The role of nitric oxide signaling in reward induced by ghrelin and alcohol in mice

Master thesis in Medicine

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Abstract

Ghrelin in known to regulate energy balance. Growing evidence on common mechanisms involved in ghrelin's, alcohol's as well as other drugs' rewarding properties has been found. A nitric oxide signaling pathway has shown to be of importance for ghrelin induced feeding as well as the rewarding effects of morphine and cocaine. We therefore hypothesize that the rewarding properties of ghrelin and alcohol might involve the same signaling pathway. Alcohol addiction is a chronic relapsing brain disease with major costs for individuals, families as well as society. The current treatment has proven to be insufficient. A key feature in development of addiction is the activation of the brain's reward system. The hunger hormone ghrelin has recently shown to be of importance for the rewarding properties of alcohol as well as other addictive drugs. Furthermore, alcohol and ghrelin has shown great neurochemical similarities, as well as having similar reward mechanisms. The nitric oxide involving reaction ending in a cGMP cascade is of importance for ghrelin induced feeding as well as the rewarding properties of cocaine and morphine. Therefore, the effects of the nitric oxide synthase inhibitor L-name on the rewarding properties of alcohol and ghrelin was studied. A model for reward measurement called Conditioned Place Preference (CPP) was used where half of the mice received L-name during conditioning and half received it acutely, after conditioning. Mice receiving L-name was compared to mice receiving vehicle (placebo) using an uncoupled T-test. L-name does not have any intrinsic rewarding effects. The rewarding effects of alcohol are not affected by L-name administration. Chronic administration of L-name induces a CPP in ghrelin conditioned mice. Thus suggesting a role for nitric oxide signaling mediating the rewarding properties

of ghrelin. Taken together with ghrelin's hunger stimulating effects, nitric oxide signaling proves to be of importance for binge eating.

Introduction

Addiction

Addiction is a chronic relapsing brain disease, characterized by behaviors such as compulsive intake of the drug, relapse, loss of control in addition to continued intake regardless of negative consequences (Hunt et al., 1971; Leshner, 1997)). Thus, addiction should be compared with other diseases of chronic nature such as hypertension and hence also ought to be treated accordingly. As a result, the treatment is therefore more about management of consequences rather than of curative essence. Substance use disorders are patterns of symptoms resulting from use of a substance, which the individual continues to take, despite experiencing problems as a result (APA 2013). Here the diagnostic criteria from The American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM 5) are presented (table 1).

Table 1. DSM criteria for substance use disorder where 2-3 applicable symptoms constitute a mild disorder whereas 6+ applicable symptoms constitute a severe disorder.

1. Taking the substance in larger amounts or for longer time than intended
2. Wanting to cut down or stop using the substance but not managing to
3. Spending a lot of time getting, using, or recovering from use of the substance
4. Cravings and urges to use the substance
5. Not managing to do what should be done at work, home (family and household) or school, because of substance use
6. Continuing to use, even when it causes interpersonal relationship problems
7. Giving up or reducing important social, occupational or recreational activities because of substance use
8. Using substances repeatedly, even when it puts the user in physical danger
9. Continued use, while aware of physical or psychological problem that have been caused or made worse by the substance (for example smoking and COPD)
10. Needing increased amount of substance to get the wanted effect (tolerance)
11. Occurrence of withdrawal symptoms, relieved by taking more of the substance.

Different theories concerning the causes of addiction have been proposed, among those the drug-centered and the individual-centered theories. The drug-centered theory proposes that addiction is a result of molecular changes in the brains rewards systems due to chronic drug use (Berke and Hyman, 2000; Deroche-Gamonet et al., 2004; Nestler, 2001), whereas the individual-centered theory instead clings to the idea that hereditary related constitutions of the reward system, more specifically the mesolimbic dopaminergic reward system, are causing the addiction (Wolfe and Maisto, 2000).

In addition to the association with alcohol and other drugs of abuse, compulsive behavior such as gambling can according to DSM V be applied to the term addiction. This stems from the big similarities in behavior observed in substance addicts and those with compulsive behaviors. Internet addiction just barely failed to make the cut, and including hypersexuality as well as binge eating into the definition has also been proposed, due to the fact that the they share brain projection pathways with drug addiction, pathways with similar dynamic brain activating patterns (Grant et al., 2006; Potenza et al., 2003; Volkow and Li, 2004; Wang et al., 2004). The brain can be changed over time; this is due to the so-called plasticity, a procedure directed by the individual's actions and experiences, causing synaptic changes (Lamprecht and LeDoux, 2004). For instance repeated exposure to a drug leads to long lