involved in energy balance regulation, the role for ghrelin in brain reward was investigated in Papers II, III, IV. It was demonstrated that intracerebroventricluar administration of ghrelin increases locomotor activity and accumbal DA overflow in mice, suggesting that ghrelin activates the mesolimbic DA system. In subsequent experiments it was found that administration of ghrelin

into either the LDTg or the VTA (reward nodes expressing growth hormone secretagougue receptors (GHSR-1A)) increases the locomotor activity as well as accumbal DA overflow. Thus indicating that ghrelin, via GHSR-1A in the LDTg and/or VTA, activates the cholinergic-dopamine reward link. Further, the stimulatory and DA enhancing properties of ghrelin (intracerebroventricluar) were antagonized by systemic administration of the unselective nicotinic antagonist, mecamylamine, implying that cholinergic mechanisms are involved in mediating the stimulatory and DA enhancing effects of ghrelin. Additionally, it was showen that the stimulatory and DA enhancing effects of ghrelin administration (into either the VTA or LDTg) were mediated via αCtxMII-sensitive nAChRs, *i.e.* the $\alpha_3\beta_2$* and/or β_3* subtypes, in the VTA, implying neurochemical analogies between ethanol and ghrelin. These findings provide the first indication that ghrelin has a role in brain reward and that ghrelin is a part of the neurochemical overlap between systems regulating energy balance and reward. We hypothesize that ghrelin stimulates the cholinergic-dopaminergic reward link and thereby increases the incentive values of signals associated with motivated behaviours such as food searching/foraging. Thus ghrelin drives animals (and man) to work and to seek for food. High plasma levels of ghrelin have been associated with some aspects of binge eating/compulsive overeating as well as alcoholism. Additionally, a deranged reward system has been implicated in overeating and alcoholism. We therefore hypothesizes that hyperghrelinemia, via activation of the cholinergic-dopaminergic reward link, may be a part of the pathophysiology of binge eating and alcoholism. The findings in the present thesis demonstrate that the $\alpha_3\beta_2$* and/or β_3* subtypes are involved in mediating the stimulatory and DA enhancing effects of ethanol and ghrelin. It is therefore suggested that these subunits might be novel pharmacological targets for treatment of compulsive overeating as well as alcoholism.

Key words: ethanol, ghrelin, reward, food-seeking, ventral tegmental area, laterodorsal tegmental area, nucleus accumbens, dopamine, nicotinic acetylcholine receptors, *in vivo* microdialysis, locomotor activity, mice, addictive behaviours.

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This thesis is based on the following research papers, which will be referred to in the text by their Roman numerals:

I. Jerlhag E, Grøtli M, Luthman K, Svensson L, Engel JA (2006) Role of the subunit composition of central nicotinic acetylcholine receptors for the stimulatory and dopamine-enhancing effects of ethanol in mice. Alcohol and Alcoholism 41(5): 486-493.

II. Jerlhag E, Egecioglu E, Dickson SL, Andersson M, Svensson L, Engel JA (2006) Ghrelin stimulates locomotor activity and accumbal dopamine overflow via central cholinergic mechanisms: implications for its involvement in brain reward. Addiction biology 11: 45-54.

III. Jerlhag E, Egecioglu E, Dickson SL, Douhan A, Svensson L, Engel JA (2007) Ghrelin administration into Tegmental Areas stimulates locomotor activity and increases extracellular concentration of dopamine in the nucleus accumbens. Addiction biology 12: 6-16.

IV. Jerlhag E, Egecioglu E, Dickson SL, Svensson L, Engel JA (2007) Alpha-conotoxin MII-sensitive nicotinic acetylcholine receptors are involved in mediating the ghrelin-induced locomotor stimulation and dopamine overflow in nucleus accumbens. Manuscript.

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List of abbreviations

ACh	Acetylcholine
αCtxMII	α-Conotoxin MII
αCtxPIA-analogue	α-Conotoxin PIA analogue
AgRP	Agouti related peptide
ANOVA	Analysis of variance
BBB	Blood brain barrier
BED	Binge eating disorder
BMI	Body mass index
CNS	Central nervous system
DA	Dopamine
DHβE	Dihydro-beta-erythroidine
DPM	Disintegrations per minute
EAA	Excitatory amino acids
EtOH	Ethanol
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GHS	Growth hormone secretagogue
GHSR	Growth hormone secretagogue receptor
HPLC-ED	High-pressure liquid chromatography with electrochemical detection
icv	Intracerebroventricular
ip	Intraperitoneally
LDTg	Laterodorsal tegmental area/grey
MC	Melanocortins
MCH	Melanin-concentrating hormone
MEC	Mecamylamine
MLA	Methyllycaconitine
mAChR	Muscarinic acetylcholine receptor
NA	Noradrenaline
N.Acc.	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartic acid
NPY	Neuropeptide Y
n.s.	Non significant
PET	Positron emission tomography
PLSD	Protected least-significant difference

POMC	Proopiomelanocortin
PPTg	Pedunculopontine tegmental area/grey
sc	Subcutaneous
S.E.M.	Standard error of the mean
5-HT	Serotonin
VTA	Ventral tegmental area

Introduction

Addiction

Addiction is a chronic, relapsing brain disorder (Hunt et al, 1971; Leshner, 1997; McLellan et al, 2000), characterized by a compulsive drug-seeking behaviour and a loss of control (Koob and Le Moal, 2001). Substance use is defined as a controlled drug intake for non-medical purposes, whereas substance abuse is a harmful drug intake that is continued despite negative effects. Substance use or abuse causes substance dependence in some, but not all, individuals. The definition for substance dependence is described in the Diagnostic and statistical manual of mental disorders 4th edition (table 1).

1. Tolerance
2. Withdrawal
3. The substance is taken in larger amounts or over a longer period than intended (*i.e.* loss of control)
4. There is a persistent desire or unsuccessful effort to cut down or control substance use (*i.e.* craving)
5. A great deal of time is spend in activities necessary to obtain and use the drug as well as recover from its effects
6. Important social, occupational or recreational activities are reduced or given up due to the substance use
7. The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance

Table 1. Diagnostic criteria for substance dependence as described in the Diagnostic and statistical manual for mental disorders.
Substance dependence is defined by the occurrence of three (or more) of the following criteria, over a continuous 12-month period.

With new perspectives and knowledge, the general idea of addiction as substance dependence has changed. Clinical studies of patients with aberrant eating behaviour have shown behavioural parallels between compulsive overeating and chemical addictions (e.g. nicotine, alcohol and psychomotor stimulants) (Davis and Claridge, 1998; Davis, 2001; Davis and Woodside, 2002). Additionally, it has been shown that food, when consumed in excess and over time, can cause the same brain neuroadaptations as drug abuse (*e.g.* Grigson, 2002). It has therefore been suggested that brain functions can be similarly derailed by natural rewards and drugs of abuse. "Behavioural" addictions, such as compulsive overeating, gambling and compulsive shopping, have

therefore been included in the definition of addiction and are together with drug dependence called addictive behaviours. Interestingly, human imaging studies have revealed that there is an underlying disruption in the reward systems in the brain (Holden et al, 2001; Knutson et al, 2001; Potenza et al, 2003; Volkow et al, 2003a; Volkow and Li, 2004; Wang et al, 2004a; Reuter et al, 2005), as well as in brain regions important for inhibitory control (Volkow et al, 2003b) in addictive behaviours.

A number of theories approach the causes for addiction, including the drug-centred and/or the individual-centred hypothesis. The drug-centred theory suggests that a chronic use of a drug or chronic disrupted behavioural patterns causes molecular changes within the brain, *e.g.* in the dopamine (DA) reward systems (*vide infra)*, which shifts the individual's behaviour from a normal approach to a state of addiction (Berke and Hyman, 2000; Hyman and Malenka, 2001; Nestler, 2001; Deroche-Gamonet et al, 2004). In contrast, the individual-centred hypothesis implies that individuals likely to become dependent are born with an increased vulnerability in the reward systems (Wolf and Maisto, 2000), such as a dopaminergic hypo- and/or hyper-function. It has been hypothesized that a dopaminergic hypo-function causes a reward deficit syndrome (Balldin et al, 1985; Volkow et al, 1990; Balldin et al, 1992; Volkow et al, 1993; Volkow et al, 1996; Wang et al, 1997; Volkow et al, 2001; Wang et al, 2004a; Bowirrat and Oscar-Berman, 2005) and hence individuals with a reward deficit syndrome excessively use rewards in a compensatory manner to increase the DA levels to an ambient level. On the other hand, augmentation of DA signalling increases the desire to eat in response to a palatable food cue (Volkow et al, 2002), implicating that increased DA function may cause an enhanced hedonic capacity (Cohen et al, 2005). This hyper-function in DA signalling makes individuals more sensitive to rewards (Davis et al, 2004). It is thus plausible these individuals will gain more gratification out of a reward than subjects with low sensitivity to reward, making them more prone to develop an addiction (Davis et al, 2004). Taken together, it may be concluded that individuals with a high sensitivity to reward are driven to excessively use rewards and that this over stimulation over time may cause a down regulation of the mesolimbic DA system. Additionally, it should be emphasized that age, genetics and hormonal status are essential factors influencing the individual's risk to develop an addiction (Engel et al, 1992). Conclusively, it is most probable that

both the drug-centred and individual-centred theories make valid points to the conceiving of addiction (Deroche-Gamonet et al, 2004). Needless to say, the plethora of hypothesis regarding the causes of addiction point at the complexity of this disease.

Alcoholism

Alcohol dependence is a chronic disorder (Garbutt et al, 1999) and is today recognized as a disease. Attempts have been made to classify this heterogeneous disease into different subtypes (*e.g.* Cloninger et al, 1988; Lesch et al, 1988). It should be taken to consideration that different neurochemical, genetical and psychological factors are involved in the development of the subtypes of alcohol use disorder, thus implying that differential treatment strategies may be applied. Alcoholism causes considerable suffering to the individual as well as to their families and society (Garbutt et al, 1999). Patient related problems include decreased health status, malnutrition, liver damage and cardiovascular problems (Bien and Burge, 1990). The direct and indirect health and social costs related to alcoholism in Sweden is annually estimated to be tens of billions of Swedish crowns, due to factors such as loss of production, social welfare and medical costs.

Alcoholism and smoking

Alcohol and nicotine are the most commonly abused drugs all and are often co-abused. Both smoking and alcoholism are major public health problems (Walton, 1972) and the combination of the two increases the risk of bad health substantially (Bien and Burge, 1990). Several clinical and epidemiological studies have demonstrated an association between high alcohol consumption and the use of tobacco and vice versa (*e.g.* Walton, 1972; Craig and Van Natta, 1977; Istavan and Matarazzo, 1984; Mello et al, 1987; Bien and Burge 1990; DiFranza and Guerra, 1990; Zacny, 1990; Miller and Gold, 1998). For instance, approximately 90 % of all alcoholics smoke, which is a much higher percentage than for the average population (Walton, 1972; Ayers et al, 1976; Bien and Burge, 1990, Batel et al, 1995; Miller and Gold, 1998). A clinical trial by Dawson (2000) showed that the prevalence for lifetime smoking is the highest among former drinkers compared to abstainers from alcohol and past-year drinkers. Additionally, smokers consume twice as much alcohol as non-smokers (Carmody et al, 1985) and alcoholism is estimated to be 10 to 14 times more common among smokers than non-smokers (DiFranza and Guerrera, 1990; Daeppen et

al, 2000). In addition, nicotine dependent individuals have a greater severity of alcohol dependence (Daeppen et al, 2000) and alcoholics fail to quit smoking to a greater extent than non-alcoholics (DiFranza and Guerrera, 1990). Interestingly, in some studies ethanol has been found to potentiate the rewarding effects of nicotine in smokers (Rose et al, 2004) and nicotine increases the motivation to consume ethanol in male, non-dependent smokers (Barrett et al, 2006). These effects may be some of the reasons for the co-abuse of ethanol and nicotine, however other contributors may be psychosocial and environmental factors. Interestingly, there also appears to be a strong correlation between an early onset of tobacco abuse and addiction to alcohol later in life (DiFranza and Guerrera, 1990; Grant, 1998). Moreover, nicotine use during pregnancy may cause alcohol dependence in the next generation (Brennan et al, 2002; for review see Hellström-Lindahl and Nordberg, 2002), indicating that early exposure to nicotinic may contribute to an increased risk of alcoholism.

Pre-clinically, it has been reported that the ethanol intake and preference increases significantly following sub-chronic nicotine treatment in rat (Potthoff et al, 1983, Blomqvist et al, 1996; Ericson et al, 2000; Lê et al, 2000; Clark et al, 2001; Olausson et al, 2001). Furthermore, nicotine reinstates the alcohol-seeking behaviour in rats during drug-free periods (Lê et al, 2003). Additionally, simultaneous administration of lower doses of ethanol and nicotine potentiates DA release in the N.Acc. (Tizabi et al, 2002), suggesting that nicotine enhances the rewarding properties of ethanol.

As discussed above the neurochemical basis underlying the development of alcoholism is still unknown. However, these clinical and pre-clinical findings, suggest that ethanol and nicotine may share important neurochemical mechanisms of action in the brain reward systems such as those involving nicotinic acetylcholine receptors (nAChR) (for review see Larsson and Engel, 2004).

Pharmacological treatment

A century ago Merck Manual recommended cocaine to remove craving for alcohol and spirit of ammonia as a substitute for alcohol. The treatment of patients with alcohol dependence has changed considerably since then. Individual and group counselling have been, and still are, basic elements in the rehabilitation of alcohol dependent patients (Kranzler, 2000). The combination of psychological and pharmacological treatment is another alternative (Garbutt et al, 1999). The drastic increase in knowledge of specific areas and

neurotransmitter systems in the brain involved in drug reinforcement has resulted in the development of new pharmaceutical agents. In animal studies such agents have been efficient in decreasing alcohol intake, whereas a few have been efficient in clinical studies (Kranzler, 2000). The first drug approved for alcohol use disorder was the deterrent drug disulfiram (Antabus®), which makes the ingestion of alcohol unpleasant (Kranzler, 2000). Two other pharmaceuticals have been approved for alcohol use disorder (acamprosate (Campral®) and naltrexone (Revia®)), although they are not fully efficient and thus the need for novel treatment strategies remains. It is therefore vitally important to further study the neurobiological mechanisms involved in alcohol use disorder and this is one of the aims of the present thesis.

Compulsive overeating/binge eating

Individuals with an imbalance between energy intake and expenditure, where the former is in favour, are often over weight and obese as a result (for review see Hellström et al, 2004). Approximately 30% of obese subjects who participate in weight loss programs have binge eating disorder (BED) (for review see Yanovski, 1993; De Zwaan et al, 1994). Subjects with BED overeat with a sense of loss of control and do not engage in inappropriate compensatory behaviour afterwards (for review see Yanovski, 1993; Yanovski, 1995). One of the most common genetic causes of obesity is the Prader-Willi syndrome, which is characterized by symptoms such as severe hyperphagia, mental retardation and hypogonadism (Holland et al, 1993; Holm et al, 1993). Aberrant patterns of eating behaviour, weight regulation as well as disturbed attitudes towards weight, shape and perception of body shape characterize both anorexia nervosa and bulimia nervosa (for review see Kaye et al, 2000). Anorexia nervosa, a fear of fatness and an obsession with fatness, can be divided into two subtypes: restrictive and binge eating. The restriction subgroup is characterized by food avoidance and malnutrition, whereas the binge eating subtype is distinguished by periods of bingeing followed by self-induced vomiting or laxative abuse (Strober, 1980). Patients with bulimia nervosa have irregular eating patterns and impaired satiety. The core features include repeated periods of binge eating followed by compensatory behaviours to counteract weight gain *e.g.* vomiting, laxative abuse or extreme exercise, keeping the body weight in a normal range. The multi-impulsive type and self-effecting type are two distinct subtypes of patients with bulimia nervosa. Interestingly, histories of substance

abuse or other addictive behaviours, *e.g.* shop lifting, and other impulse-control problems such as self-injuries are more common among the multi-impulsive type. Furthermore, they are also more likely to binge eat to regulate anger and tension. The self-effecting type are more likely to binge eat to reduce the feelings of guilt associated with weight gain (for review see Kaye et al, 2000). Interestingly, bulimic individuals have higher sensitivity to reward than the subjects with restrictive type of anorexia nervosa, indicating that bulimic patients are more sensitive to the rewarding effects of food and drugs (Davis and Woodside, 2002).

Compulsive overeating/binge eating is characterized by the persistent intake of large amounts of food during discrete periods of time and can be observed in anorexia nervosa, bulimia nervosa, Prader-Willi and obesity. Interestingly, the behaviours of binge eating individuals are very similar to that of a drug addict, including obsessive-compulsiveness, impulsivity, sensation seeking and loss of control (Davis and Claridge, 1998; Davis, 2001; Davis and Woodside, 2002; for review see Cassin and von Ranson, 2005). Thus compulsive overeating is defined as an addictive behaviour (*e.g.* Davis et al, 2004; James et al, 2004; Wang et al, 2004a; Corwin, 2006), and is thought to be caused by a dysfunction in the reward systems (Volkow et al, 2003a; Wang et al, 2004a). Furthermore, functional magnetic resonance imaging (fMRI) scans have shown that the activation of the nucleus accumbens (N.Acc.) due to ingestion of oral glucose is delayed in Prader-Willi patients (Shapira et al, 2005); indicating that a dysfunction of the reward system may be involved in the pathophysiology of Prader-Willi syndrome.

Aberrant eating patterns and drugs of abuse

A co-morbidity of substance abuse and deviant eating behaviours is well documented in the literature (for reviews see Holderness et al, 1994, Wolfe and Maisto, 2000; Bulik et al, 2004b). Nicotine use is more prevalent in individuals with eating disorders (Welsh and Fairburn, 1998; Wiseman et al, 1998). Several studies suggest that differences in smoking patterns may exist across the different subtypes of eating disorders (Bulik et al, 1991; Haug et al, 2001; Wiederman and Pryor, 1996), *e.g.* patients with bulimia nervosa have higher smoking prevalence than those with anorexia nervosa (Bulik et al, 1991; Haug et al, 2001). Additionally, women with eating disorder subtypes, that include binge eating, have been shown to score higher for nicotine

dependence (Anzengruber et al, 2006) and are more likely to smoke (Wiseman et al, 1998; Crisp et al, 1999). However, this difference was not observed in a recent community study (von Ranson et al, 2002). Patients with anorexia nervosa-restricting type showed no difference in smoking prevalence to controls (Anzengruber et al, 2006). Additionally, this subtype displays lower rates of other substance abuses, impulsivity and novelty seeking behaviours (for review see Bulik et al, 2004a; Bulik et al, 2004b; Klump et al, 2004). Dieting increases the risk of smoking in girls and the risk of smoking is positively correlated with the dieting frequency (Austin and Gortmaker, 2001). Additionally, alcohol consumption and bulimic behaviours have been suggested to be additive risk factors in smoking adolescents (Field et al, 2002). Furthermore, the abuse of alcohol is higher in patients with eating disorders than in the general population (Bulik et al, 2004a; for review see Bulik et al, 2004b). More specifically, in a review of 25 studies the prevalence of alcohol dependence in bulimic patients was estimated to 22.9% (Holderness et al, 1994). The prevalence of alcoholism varies with different subtypes of eating disorders, where alcohol abuse disorder is uncommon in the anorexia nervosa restrictive subtype but frequent in the binge eating subtype and in individuals with bulimia nervosa (Henzel, 1984; Bulik et al, 1992; for review see Bulik et al, 2004b). Interestingly, a co-morbidity of bulimia and alcohol dependence has been found to be associated with other types of substance abuse, increased novelty seeking scores and impulsivity (Bulik et al, 1997). Furthermore, the lifetime rates of alcohol/drug dependence in first degree relatives to patients with bulimia nervosa is significantly higher than in first degree relatives to control individuals (Kaye et al, 1996). On the other hand, obese women have been found to display lower rates of alcohol (Kleiner et al, 2004) and marijuana use (Warren et al, 2005). Common neurobiological mechanisms may therefore be implied to underlie addictive behaviours such as compulsive overeating, alcoholism and nicotine dependence.

The reward systems

Since ancient times mankind has been using drugs to achieve euphoria and a state of well-being. During the last decades the neuronal systems within the brain mediating these feelings have progressively been identified. As early as in the middle of the 1950s Olds and Milner serendipitously discovered that rats would work to self-administer

electrical currents into some, but not into other brain areas (Olds and Milner, 1954). Interestingly, the rats pressed the lever repeatedly and their attention to natural rewards such as eating, drinking and breeding, vanished; they had become electricity-dependent (Phillips and Fiberger, 1989). The rats had been implanted with electrodes in specific areas of the limbic system, a system involved in emotional experiences in both rodents and humans. These brain areas were later anatomically mapped in more detail and are today known to mediate reward, pleasure and euphoria and are therefore called the "reward systems". The reward systems have been identified in flatworms as well as in other primitive animals, and are hence suggested to be highly conserved and stable throughout the evolution. An important role of these systems is to stimulate and enhance the motivation of behaviours that increase the probability of survival, such as foraging and drinking, as well as the continued existence of the species, *e.g.* breeding (Hansen et al, 1991; Schultz et al, 1997). *Videlicet*, these natural rewards are known to activate the reward systems in animals as well as in humans, inducing euphoria and a state of well-being. In humans, the modulation of the reward systems is more complex and varies substantially between individuals. It has been demonstrated in humans that a monetary reward (Pappata et al, 2002), music (Menon and Levitin, 2005), a picture of a pretty face (Kampe et al, 2001) and video games (Koepp et al, 1998) can activate the reward systems. However, humans as well as animals can learn to activate the reward systems artificially with addictive drugs, such as ethanol and nicotine, as well as by engaging in compulsive behaviours, *e.g.* compulsive shopping and compulsive overeating (for review see Miller, 1980; Holden, 2001). Direct electrical stimulation and habit-forming drugs are more powerful rewards than natural rewards and are hypothesized to hijack the reward systems (Wise and Rompre, 1989). A long-term consequence of such artificial activation may be a loss of interest for natural rewards and may thus cause drug/behavioural dependence.

The mesocorticolimbic dopamine system

Several brain regions involved in the reward systems have been identified and include the prefrontal cortex, hippocampus and N.Acc. (*i.e.* the ventral striatum). Through investigations of physiological responses to un-sensed incentives, such as intra-cranial stimulation and intra cranial drug injections, the mesocorticolimbic DA system has been identified as a common denominator of the reward systems (Wise

and Rompre, 1989). The mesocorticolimbic DA system consists of dopaminergic neurons originating in the ventral tegmental area (VTA) projecting via the medial forebrain bundle to several limbic structures, such as N.Acc. and the amygdala, and to cortical structures, *e.g.* the prefrontal cortex (see *e.g.* Dahlström and Fuxe, 1964; Ungerstedt, 1971; Engel et al, 1988; Koob, 1992a; Nestler et al, 2001). The dopaminergic projections to these areas are likely to differ with regard to neurobiology, electrophysiology and function and could therefore be divided into several different systems, such as the mesolimbic and the mesocortical DA system. More specifically, the mesolimbic DA system, *i.e.* the dopaminergic neurons projecting from the VTA to the N.Acc., have been suggested to create the most central part of the reward systems (see *e.g.* Koob, 1992a, Koob, 1992b) (Fig. 1), and are implicated in the shaping of goal-oriented behaviours driven by conscious or unconscious motivation (Schultz, 1998). The N.Acc. can be divided into two distinct anatomically and functionally different regions (Graybiel and Ragsdale, 1978), the central core and the surrounding shell (Voorn et al, 1989; Heimer et al, 1991; Zahm and Brog, 1992; Zahm, 1999).

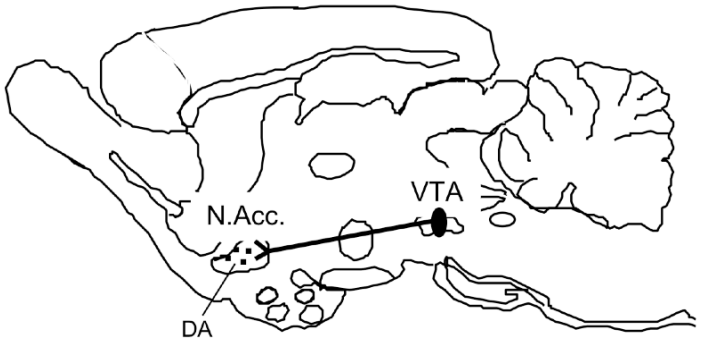


Fig. 1. The mesolimbic dopamine system.
The dopaminergic cell bodies originate in the ventral tegmental area (VTA) and project to the nucleus accumbens (N.Acc.). Upon stimulation dopamine (DA) is released in the N.Acc..

The reinforcing effects of addictive drugs and palatable foods are dependent on activation of the mesocorticolimbic DA system (*e.g.* Engel et al, 1999; Risinger et al, 2000; Di Chiara et al, 2004), and it has been suggested that this system cannot differentiate among rewards provoked by natural reinforcers *e.g.* food, addictive drugs such as ethanol, or behaviours like gambling (Kelley et al, 2005).

Firing of the dopaminergic neurons in the VTA

The DA neurons in the VTA typically display two basic modes of firing; a single spike-firing mode and a burst-firing mode (Grace and Bunney, 1984a; Grace and Bunney, 1984b; Grenhoff et al, 1986). Under normal conditions the DA neurons in the VTA are quiescent or fire with single spikes. When triggered by appropriate input the DA neurons can switch back and forth between the two modes. A change from single spikes to burst firing enhances and prolongs the signal strength, which in turn increases the DA levels in N.Acc. (Gonon, 1988; Wightman and Zimmerman, 1990). This shift appears to originate from the excitatory amino acid inputs to the VTA (Overton and Clark, 1997) and may also cause a release of co-localized peptides (Bean and Roth, 1991). The functional relevance of burst firing in the reward circuits is not fully understood, however it is plausible that the switch from spikes to burst-firing is used to enhance the signals from salient events and dampen the signals from non-salient rewards. More specifically, it has been demonstrated that the DA neurons show phasic bursting activity in response to unexpected presentation of a novel food reward (Schultz, 2001) as well as of a conditioned stimulus that has been associated with food reward (Schultz et al, 1993). Furthermore, during the course of training, as the reward becomes expected, the DA neurons loose the phasic bursting. Interestingly, it has been shown that moderate food restriction causes a prolonged increase in the basal impulse activity and firing of the DA neurons, making the cells more attentive during particular motivational states. Given that spontaneous burst firing does not occur in brain slice preparation of DA neurons, it has been implied that the firing of these neurons is driven by afferent inputs (for review see Sanghera et al, 1984).

Reward and dopamine

Accumbal DA release is widely believed to be involved in mediation of the rewarding, hedonic feelings of incentives, natural as well as artificial (*e.g.* Taylor and Robbins, 1984; Taylor and Robins, 1986; Wise and Bozarth, 1987; Engel et al, 1988; Cador et al, 1991; Robinson and Berridge, 1993), thus indicating that accumbal DA overflow is a common response to various reinforcers. Moreover, a role for DA in incentive salience ("wanting"), a motivated behaviour for reward, *e.g.* food searching, has been implied (for review see Berridge and Robinson 1998; Cannon and Palmiter 2003; Berridge 2004;

Kringelbach 2004). However, the theory of reward and DA has been challenged. Selective DA enhancement in the N.Acc. has been observed in associative learning in absence of biological reward, suggesting that the mesolimbic DA system is involved in associative learning in general, and not specifically in learning related to rewards (Spanagel and Weiss, 1999).

Drugs of abuse, dopamine and reward

The first evidence demonstrating a causal relationship between ethanol-induced stimulation of behaviour and catecholaminergic activity was that the catecholamine-synthesis inhibitor, α-methyltyrosine antagonized ethanol-induced stimulation as well as euphoria in humans (Ahlenius et al, 1973) and locomotor stimulation in rats (Engel et al, 1974). Microdialysis studies in freely moving rats showed that most drugs of abuse, including amphetamine, opiates and cocaine, increase the extracellular DA levels predominantly in the N.Acc. (Di Chiara and Imperato, 1988). Furthermore, it has been found that both ethanol (*e.g.* Imperato and Di Chiara, 1986; Engel et al, 1988; Di Chiara and Imperato, 1988; Blomqvist et al, 1993; Olive et al, 2000; Larsson et al, 2002; Larsson et al, 2004) and nicotine (*e.g.* Di Chiara and Imperato, 1988; Benwell and Balfour, 1992; Nisell et al, 1994; Larsson et al, 2002; Ericson et al, 2003) increase the accumbal DA overflow *in vivo* in rodents. More specifically, increased DA levels are observed in the N.Acc. shell but not in the core (Cadoni et al, 2000; Iyaniwura et al, 2001; Bassareo et al, 2003). Furthermore, it has been observed that voluntary oral ethanol-intake increases the accumbal DA levels in rats (Ericson et al, 1998; Doyon et al, 2003; Larsson et al, 2005), in a dose-dependent manner (Weiss et al, 1993). Additionally, intravenous administration of low doses of ethanol produces a dose-dependent increase in the firing rate of DA neurons in the VTA, which project to the ventral striatum (*i.e.* N.Acc.). On the other hand, the DA neurons, which project to the dorsal striatum, were activated to a lesser extent (Gessa et al, 1985). It has been proposed that this accumbal DA overflow represents the pleasure and euphoria experienced by alcohol consumption (*e.g.* Engel, 1977; Engel and Carlsson, 1977, Wise and Rompre, 1989). Interestingly, DA antagonists suppress the ethanol-induced locomotor stimulation (Liljequist et al, 1981). Moreover, several studies have reported alterations in ethanol intake, preference and oral self-administration following systemic and intra-accumbal administration of DA agonists or antagonists (Pfeffer and Samson, 1985a; Pfeffer and Samson, 1985b;

Pfeffer and Samson 1988; McBride et al, 1988; McBride et al, 1990; Weiss et al, 1990; Samson et al, 1991; Rassnick et al, 1993a; Rassnick et al, 1993b; Rassnick et al, 1993c; Samson et al, 1993; Hodge et al, 1997; Nowak et al, 2000). A role for accumbal DA in alcohol self-administration may therefore be suggested. However, this theory has been challenged by several reports that describe the complex interaction between accumbal DA in ethanol consumption and seeking (Czachowski et al, 2001; Samson and Chappell, 2004). Additionally, the rewarding effects of addictive drugs are attenuated by a decreased dopaminergic neurotransmission (Engel, 1977; Wise, 1996; Maldonado et al, 1997; Risinger et al, 2000). A positron emission tomography (PET) study in humans demonstrate that oral ethanol consumption, in intoxicating doses, promotes DA release in the ventral striatum (Boileau et al, 2003). Similarly, psychostimulants (Carson et al, 1997; Schlaepfer et al, 1997; Drevets et al, 2001; Volkow et al, 2001; Leyton et al, 2002; Martinez et al, 2003) as well as rewarding behavioural tasks (Koepp et al, 1998) increase the DA activity in ventral striatum in humans measured by PET. Interestingly, the amount of released DA in the ventral striatum correlates with self-reported behavioural measures of euphoria or drug wanting (Volkow et al, 1997; Drevets et al, 2001; Leyton et al, 2002; Martinez et al, 2003). Furthermore, drugs that are not rewarding and not abused by humans do not modify synaptic accumbal DA levels (Di Chiara and Imperato, 1988). Taken together, it may be implied that accumbal DA is strongly associated with reward. It should be emphasized that increased DA levels in other areas are not always associated with reward; hence increased DA utilization in the prefrontal cortex is associated with foot shock, swim stress or conditioned fear (Le Moal and Simon, 1991; Westerink 1995).

In addition to the rewarding properties of drugs, accumbal DA has been suggested to be involved in the expectation of reward. Anticipation of ethanol self-administration enhances the accumbal DA overflow in rats (Weiss et al, 1993; Gonzales and Weiss, 1998; Katner and Weiss, 1999; Melendez et al, 2002), an effect that is pronounced in alcohol-preferring rats (Katner et al, 1996). Additionally, it has been demonstrated that alcohol-associated cues activates the ventral striatum in abstinent high-risk drinkers and alcoholics (Braus et al, 2001; Kareken et al, 2004). Similarly, in rats the firing of neurons in the N.Acc. increases during reward anticipation (Martin and Ono, 2000). Neuroleptics have been found to impair the response to a drug-associated cue (Wise, 1996) and low doses of DA D_2 receptor

antagonists reduce ethanol-seeking behaviour, but not ethanol consumption (Czachowski et al, 2001; Czachowski et al, 2002); thus raising the possibility for a role of DA in incentive motivational processes, such as drug-seeking behaviour. Interestingly, this accumbal DA overflow is thought to activate appropriate motor stimulation and motivation programs for reward-seeking behaviour and consumption (Engel and Carlsson, 1977; Wise and Bozarth, 1987, Wise, 1987; for review see Le Moal and Simon, 1991; Hodge et al, 1994; Hoshaw and Lewis, 2001). Conclusively, DA may be important in processes involved in "wanting" (measured *e.g.* by voluntary intake or preference tests) (Berridge and Robinson, 1998) as well as in "liking" (hedonic affective reaction measured by taste reactivity tests) (*e.g.* Volkow et al, 1997; Drevets et al, 2001; Leyton et al, 2002; Martinez et al, 2003).

It should also be emphasized that addictive drugs affect several other neurotransmitters, *e.g.* gamma-aminobutyric acid (GABA), acetylcholine (ACh), serotonin (5-HT), noradrenaline (NA) and opioids, and that DA alone will only explain some of the rewarding effects of addictive drugs (*e.g.* Engel et al, 1988; Engel et al, 1992; Little, 1999; Engel et al, 1999). The pharmacological properties of ethanol may also be mediated by peptides and hormones (Fig. 2) (Engel et al, 1999).

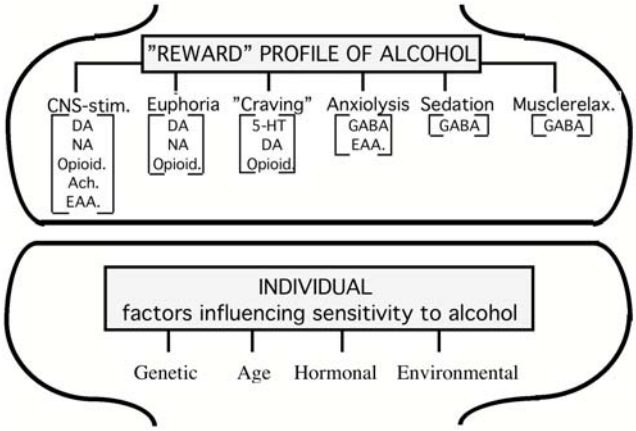


Fig. 2. The "reward" profile of ethanol.
Several different neurotransmitters, neuromodulators and individually related factors collectively orchestrate the rewarding profile of addictive drugs, *e.g.* ethanol. DA, dopamine; NA, noradrenaline; ACh, acetylcholine; 5-HT, serotonin; EAA, excitatory amino acids (Engel et al, 1992).

Natural rewards and dopamine

The mesolimbic DA system can be activated by natural rewards such as sex (Mas et al, 1990; Pleim et al, 1990; Damsma et al, 1992), water (Roop et al, 2002) and food (even in non-starving animals) (Hernandez and Hoebel, 1988; Hernandez and Hoebel, 1990; Martel and Fantino, 1996), thereby causing an increase in the extracellular concentration of accumbal DA (Yoshida et al, 1992; for review see Horvitz, 2000). However, drugs of abuse are three to five times more potent in their ability to stimulate accumbal DA release than natural rewards (Wise, 2002). Interestingly, standard food increases the extracellular levels of DA in the core of N.Acc (Bassareo and Di Chiara, 1997; Bassareo and Di Chiara, 1999), whereas palatable food, such as salty snacks (Bassareo and Di Chiara, 1997), Fonzies (Tanda and Di Chiara, 1998) or chocolate (Bassareo et al, 2002) enhances the DA levels in N. Acc. shell.

Since ensuring an adequate nutritional state is essential for the animal's survival, it is most likely that several different neural systems driving feeding behaviour have been developed. The behaviours driving animals (and man) to work and to seek for food needs to be highly motivated and to some extent rewarding; thus implicating a role for the mesocorticolimbic DA system in food-seeking behaviour and food reward (for review see Saper et al, 2002). Interestingly, lesions of the DA reward system does not affect food intake *per se*, but reduces the willingness of the animal to engage in behavioural actions aimed at anticipation or searching for food (for review see Berridge, 1996). DA has therefore been proposed to play a role in incentive salience ("wanting") *such as* food-seeking, rather than finding the sweet taste of sucrose ("liking") rewarding (for review see Berridge and Robinson 1998; Cannon and Palmiter 2003; Berridge 2004; Kringelbach 2004). In support of this hypothesis are findings associating ventral striatal DA release to the desire for food during presentation of palatable food stimuli in humans (Volkow et al, 2002) as well as with appetizing food (Beaver et al, 2006). Similarly, primates trained to associate a cue with a pleasurable experience (food), will show an increase in accumbal dopaminergic activity as a response to the cue and not to the food (Schultz, 2001). An *in vivo* voltametry study has further demonstrated that food-predicting cues increase firing of accumbal DA neurons (Roitman et al, 2004). Moreover, in rats DA transients in the ventral striatum are more frequent during brief conspecific introduction of a female than during copulation (Robinson et al, 2002). Further, the

activity of the DA neurons increases more potently before the potential delivery of an uncertain reward (related to gambling) than during gambling *per se* (Fiorillo et al, 2003).

The food-induced DA overflow in the N.Acc. shell is blunted at repeated exposures to palatable foods (Bassareo and Di Chiara, 1997; Bassareo et al, 2002), implying an important role for novelty in reward responses (Spanagel and Weiss, 1999). Interestingly, the increased DA levels in N.Acc. shell is not blunted following subsequent administrations of addictive drugs (Di Chiara, 2002) or following repeated exposures to palatable foods in the case of disrupted eating behaviours (Di Chiara, 2005; Rada et al, 2005). Furthermore, the constant challenge to the mesolimbic DA system by the excessive use of rewards *e.g.* addictive drugs or changed eating behaviour, might cause neuroadaptive changes. Accordingly, a decreased number of DA D_2- receptors has been demonstrated in cocaine (receptors that recover following a drug-free interval) (Volkow et al, 1990; Volkow et al, 1993) and methamphetamine abusers (Volkow et al, 2001) as well as in opiate (Wang et al, 1997) and ethanol dependent individuals using PET studies (Volkow et al, 1996) or using neuroendocrine tests (Balldin et al, 1992). Comparatively to drug addiction, a lower density of DA D_2- receptors have been demonstrated in patients suffering from compulsive overeating (Volkow et al, 2003a, Wang et al, 2004a). Furthermore, ethanol withdrawal causes a reduction in the firing of DA neurons in the VTA as well as in accumbal DA levels (Diana et al, 1992; Rossetti et al, 1992). The use of addictive drugs or overeating has been suggested to reflect a compensatory behaviour for this impaired DA transmission and those individuals who do not show recovery to the normal number of DA D_2- receptors at withdrawal are more likely to relapse. Taken together, a role for accumbal DA release in the hedonic feeling of incentives, natural as well as artificial, and in motivated behaviours such as drug- and food-seeking behaviour may be implicated.

The major afferents that modulate the activity of dopamine neurons in the ventral tegmental area

Accumbal DA release is modulated by various afferents to the VTA (*e.g.* Kalivas, 1993; Wise, 2002;), such as the glutamatergic input from precortical areas, which mainly has been shown to be tonic, excitatory

and N-methyl-D-aspartic acid (NMDA)-dependent (Schilström et al, 1998a: for review see Kitai et al, 1999; Carr and Sesack, 2000; Sesack et al, 2003). Additional glutamatergic afferents to the VTA originate in the lateral hypothalamus (Rosin et al, 2003), bed nucleus of stria terminalis (Georges and Aston-Jones, 2002) and the superior coliculus (Geisler and Zahm, 2005). The GABAergic input, *e.g.* from the N.Acc. shell and medial part of ventral pallidum, (Conrad and Pfaff, 1976; Walaas and Fonnum, 1980; Kalivas et al, 1993) and the GABAergic interneurons within the VTA modulate the activity of ventral tegmental dopaminergic neurons (Sesack and Pickel, 1995). Activation of the GABAergic neurons in the VTA inhibits the release of accumbal DA (see *e.g.* Koob, 1992b). Additionally, these striatal projections may co-contain peptides such as substance P and dynorphins (Fallon et al, 1985; Lu et al, 1998). 5-HT afferents from the dorsal and medial raphe (Parent et al, 1981; Herve et al, 1987) and the hypothalamic opioidic provide input via the ventral tegmental GABAergic interneurons (Sesack and Pickel, 1995; Greenwell et al, 2002) have also been demonstrated to modulate the activity of the DA neurons in VTA. The activity of dopaminergic neurons in the VTA can also be modulated by noradrenergic afferents from the locus coeruleus (Grenhoff et al, 1993). Moreover, the orexin containing projections from lateral hypothalamus to VTA have been suggested to regulate ventral tegmental DA neurons (Semba and Fibiger, 1992; Fadel and Deutch, 2002; Korotkova et al, 2003). Additionally, the GABAergic as well as the excitatory cholinergic and glutamatergic input from the mesopontine area appears to have an important modulatory role (Clements and Grant, 1990; Clements et al, 1991; Semba and Fibiger, 1992; Futami et al, 1995; for review see Kitai et al, 1999; Larsson et al, 2005) (Fig. 3).

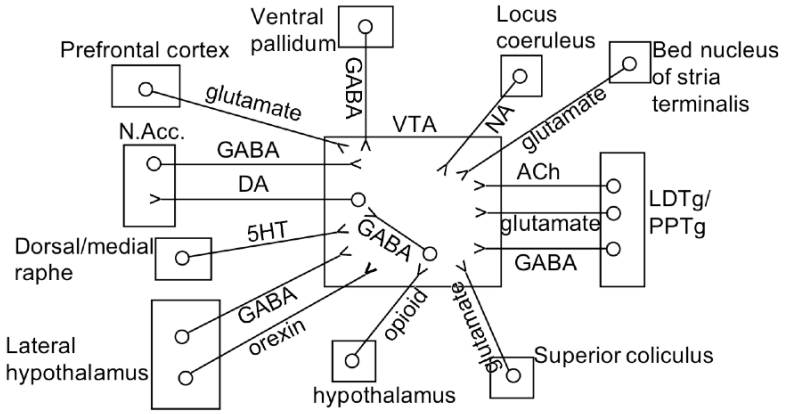


Fig. 3. The major afferents that modulate the activity of the dopaminergic neurons in the ventral tegmental area (VTA).
ACh, acetylcholine; GABA, gamma-aminobutyric acid; NA, noradrenaline; 5-HT, serotonin, MC; melanocortins; N.Acc, nucleus accumbens, LDTg, laterodorsal tegmental area.

Cholinergic regulation of the ventral tegmental area

Cholinergic neurons are widely distributed throughout the brain (Butcher and Woolf, 2003; Woolf, 1991) and have been suggested to play important roles in cognitive functions such as learning (Fine et al, 1997), memory (Hasselmo et al, 1992) and attention (Bucci et al, 1998). Interestingly, cholinergic neurons have been identified in the mesopontine area, *i.e.* the pedunculopontine tegmental area (PPTg) and laterodorsal tegmental area (LDTg). These neurons project to various brain regions, such as the thalamus, hypothalamus, basal forebrain, substantia nigra and medial limbic cortex (Mesulam et al, 1983). Additionally, the cholinergic neurons in the mesopontine area provide the only known cholinergic projections to the VTA (Butcher and Woolf, 2003). Specifically, the cholinergic input to the VTA, originates primarily in the LDTg, whereas the PPTg mainly projects to substantia nigra (Berninato and Spencer, 1987; Clarke et al, 1987; Futami et al 1995; Oakman et al, 1995; Blaha et al, 1996a; for review see Winn et al, 1997). The LDTg has been suggested to regulate the activity of ventral tegmental DA neurons projecting to the ventral striatum (*i.e.* accumbal DA) (Forster and Blaha, 2000b; Forster et al, 2001; Forster and Blaha, 2003), via activation of ventral tegmental nAChR, muscarinic acetylcholine receptors (mAChR) as well as glutamatergic receptors (Blaha et al, 1996a; for review see Winn et al,

1997; Forster and Blaha, 2000b; Forster and Blaha, 2003). Similarly, activation of nAChRs, in particular in the ventral tegmental area, have been found to increase DA in the N.Acc. (Clarke et al, 1988; Mifsud et al, 1989; Benwall and Balfour, 1992; Nisell et al, 1994). The cholinergic neurons originating in the PPTg regulate the activity of dorsal striatum (for review see Winn et al 1997; Forster and Blaha, 2000a; Forster and Blaha, 2003). However, it is unlikely that these relationships are wholly exclusive, as cholinergic projections from the PPTg, preferably the medial part, to the VTA have been identified (Jackson and Crossman, 1983; Fujimoto et al, 1990; Yeomans et al, 1993; Oakman et al, 1995; for review see Laviolette and van der Kooy, 2004).

The cholinergic input to the VTA has previously been assumed to involve regulation of the GABAergic, rather than dopaminergic neurons (Garzone et al, 1999; Fiorillo and Williams, 2000). However, lately it has been shown that the cholinergic drive from the LDTg innervates the dopaminergic, rather than the GABAergic mesoaccumbal neurons in the VTA (Omelchenko and Sesack, 2005; Omelchenko and Sesack, 2006). Our research group has denominated the cholinergic projection, preferably from the LDTg, together with the mesolimbic DA system the cholinergic-dopaminergic reward link (Fig. 4).

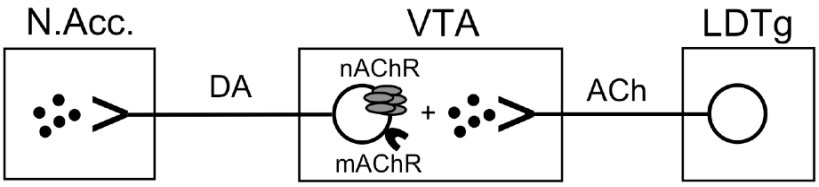


Fig. 4. The cholinergic-dopaminergic reward link.
This link is composed of the cholinergic projection from the laterodorsal tegmental area (LDTg) to the ventral tegmental area (VTA) and the mesolimbic dopamine (DA) system projecting from the VTA to the nucleus accumbens (N.Acc.). Activation of the LDTg causes a release of acetylcholine (ACh) in the VTA which by interactions with nicotinic ACh receptors (nAChR) and/or muscarinic ACh receptors (mAChR) stimulates the mesolimbic DA system causing a release of DA in N.Acc..

The cholinergic-dopaminergic reward link

It is assumed that the mesopontine cholinergic projection, at least in part, constitute an excitatory input to the mesoaccumbal dopaminergic neurons; thus a ventral tegmental release of ACh causes an increase in accumbal DA (Blaha et al, 1996a; for review see Winn et al, 1997; Forster and Blaha, 2000b; Forster et al, 2001; Forster and Blaha, 2003; Larsson et al, 2005). The cholinergic projections via mAChRs exert a tonic excitatory influence on the dopaminergic neurons in the VTA (Yeomans et al, 1985; Kofman and Yeomans, 1989; Kofman et al, 1990; Yeomans and Biptista, 1997). Moreover, since inhibition of nAChRs in the VTA does neither affect locomotor activity nor accumbal DA overflow *per se*, it has been suggested that the ventral tegmental nAChRs are involved in phasic rather than tonic influence on the mesolimbic DA system (Westerink et al, 1996; Ericson et al, 1998; Grillner and Svensson, 2000; Larsson et al 2002; Larsson et al, 2004).

Involvement of cholinergic neurons in drug-induced as well as natural reward has been implied (*vide infra*). Infusion of a cholinergic agonist into the VTA increased the extracellular DA levels in the N.Acc. (Westerink et al, 1996) and has rewarding effects as measured by a conditioned place preference task (Yeomans et al, 1985). Furthermore, it has been shown that hypothalamic self-stimulation, food or water intake (Rada et al, 2000) or electrical self-stimulation of the medial forebrain bundle (Nakahara et al, 2001) increases the extracellular levels of ACh in the VTA. In addition, lesion of the PPTg decreases nicotine and cocaine self-administration (Lanca et al, 2000; Corrigall et al, 2002) and inhibits the motivational effects of opiates (Bechara and van der Kooy, 1992). Similarly, lesions of the PPTg attenuates the intake of saccharin and water in rats (Stefurak and van der Kooy, 1994), blocks the rewarding properties of food (Bechara and van der Kooy, 1992) and impairs copulation in naïve male rats (Kippin and van der Kooy, 2003). Lesioning the medial PPTg, the part of PPTg projecting to the VTA, blocks conditioned place preference for morphine and amphetamine (Bechara and van der Kooy, 1989). Furthermore, it has been shown that ethanol intake in high ethanol-preferring rats causes a concomitant, and almost time-locked, increase in ventral tegmental ACh and accumbal DA (Larsson et al, 2005). Hence, a role for the cholinergic-dopaminergic reward link in natural as well as drug-induced reinforcement may be suggested.

Afferents modulating the activity of the mesopontine cholinergic neurons

It has been shown that different inputs to the mesopontine area modulate the activity of cholinergic neurons. The major origins for the mesopontine afferents are the substantia nigra (*e.g.* Beckstead et al, 1979; Semba and Fibiger, 1992), the subthalamic nucleus (*e.g.* Granata and Kitai, 1989) and the globus pallidus (*e.g.* Kim et al, 1976). Additionally, non-dopaminergic projections, presumably GABAergic, from the VTA and N.Acc. to the mesopontine area have been identified (Walaas and Fonnum, 1980; Swanson, 1982; Goldsmith and van der Kooy, 1988; Semba and Fibiger, 1992).

Nicotinic acetylcholine receptors

ACh exerts its effects through both nAChRs and mAChRs. These receptors were classified by Sir Henry Dale (for review see Gaudenz Waser, 1986) and it has been shown that the expression levels of nAChRs are lower then those of mAChRs (Wada et al, 1989; Sargent, 2000). There are five different subtypes of the mAChRs, m1-m5, and they belong to the super-family of G-protein coupled receptors. The mAChRs, coupled to second messenger systems, mediate the slow effects of ACh, whereas the nAChRs, as ligand-gated ion channels, mediate the fast synaptic transmission of the neurotransmitter. The nAChRs, together with the $GABA_A$, glycine and $5\text{-}HT_3$ receptors, belong to the gene super-family of ligand-gated ion channels (*e.g.* McGehee and Role, 1995; Sieghart and Sperk, 2002; Lynch, 2004). The neuromuscular nAChRs were described by Langley (1907), whereas the neuronal nAChRs were not identified until decades later (Caulfield and Higgins, 1983; Clarke et al, 1984; Clarke and Pert, 1985; Clarke et al, 1985; Collins et al, 1996). Both pre-synaptic and post-synaptic nAChRs have been show to be widely distributed in the brain (Clarke and Pert, 1985; Lindstöm, 1997). An important modulatory role for pre-synaptic nAChRs, located on *e.g.* glutamatergic, GABAergic and noradrenergic neurons, have been demonstrated (McGehee and Role, 1995; Wonnacott, 1997), although a similar role has also been assigned to post-synaptic nAChRs (Ullian et al, 1997). Several different brain functions such as cognition, learning, memory, arousal and motor function have been linked to the neuronal nAChRs (Arneric and Brioni, 1999; Lukas et al, 1999; Paterson and Nordberg, 2000; Levin, 2002).

Subtypes of the nicotinic acetylcholine receptor

The nAChR consist of five subunits that form a pentameric ion-channel. All subunits have a similar structure, *i.e.* two hydrophilic and four transmembranic domains (M1-M4). The M2 domains from each subunit form the wall of the central aqueous pore (Cartaud et al, 1973), which allows the cations Na^+, K^+ and Ca^{2+} to flux through the receptor (Changeux et al, 1998). The subunits expressed in the central nervous system (CNS) are the α_2-α_{10} and β_2-β_4 (Lukas et al, 1999). More specifically, the mRNA expression of the α_2-α_7 and β_2-β_4 subunits in VTA have been identified with reverse transcriptase-polymerase chain reaction (Charpantier et al, 1998) and the α_3-α_6 and β_2-β_3 subunits in VTA with *in situ* hybridization (Lena and Changeux, 1997; Le Novere et al, 2002). The subunits of α_3-α_7 and β_2-β_4 have been localized on dopaminergic cell bodies and on non-dopaminergic neurons in the VTA (Klink et al, 2001). The different subunit combinations form a large variety of nAChRs, *i.e.* subtypes, either as heteromeric or homomeric receptors (Fig. 5). The subtypes have different distribution patterns and may in all probability have various functional roles (for review see Nicke et al, 2004). Interestingly, the different nAChR subtypes are characterized by significant differences in properties such as ligand pharmacology, activation and desensitization kinetics and cation permeability (Chavez-Noriega et al, 1997).

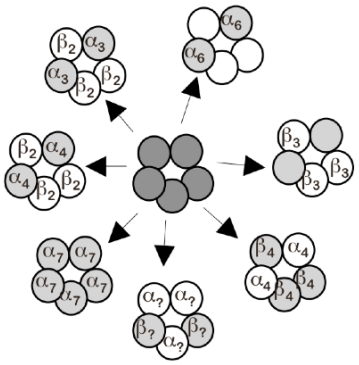


Fig. 5. The pentameric nicotinic acetylcholine receptor (nAChR).
The nAChR can be a homeomeric or heteromeric receptor, and these have different functional roles and different distribution pattern.

The homomeric nAChRs are composed of the α_7, α_8 or α_9 subunits (Arneric and Brioni, 1999). The heteromeric subtypes of the nAChRs are formed from the α_2-α_6 and β_2-β_4 subunits (Nelson et al, 2003). The α_9 and α_{10} subunits have not been found in the VTA and the α_8 subunit has only been found in chick.
Interestingly, not all subunits can be combined and form functional receptors (for review see Nicke et al, 2004). More specifically, it has been shown in Xenopus oocytes or mammalian cell lines that the subunits α_2, α_3 and α_4 all can form functional subtypes with both the β_2 and the β_4 subunits. Contrarily, the α_6 subunit can only form functional receptors with the β_4 but not with the β_2 subunit (Chavez-Noriega et al, 1997; Stauderman et al, 1998; Lukas et al, 1999; Chavez-Noriega et al, 2000; Kuryatov et al, 2000; Dowell et al, 2003). The α_5 and β_3 subunits are structural subunits and can therefore be used to form more complex nAChRs, however, since they lack amino acid residues important for agonist binding they cannot be involved in the formation of the binding site (Ramirez-Latorre et al, 1996; Boorman et al, 2003). The asterisk used in the receptor nomenclature indicates that other subunits might be present in the receptor complex.

The most common subunits in the CNS are the α_4, β_2 and α_7, which are widely distributed in the brain (Clarke et al, 1985; Wada et al, 1989; Séguéla et al, 1993; for review see Lindstöm et al, 1995; Paterson and Nordberg, 2000). In fact, approximately 90% of all nAChRs in the CNS have been identified as the $\alpha_4\beta_2$* receptor, known as the high-affinity nicotine binding site, and the second most common nAChRs is the α_7* receptor. The expression of other subtypes is more limited (Wada et al, 1989; Flores et al, 1992; Séguéla et al, 1993). However, there is no democracy in the brain and less common subtypes can have essential functional roles.

Selective antagonists for the different subunits of the nicotinic acetylcholine receptor

The functional importance of the different subunits can be studied by using *e.g.* subtype-specific nicotinic agonists/antagonists and gene-manipulated mice. The availability of selective pharmacological tools (that can distinguish between different nAChR subtypes) is still limited.

Nicotinic antagonist	Subunit selectivity
Mec	non-selective for neuronal nAChR
DHβE	$\alpha_4\beta_2^*$
MLA	α_7^*, α_6^*
αCtxMII	$\alpha_3\beta_2^*$, β_3^*, α_6^*
αCtxPIA-analogue	α_6^*

Fig. 6. The subunit selectivity of different nicotinic antagonists used in the present thesis.
Mecamylamine (MEC); Dihydro-β-erythroidine (DHβE); methyllycaconitine (MLA); α-Conotoxin MII (αCtxMII); α-Conotoxin PIA-analogue (αCtxPIA-analogue).

Several different nicotinic antagonists were used in the present thesis (Fig. 6). Mecamylamine (MEC) is an unselective negative allosteric modulator of the nAChR and binds in the aquatic pore of the receptor. MEC has previously been used clinically as an antihypertensive drug but was removed from the market due to unpleasant side effects such as dry mouth, constipation and ortostatic hypotension (Young et al, 2001). MEC may act as a non-selective, non-competitive antagonist of NMDA receptors (O'Dell and Christensen, 1988; Papke et al, 2001). Dihydro-β-erythroidine (DHβE) is a rather selective competitive antagonist for the $\alpha_4\beta_2$* subunits (Alkodon and Alberquerque, 1993; Dwoskin and Crooks, 2001; Khiroug et al, 2004). However, at higher doses DHβE displays affinity for numerous other subunits than (see *e.g.* Buisson et al, 1996). The plant alkaloid, methyllycaconitine (MLA) has experimentally been shown to be selective for the α_7* subunits (Macallan et al, 1988; Ward et al, 1990; Alkondon et al, 1992; Wonnacott et al, 1993; Holladay et al, 1997; Davies et al, 1999) as well as to display a low affinity for the α_6 * subtype (Vailati et al, 1999). Nevertheless, it was recently shown that MLA, especially in higher doses, blocks the α_3* and/or $\alpha_3\beta_2\beta_3$* subunits (Klink et al, 2001; Mogg et al, 2002; Salminen et al, 2004). Both DHβE (Bowman and Rand, 1980) and MLA (Turek et al, 1995) have been shown to pass the blood brain barrier (BBB). α-Conotoxin MII (αCtxMII) is a venom from a predatory marine cone shell, *Conus magus* (McIntosh et al, 1999). It is a 16 amino acid peptide with two disulfide bridges that together form a complex three-dimensional structure and is proposed to be a selective competitive antagonist for the $\alpha_3\beta_2$* (Cartier et al, 1996; Grady et al, 2001), β_3* (Cui et al, 2003) and α_6 * (Vailati et al, 1999; Champtiaux et al, 2002) containing nAChRs. α-Conotoxin PIA (αCtxPIA), another venomous peptide from a marine cone shell, *Conus*

purpurascens, is composed of 18 amino acids and two disulfide bridges, together forming a complex three-dimensional structure. The amino acid sequence is quite homologous compared to αCtxMII, however, the nAChR subunit selectivity is different. It has been reported that αCtxPIA shows a significantly higher affinity for the α_6 * subunit rather than the $\alpha_3\beta_2$* and β_3* subunits (Dowell et al, 2003). Both αCtxMII and αCtxPIA belong to the super-family of 4/7 α-conotoxins, due to the positions of the disulfide bridges. The nAChR subunit selectivity of 4/7 α-conotoxins has been reported to reside to the central and the *C*-terminal part of the peptide, whereas the *N*-terminal amino acids appear to be of less importance (McIntosh et al, 1999; Arias and Blanton, 2000; Dutertre and Lewis, 2004; Everhart et al, 2004; Dutertre et al, 2005). Specifically, the bulky charged *N*-terminal protrusion of αCtxPIA has been suggested to be of less importance for its interaction with the α_6 subunit of the nAChR (Chi et al, 2005). The subunit selectivity of the αCtxPIA-analogue is therefore assumed to be similar to the one for αCtxPIA (Fig. 7). Due to the size of the peptides, such as those used in the present thesis, it is unlikely that they pass the BBB.

α-conotoxin	nAChR selectivity	amino acid sequence
αCtxMII	α3β2*, β3*, α6*	G**CCSNPVC**HLEHSNL**C**
αCtxPIA	α6*	RDP**CCSNPVC**TVHNPQI**C**
αCtxPIA-analogue	α6*	DP**CCSNPVC**TVHNPQI**C**

Fig. 7. Amino acid sequence and nAChR subunit selectivity of three different α-conotoxins.
α-Conotoxin MII (αCtxMII); α-Conotoxin PIA (αCtxPIA); α-Conotoxin PIA (αCtxPIA-analogue)

Functional roles for the different subunits of the nicotinic acetylcholine receptor

The different subtypes of the nAChRs show a regional distribution, regulate various physiological processes and are associated with a number of brain disorders *e.g.* $\alpha_4\beta_2$* and epilepsy or α_7* and schizophrenia (for review see Picciotto et al, 2001). It has been reported that the central nAChRs, especially those located in the VTA, play a role in mediating the accumbal DA enhancing, stimulatory and rewarding properties of nicotine (Clarke et al, 1988; Wonnacott et al, 1990; Corrigall et al, 1994; Di Chiara, 2000) and ethanol (Blomqvist et

al, 1997; Larsson et al, 2002; Ericsson et al, 2003) as well as in food-induced accumbal DA overflow (Schilström et al, 1998b). It may be hypothesized that certain subtypes of the nAChRs are involved in mediating the reinforcing properties of addictive drugs, *e.g.* nicotine or ethanol, as well as of natural rewards. Thus, results from genetically modified mice, suggesting that the α_4 and β_2 subunits appear to be involved in the development of nicotine addiction (Picciotto et al, 1998; Tapper et al, 2004). Additionally, DHβE, antagonizes nicotine-induced hypomotility in mice (Damaj et al, 1995) and shifts the dose-response curve for the discriminative stimulus effect of nicotine to the right (Gommans et al, 2000). Similarly, DHβE blocks nicotine-induced locomotor stimulation (Stolerman et al, 1997), nicotine-induced accumbal DA overflow as well as nicotine self-administration (*e.g.* Alkondon and Alberquerque, 1993; Corrigall et al, 1994; Grillner and Svensson, 2000; Dwoskin and Crooks, 2001; Larsson et al, 2002; Ericsson et al 2003; Khiroug et al, 2004). Furthermore, MLA blocks nicotine-induced accumbal DA overflow (Schilström et al, 1998b; Larsson et al, 2002), suggesting that the $\alpha_4\beta_2$* and α_7* subunits are important for behavioural and neurochemical effects of nicotine. In addition, the α_6* subunit has been suggested to play an important role in nicotine-induced locomotor stimulation (Le Novere et al, 1996) and the $\alpha_6\beta_2\beta_3$* subunits have been implicated in mediation of the DA enhancing properties of nicotine (Champtiaux et al, 2002). Furthermore, administration of MLA into the VTA attenuates the food-induced accumbal DA overflow (Schilström et al, 1998b), implying a role for the α_7 subunit, in food related reward. Contrarily, DHβE does neither modify ethanol self-administration (Lê et al, 2000), nor ethanol-induced accumbal DA overflow in rats (Ericson et al, 2003). In a recent study systemic administration of either DHβE or MLA had no effect on ethanol-induced locomotor activity and dopamine overflow in N.Acc. in mice (Larsson et al, 2002). Furthermore, administration of αCtxMII into the VTA antagonizes ethanol-induced locomotor stimulation and accumbal DA overflow in mice as well as ethanol intake and preference in mice and rats (Larsson et al, 2004). Taken together, this indicate that $\alpha_3\beta_2$*, β_3* and/or α_6*, rather than the $\alpha_4\beta_2$*, and/or α_7*, subunits of the nAChR, are involved in mediating these neurochemical and behavioural effects of ethanol (Fig. 8).

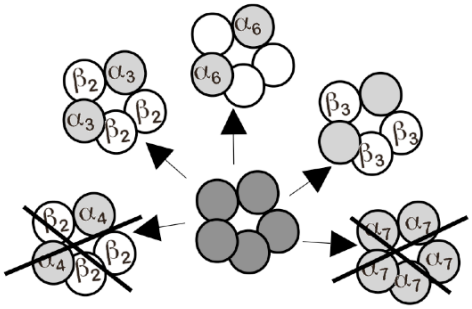


Fig. 8. The $\alpha_3\beta_2$*, β_3* and/or α_6*, rather than the $\alpha_4\beta_2$* or α_7*, subtypes of the nicotinic acetylcholine receptor appear to be involved in mediating the stimulatory and dopamine-enhancing effects of ethanol.

Ethanol

It has been known since the 1940s that rodents voluntarily drink ethanol in a laboratory setting (Richter and Campbell, 1940). This drinking behaviour has thereafter been observed in freely living animals, who intoxicate themselves by eating rotten fruit (for review see Spanagel, 2000).

Ethanol is a small molecule with both lipophilic and hydrophilic characteristics and when ingested, ethanol spreads quickly and passes the BBB into the CNS (*e.g.* Barry, 1991). Ethanol has a complex and diverse pharmacological profile including effects such as euphoria, locomotor stimulation, sedation, anxiolysis and muscle relaxation. In addition, ethanol is a low potent drug, *i.e.* several grams are needed for an effect.

Ethanol and ligand-gated ion channels

Ethanol was previously believed to produce its effects via a direct action on the cell membrane's lipid bi-layer (Meyer, 1899). However, this theory has been revised and a large body of evidence indicates that ethanol, in rather small doses, interacts directly with ligand-gated ion channels such as 5-HT_3, $GABA_A$, glycine, NMDA, and nACh receptors (Narahashi et al, 1991; Grant, 1994; Lovinger, 1997; Lovinger, 1999; Narahashi et al 2001; for review see Larsson and Engel, 2004), as well as Ca^{2+} channels (Davies, 2003). Ethanol can

either stimulate or inhibit these receptors, depending on the receptor type or subtype (Harris, 1999).

In vitro studies have shown that ethanol might act as a co-agonist for the 5-HT_3 receptor (Lovinger and White, 1991; Machu and Harris, 1994; Jenkins et al, 1996) and that ethanol potentiates the action of 5-HT on this receptor (Lovinger and Zhou, 1994). Additionally, ethanol augments the effects of GABA on the $GABA_A$ receptor by increasing the influx of Cl^-; thus it has been suggested that ethanol acts as an allosteric modulator of the $GABA_A$ receptor (Suzdak et al, 1986). The interaction between ethanol and $GABA_A$ receptors may cause hypnosis, sedation, anxiolysis and muscle relaxation (Liljequist and Engel, 1982; Liljequist and Engel, 1983). Furthermore, ethanol, glycine and other glycine agonists act synergistically at the strychnine-sensitive glycine receptors (Mascia et al, 1996). Ethanol acutely inhibits the NMDA glutamate receptor (Hoffman et al, 1989; Lovinger et al, 1989). Interestingly, clinical and pre-clinical studies show that manipulation of these systems affects the ethanol intake and therefore might be efficient as additional pharmaceuticals for treatment of alcoholism (*e.g.* Engel, 1977; Blaha et al, 1996b; LeMarquand et al, 1994a; LeMarquand et al, 1994b; Johnson et al, 2000; Koob et al, 2002; Addolorato et al, 2002; Molander et al, 2005)

The first evidence for a possible interaction between ethanol and nAChRs were results from our group showing that chronic ethanol consumption produces changes in the B_{max} for ^{3}[H]-nicotine in different regions of the rat brain (Yoshida et al, 1982). Additionally, long-term ethanol treatment in mice increases ^{3}[H]-nicotine binding in the thalamus (Booker and Collins, 1997). Given that ethanol can stabilize the open state of the Torpedo nAChR (Wu et al, 1994; Forman and Zhou, 1999), increase the agonist affinity for this receptor (Forman et al, 1989) and enhance the response to nicotine (Marszalec et al, 1999), it may be suggested that ethanol acts as a co-agonist with ACh on the nAChRs.

In subsequent studies it has been found that ethanol intake and preference as well as ethanol-induced locomotor stimulation and accumbal DA overflow may involve central nAChR, especially those located in the VTA (Blomqvist et al, 1992; Blomqvist et al, 1993; Blomqvist et al, 1997; Ericsson et al, 1998; Nadal et al, 1998; Lê et al, 2000; Larsson et al, 2002; Tizabi et al, 2002; Ericson et al, 2003;

Larsson et al, 2004). More specifically, αCtxMII-sensitive, *e.g.* $\alpha_3\beta_2$*, β_3* and/or α_6* subtypes of the nAChR in the VTA, have been demonstrated to be involved in mediating the locomotor stimulatory, rewarding and DA enhancing effects of ethanol (see *Functional roles for the different subunits of the nicotinic acetylcholine receptor).*

Neurochemical overlap between the reward systems and those regulating energy balance

Several studies have suggested that there is a neurochemical overlap between the reward systems and the systems regulating energy homeostasis (for reviews see DiLeone et al, 2003; Thiele et al, 2003; Thiele et al, 2004), implicating especially peptides such as leptin, orexin, neuropeptide Y (NPY) and galanin.

Relation between food and substance abuse

The interconnection between neural networks involved in feeding and drug reward is further demonstrated by studies showing that food deprivation enhances the rewarding properties of addictive drugs including heroin, amphetamine and cocaine (Carroll et al, 1979; Cabeza de Vaca and Carr, 1998; Stuber et al, 2002) and increases drug craving in humans (for review see Grigson, 2002). Furthermore, food restriction potentiates the threshold-lowering effect on self-stimulation in the lateral hypothalamus exerted by drugs (Cabeza de Vaca and Carr, 1998), augments the reinforcing value of electrical self-stimulation of the brain reward system (Fulton et al, 2000) and increases the cocaine-induced conditioned place preference and locomotor stimulation (Bell et al, 1997). This may be related to altered levels of orexigenic/anorexigenic peptides, even though an influence of other causes such as stress cannot be excluded. Moreover, high-ethanol preferring rats as well as alcoholics show a high preference for sweets (Kampov-Polevoy et al, 1999; for review see Grigson, 2002) and there is a co-morbidity between eating disorders and drug or alcohol abuse (Wolfe and Maisto, 2000). Although similarities between natural and drug-induced reward may be assumed, it has to be emphasized that food and drugs partly activate different neurons in the VTA (for review see Grigson, 2002).

The orexigenic/anorexigenic peptides and their effects on reward and drugs of abuse

Several peptides, such as ghrelin, NPY, galanin, orexin and melanin-concentrating hormone (MCH) stimulate feeding whereas other peptides including leptin, melanocortins (MC), cholecystokinin and corticotrophin-releasing factor, inhibit food intake (for review see Arora and Anubhuti, 2006*)*. Additionally, there is an increasing body of evidence indicating that these peptides also act on the reward systems and have a role in addictive behaviours, such as drug addiction (*vide infra)*.

Centrally acting NPY has DA enhancing properties in the N.Acc. (Salin et al, 1990) and has been found to reduces voluntary ethanol consumption in rodents, especially in those bread to consume ethanol (for review see Kiefer and Wiedemann, 2004; Thorsell et al, 2006). Additionally, MCH acts centrally to increase alcohol or sucrose intake in rats (Duncan et al, 2005). A microinjection of orexin into the VTA of rats increases the DA levels in the N.Acc. (Narita et al, 2006). Further, central infusion of orexin causes a dose-related reinstatement of cocaine seeking without changing cocaine intake (Boutrel et al, 2005) and reinstates morphine seeking (Harris et al, 2005). Moreover, orexin has in situations of nutritional depletion been suggested to stimulate arousal and motivation to reinforce food-seeking/feeding pathways and thereby ensure survival (for review see Sakurai, 2005). Hypothalamic administration of galanin increases DA and decreases ACh levels in the N.Acc. (Rada et al, 1998), possibly mediated via galanin receptors in the VTA (Hawas and Picciotto, 2004). Central administration of galanin has also been seen to increase ethanol intake in rats (Lewis et al, 2004; Rada et al, 2004a). The anorexigenic MC peptides decrease voluntary alcohol drinking in rats (Ploj et al, 2002). Furthermore, leptin into the lateral ventricle can suppress the basal levels of accumbal DA as well as the feeding-induced increase in accumbal DA (Krugel et al, 2003). Elevated leptin levels have been demonstrated in male alcoholics (Nicolas et al, 2001) and high ethanol-preferring mice (Obradovic and Meadows, 2002) and have been associated with alcohol craving during withdrawal (Kraus et al, 2005), effects tentatively mediated via leptin receptors expressed in the VTA (Figlewicz et al, 2003). Similarly, inhibition of leptin decreases ethanol preference in mice (Blednov et al, 2004) and leptin increases the ethanol intake (Kiefer et al, 2001) as well as enhances the motivation

for alcohol consumption in habituated mice after alcohol withdrawal (Kiefer et al, 2005). Nevertheless, the present thesis is focused on another energy balance regulating peptide, ghrelin.

Ghrelin

Ghrelin was isolated from rat stomach and was identified as the first endogenous ligand for the growth hormone secretagogue receptor (GHSR). It was given the name ghrelin; "ghre" as the etymological root for growth and the suffixes "GH" and "relin" as an abbreviation for "growth-hormone release", a characteristic effect of ghrelin (Kojima et al, 1999; Hosoda et al, 2000b; Casanueva and Diéguez, 2002; Schmid et al, 2005). Ghrelin is highly conserved among different species, particularly among mammals (Kojima et al, 1999; Kaiya et al, 2001; Saito et al, 2002; Hosoda et al, 2003; Kaiya et al, 2003; van der Lely et al, 2004; Kojima and Kangawa, 2005). It is a twenty-eight amino acid peptide acylated at the third serine position, which is essential for the activity of the peptide (Kojima et al, 1999). The acylated ghrelin, used in the present thesis, has been recognized as an orexigenic peptide (Date et al, 2000; Hosoda et al, 2000a), whereas the *des*-acyl ghrelins mainly have different or opposite effects to the acylated forms (Asakawa et al, 2005; Chen et al 2005a, Chen et al, 2005b; Ukkola, 2005).

Ghrelin is mainly produced in and secreted from the stomach (Kojima et al, 1999; Date et al, 2000; Sakata et al, 2002), however, smaller amounts are also produced in other gastrointestinal segments (Hosoda et al, 2000a), in peripheral tissues such as the pancreas, kidney, pituitary and spleen (Gnanapavan et al, 2002; for review see Korbonits and Grossman, 2004) and in the brain (Cowley et al, 2003; Mondal et al, 2005; Sato et al, 2005). Based on the assumption that the effects of growth hormone secretagouges (GHS; ghrelin mimetics) on food intake and GH release are thought to be mediated via central mechanism (Lall et al, 2001; for review see Smith, 2005), it was suggested that peripherally produced ghrelin also may have central effects. However, bioactive ghrelin is foremost known to be transported from the brain across the BBB, rather than the opposite direction (Banks et al, 2002). On the other hand, ghrelin containing cells have been identified in the hypothalamus (Lu et al, 2001) and ghrelin mRNA has been found adjacent to the third ventricle in the brain (Cowley et al, 2003), the arcuate nucleus (Mondal et al, 2005) as

well as in the hypothalamus (Sato et al, 2005), implicating that centrally produced ghrelin may be of importance.

Two types of the G-protein coupled GHSRs have been identified: GHSR-1A and GHSR-1B. GHSR-1A is activated by ghrelin and GHS, whereas GHSR-1B is not. The expression of mRNA for GHSR-1A in the human brain appears to resemble that of the rat brain, where the main expression is observed within different hypothalamic nuclei, the arcuate nucleus among others. However, the expression of GHSR-1A is not restricted to the hypothalamus. It has been identified *e.g.* in the pituitary, hippocampus, VTA and the LDTg (Howard et al, 1996; Guan et al, 1997). Interestingly, the two latter nuclei have central roles in the endogenous reward systems (see *The mesocorticolimbic dopamine system*). In addition to the CNS mRNA for ghrelin and its receptor is widely distributed within the human body, *e.g.* stomach, pancreas, heart and kidney (Howard et al, 1996; Gnanapavan et al, 2002; Gaytan et al, 2004; Sun et al, 2004), suggesting that ghrelin has multiple physiological functions. More specifically, it has been demonstrated that ghrelin is involved in mediation of sleep, memory and anxiety-like behaviour in rats (for reviews see Einstein and Greenberg, 2003; van der Lely et al, 2004; Ghigo et al, 2005).

Role of ghrelin in energy homeostasis

It may be suggested that ghrelin plays a role in appetite and meal initiation as well as in consummatory feeding behaviours. Numerous studies have demonstrated that central as well as peripheral administration of ghrelin increases food intake in rats and humans (Tschöp et al, 2000; Nakazato et al, 2001; Wren et al, 2001a; Wren et al, 2001b). Furthermore, chronic intracerebroventricular (icv) injections of ghrelin increase cumulative food intake in mice (Theander-Carrillo et al, 2006). The role of ghrelin in feeding is further supported by studies demonstrating that anti-ghrelin antibodies or GHSR antagonists suppress dark phase feeding as well as starvation-induced food intake (Nakazato et al, 2001; Asakawa et al, 2003; Bagnasco et al, 2003). Moreover, ghrelin-induced food intake is ablated in GHSR knockout mice (Sun et al, 2004), indicating that feeding stimulatory effect of ghrelin is mediated via GHSR. Additionally, chronic ghrelin administration induces adiposity, supposedly by interaction with hypothalamic circuits controlling energy homeostasis (Tschöp et al, 2000).

Plasma ghrelin and ghrelin mRNA expression increase pre-prandially, *i.e.* anticipatory to meal initiation, and decrease post-prandially (Tschöp et al, 2000; Asakawa et al, 2001; Cummings et al, 2001; Toshinai et al, 2001; Tschöp et al, 2001a; Ariyasu et al, 2002; Cummings et a, 2002b). Arvat and co workers (2000) discovered that a peripheral ghrelin injection increases hunger in healthy volunteers and subsequent studies have confirmed that ghrelin induces sensations of hunger and appetite (Horvath et al, 2001; Nakazato et al, 2001; Wren et al, 2001a; Eisenstein and Greenberg, 2003). Similarly, an intravenous administration of ghrelin to (healthy) humans increases the imagination of food (Schmid et al, 2005). Moreover, ghrelin increases behaviours such as sniffing and foraging for food (Keen-Rhinehart and Bartness, 2004). Moreover, administration into the brainstem reduces the latency to begin eating and stimulates additional meals, but does not alter meal size in animals (Faulconbridge et al, 2003); thus indicating that ghrelin has a role in meal initiation.

Ghrelin and deviant eating behaviours

Several studies have demonstrated an association between anomalous plasma ghrelin levels and aberrant eating patterns (*vide infra*). It has been suggested that a hyperghrelinemia may have a role in the pathophysiology of binge eating (*i.e.* compulsive overeating), at least in bulimic, anorectic and Prader-Willi patients. Children with Prader-Willi syndrome, who have not yet developed the core symptoms, have normal plasma levels of ghrelin (Erdie-Lalena et al, 2006), whereas the ghrelin levels are increased along with development of hyperphagia and obesity (Cummings et al, 2002a; DelParigi et al, 2002; Haqq et al, 2003; Erdie-Lalena et al, 2006). Similarly, anorectic and bulimic patients of the binge type have higher levels of ghrelin in the plasma than their non-binging counterparts, and the frequencies of bingeing correlate positively with plasma ghrelin levels (Tanaka et al, 2003a).

Peripheral ghrelin levels are elevated in anorectic and bulimic subjects (Ariyasu et al, 2001; Otto et al, 2001; Shiiya et al, 2002; Tanaka et al, 2002; Monteleone et al, 2003; Nedvidkova et al, 2003; Tanaka et al, 2003b; Tolle et al, 2003; Soriano-Guillen et al, 2004; Stock et al, 2005) and decreased in obese, compared to lean, subjects (Tschöp et al, 2001b; Wren et al, 2001b; Shiiya et al, 2002; Rosická et al, 2003; Soriano-Guillen et al, 2004; Stock et al, 2005). Moreover, obese individuals with BED have lower plasma levels of ghrelin than those without BED (Geliebter et al, 2004; Geliebter et al, 2005; Monteleone

et al, 2005). Additionally, following dietary inventions in obese and anorectic individuals the plasma ghrelin levels increase or decrease, respectively (Cummings et al, 2002b; Hansen et al, 2002; Soriano-Guillen et al, 2004). However, it should be considered that the ghrelin levels in plasma may not correspond positively to the levels of ghrelin in brain areas such as the hypothalamus, VTA and LDTg.

Various polymorphisms in the GHSR have been associated to bulimia nervosa, obesity (Wang et al, 2004b; Baessler et al, 2005; Holst and Schwartz, 2006; Miyasaka et al, 2006) and alterations in eating patterns (*i.e.* the proclivity to "gaze" versus "binge") (Korbonits et al, 2004). Moreover, different pro-ghrelin gene polymorphisms have been linked to obesity, bulimia nervosa purging individuals (Ukkola et al, 2001; Korbonits et al, 2002; Vivenza et al, 2004; Ando et al, 2006; Vartiainen et al, 2006) as well as methamphetamine withdrawal (Yoon et al, 2005).

Central nervous system targets

The effects of ghrelin on energy balance have been shown to be mediated, at least in part, via the hypothalamus (Nakazato et al, 2001; Shuto et al, 2002). Specifically, the ghrelin-induced feeding is mediated via the hypothalamic arcuate nucleus (Tamura et al, 2002). NPY and agouti related peptide (AgRP) containing neurons in the arcuate nucleus have been demonstrated to mediate the orexigenic effects of ghrelin (Kamegai et al, 2001; Nakazato et al, 2001; Shintani et al, 2001; Wang et al, 2002; Olszewski et al, 2003; Toshinai et al, 2003; Chen et al, 2004; Riediger et al, 2004; Tang-Christensen et al, 2004; Gropp et al, 2005). Further, an electrophysiology study has shown that ghrelin decreases the firing in proopiomelanocortin (POMC) neurons in the arcuate nucleus (Cowley et al, 2003). Moreover, it has been demonstrated that the ghrelin-induced feeding is mediated via central MC signalling (Tschöp et al, 2002; Chen et al, 2004) as well as orexin containing neurons (Toshinai et al, 2003). Taken together, the feeding-stimulatory effects of ghrelin in the arcuate nucleus may possibly collectively be mediated via NPY/AgRP neurons and *e.g.* POMC, orexin and the MC systems.

Aim of the present thesis

The overall aim of the present thesis was to investigate the mechanisms of action for ethanol and ghrelin on the DA reward

systems, and thereby pin point novel targets for treatment of addictive behaviours.

Initially, the functional role the different nAChRs subunit compositions for the stimulatory and DA enhancing effects of ethanol was explored. In addition, the possibility that the orexigenic peptide ghrelin activate the reward systems, specifically the cholinergic-dopaminergic reward link, was elucidated. In subsequent experiments the role of cholinergic mechanisms in mediation of the ghrelin-induced stimulation and DA overflow was studied.

Materials and methods

To explore our hypotheses in the present thesis the following methods and experimental setups were used.

Methods	Paper
Peptide synthesis	I
Locomotor activity recording	I, II, III, IV
In vivo microdialysis	I, II, III, IV
Radioligand binding assay	IV

Peptide Synthesis

The availability of α-conotoxins is limited and in order to synthesis αCtxMII analogues with various nAChR subunit selectivity, a slightly modified method described my McIntosh and co-workers (Cartier et al, 1996) was used to synthesize α-conotoxin peptides (*i.e.* αCtxMII and the αCtxPIA-analogue).

Linear Peptides

The peptides were synthesized on a Rink amide resin (loading 1.20 mmol/g) using *N*-9-fluorenylmethoxycarboxyl (Fmoc) chemistry and *O*-(7-benzotriazole-1-yl)-1,1,3,3 tetramethyluronium tetrafluoroborate (TBTU) and *N,N*-diisopropylethyl amine (DIPEA) activation. The peptides were synthesized on a 0.24 mmol scale with standard amino acid side chain protection (Glu and Ser [*t*-Bu]; Asn and His [trityl] except on cysteine residues. Cysteine residues were protected in pairs with *S*-trityl on the first and third cysteines and *S*-acetamidomethyl on the second and fourth cysteines. Each residue was used in a five-fold excess and coupled for 60 min.
Following completion of the synthesis the resin was washed with methanol (3 x 10ml) and dried under reduced pressure.

The linear peptide amide was cleaved of the resin by treatment with 6 ml of trifluoroacetic acid/H_2O/ethanedithiol /thioanisole (94.5/2.5/ 2.5/1 by volume) for 6h at 20 °C. The resin was rinsed twice with 6 ml of the same solution. The combined filtrate and washings were pooled and concentrated to dryness. The residue was washed two times with diethyl ether. The supernatant was discarded and the precipitated peptide powder was dried on the vacuum line.

Peptide Cyclization

To form a disulfide bridge between Cys and Cys (*i.e.* the first and third cysteines), the pelleted peptide was dissolved in 1.2 ml of B-Buffer (H_2O/acetonitrile/trifluoroacetic acid (95/5/0.1 by volume)) and acetonitrile (40/60 by volume), with gentle swirling (to avoid foaming). The linear peptide solution was added drop-wise into 57 ml of H_2O (pH 7.6 by solid Tris base). The solution was gently swirled at room temperature for 45 h when the reaction was judged to be complete by analytical HPLC using a Genesis C18 column (4μ, 15cm x 4.6mm; Jones Chromatography, Hengoed, USA) and a gradient from 5% to 95% of B-Buffer in acetonitrile as eluent (flow rate at 1 ml/min). The pH of the solution was adjusted to 2-3 by the addition of trifluoroacetic acid and the solution was freeze-dried. The monocyclic peptide was then purified by preparative HPLC using an Ace 5AQ column (25cm x 21.2mm: Advanced Chromatography Technologies, Aberdeen, United Kingdom) and a gradient from 5% to 95% of B-Buffer in acetonitrile as eluent (flow rate at 10 ml/min). The fractions containing product were pooled and freeze-dried.

Removal of the S-acetamidomethyl groups and formation of the second disulfide bridge (Cys-Cys, *i.e.* the second and fourth cysteines) was carried out simultaneously by iodine oxidation. The monocyclic peptide was diluted in 3.5 ml of B-Buffer and added drop-wise to 3.5 ml of a rapidly stirred solution of 20 mM iodine in H_2O/trifluoroacetic acid/acetonitrile/MeOH (50/20/20/10 by volume) over 5.5 minutes at room temperature. This reaction was allowed to proceed for another 90 minutes and was thereafter terminated by the addition of a diluted aqueous solution of ascorbic acid (1M, two drops). The solution was freeze-dried over night and the peptide was obtained as a powder. The peptide was dissolved in 450 μl of B-Buffer and was purified by preparative HPLC as described above. The purity of the peptide was analyzed by analytical HPLC as described above. The peptide was thereafter freeze-dried and the amino acid sequence was analyzed by fast atom bombardment mass spectrometry (FAB-MS) (Einar Nilsson, Department of Organic Chemistry, Lund University, Sweden). The peptide was thereafter used in the animal experiment.

Animals

Adult male NMRI mice weighing approximately 25-40 g, purchased from either Charles River (Sulzfeld, Germany (paper I, II, III)) or

B&K Universal AB (Sollentuna, Sweden (Paper III, IV)) were used for the locomotor activity and microdilaysis experiments. Adult male Sprague Dawly rats weighing approximately 220 g, purchased from B&K were used for the radioligand binding experiments (Paper IV). Upon arrival the animals were allowed to habituate in groups of eight mice or six rats, in standard cages (Macrolon III: 400 x 250 x 150 mm (mice), Macrolon IV: 550 x 350 x 200 mm (rats)), for at least a week before initiation of the experiment. Standard feed (Harlan Teklad, Norfolk, England) and tap water were freely supplied from the arrival until the day of experiment. The cages and bedding material (wood-cuttings) were changed once a week. A temperature of 20°C, humidity of 50% and a 12/12 hour light/dark cycle (light switched on 7 am) was maintained in the animal room. The present studies were approved by the Ethics Committee for Animal Experiments in Göteborg, Sweden.

Locomotor activity procedure

Most drugs of abuse cause locomotor stimulation, an effect, at least in part, mediated by their ability to enhance the extracellular concentration of accumbal DA (Engel and Carlsson, 1977; Wise and Bozarth, 1987) and may therefore be suggested to be a putative endophenotype for drugs of abuse (Gabby, 2005). Interestingly, low doses of ethanol stimulates the locomotor activity in alcohol-preferring but not in non-alcohol preferring rats (Waller et al, 1986) and the behavioural effects of ethanol in rats are closely time-locked with accumbal DA release (Imperato and Di Chiara, 1986). In addition, the locomotor and the positive reinforcing effects of addictive drugs has been suggested to be homologous effects evolving from an activation of a common mechanism in rodents and humans, *i.e.* the dopaminergic reward systems (Wise and Bozarth, 1987). Furthermore, locomotor activity is an important component of food-seeking behaviour and thus essential for survival.

Device for measuring locomotor activity

Locomotor activity was registered in eight sound attenuated, ventilated and dimly lit locomotor boxes (420 x 420 x 200 mm, Plexiglas®). Five by five rows of photocell beams at the floor level of the box allowed a computer-based system to register the activity of the mice (Kungsbacka mät- och reglerteknik AB, Fjärås, Sweden). The mice were allowed to habituate to the environment in the box for one hour before drug challenge and initialization of the experiment. This

because naïve animals initially display a high exploratory activity which is followed by a decline in locomotor activity. To reduce the influence of injection-induced hyper-motility, the registration of locomotor activity started 5 minutes after the drug administration. Locomotor activity was defined as the accumulated number of new photocell beams interrupted during a 30-minute (Papers I, II) or 60-minute (Papers III, IV) period.

Guide cannula implantation

In papers I and IV, αCtxMII and/or the αCtxPIA-analogue were administered into the VTA, as these peptides may not pass the BBB. On the day of the locomotor experiment (Papers II, III and IV) the animals were challenged with a ghrelin injection into the third ventricle (from which drug distribution to other parts of the brain is known to occur) or injections into the VTA and/or the LDTg (for regional distribution). To facilitate this administration, bilateral and/or unilateral guide cannula/s, aiming at the VTA, the third ventricle or LDTg, was surgically implanted four days prior to the experiment using the method described below.

The mice were anesthetized with isofluran (Isofluran Baxter: Univentor 400 Anaesthesia Unit, Univentor Ldt., Zejtun, Malta), placed in a stereotaxic frame (David Kopf Instruments: Tujunga, CA, USA) and kept on a heating pad to prevent hypothermia. The skull bone was exposed and one or two holes for the guide cannulas (stainless steel, length 10 mm, outer/inner diameter of 0.6/0.45 mm) and one for an anchoring screw were drilled. Following surgery, 1 ml of saline was injected subcutaneously (sc) to avoid dehydration.

The coordinates for the VTA relative to the bregma were: posterior – 3.4 mm, lateral to midline ±0.5 mm, for the third ventricle: posterior -0.9 mm, lateral to midline ±0.0 mm, and for the LDTg: 5.0 mm posterior to bregma, ±0.5 mm lateral to the midline (Franklin and Paxinos, 1996). The guide cannula/s were lowered 1.0 mm below the surface of the brain and was fixed to an anchoring screw and the scull with dental cement (DENTALON® plus: AgnTho's AB, Lidingö, Sweden). After surgery the mice were kept in individual cages (Macrolon III (Paper I), Sealsafe IVC 2L, 365 x 207 x 140 mm (Papers II, III, IV)), with food and water supplied *ad libitum* and allowed to recover for four days before the locomotor activity test.

At time of the experiment the infusion cannula was inserted and extended another 3.8 mm, 1.1 mm, 2.2 mm ventrally beyond the base

of the guide cannula, aiming at the VTA, third ventricle and LDTg respectively.

Locomotor experiment procedure

On the day of the experiment the guide cannula was used to administer drug into the VTA, third ventricle or LDTg. Before initiating the experiment, a dummy cannula was carefully inserted into the guide cannula and then retracted to remove clotted blood and to hamper spreading depression. Spreading depression occurs as a result of injuries to brain tissues, causing release of ions and other compounds, and thereby interfering with the animal's normal function. Previous studies from the present laboratory indicate that the probability of a second spreading depression is largely reduced by pre-insertion of a dummy cannula.

Paper I: Either αCtxMII (5 nmol in 1μl), the αCtxPIA-analogue (5, 10, 20 nmol in 1μl) or an equal volume of vehicle (Ringer solution) was carefully administered bilaterally into the VTA to one side at the time. Twenty minutes later ethanol (1.75 g/kg) or vehicle (saline) was administered intraperitoneally (ip).

Paper II: In the first locomotor activity experiment, ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) was administered into the third ventricle. In the second locomotor activity experiment the mice were first challenged with MEC (2.0 mg/kg) or vehicle (saline) ip. Ten minutes later ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) solution was administered into in the third ventricle.

Paper III: In these experiments ghrelin (1 μg in 1 μl) or an equal volume of vehicle solution (Ringer solution) was administered bilaterally either into the VTA or the LDTg.

Paper IV: In the first series of experiments, mice were unilaterally pre-treated with MEC (5 pmol in 1 μl), αCtxMII (50 pmol in 1 μl) or an equal volume of vehicle (Ringer solution) into the VTA and 10 or 20 minutes later ghrelin (2 μg in 1 μl) or an equal volume of vehicle (Ringer solution) were injected into the LDTg. In the second series of experiments, mice were pre-treated with DHβE (0.5 mg/kg, sc), MLA (2 mg/kg, ip) or vehicle (saline) and 10 minutes later ghrelin (1 μg/ in

μl) or an equal volume of vehicle (Ringer solution) were injected bilaterally into the LDTg. In the third series of experiments, mice were bilaterally pre-treated with MEC (5 pmol in 1 μl), αCtxMII (50 pmol in 1 μl) or an equal volume of vehicle (Ringer solution) into the VTA and either ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) into the VTA. In the fourth series of experiments, mice were pre-treated with DHβE (0.5 mg/kg, sc), MLA (2 mg/kg, ip) vehicle (saline) and 10 minutes later ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) were injected bilaterally into the VTA.

In all the locomotor activity experiments the mice were returned to the locomotor activity boxes after each drug administration. All mice received drug treatment only once. In all experiments with local drug administrations the drug was administered for one minute, the cannula was left in place for another minute, and was then retracted. A cannnula connected to a 5 μl syringe (Kloehn microsyringe: Skandinaviska Genetec AB, V. Frölunda, Sweden) was used for the local drug administrations. Neither water nor food was available to the animal during the locomotor experiments.

Statistical analyses for locomotor activity

All data obtained from the experiments were statistically analyzed using SAS Statview 5.01 computer software (Eudorex Sales AB, Stockholm, Sweden). A probability value (*p*) less than 0.05 was considered as statistically significant. Error bars in the figures represent standard error of the mean (S.E.M).

The data were evaluated by one- or two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test (PLSD) for comparisons between treatments (Papers I, II, IV). In Paper III the Bonferroni procedure was used for post-hoc comparisons between different treatments. Unpaired t-test was also used to analyse the locomotor activity data.

Microdialysis procedure

Ungerstedt (Ungerstedt, 1983) and Johnson and Justice (Johnson and Justice 1983) introduced *in vivo* microdialysis in awake and freely moving animals, and the technique is now well established and commonly used. Previous work from the present laboratory has

adapted the *in vivo* microdialysis method from rats to mice. The microdialysis technique enables measurements of extracellular neurotransmitter levels in the brain in awake, freely moving mice. The method is based on the movement of substances from the outside the probe to the inside. High extracellular DA concentrations may depend on rapid saturation of the DA transporter, slow enzymatic degradation, DA released from other cells, diffusion from other areas, slow autoreceptor activation and/or burst firing. The method cannot distinguish between these different origins of DA. In the present experiments the technique was used to monitor the drug-induced changes in extracellular levels of dopamine in N.Acc.. Dialysates were sampled at 20 minutes intervals, and thus, an obvious limitation was that real-time estimations of alterations in transmitter levels could not be estimated. It should therefore be noted that the technique does not reflect synaptic release, but an averaged "overflow" of the extracellular DA that escapes reuptake and breakdown mechanisms.

Probe manufacturing

All probes were manufactured on site, where a modified version of the microdialysis probes described by Santiago and Westerink (Santiago and Westerink, 1990) was used. The probe production was facilitated by specific stands (Medi Tech, Göteborg University, Sweden). The inlet and the outlet of the probe were made of PE20 polyethylene tubing (outer/inner diameter of 1.09/0.38 mm: Becton Dickinson and Company, Sparks, MD, USA). A fused silica extended 5 mm from the tip of the probe. A glass rod was centred between the inlet and outlet and was attached by Super Epoxy (Loctite, Göteborg, Sweden). The dialysis membrane (20 000 kDa cut off with an outer/inner diameter of 310/220 μm: HOSPAL, Gambro, Lund, Sweden), prepared from a co-polymer of polyacrylonitrile and sodium methallyl sulfonate, was sealed with a glue plug and thereafter tread on the fused silica. The exposed tip of the dialysis membrane of the probe was 1 mm; the remaining membrane area was covered with silicone glue (CAF 3: Rhodorsil Silicones, Saint-Fons Cedex, France). Each probe was controlled to ensure that is was straight and that the flow was correct. The probes were thereafter connected to a microperfusion pump (U-864 Syringe Pump: AgnThós AB) and firstly perfused with an ethanol solution (70% vol./vol.; 100 μl) and secondly with an artificial cerebrospinal fluid (Ringer solution; 200μl) at a rate of 4 μl/min. The

probes were sealed by heating and stored in Ringer solution for maximum 1 day in 6°C before implantation.

Surgical procedure

The mice were implanted with a microdialysis probe (Waters et al, 1993) positioned in the N.Acc. for measurement of extracellular DA levels, and a guide cannula, aiming at the VTA, the third ventricle and/or the LDTg (to enable drug administration). The location of the probe and guid cannula was ipsilateral and alternated to both the left and right side of the brain. The surgical procedure was performed as described above (see *Guide cannula implantation*), where the probe was slowly lowered into position and anchored to the screw in the skull bone with dental cement (Dentalon Plus; Angthós AB). Thereafter the guide cannulas were lowered into position and anchored to the probe with dental cement (Dentalon Plus: Angthós AB).
The coordinates for the N.Acc. were: relative to the bregma +1.5 mm anterior, lateral to midline ±0.8 and ventral –4.7 mm. The coordinates for the VTA, third ventricle and the LDTg were the same as above (see *Guide cannula implantation*) (Franklin and Paxinos, 1996). At time of the experiment the cannula was inserted and extended another 3.8 mm, 1.1 mm, 2.2 mm ventrally beyond the base of the guide cannula, aiming at the VTA, third ventricle and LDTg respectively.

After surgery the mice were housed in individual cages (Macrolon III (Paper I), Sealsafe IVC 2L, 365 x 207 x 140 mm (Paper II, III, IV) with the food and water supplies *ad libitum*. The animals were allowed to recover for four days before the microdialysis experiment. To ensure a good health status of the mice, the weight of the mice was registered prior to the experiment and after.

Microdialysis drug treatment paradigm

On the day of the experiment and immediately before start of the experiment, a dummy cannula was carefully inserted into the guide cannula and thereafter retracted to remove clotted blood and to hamper spreading depression (see *Locomotor experiment procedure*). The probe was then connected to a microperfusion pump (U-864 Syringe Pump: AgnThós AB) and perfused with vehicle (Ringer solution) at a rate of 1.5 μl/min. The mice were connected to the microdialysis apparatus via a liquid swivel (CMA/Miceodialysi AB, Stockholm, Sweden) and were able to move freely during the experiment. After

one hour of habituation to the microdialysis perfusion set up, perfusion samples (30 μl) were collected every 20 minutes. Five samples were collected prior to the first drug challenge. The baseline DA level was defined as the averaged concentration of the three consecutive samples before the first drug challenge.

Paper I. Ethanol (1.75 g/kg, ip) was administered at time 0 minutes. Three hours later the αCtxPIA-analogue (5 nmol in 1μl) or an equal volume of vehicle (Ringer solution) was administered into the VTA. Twenty minutes after the local drug administration an additional ethanol (1.75 g/kg, ip) or vehicle (saline, ip) injection followed, and another four samples were collected.

Paper II. In the first microdialysis experiment ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) was administered into the third ventricle at 10 minutes. In the second microdialysis experimental setup the unselective nicotinic antagonist MEC (2 mg/kg, ip) or vehicle (saline, ip) was administered at 0 minutes and 10 minutes later ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) were administered into the third ventricle.

Paper III. In this microdialysis experimental setup, at 10 minutes, ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) were administered into the VTA or LDTg.

Paper IV. In this microdialysis experimental setup, αCtxMII (50 pmol in 1 μl) or an equal volume of vehicle (Ringer solution) were administered into the VTA. 20 minutes later, ghrelin (1 μg in 1 μl) or an equal volume of vehicle (Ringer solution) was administered into the VTA or LDTg. Mice only received a ghrelin injection into VTA or LDTg

In papers II, III and IV, perfusion samples were collected for three hours (9 samples) after the last drug injection. At the end of the experiment amphetamine (2 mg/kg, ip) was administered; mice without amphetamine-induced DA overflow in the N.Acc. were excluded from the statistical analysis. The experiments was then terminated. In all microdialysis experiment the mice were returned to microdialysis cage after each drug administration. All mice received drug treatment only once. In all experiments with local drug administrations the drug was administered for one minute, the cannula

was left in place for another minute, and was then retracted. Neither water nor food was available to the animals during the microdialysis experiments.

Biochemical assay

The DA levels in the dialysates were determined by means of HPLC with electrochemical detection (HPLC-EC). A pump (Gyncotec P580A: Kovalent AB, V. Frölunda, Sweden), an ion exchange column (2.0 x 100 mm, Prodigy 3 μm SA, Skandinaviska GeneTec AB) and a detector (Antec Decade: Antec Leyden, Zoeterwoude, The Netherlands) equipped with a VT-03 flow cell (Antec Leyden) was used. The mobile phase (pH 5.6), consisting of sulfonic acid (10 mM), citric acid (200 mM) sodium citrate (200 mM), 10% EDTA, 30% MeOH, was vacuum filtered by using a 0.2 μm membrane filter (GH Polypro: PALL Gelman Laboratory, Lund, Sweden). The mobile phase was delivered at a flow rate of 0.2 ml/min passing a degasser (Degas Populaire: Kovalent AB), and the analyte was oxidized at +0.4 V. The analysis was kept under constant temperature (+ 25°C).

An external DA standard with a concentration of 3.26 nM was used to identify the DA peak. The limit of detection at a signal/noise ratio 3:1 was 4 fmol DA/20 μl injection. Basal, average baseline levels of the extracellular the DA concentration were approximately 1 nM.

Statistical analyses of the microdialysis experiments

All data obtained from the experiments were statistically analyzed using SAS Statview 5.01 computer software (Eudorex Sales AB). A probability value (p) less than 0.05 was considered as statistically significant. Error bars in the figures represent S.E.M. The baseline DA level was defined as the averaged concentration of the three consecutive samples before the first drug challenge (time point 0 min).

Paper I: The microdialysis data were analyzed by paired or unpaired t-tests. The baseline DA concentration preceding the first ethanol-induced increase in DA overflow was defined as the averaged concentration of the two consecutive samples obtained before ethanol challenge (time point 0 min) and the peak increase as the averaged concentration of the two consecutive samples obtained at 80 and 100 min. The baseline DA concentration preceding the second ethanol-induced increase in DA overflow was defined as the averaged

concentration of the two consecutive samples obtained at 160 and 180 min and the peak increase as the averaged concentration of the two consecutive samples obtained at 220 and 240 min. Paired t-tests were used to investigate the maximum DA increase for the respective peaks in each treatment group. Differences between treatment groups in ethanol-induced DA overflow were analyzed by unpaired t-tests.

Paper II: The microdialysis data were analyzed with a two-way ANOVA, with treatment as between-subjects factor and time as within-subjects factor, followed by Fisher's PLSD test for comparison between treatments. Changes from baseline between treatment groups in ghrelin-induced DA overflow at every time point were analyzed by unpaired t-tests. A paired t-test was used to analyze the effect of ghrelin compared to its own baseline.

Paper III: Two-way ANOVA was performed on the data obtained with treatment as between-subjects factor and time as within-subjects factor. The Bonferroni procedure was used for post-hoc comparisons between different treatments.

Paper IV: The microdialysis data were analyzed with a two-way ANOVA, with treatment as between-subjects factor and time as within-subjects factor, followed by Fisher's PLSD test for comparison between treatments. Changes from baseline within the representative treatment groups in ghrelin-induced DA overflow at the different time points were analyzed by Fisher's PLSD, following a significant two-way ANOVA.

Verification of probe and/or guide cannula/s placement

After the locomotor activities as well as the microdialysis experiments were completed, the locations of the probe and/or cannula/s were verified. The mice were decapitated, probes were perfused with pontamine sky blue 6BX to facilitate probe localization, and the brains were mounted on a vibroslice device (752M Vibroslice: Campden Instruments Ltd., Loughborough, UK). The brains were cut in 50 μm sections and the location of the probe and/or cannula was determined by gross observation using light microscopy. Only mice with guide cannula/s placement in the third ventricle (Fig. 9A), VTA (Fig. 9B),

LDTg (Fig. 9C) and/or probe placement in the N.Acc. (Fig. 9D) were included in the statistical analysis.

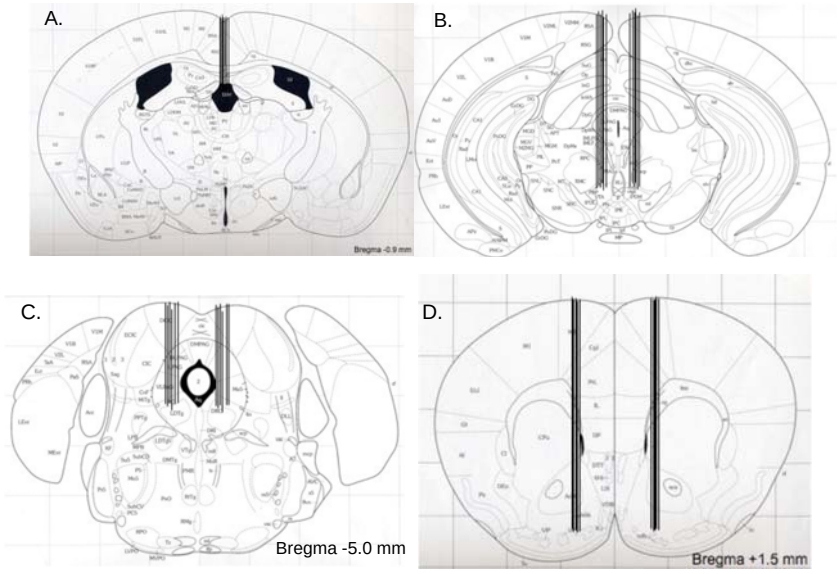


Fig. 9. Coronal mouse brain sections showing guide cannula/s and/or probe placement.
A. Cannula placements within the third ventricle of ten representative mice.
B. Cannula placements within the ventral tegmental area of six representative mice.
C. Cannula placements within the laterodorsal tegmental area of six representative mice.
D. Probe placements within the nucleus accumbens of ten representative mice. All brain sections are reprinted from *The Mouse Brain in Stereotaxic Coordinates*, K. B. J. Franklin and G. Paxinos, figure 17, 18, 19. Copyright (1996), with permission from Elsevier.

Radioligand binding assay

Tissue preparation

The rats (n=26) were decapitated, the brains rapidly removed and dissected on ice. Bilateral VTAs, from each rat, were saved and stored at -70 °C until use. On the day for the binding experiment, the brain tissue was homogenized in 50 mM TRIS pH 7.7 (approximately 0,2 g tissue in 20 ml TRIS) with a glass/Teflon homogeniser. The homogenized tissue was centrifuged (Beckman, Model J2-21 centrifuge) at 16,000 g for 25 min and +4 °C. The pellet was recovered

and dissolved in 20 ml distilled water. 60 minutes later the solution was centrifuged (Beckman, Model J2-21 centrifuge) at 16,000 g for 25 min and +4 °C. The pellet was recovered and dissolved in 20 ml 50 mM TRIS pH 7.7 and was centrifuged again at 16,000 g for 25 min and +4 °C. Finally the pellet was recovered and re-suspended in 8.6 ml 50 mM TRIS pH 7.7.

Binding assay

The total vial volume for the binding assay was 500 µl: 300 µl of tissue suspension, 100 µl of 5 nM [3H]-nicotine, 50 µl of volume adjustment and 50 µl of the different competing drugs used (nicotine or ghrelin) at increasing concentrations (0-200 µM) dissolved in 50 mM TRIS pH 7.7. For each drug, eight different concentrations were used. The samples were incubated for 90 minutes at +4 °C and thereafter filtered with a cell harvester (Brandel, Biomedical Research and Developmental Laboratories, Gaitherburg, USA). Glass microfibre filter (GF/B; Whatman, Maidstone, England) and ice-cold 50 mM TRIS pH 7.7 were used. The filters were rinsed twice with 4.5 ml 50 mM TRIS pH 7.7, thereafter 5 ml scintillation liquid (Optiphase "Hi Safe" 2; NEN Life Science, Sollentuna, Sweden) was added and the samples were incubated shaking over night. Thereafter the samples were counted in a multi-purpose scintillation counter (Beckman LS 6500, Beckman Instruments). All determinations were performed in duplicates.

Drugs

αCtxMII and the αCtxPIA-analogue were dissolved in Ringer solution (NaCl 140 mM; CaCl2 1.2 mM; KCl 3.0 mM and MgCl2 1.0 mM) (Merck KGaA, Darmstadt, Germany).

Paper I: The dose of αCtxMII (5 nmol in 1 µl) used, was selected on the bases of pervious dose-finding experiments to select the highest dose that did not affect locomotor activity *per se* (data not shown). In a similar manner 5 nmol in 1 µl for the αCtxPIA-analogue was used.

Paper IV: The dose of αCtxMII (50 pmol in 1 µl) used, was selected on the bases of previous experiments showing that the operant leaver response for ethanol and the alcohol deprivation effect could be

antagonized by a lower dose of αCtxMII (Kuzmin, Jerlhag, Liljequist, Engel, unpublished data).

Ethanol (VWR International AB, Stockholm, Sweden) was diluted in a 0.9% sodium chloride solution to a 15% (weight/volume) concentration and was administrated ip. MEC hydrochloride (Sigma-Aldrich Sweden AB, Stockholm, Sweden) was dissolved in sodium chloride solution (0.9 %) (Paper II) or Ringer solution (Paper IV) and administered ip (2 mg/kg, 10 ml/kg body weight) (Paper II) or into the VTA (5 pmol in 1 µl) (Paper IV). Saline vehicle contained 0.9% sodium chloride solution and was administered 12 ml/kg. The dose used for MEC was base on previous dos-response experiments in our research group and found to be the highest dose with no effect *per se* on locomotor activity (data not published). Active (acylated) rat ghrelin (Bionuclear, Bromma, Sweden) was dissolved in Ringer solution. The selected dose of ghrelin was used since this dose has been reported to statistically significantly increase the food intake in mice (Bjursell et al, 2005). Methyllycaconitine HCl or dihydro-β-erythroidine HBr (Sigma-Aldrich Sweden AB) were dissolved in sodium chloride solution (0.9 %) and were administered ip or sc, respectively. The selected doses were used since they have been shown to antagonize the locomotor stimulatory and DA enhancing effects of nicotine (Larsson et al, 2002). Dex-amphetamine sulphase (RBI, Natick, USA) was dissolved in sodium chloride solution (0.9 %) and administered ip (2 mg/kg, 10 ml/kg body weight). The drug challenges were randomized in all experiments. All drugs were administered as the weight of the salt.
[3H]-nicotine (NEN Life Science Products), nicotine (Sigma-Aldrich Sweden AB), TRIS (Trisma pre-set crystals; Sigma-Aldrich Sweden AB) and scintillation liquid (Optiphase "Hi Safe" 2; NEN Life Science Products) were used for the radio-ligand binding experiments.

Methodological considerations

Ghrelin and ethanol increases the locomotor activity and accumbal DA (Papers I, II, III, IV), indicating that both these drugs have rewarding properties. However, it should be emphasized that these measures do not directly reflect reward but are rather mechanisms associated with reward. Given that *in vivo* microdialysis enables measurements of the extracellular concentration of DA and that the N.Acc. core and shell are closely associated (especially in a small animal like the mouse), it may be suggested that in the present experiments we measure DA

levels in both these areas. However, this appears less likely since dorsal and/or lateral placement of the probe does not demonstrate an increase in accumbal DA upon ethanol or ghrelin administration. Most drugs of abuse cause locomotor stimulation (Engel and Carlsson, 1977), an effect mediated by the ability of these drugs to enhance accumbal DA overflow. Locomotor activity is therefore a putative endophenotype for drugs of abuse. In addition, the locomotor and the positive reinforcing effects of addictive drugs has been suggested to be homologous derived from an activation of a common mechanism, i.e. the dopaminergic reward systems (Wise and Bozarth, 1987). The duration of the locomotor stimulation was shorter than that for accumbal DA overflow (Papers I, II, III, IV). This may be due to the fact that the *in vivo* microdialysis method measures the extracellular DA levels, which does not reflect synaptic release, but rather an averaged "overflow" of the extracellular DA concentration. This may be elucidated by methods such as *in vivo* electrochemistry or non-net flux. Additionally, an obvious limitation to the experimental techniques used in the present thesis is that real-time measurements of alterations in transmitter levels are not possible. However, *in vitro* electrophysiological studies on slices from the VTA show that ghrelin increases the firing rate of dopaminergic neurons in the VTA and systemic administration of ghrelin increases the *in vitro* turnover of DA in the N.Acc. (Abizaid et al, 2006a) Taken together, we suggest that ghrelin activates the mesolimbic DA system and therefore is a part of the neruochemical overlap between the reward systems and those regulating energy balance.

It should be emphasized that the mouse brain may not represent the human brain. However, we believe that our animal models have validity.

Results and discussion

α-Conotoxin MII and the α-conotoxin PIA-analogue can be efficiently synthesized (Paper I)

The method used in Paper I was slightly modified from a method by McIntosh and co-worker (Cartier et al, 1996). These were modifications in reaction times, volumes as well as different purification columns. Results from analytical HPLC and FAB-MS analysis confirmed the purity and the identity, respectively, of the synthesized αCtxMII and our αCtxPIA-analogue. Furthermore, co-injection of our in house-synthesized and in purchased αCtxMII showed an identical eluation profile and both peptides attenuated the ethanol-induced locomotor stimulation (Larsson et al, 2004; Paper I). Taken together, this indicates that we efficiently could synthesize the αCtxMII and its peptide analogues.

The αCtxPIA has been reported to have higher selectivity for the α_6* than for the $\alpha_3\beta_2$* and/or β_3* subunits (Dowell et al, 2003). In the present series of experiments an analogue to αCtxPIA was synthesized in which the *N*-terminal arginine had been removed whereas the remaining part including the central amino acids and the *C*-terminal of the peptide was left intact. It cannot be excluded that the synthesized αCtxPIA-analogue is different from the native αCtxPIA in subunit selectivity. However, this appears less likely since the nAChR subunit selectivity of 4/7 α-conotoxins has been reported to involve the amino acid residues in the central and the *C*-terminal part of the peptide, whereas the *N*-terminal appears to be of less importance (McIntosh et al, 1999; Arias and Blanton, 2000; Dutertre and Lewis, 2004; Everhart et al, 2004; Dutertre et al, 2005). Specifically, the bulky charged *N*-terminal protrusion of αCtxPIA has been suggested to be of less importance for its interaction with the α_6* subunit (Chi et al, 2005).

α-Conotoxin MII-sensitive receptors are involved in mediating the ethanol-induced locomotor stimulation (Paper I)

In Paper I it was showed that bilateral administration of our synthesized αCtxMII into the VTA significantly antagonized the ethanol-induced locomotor stimulation. This is in agreement with our previous experiments with purchased αCtxMII (Larsson et al, 2004). It is important to emphasize that only the acute effects of ethanol was investigated in the paper and this does not represent the neurochemical alterations observed in alcohol dependent patients. However, we have previously shown that VTA administration of αCtxMII reduces ethanol intake and preference in high alcohol-preferring mice and rats (Larsson et al, 2004). The dose (5 nmol) used in the present experiments may be high, and therefore unselective, raising the possibility that other subunits than the $\alpha_3\beta_2$*, β_3* and/or α_6* may be involved in mediating the behavioural and neurochemical effects of ethanol. In preliminary experiments we have found that administration of our synthesized αCtxMII into the VTA in a lower dose than previously used (2.5 pmol) also antagonizes ethanol-induced locomotor stimulation in mice (Fig. 10, unpublished data).

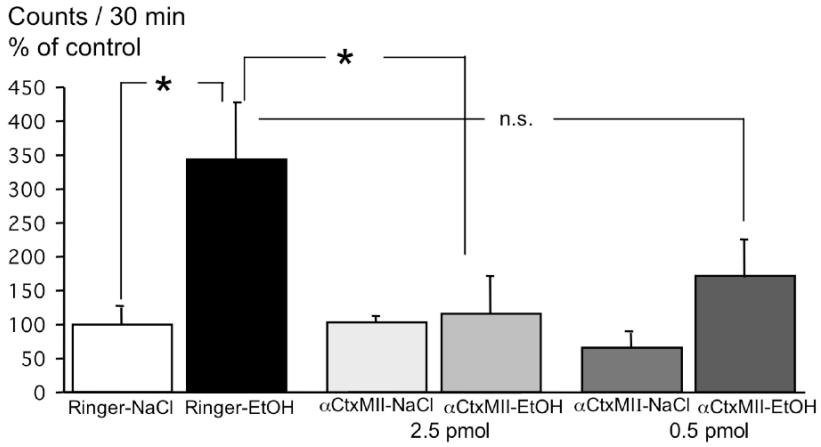


Fig. 10. A low dose of α-conotoxin MII (2.5 pmol in 1 µl), bilaterally administered into the ventral tegmental area antagonizes the ethanol-induced (EtOH, 1.75 g/kg, ip) locomotor stimulation in mice.
Shown are the mean values ± S.E.M. of 6 observations in each group. *$p<0.05$, Fisher's PLSD, following significant ANOVA.

A role of these subtypes in ethanol-induced reward is further supported by results demonstrating that infusion of a low dose of αCtxMII into the VTA (1) diminishes the conditioned reinforcing properties of alcohol cues (Löf, 2006) and (2) blocks the operant lever pressing for alcohol and the excessive alcohol consumption following a period of alcohol deprivation (Kuzmin, Jerlhag, Liljequist, Engel, unpublished data). In summary, this implies that αCtxMII-sensitive nAChRs, $\alpha_3\beta_2$*, β_3* and/or α_6* subtypes, are involved in mediating several different aspects of ethanol addiction.

α-Conotoxin PIA-analogue-sensitive receptors are neither involved in mediating the ethanol-induced locomotor stimulation nor accumbal dopamine overflow (Paper I)

Given that the different subunits can be expressed in various combinations in either heteromeric or homomeric receptors with different distribution patterns and functional roles (for review see Nicke et al, 2004), it is possible that particular subunit combinations of the nAChR may be involved in mediating the stimulatory and DA enhancing effects of ethanol. Interestingly, it has previously been demonstrated that the $\alpha_3\beta_2$*, β_3* and/or α_6* (using αCtxMII) rather than the $\alpha_4\beta_2$* or α_7* (using DHβE or MLA respectively) nAChRs are involved in mediating the stimulatory, rewarding and DA enhancing effects of ethanol (Larsson et al, 2002; Larsson et al, 2004). In Paper I we provided additional information on the functional role of the α_6* subtype. It was found that bilateral administration of our synthesized αCtxPIA-analogue (selective for the α_6* subtype) into the VTA neither antagonized the ethanol-induced locomotor stimulation nor the increase of DA in the N.Acc. (Fig. 11). Tus indicating that the stimulatory and DA enhancing effects of ethanol might be mediated via αCtxMII- rather than αCtxPIA-analogue-sensitive nAChRs in the VTA, *i.e.* $\alpha_3\beta_2$*, β_3* nAChRs.

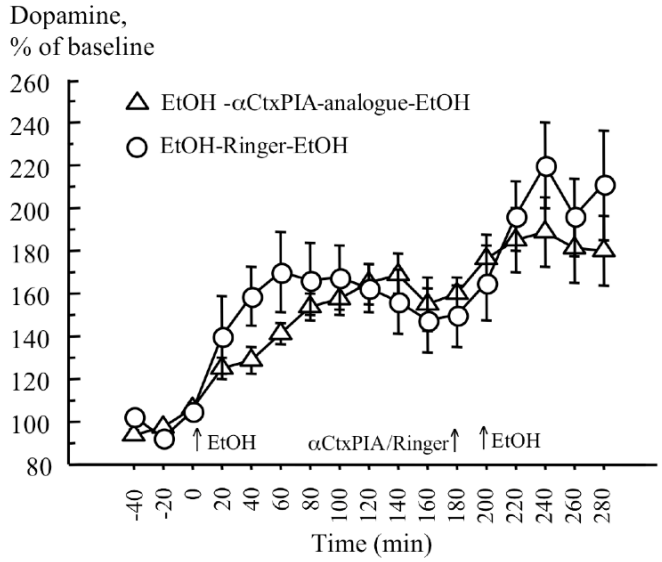


Fig. 11. Our in house-synthesized α-conotoxin PIA-analogue (5 nmol), unilaterally administered into the ventral tegmental area, does not antagonize the ethanol-induced (EtOH, 1.75 g/kg, ip) dopamine overflow in nucleus accumbens in mice measured by *in vivo* microdialysis.
Ethanol was administered the first time at 0 minutes, vehicle or α-conotoxin PIA-analogue was administered at 180 minutes. After 20 minutes, from the beginning of drug administration followed the second ethanol (EtOH, 1.75 g/kg, ip). Shown are mean values ± S.E.M.

In line with this hypothesis, the expression of the α_3 subunit by ventral tegmental DA neurons has been reported (Klink et al, 2001). Additionally, 18-methoxycoronaridine, selective for the $\alpha_3\beta_4$* nAChR, reduces ethanol intake in rats (Rezvani et al, 1997; Maisonneuve and Glick, 2003). Furthermore, MEC has been reported to have affinity for the $\alpha_3\beta_4$* and $\alpha_3\beta_2$* at least when expressed in *Xenopus* oocytes (Papke et al, 2001) and MEC antagonizes the stimulatory, rewarding and DA enhancing effects of ethanol (*e.g.* Blomqvist et al, 1997; Ericsson et al, 1998; Larsson et al, 2002). MLA may, in addition to the α_7, interact with the α_6 subunit (Vailati et al, 1999). When it is taken to account that MLA does neither antagonize ethanol-induced locomotor stimulation nor accumbal DA overflow (Larsson et al, 2002), it may be implied that the α_3* subtype, rather than the α_6* containing nAChRs, is involved in mediation of these effects of ethanol.

Considering that voluntary ethanol concomitantly increases ventral tegmental ACh and accumbal DA levels (Larsson et al, 2005) and that nAChRs in the VTA mediate the stimulatory, rewarding and DA enhancing effects of ethanol (Blomqvist et al, 1997; Ericsson et al, 1998; Larsson et al, 2002; Larsson et al, 2004), it is likely that ethanol activates the cholinergic (LDTg/PPTg)-dopaminergic (VTA) reward link. Natural rewards such as food has been shown to activate this link (Lanca et al, 2000; Rada et al, 2000; for review see Wise, 2002) and this prompted us to investigate the effects of the orexigenic peptide ghrelin on this system.

The orexigenic peptide ghrelin increases locomotor simulation and accumbal dopamine overflow in mice (Paper II and III)

Here we provide the first indication that the orexigenic peptide ghrelin activates the mesolimbic DA system, since administration of ghrelin into the third ventricle increases locomotor activity (Fig. 12A) and accumbal concentrations of DA (Fig. 12B).

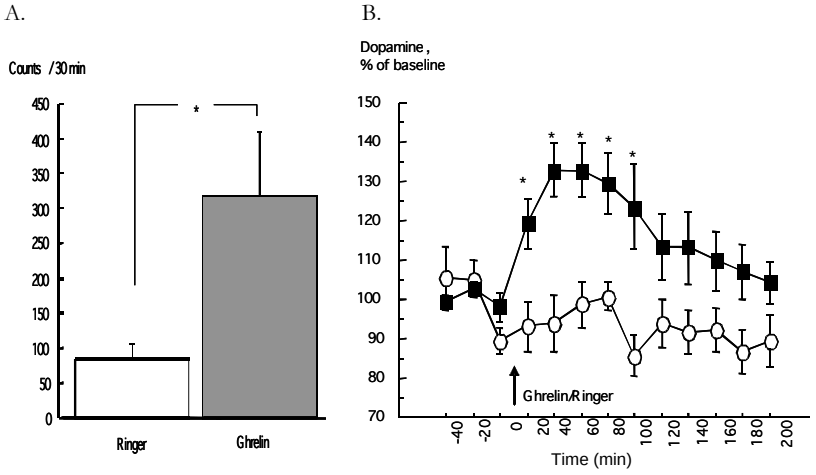


Fig. 12. Ghrelin (1μg in 1 μl), administered into the third ventricle, increases the locomotor activity (A) and accumbal dopamine overflow (B) in mice.
A. Data are presented as the accumulated counts during the entire 30 minute-period. Shown are the mean values ± S.E.M. of 7-8 observations in each group.
B. Ghrelin was administered at 10 minutes. Shown are mean values ± S.E.M. of 4-5 observations in each group. Filled squares represent ghrelin and unfilled circles represent vehicle treatment.
* $p<0.05$ Fisher PLSD, following significant ANOVA.

This suggests that ghrelin activates the DA reward systems and may constitute a part of the neurochemical overlap between the reward systems and the systems regulating energy balance. Experiments on hypothalamic synaptosomes have, however demonstrated that ghrelin do not modify the release of DA or NA but rather inhibits the 5-HT release (Brunetti et al, 2002). Even though this experimental design differs significantly from our it may be suggested that ghrelin has DA enhancing properties in the N.Acc. but not in the hypothalamus. In line with our hypothesis, systemic administration of ghrelin increases the *ex vivo* DA turnover in N.Acc. (Abizaid et al, 2006a). Additionally, icv administration of ghrelin markedly increased spontaneous locomotor activity in rats, an effect blocked by pre-treatment with the DA antagonist haloperidol (Jászberényi et al, 2006). Contrarily, locomotor activity has been reported to decrease upon icv ghrelin infusion (Tang-Christensen et al, 2004). This apparent inconsistency could be explained by differences in the availability of food. In Paper II, increased locomotor activity was observed following an acute icv ghrelin injection to mice without food present during the experiments, possibly leading to food-seeking (locomotor activation) behaviour. By contrast, *ad libitum* access to food during the study of Tang-Christiansen et al would be expected to lead to satiety and hence, less food-seeking and locomotor activity.

In rat and humans the GHS-R1As are expressed in the VTA as well as the LDTg (important reward nodes) (Guan et al, 1997) raising the possibility that the stimulatory and DA enhancing effects of ghrelin may involve activation of one or of both these sites. In fact, we have found that administration of ghrelin into the LDTg (Fig. 13A) or the VTA (Fig. 13B) increases locomotor activity and accumbal DA overflow in mice (Paper III).

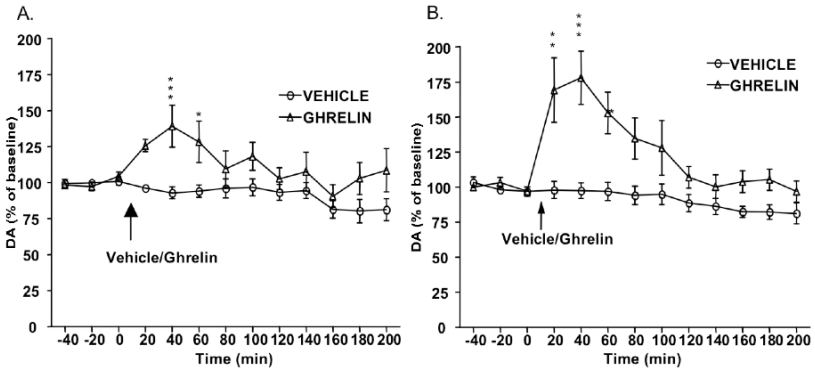


Fig. 13. Ghrelin (1 μg in 1 μl), administered into (A) the laterodorsal tegmental area or (B) ventral tegmental area, increases the extracellular concentration of dopamine in nucleus accumbens in mice measured by *in vivo* microdialysis.
Ghrelin was administered at 10 minutes. Shown are mean values ± S.E.M. $p < 0.001$, * $p<0.05$, ** $p<0.01$, *** $p<0.01$, Bonferroni post-hoc test, following a significant ANOVA.

It should be emphasized that ghrelin, in Paper III (and IV), was locally injected into the VTA and/or LDTg at a volume of 1μl, raising the concern that some solution might spread throughout the brain and thereby reach and activate other important areas such as the closely located PPTg. A role for PPTg in the rewarding and motivational properties of addictive drugs as well as in the rewarding properties of natural rewards has been found (for references see *Cholinergic regulation of the ventral tegmental area*). Additionally, in C57Bl mice the expression of GHSR-1A was observed in the PPTg, but not in the LDTg (Zigman et al, 2006). Even though the involvement of PPTg cannot be excluded, it appears less likely since microinfusions of ghrelin into regions dorsal, ventral and lateral LDTg were preformed (*i.e.* in animals where the guid cannula was misplaced). In these cases no effect of ghrelin on either locomotor activity or extracellular concentration of accumbal DA was observed.

The stimulatory and dopamine-enhancing effects of ghrelin are mediated via central nicotinic acetylcholine receptors (Paper II)

Taken together, the results from Paper II and III suggest that ghrelin activates the cholinergic-dopaminergic reward link. The fact that

ethanol also has been shown to activate this reward link (*e.g.* Larsson et al, 2005; Paper I), indicates neurochemical analogies between ethanol and ghrelin. To this end, we have investigated the role of nAChRs, *e.g.* those in the VTA, for the stimulatory and DA enhancing effects of ghrelin.

We have demonstrated (Paper II) that systemic pre-treatment with the unselective nicotinic antagonist, MEC, inhibits the stimulatory and DA enhancing effects of icv administration of ghrelin (Fig. 14), indicating that these effects of ghrelin are mediated via cholinergic mechanisms *e.g.* in the brain.

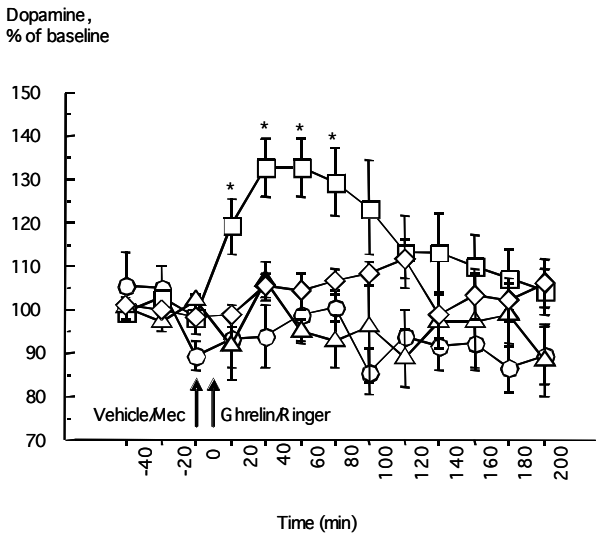


Fig. 14. Systemic administration of the unselective nicotine antagonist MEC (2.0 mg/kg) antagonizes the ghrelin (1μg in 1μl)-induced accumbal dopamine overflow in mice measure by *in vivo* microdialysis.
MEC was administered at 0 minutes and ghrelin at 10 minutes. Shown are mean values S.E.M. of 4-5 observations in each group. The squares represent vehicle-ghrelin, the circles represent vehicle-vehicle, the triangles mecamylamine-vehicle and rhomb represent mecamylamine-ghrelin treatment. $p<0.05$, unpaired t-test, following a significant ANOVA.

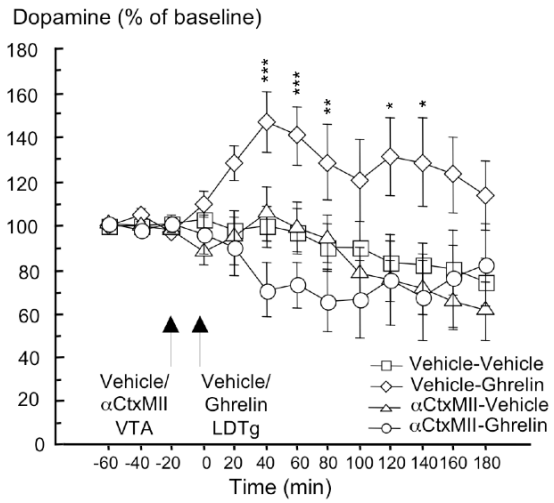
Further support for the involvement of cholinergic mechanisms is the results demonstrating that smoking or alcohol consumption in adolescent has been positively correlated to apparent eating patterns (*e.g.* Welsh and Fairburn, 1998; Wiseman et al, 1998; Anzengruber et al, 1996). Given the neurochemical analogy between ethanol and ghrelin

and that αCtxMII-sensitive receptors in the VTA appear to be involved in mediating the behavioural and neurochemical effects of ethanol (Larsson et al, 2004; Löf, 2006; Paper I), it may be implicated that these effects of ghrelin are mediated via similar nAChR subunits. This was further investigated in Paper IV.

The stimulatory and dopamine-enhancing effects of ghrelin are mediated via the $\alpha_3\beta_2$*, β_3* and/or α_6* rather than the $\alpha_4\beta_2$* or α_7* subtypes (Paper IV)

We showed (Paper IV) that infusion of the unselective nicotinic antagonist, MEC, into the VTA inhibits the stimulatory effects of ghrelin administered into the VTA or the LDTg. Further, neither systemic administration of DHβE (selective for the $\alpha_4\beta_2$* subunits) nor MLA (selective for the α_7* subunit), blocked the stimulatory effects of ghrelin infused into the VTA or the LDTg. Additionally, it was demonstrated that administration of αCtxMII (selective for the $\alpha_3\beta_2$*, β_3* and α_6* subunits), into the VTA antagonizes the stimulatory and DA enhancing effects of administration of ghrelin into either the LDTg (Fig. 15A) or the VTA (Fig. 15B). This suggests that the behavioural and neurochemical effects of ghrelin are mediated via αCtxMII-sensitive receptors, preferably the $\alpha_3\beta_2$*, β_3* and/or α_6*, rather than the $\alpha_4\beta_2$* and the α_7*, nAChRs in the VTA. This raises the possibility that ghrelin activates the cholinergic-dopaminergic reward link, via GHSR-1A in the LDTg as well as in the VTA.

A.



B.

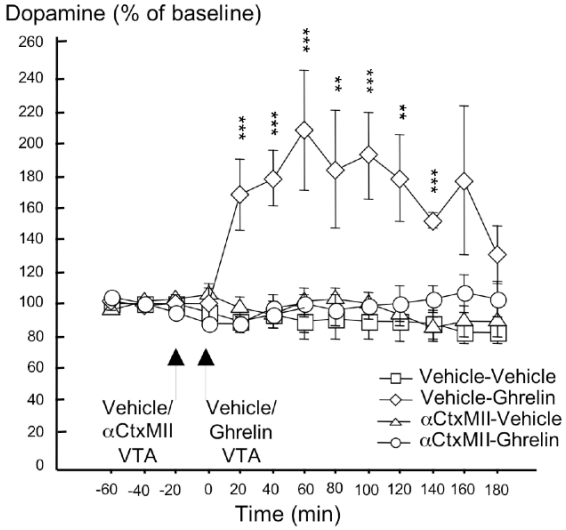


Fig. 15. α-conotoxin MII (50 pmol in 1 μl) antagonizes the stimulatory effects of ghrelin administered into the ventral or laterodorsal tegmental area
A. Unilateral administration of α-conotoxin MII (αCtxMII; 50 pmol in 1 μl) into the ventral tegmental area (VTA) antagonizes the stimulatory effects of ghrelin (2 μg in 1 μl) administered into the laterodorsal tegmental area (LDTg), on the extracellular concentration of dopamine (DA) in nucleus accumbens (N.Acc.) in mice measured by *in vivo* microdialysis. B. Unilateral administration of αCtxMII (50 pmol in 1 μl) into the VTA antagonizes the stimulatory effects of ghrelin (2 μg in 1 μl) administered into the VTA, on the extracellular concentration of DA in N.Acc. in mice measured by *in vivo* microdialysis.
αCtxMII was administered at -20 minutes and ghrelin at 0 minutes. Shown are mean values ± S.E.M. Two-way ANOVA showed statistically significant effects of treatment, ** $p<0.01$, *** $p<0.001$ Fisher post-hoc test.

The lack of effects of DHβE or MLA on ghrelin-induced locomotor stimulation are in line with the previous results on ethanol showing that neither DHβE nor MLA antagonized the ethanol-induced locomotor stimulation and accumbal DA overflow (Larsson et al, 2002) and that DHβE did not reduce ethanol consumption in rats in a limited access paradigm (Lê et al, 2000). However, it may be argued that the these drug do not pass the BBB or that the doses used were two low. The first possibility seems less likely since both DHβE (Bowman and Rand, 1980) and MLA (Turek et al, 1995) have been shown to penetrate the blood–brain barrier. Additionally, the $\alpha_4\beta_2$*

(using DHβE) and α_7* (using MLA) subunits have been demonstrated to play a critical role in behavioural and neurochemical effects of nicotine (see *e.g.* Stolerman et al, 1997; Schilström et al, 1998b; Larsson et al, 2002; Ericsson et al, 2003); thus indicating that the doses used are relevant and that the drugs pass the BBB. Moreover, by increasing the doses of either MLA or DHβE the subunit selectivity may be lost (see *e.g.* Buisson et al, 1996).

The stimulatory and DA enhancing effects of ethanol are mediated via $\alpha_3\beta_2$* and/or β_3*, rather then the $\alpha_4\beta_2$*, α_7* and α_6*, subtypes (Larson et al, 2002; Larsson et al, 2004; Paper I). Moreover, the ventral tegmental $\alpha_3\beta_2$*, β_3* and/or α_6*, rather then the $\alpha_4\beta_2$* and α_7*, subtypes appear to have a central role for ghrelin-induced locomotor stimulation and accumbal DA overflow. Given that both ethanol and ghrelin activate the cholinergic-dopaminergic reward link, neurochemical analogies between ghrelin and ethanol may be implicated. It may therefore be hypothesized that the $\alpha_3\beta_2$* and/or β_3*, rather than the α_6*, α_7* and $\alpha_4\beta_2$* nAChRs, may be a common denominator for the stimulatory and DA enhancing effects of ethanol as well as ghrelin.

Nicotine, but not ghrelin, displace the [3H]-nicotine binding in tissues from the ventral tegmental area (Paper IV)

Given that ghrelin into the VTA increases the locomotor activity and accumbal DA overflow (Paper III, IV) and that these effects are mediated via ventral tegmental nAChRs (Paper IV) the possibility that ghrelin interacts directly with nAChRs in the VTA and thereby activates the mesolimbic DA system should be raised. In the present paper we showed that nicotine, but not ghrelin, displaces the [3H]-nicotine binding in tissues form the VTA (Fig. 16), indicating that ghrelin does not interact directly with ventral tegmental nAChRs.

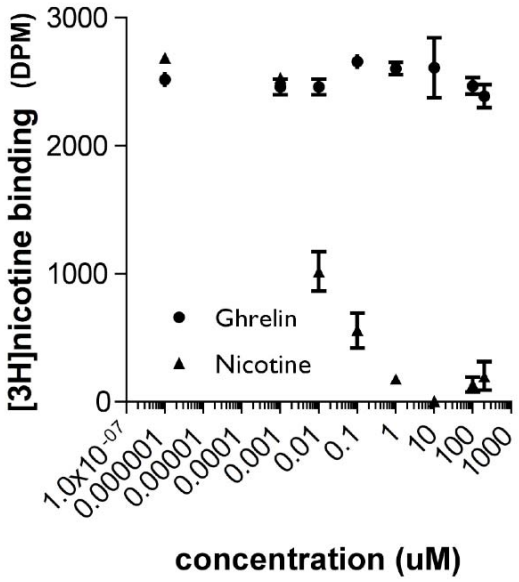


Fig. 16. Nicotine, but not ghrelin, displaces the binding of [3H]-nicotine in tissue from rat ventral tegmental area.
Data poled from two experiments.

In summary, the stimulatory and DA enhancing effects of ethanol as well as ghrelin appear to be mediated via αCtxMII-sensitive nAChRs, *e.g.* $\alpha_3\beta_2$* and/or β_3* subtypes, in the VTA. This further supports the existence of a neurochemical analogies between ethanol and ghrelin, *i.e.* their ability to activate the cholinergic-dopaminergic reward link.

Summary of results

- A slightly modified method for synthesizing αCtxMII peptide analogues was established.
- αCtxMII-sensitive, rather than α-CtxPIA-analogue-sensitive nAChRs, *i.e.* the $\alpha_3\beta_2$* and/or β_3*, rather than the subunits α_6*, in the VTA are involved in mediating the stimulatory and DA enhancing effects of ethanol in mice.
- Administration of the orexigenic peptide ghrelin increased locomotor activity and the extracellular concentration of DA in the N.Acc. in mice. These results indicate that ghrelin

activates the mesolimbic DA system (*i.e.* the reward systems) and is a part of the neurochemical overlap between the reward systems and those regulating energy balance.

- Administration of ghrelin into the VTA or LDTg (important brain reward nodes expressing GHSR-1A) increased the locomotor activity and accumbal DA overflow in mice, suggesting that ghrelin activates the cholinergic-dopaminergic reward link, probably via GHSR-1A in the VTA and LDTg. It is thus hypothesized that ghrelin may have incentive value for motivated behaviours such as meal initiation and/or food-seeking.
- Systemic administration of the unselective nicotinic antagonist, MEC, significantly inhibited the stimulatory and DA enhancing effects of ghrelin (icv), implying that cholinergic mechanism in the brain, *i.e.* nAChRs, are involved in mediating these behavioural and neurochemical effects and that ghrelin activates the cholinergic-dopaminergic reward link.
- Infusion of the unselective nicotinic antagonist, MEC, into the VTA inhibited the stimulatory effects of ghrelin injected into the VTA or the LDTg. However, neither systemic administration of DHβE (selective for the $\alpha_4\beta_2$* subunits) nor MLA (selective for the α_7* subunit), blocked the stimulatory effects of ghrelin infused into VTA or the LDTg, indicating that ventral tegmental nAChR, but not the $\alpha_4\beta_2$* or the α_7* subunits, appear to be involved in mediating the stimulatory effects of ghrelin.
- Administration of αCtxMII (selective for the $\alpha_3\beta_2$*, β_3* and α_6* subunits), into the VTA antagonized the stimulatory and accumbal DA enhancing effects of ghrelin injected into the VTA or the LDTg. Neurochemical analogies between ethanol and ghrelin can be implicated, suggesting that the behavioural and neurochemical effects of ghrelin, as well as ethanol, are mediated via αCtxMII-sensitive receptors, preferably the $\alpha_3\beta_2$* and/or β_3* subtypes, in the VTA.
- In a radioligand-binding assay ghrelin does not displace the binding of [3H]-nicotine in tissue from the VTA, suggesting that ghrelin does not interact directly with ventral tegmental nAChRs.

General discussion

Ethanol and the cholinergic-dopaminergic reward link

The stimulatory and DA enhancing effects of ethanol were found to be mediated via αCtxMII-sensitive, rather than αCtxPIA-analogue-sensitive, nAChRs in the VTA (Paper I). From the present experiments it cannot be determined whether these effects of ethanol are mediated by direct and/or indirect mechanisms in the VTA. Considering the first possibility, electrophysiological studies have suggested that ethanol acts as a co-agonist to ACh on nAChRs (Forman et al, 1989; Wu et al, 1994; Forman and Zhou, 1999; Marszalec et al, 1999). This effect is dependent on the α-subunit (Zou et al, 2002), implying that ethanol may interact directly with nAChRs, possibly the α_3 subunit, on dopaminergic cell bodies in the VTA. However, this appears less likely since it has been found that acute ethanol application has no effect at least on human recombinant $\alpha_3\beta_2$* and $\alpha_3\beta_4$* nAChRs expressed in oocytes (Cardoso et al, 1999). Considering that rats demonstrate a concomitant increase in ventral tegmental ACh and accumbal DA overflow during voluntary ethanol consumption (Larsson et al, 2005), and that nAChR in the VTA mediate the stimulatory, rewarding and DA enhancing effects of ethanol (*e.g.* Blomqvist et al, 1997; Ericsson et al, 1998; Larsson et al, 2002; Larsson et al, 2004; Paper I), it may be inferred that ethanol activates the cholinergic-dopaminergic reward link. More specifically, ethanol may, via activation of the mesopontine cholinergic input to the VTA, cause release of ACh in the VTA and thereby, via $\alpha_3\beta_2$* and/or β_3* nAChRs, excite the mesolimbic DA system. Additionally, administration of MEC into the VTA blocks the increased accumbal DA caused by anticipation of ethanol (Löf, 2006), implying that alcohol seeking behaviour may also involve the cholinergic projections to the VTA.

In all probability, ethanol may activate the mesolimbic DA system at several different levels, such as the N.Acc., the VTA and the LDTg. Reverse microdialysis of ethanol into the N.Acc. increases DA overflow in the N.Acc. (Ericson et al, 2003; Tuomianen et al, 2003, Löf et al, 2007b). However, ethanol-induced DA overflow in the N.Acc. is not directly related to the concentration of ethanol that

reaches the N.Acc. (Doyon et al, 2003; Löf et al 2007a). On the other hand, ethanol infusion into the anterior VTA does not affect accumbal DA levels (Ericson et al, 2003; Tuomianen et al, 2003; Löf et al, 2007b). The VTA is nonetheless a heterogeneous brain structure, which integrates various afferents from several brain regions. The anterior versus the posterior part of the VTA differ in dopaminergic cell morphology, topography of their efferent projections, and presumably in function. In support of this hypothesis, it has been demonstrated that caudoventral DA neurons are more active during the rat's active dark period, whereas rostrodorsal DA neurons are active during the light period (Luo and Aston-Jones, 2006). Additionally, we have found that perfusion of ethanol into the posterior VTA, but not into the anterior VTA, dose-dependently increased the extracellular levels of DA in N.Acc. (unpublished data). Similarly, rats voluntarily self-administer ethanol, cholinergic agonists or acetaldehyde into the posterior, but not anterior, part of the VTA (Gatto et al, 1994; Rodd-Henricks et al, 2000; Ikemoto and Wise, 2002; Rodd et al, 2004; Rodd et al, 2005). Co-administration of ethanol with the DA D_2 receptor agonist quinpirole, which selectively inactivates dopaminergic neurons, into the posterior, but not anterior, VTA extinguished the maintenance of ethanol self-infusion (Rodd et al, 2004). Furthermore, administration of quinpirole into the posteriomedial, but not the interolateral, VTA disrupted condition place preference induced by food rewards, whereas no effect on food intake *per se* was observed (Liu and Ikemoto, 2006). It may therefore be suggested that dopaminergic neurons in the posteriomedial, rather than anterior, part of the VTA are involved in the mediation of natural as well as drug-induced reinforcement.

Ghrelin and the cholinergic-dopaminergic reward link

The present thesis shows that ghrelin stimulates the cholinergic-dopaminergic reward link via GHSR-1A in the VTA and/or LDTg. With regard to the possibility of ghrelin interactions in the LDTg, we suggest that ghrelin, via GHSR-1A in the LDTg, activates the cholinergic input to the VTA, causing a release of ACh which stimulates ventral tegmental nAChRs, preferentially of the $\alpha_3\beta_2$* and/or β_3* subtypes, thereby increasing mesoaccumbal DA levels and locomotor activity. However, this remains to be verified by *e.g.* measuring ACh release in the VTA after ghrelin administration into

the LDTg. In fact, the cholinergic LDTg projection to the VTA has been found to be involved in the regulation of the activity of the mesolimbic DA system as measured by locomotor stimulation and accumbal DA overflow. Thus, lesions of the LDTg blunt the stimulatory effects of nicotine (Alderson et al, 2005) as well as amphetamine-induced locomotor stimulation and increased accumbal DA overflow (Forster et al, 2002). Further support for this idea is provided by the finding that MEC antagonizes cocaine self-administration (Levin et al, 2000) as well as cue-elicited cocaine craving in humans (Reid et al, 1999).

In addition to GHSR-1A in the LDTg, we found that GHSR-1A in the VTA may be involved in the effects of ghrelin on the cholinergic-dopaminergic reward link. Double-labelling studies in rats and mice have demonstrated both pre- and post-synaptic GHSR-1A in the VTA (Abizaid et al, 2006a). From the present series of experiments it cannot be determined whether pre- and/or post-synaptic GHSR-1As are involved. Ghrelin may act on pre-synaptic GHSR-1A situated on cholinergic or other excitatory *e.g.* the glutamatergic afferents. Thus, the competitive glutamate antagonist AP5 blocks the ghrelin-induced DA-firing in VTA slices (Abizaid et al, 2006b). However, the possibility that ghrelin interacts with post-synaptic GHSR-1A located on the DA cell bodies should also be taken into consideration (Abizaid et al, 2006a). With regards to this possibility, it should be emphasized that functional cholinergic projections to the VTA appear to be essential for the ability of drugs of abuse to activate the mesolimbic DA system (Reid et al, 1999; Levin et al, 2000; Forster et al, 2002; Alderson et al, 2005).

Ghrelin stimulates a food-seeking behaviour

Ghrelin increases food intake (Kamegai et al, 2001; Wren et al, 2001b; Theander-Carrillo et al, 2006) and stimulates food-seeking behaviour (Paper II; Paper III; Paper IV), effects possibly mediated via different neuronal systems. The ghrelin-induced feeding has been found to depend on GHSR-1A in the arcuate nucleus (Wren et al, 2001b; Rüter et al, 2003) and is likely to be mediated via NPY/AgRP projections (Kamegai et al, 2001; Chen et al, 2004). Additionally, GH-receptors (Egecioglu et al, 2006), orexin (Toshinai et al, 2003), POMC (Cowley et al, 2003) and MC containing (Chen et al, 2004) neurons seem to be involved. Nevertheless, a role of ghrelin in hunger, meal initiation (*e.g.*

Arvat et al, 2000; Tschöp et al, 2000; Horvath et al, 2001; Nakazato et al, 2001; Schmid et al, 2005) and foraging (Keen-Rhinehart and Bartness, 2004) has been suggested. We propose that ghrelin stimulates food-seeking behaviour via activation of the cholinergic-dopaminergic reward link. In fact, accumbal DA is involved in mediating the willingness of an animal to engage in motivated behaviours, *e.g.* foraging (for review see Berridge, 1996; Berridge and Robinson, 1998; Kringelbach, 2004; Kalivas and Volkow 2005). It is therefore conceivable that ghrelin has, at least in situations without food, incentive value for goal-directed behaviours such as food-seeking behaviour.
Additionally, GHSR-1A in the VTA, via activation of the mesolimbic DA system, may regulate elemental feeding behaviour (Naleid et al, 2005; Abizaid et al, 2006a). Furthermore, our preliminary data show that food presentation reduces ghrelin (icv)-induced locomotor stimulation and increases food intake (unpublished data). It may therefore be suggested that ghrelin induces foraging in animals without access to food, whereas animals presented with food choose to consume rather than seek food. Thus, centrally acting ghrelin appears to regulate energy homeostasis by increasing food intake via the arcuate nucleus, and by stimulating food-seeking behaviour via the cholinergic-dopaminergic reward link (Fig. 17).

The DA reward systems are interconnected with a number of brain regions, which may be involved in the present effects. One possibility is that ghrelin induces foraging via projections between the cholinergic-dopaminergic reward link and the lateral hypothalamus. Orexin containing projections from the lateral hypothalamus to the VTA have been demonstrated to regulate the activity of ventral tegmental DA neurons (Fadel and Deutch, 2002) and they are essential for the acquisition of morphine-induced condition place preference (Wimmer et al, 2006). Additionally, orexin in the VTA is involved in the mediation of reinstatement of cocaine or morphine seeking as well as in the development of cocaine-mediated behavioural sensitization (Boutrel et al, 2005; Harris et al, 2005; Borgland et al, 2006). It is therefore plausible that ghrelin, via orexin projections to the VTA, activates the mesolimbic DA system and causes food-seeking behaviour, thus integrating functions of homeostatic and hedonic sites (Fig. 17). However, this needs to be further elucidated, *e.g.* by studying the effects of ghrelin into the arcuate nucleus on food-seeking behaviour. Furthermore, chronic food deprivation in rats induces

weight loss (Pothos et al, 1995) and increases drug-seeking behaviour (Carroll et al, 1979; Carroll and Stoltz, 1983). Food restriction enhances the plasma levels of ghrelin (Gualillo et al, 2002), implying that ghrelin may induce drug-seeking behaviour via activation of the cholinergic-dopaminergic reward link. Further support for a role of ghrelin in drug-seeking behaviour may be derived from the recent data showing a correlation between serum ghrelin levels and cocaine-seeking behaviour (Tessari et al, 2007).

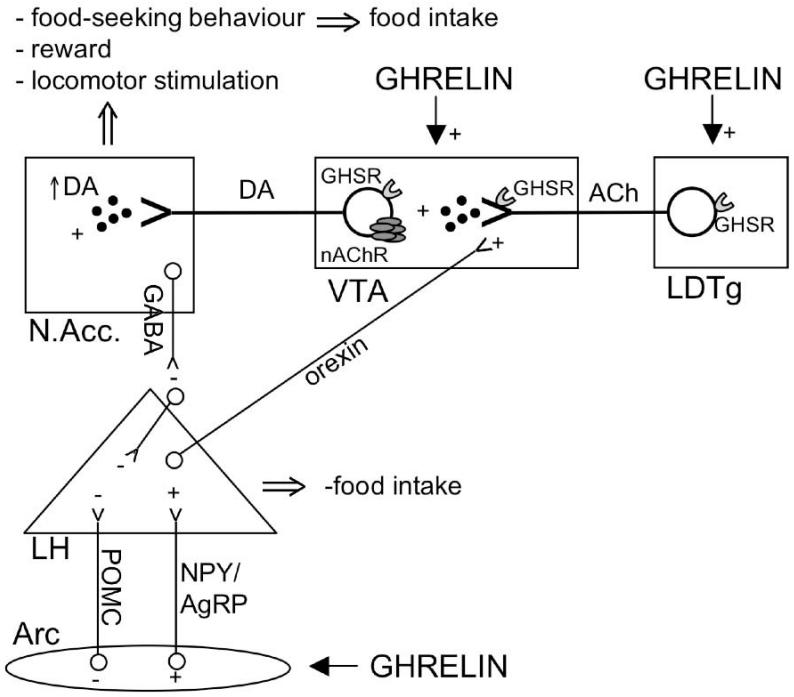


Fig. 17. Ghrelin may regulate food-seeking behaviour and food intake via different neuronal systems.
ACh, acetylcholine; AgRP, agouti-related peptide; DA, dopamine; GABA; gamma-aminobutyric acid; NPY, neuropeptide Y; POMC, proopiomelanocortin; LDTg, laterodorsal tegmental area; VTA, ventral tegmental area; N.Acc., nucleus accumbens, LH, lateral hypothalamus; Arc, arcuate nucleus; GHSR, growth hormone secretagogue receptor; nAChR, nicotinic ACh receptor.

Ghrelin and compulsive overeating

If feeding was controlled solely by homeostatic mechanisms, our ideal body weight would be easily maintained (for review see Saper et al, 2002). However, many anorexigenic/orexigenic peptides, including ghrelin, are also integrated in the reward systems (Thiele et al, 2003; Thiele et al, 2004; Paper II Paper III; Paper IV), intrinsically reinforcing "non-homeostatic" food-consumption (for review see Saper et al, 2002) and thereby increasing the complexity of body weight regulation. Peripheral hyperghrelinemia is associated with binge eating in bulimic/anorectic subjects (Tanaka et al, 2003a) and hyperphagia in Prader-Willi patients (Cummings et al, 2002a; for review see Chanoine 2005). Additionally, high ghrelin protein levels, as well as mRNA expression in the hypothalamus, have been demonstrated in patients with obesity (Couce et al, 2006), implying that peripheral and/or central hyperghrelinemia might be involved in the pathophysiology of compulsive overeating in anorexia/bulimia /Prader-Willi and/or in obesity respectively. When taken into account that compulsive overeating has been associated with aberrations in the central DA system (*e.g.* Volkow et al, 2003a; Shapira et al, 2005) and that ghrelin activates the mesolimbic DA system (Naleid et al, 2005; Paper II; Abizaid et al, 2006a; Paper III; Paper IV), it might be suggested that a ghrelin dysbalance/dysfunction causes abnormalities in the mesolimbic DA system and thereby induces compulsive overeating (*i.e.* addictive behaviour).

Nicotine, amphetamine and cocaine all induce satiety (Hollister, 1971; Grunberg et al, 1986; Mark et al, 1999), whereas ethanol (Hollister, 1971; Yeomans et al, 1999) and ghrelin (*e.g.* Arvat et al, 2000) increase appetite and hunger. Satiety has been suggested to be associated with increased ACh (Rada et al, 2005), together with high DA (Rada et al, 2001) levels in N.Acc.. These effects have been observed after food intake (*e.g.* Hernandez and Hoebel, 1988; Mark et al, 1992) as well as after nicotine, amphetamine or cocaine administration (Di Chiara and Imperato, 1988; Lindefors et al, 1992; Mark et al, 1999; Rada et al, 2001). On the other hand, hypothalamic infusion of the orexigenic peptide galanin decreases accumbal ACh and increases accumbal DA (Rada et al, 1998). In contrast to this, ethanol has no effect on ACh in the N.Acc. *in vivo* (Rada et al, 2004b). Given the neurochemical analogies between ethanol and ghrelin, it may be speculated that ghrelin has no effect on accumbal ACh and thus does not induce

satiety. However, this needs to be further investigated. In addition, a role for 5-HT in satiety has been suggested (Gamaro et al, 2003). Thus, drugs that enhance 5-HT in the dorsal raphe reduce food intake, whereas drugs that inhibit the release of 5-HT stimulates food intake (Ohliger-Frerking et al, 2002). The ghrelin-induced feeding may thus partly be due to its ability to reduce the release of 5-HT in hypothalamic synaptosomes (Brunetti et al, 2002). It is also plausible that satiety, food intake and food-seeking behaviour are mediated via separate neuronal systems in the brain.

Ghrelin and drugs of abuse

An association between drugs of abuse and ghrelin has been shown. Smoking or methamphetamine administration acutely increases the plasma levels of ghrelin (Bouros et al, 2006; Fagerberg et al, 2003; Crowley et al, 2005). Further, systemic ghrelin enhances cocaine-induced locomotor stimulation as well as condition place preference in rats (Wellman et al, 2005; Davis et al, 2007). High plasma ghrelin levels have been positively linked to craving during ethanol withdrawal (Addolorato et al, 2006), preferably in patients of Lesh's type 1 (Hillemacher et al, 2007). Individuals with high craving rates are more likely to relapse, implicating that high ghrelin levels during ethanol withdrawal may be involved in craving and thus cause relapse. An increased desire for sweet-tasting and high caloric food, have been shown to occur as a consequence of withdrawal in human alcoholics (for review see Kampov-Polevoy, 1999; Junghanns et al, 2000), especially in those with a family history of alcoholism (Jughanns et al, 2005). High ghrelin levels in ethanol withdrawal (Kim et al, 2005) may cause food-seeking behaviour and thus increase the intake of high caloric food. The possibility that patients compensate for the lack of drug-induced reward by increasing their consumption of other rewards such as sucrose cannot be excluded (*e.g.* Junghanns et al, 2000). However, it should be considered that several substrates collectively enhance the desire to consume sweets during withdrawal.

The plasma levels of ghrelin are elevated in alcohol dependent patients (Kim et al, 2005; Kraus et al, 2005), suggesting that ghrelin may be involved in the mediation of ethanol consumption and/or seeking. We recently conducted preliminary experiments showing that icv administration of ghrelin increases ethanol intake (Fig. 18) and preference and decreases water intake in a free choice limited access

paradigm in mice. The ghrelin system may thus constitute a novel potential target for future treatment of alcoholism.

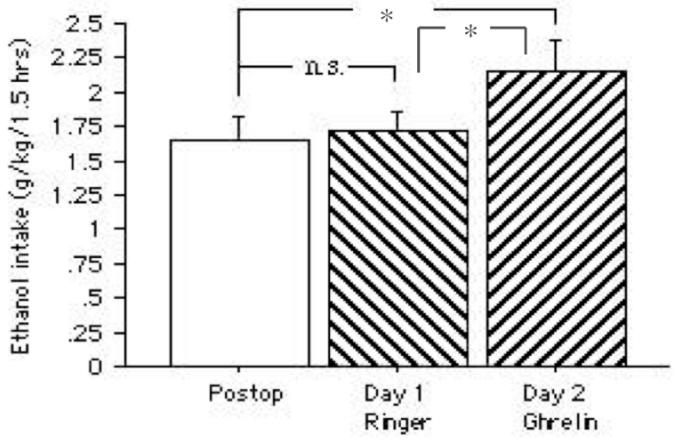


Fig. 18. The effects of ghrelin (2 µg) or an equal volume (1 µl) of vehicle solution administered into the third ventricle on ethanol intake in a limited access paradigm.
Ghrelin significantly increases the ethanol intake compared to baseline (postop) as well as vehicle solution. $p < 0.05$, paired t-test.

Given that ghrelin modulates ethanol intake it may be implied that this may be due to the high caloric properties of ethanol and that mice consume calories from ethanol instead of food. However, alcohol drinking acutely attenuates circulating ghrelin levels (Calissendorff et al, 2005; Zimmermann et al, 2007), an effect more pronounced than what would be expected from the calories ingested with alcohol (Calissendorff et al, 2006). Additionally, there is minimal evidence for any compensatory reduction in food intake in response to energy ingested as alcohol (for review see Yeomans, 2004). Alternatively, ghrelin could, via activation of the HPA-axis, augment the sensitivity of the mesolimbic DA system and thereby increase the ethanol intake. Intriguingly, ghrelin induces a prominent activation of the HPA axis (for review see Otto et al, 2005) such as elevating the plasma concentration of corticosterone (Jászberényi et al, 2006). It has previously been demonstrated that high plasma levels of corticosterone enhances DA release in the N.Acc. (Gianoulakis, 1998) and increases ethanol consumption (Fahlke et al, 1994; Hansen et al, 1994; Brady and Sonne, 1999).

Muscarinic acetylcholine receptors

In addition to nAChR, ventral tegmental mAChRs appear to be involved in the ability of the LDTg to activate the DA neurons in the VTA (for review see Winn et al, 1997) and in natural reinforcement (Yeomans and Baptista, 1997; Rada et al, 2000; Sharf et al, 2006) and could possibly be involved in mediating the stimulatory and DA enhancing effects of ethanol as well as ghrelin. Our preliminary results show that the mAChR antagonist p-fluoro-hexaydro-silia-difendiol hydrochloride (selective for the M3/M5 subtypes), administered into the VTA reduces ethanol-induced locomotor stimulation. Furthermore, Western immunoblotting experiments demonstrate that the M5 subtype is expressed at approximately 20 % higher frequency in ethanol high-preferring than in low-preferring rats (unpublished data). The muscarinic antagonist, atropine, has further been found to decrease ethanol intake in rats (*e.g.* Wahlström and Nordberg, 1992). On the other hand, systemic injection of the unselective muscarinic antagonist, scopolamine, does not block the ghrelin-induced (icv) locomotor stimulation (unpublished data). Thus, mAChRs may also be involved in mediating the stimulatory effects of ethanol, but not of ghrelin, indicating that there are some differences in mode of action of the two substances. Furthermore, it should be emphasized that several other neurotransmitters besides DA, such as GABA, 5-HT, glutamate and opioid peptides, collectively orchestrate the rewarding properties of ethanol (see *e.g.* Engel et al, 1992), and possibly of ghrelin. Needless to say, other areas in the brain may also be involved in mediating the reinforcing properties of addictive drugs and natural rewards. A role for the lateral hypothalamus in brain reward has previously been suggested as rats self-administer electro-stimulation (Olds, 1958) or morphine (Olds, 1979) into this area. Similarly, it has been demonstrated that hypothalamic electro-stimulation (Hernandez and Hoebel, 1988; Phillips et al, 1989) increases accumbal DA. Additionally, areas such as amygdala, hippocampus, orbifrontal cortex and dorsal striatum may have an important role in brain reward and drug-seeking behaviour (Volkow and Fowler, 2000; Volkow et al, 2006; Zhao et al, 2006).

Neurochemical analogies

The $\alpha_3\beta_2$* and/or β_3*, rather than the α_6*, α_7* and $\alpha_4\beta_2$* nAChRs, may be a common denominator for the stimulatory and DA enhancing

effects of ethanol as well as ghrelin (see *Results and discussion*), implying neurochemical analogies. It should be considered that the αCtxMII-sensitive nAChRs, *e.g.* $\alpha_3\beta_2$* and/or β_3* subtypes, might serve as novel pharmacological targets for development of treatment strategies for addictive behaviours, such as binge eating and alcoholism. Interestingly, it has been shown that MEC reduces the positive, *e.g.* stimulant and euphoric effects of ethanol drinking in healthy volunteers (Blomqvist et al, 2002; Chi and de Wit, 2003), but given that MEC is an unselective nicotinic antagonist it is not surprising to note that the number of side effects is large. On the other hand a selective antagonist at the $\alpha_3\beta_2$* and/or β_3* nAChRs, may be more efficient and may produce less side effects. However, αCtxMII is a 16 amino acid peptide and is unlikely to pass the BBB thereby excluding it as a useful pharmaceutical agent. However, by elucidating the structure-activity relationship for αCtxMII, *e.g.* by an alanine walk, a smaller compound with similar subunit selectivity could be developed.

In summary, the present thesis suggests that both ethanol and ghrelin activate the cholinergic-dopaminergic reward link, implying neurochemical analogies. Specifically, we hypothesize that ethanol via the PPTg/LDTg and/or VTA stimulates the cholinergic projections to the VTA. We also suggest that ghrelin activates the cholinergic LDTg-VTA projection via GHSR-1A in the LDTg as well as in the VTA. This causes a release of ACh which activates ventral tegmental nAChRs, preferably the $\alpha_3\beta_2$* and/or β_3* subtypes, and thereby increases mesoaccumbal DA levels and locomotor activity. The DA enhancing properties of ethanol may represent the rewarding feelings of ethanol. Moreover, the ghrelin-induced accumbal DA overflow causes locomotor stimulation, which may reflect a rewarding food-seeking behaviour (Fig. 19).

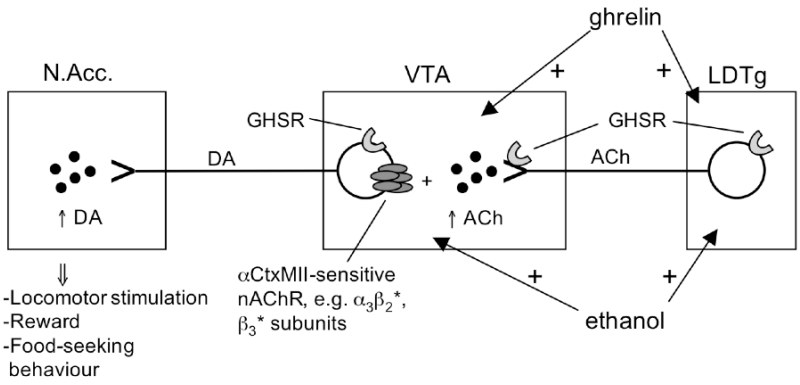


Fig. 19. Ethanol as well as ghrelin activate the cholinergic LDTg-VTA projection and thereby cause an increase in ventral tegmental ACh. This activates the mesolimbic DA system via αCtxMII-sensitive receptors (*e.g.* $\alpha_3\beta_2$* and/or β_3* subtypes) resulting in an accumbal DA release, which causes a locomotor stimulation, reward and/or food-seeking and drug-seeking behaviour.

Ach, acetylcholine; DA, dopamine; LDTg, laterodorsal tegmental area; VTA, ventral tegmental area; N.Acc., nucleus accumbens; GHSR, growth hormone secretagogue receptor; nAChR, nicotinic ACh receptor.

Concluding remarks

Patients with alcoholism or aberrant eating patterns can be divided into defined subtypes, characterized by specific phenotypes such as the restrictive and binge eating types. Furthermore, bingeing individuals have higher scores for nicotine dependence and are more likely to smoke than their non-bingeing counterparts. Different pathophysiological mechanisms may in all probability underlie the characteristics of these various subtypes, *e.g.* ghrelin correlates with craving in Lesch's type 1, but not type 2. Conceivably, the treatment strategies should be adjusted depending on the neurobiological basis of the patient's subtype.

Results presented in this thesis suggest that ghrelin and ethanol activates the cholinergic-dopaminergic reward link. Moreover, αCtxMII-sensitive ($\alpha_3\beta_2$* and/or β_3*) nAChRs may mediate the behavioural and neurochemical effects of ethanol as well as of ghrelin. We hypothesize that these mechanisms also are involved in alcoholism and compulsive overeating. It is therefore intriguing to speculate that $\alpha_3\beta_2$* and/or β_3* containing nAChRs may serve as potential novel pharmacological targets for treatment of some subtypes of alcoholism and deviant eating behaviours.

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Swedish summary

På 1950-talet upptäckte Olds och Milner att råttor självadministrerade elektrostimulering till vissa områden i hjärnan men inte till andra. Dessa djur slutade fokusera på naturliga belöningar såsom mat, och sex; de hade blivit elberoende. Områden involverade i detta fenomen identifierades och kom att kallas för hjärnans belöningssystem. Forskning har därefter visat att dessa system förmedlar de belönande egenskaperna hos naturliga belöningar, såsom mat, samt hos beroendeframkallande droger, t.ex. alkohol. Det har också demonstrerats att dessa system är viktiga för att förstärka och öka motivationen för inlärning av beteenden som i förlängningen ökar individens överlevnad, såsom att söka efter mat. En viktig del av hjärnans belöningssystem är det mesolimbiska dopaminsystemet. Detta består av dopaminneuron löpande från ventrala tegmentala arean (VTA) till nucleus accumbens (N.Acc.). Vidare verkar även de kolinerga projektionerna till VTA vara viktiga för de belönande egenskaperna hos droger och hos naturliga belöningar. Den idag kända kolinerga projektionen till VTA utgår framförallt från laterala dorsala tegmentala arean (LDTg) och den har föreslagits vara en viktig komponent i hjärnans belöningssystem. Tillsammans kallar vi detta för den kolinerga-dopaminerga belöningslänken (Fig. 20). Vid en obalans/dysfunktion i belöningssystemen kan ”addictive behaviours”, så som drogberoende, ätstörningar, sex- och spelmissbruk, utvecklas.

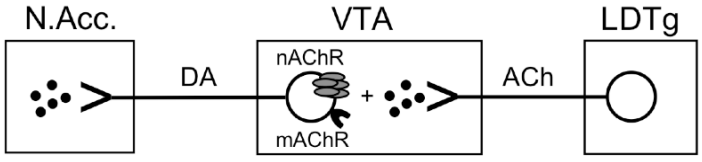

Fig. 20. Den kolinerga-dopaminerga belöningslänken.
Den kolinerga-dopaminerga belöningslänken består av en kolinerg projektion från laterodorsal tegmentala arean (LDTg) till ventrala tegmentala arean (VTA) och därefter det mesolimbiska dopaminsystemet, d.v.s. dopaminprojektionen från VTA till nucleus accumbens (N.Acc.). Aktivering av LDTg orsakar en frisättning av acetylkolin i VTA, vilket via påverkan av nikotinreceptorer och/eller muskarinreceptorer stimulerar det mesolimbiska dopaminsystemet och orsakar en frisättning av dopamin i N.Acc..

Alkoholism är idag ett stort samhällsproblem och de alkoholrelaterade kostnaderna i Sverige uppskattas till tiotals miljarder kronor per år. Ett

äldre (Antabus®) och två nyare läkemedel (Campral® och Revia®) är idag godkända för behandling av alkoholberoende. Det har i kliniska studier visats att dessa dock inte fungerar optimalt och ett stort behov för ytterligare behandlingsstrategier finns. Genom att öka förståelsen av de mekanismer som är involverade i alkoholberoende kan nyare och bättre läkemedel utvecklas, vilket var ett av målen med denna avhandling.

Tidigare forskning har visat att alkoholens stimulerande och belönande egenskaper är förmedlade via det mesolimbiska dopaminsystemet, vilket delvis representeras av en dopaminökning i N.Acc.. Verkningsmekanismen för alkohol är relativt okänd, men det har visats att alkohol interagerar med ligandstyrda jonkanaler i hjärnan, exempelvis nikotinreceptorn. Intressant nog, har det visat sig att det finns ett samband mellan alkohol- och nikotinintag bland alkoholister samt i normalpopulationen. Man har bland annat funnit att 80-90% av alla alkoholister också är storrökare och att alkoholism är cirka 10 gånger mera vanligt hos rökare än icke-rökare. Dessutom har studier på friska frivilliga visat att alkohol förstärker nikotinets belönande egenskaper samt att en oselektiv nikotinreceptorblockerare (mekamylamin) minskar de positivt förstärkande effekterna av alkohol. Vår forskargrupp har tidigare visat att kronisk tillförsel av alkohol förändrade nikotininbindningen i hjärnan samt att nikotinreceptorer, framför allt de som finns i VTA, är involverade i att förmedla alkoholens stimulerande, dopaminökande och belönande egenskaper.

Nikotinreceptorn består av fem stycken subenheter, som sitter ihop i en ringformation. Det finns många subenheter såsom α, β och γ, vilka i sin tur finns i olika former (t.ex. α3-α10 och β2-β4). De olika subenheterna kan kombineras på många olika sätt och därmed bilda så kallade subtyper av nikotinreceptorn. Eftersom de olika subtyperna av nikotinreceptorn har olika funktion, postulerar vi att vissa, men inte andra, subtyper är involverade i alkoholens belöningsprofil. Vi har därför tidigare genomfört en serie försök där vi med hjälp av olika nikotinreceptorblockerare, med selektivitet för olika subtyper, utrett vilken sammansättning av nikotinreceptorn som är av störst betydelse för alkoholens stimulerande, belönande och dopaminökande egenskaper. I tidiga försök har vi funnit att nikotinreceptorns $\alpha_3\beta_2$*, β_3* och/eller α_6* (genom att använda α-conotoxin MII), men inte $\alpha_4\beta_2$* (genom att använda dihydro-β-erythroidine) eller α_7* (genom att använda methyllycaconitine) subenheter är involverade i alkoholens ovan nämnda effekter.

I delarbete I ville vi undersöka vilka av de ovannämnda subenheterna ($\alpha_3\beta_2$*, β_3* och/eller α_6*) som är involverade i alkoholens stimulerande och dopaminökande egenskaper. I ett samarbete med läkemedelskemisterna professor Kristina Luthman och Morten Grøtli på Naturvetenskapliga fakulteten på Göteborgs universitet har vi utvecklat en metod för att göra nikotinreceptorblockerare med olika subenhetselektivitet. Först tillverkade vi nikotinreceptorblockeraren α-conotoxin MII, och fann att administration av vårt α-conotoxin MII till VTA blockerade alkoholens stimulerande effekter, vilket stämmer överens med våra tidigare data med kommersiellt α-conotoxin MII. Detta pekar att på att α-conotoxin MII-känsliga nikotinreceptorer, förslagsvis $\alpha_3\beta_2$*, β_3* och/eller α_6* subenheterna, i VTA är involverade i att förmedla alkoholens stimulerande egenskaper samt att vår syntesmetod fungerade tillfredställande. Vi syntetiserade också en annan nikotinreceptorantagonist med selektivitet för nikotinreceptorns α_6* subenhet, en α-conotoxin PIA-analog. Vi fann att administration av vår α-conotoxin PIA-analog till VTA inte blockerade alkoholens stimulerande och dopaminökande egenskaper. Sammantaget tyder detta på att nikotinreceptorns $\alpha_3\beta_2$* och β_3*, men inte α_6*, subenheter i VTA är involverad i alkoholens stimulerande och dopaminökande egenskaper. Tidigare försök från vår forskargrupp har visat att råttor som dricker alkohol får en frisättning av acetylkolin i VTA samtidigt med dopamin i N.Acc.. Vår övergripande arbetshypotes är därför att alkohol, via aktivering av den kolinerga-dopaminerga belöningslänken, orsakar en frisättning av acetylkolin i VTA vilket via interaktion med $\alpha_3\beta_2$* och/eller β_3* subenheterna i VTA stimulerar det mesolimbiska dopaminsystemet.

Denna belöningslänk anses också vara av central betydelse för de belönande egenskaperna hos naturliga belöningar, såsom mat. Det är idag allmänt accepterat att det finns ett neurokemiskt överlapp mellan belöningssystemen och de system som reglerar energibalansen, d.v.s. endogena substanser som både påverkar belöningssystemen och energibalansen. Ghrelin är en aptitökande peptid som till största delen bildas till i magsäcken, men mindre mängder produceras också i hjärnan. Eftersom ghrelin reglerar energibalansen ville vi i delarbete II, III och IV, undersöka om och hur ghrelin kan aktivera hjärnans belöningssystem.

I samarbete med professor Suzanne Dicksons forskargrupp, har vi funnit att infusion av ghrelin till tredje ventrikeln hos möss (vilket gör att ghrelin kan ta sig in i hjärnan) ökar aktiviteten samt orsakar en frisättning av dopamin i N.Acc.. Detta har ghrelin gemensamt med

alkohol vilket tyder på att ghrelin är en spelare på belöningsplanen. Med utgångspunkt i att ghrelinreceptorer uttrycks i LDTg och VTA (områden förknippade med belöning och motivationshöjande beteende) ville vi undersöka om ghrelin stimulerar belöningssystemen via en aktivering av ghrelinreceptorer i dessa områden. Vi fann då att lokal injektion av ghrelin i LDTg eller VTA orsakar en motorisk stimulation samt en dopaminökning i N.Acc.. Detta tyder på att ghrelin stimulerar den kolinerga-dopaminerga belöningslänken. Denne effekt har ghrelin gemensamt med alkohol, vilket talar för neurokemiska likheter mellan ghrelin och alkohol.

Eftersom nikotinreceptorer är involverade i alkoholens belöningsprofil föreslår vi att nikotinreceptor också medierar ghrelins stimulerande och dopaminökande egenskaper. Vi fann i en serie försök att en oselektiva nikotinblockerare (mekamylamin) blockerar ghrelinets ovannämnda egenskaper. Med bakgrund i våra tidigare försök med alkohol, som visar att alkoholen belöningsprofil är förmedlade via αCtxMII-känsliga nikotinreceptorer, hypotiserade vi att ghrelins stimulerande och dopaminökande egenskaper också är förmedlade via αCtxMII-känsliga nikotinreceptorer i VTA. I en serie försök fann vi att varken nikotinreceptorns $\alpha_4\beta_2$* (genom att använda dihydro-β-erythroidine) eller α_7* (genom att använda methyllycaconitine) subenheter är involverade i ghrelins stimulerande egenskaper. Vidare fann vi att injektion av αCtxMII till VTA blockerar ghrelinets stimulerande och dopaminökande egenskaperna. Våra resultat vittnar om att ghrelin stimulerar den kolinerga-dopaminerga belöningslänken. Vi föreslår därför att ghrelin via aktivering av ghrelinreceptorer i LDTg och VTA, orsakar en frisättning av acetylkolin i VTA som i sin tur interagerar med αCtxMII-känsliga nikotinreceptorer, förslagsvis $\alpha_3\beta_2$*, β_3* och/eller α_6* subenheter, och därmed stimulerar det mesolimbiska dopaminsystemet. Möjligheten att ghrelin interagerar direkt med nikotinreceptorer uteslöts eftersom ghrelin inte påverkar inbindningen av nikotin till nikotinreceptorer.

Eftersom belöningssystemen framför allt är involverade motivations höjande beteenden så som aktivt sökande efter mat föreslår vi att ghrelin har ett förstärkande värde för motivations höjande beteenden. Vikten av detta är förslagsvis att ghrelin orsakar ett aktivt sökande efter mat (lokomotorstimulation) samt att sökandet i sig själv är belönande (dopaminökning i N.Acc.), vilket ökar sannolikheten för individens överlevnad (Fig. 21).

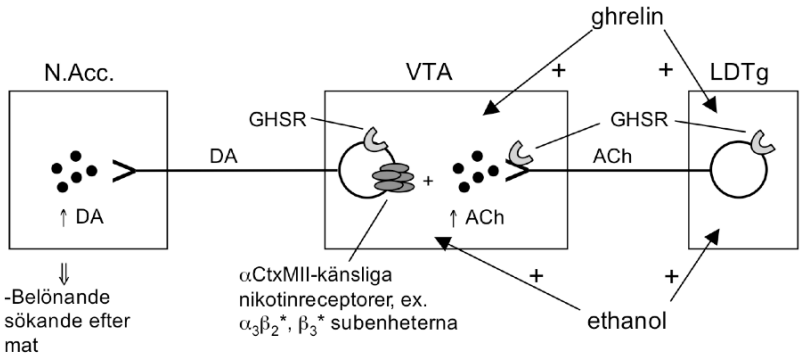

Fig. 21. Ghrelin framkallar ett belönande sökande efter mat via aktivering av den kolinerga-dopaminerga belöningslänken.

Ghrelin aktiverar, via ghrelinreceptorer i laterodorsala tegmentala arean (LDTg) och ventrala tegmentala arean (VTA), belöningssystemen, mer specifikt den kolinerga-dopaminerga belöningslänken. Detta orsakar i sin tur en frisättning av acetylkolin i VTA vilket via interaktion med αCtxMII-känsliga nikotinreceptorer, förslagsvis $\alpha_3\beta_2$* och/eller β_3* subenheter, stimulerar det mesolimbiska dopaminsystemet. Denna stimulering leder till ett födosökande beteende (lokomotorstimulation) som i sig är belönande.

Sammanfattningsvis pekar detta på att ghrelin liksom alkohol aktiverar den kolinerga-dopaminerga belöningslänken samt att αCtxMII-känsliga nikotinreceptorer i VTA har en central betydelse för ghrelins och alkohols effekter. Detta tyder på neurokemiska likheter mellan ghrelin och alkohol.

Eftersom förändrade ghrelinnivåer i blodet är kopplade till hetsätningar (som är inkluderade i ”addictive behaviours”) samt att hetsätningar är associerade till belöningssystemen, föreslår vi att ghrelins aktivering av belöningssystemen kan vara en del av hetsätningens patofysiologi. Sammantaget öppnar detta möjligheten för att αCtxMII-känsliga nikotinreceptorer, förslagsvis $\alpha_3\beta_2$* och/eller β_3* subenheterna, skulle kunna vara ett potentiellt mål för utveckling av nya behandlings-strategier för ”addictive behaviours”, såsom hetsätningar och alkoholism.

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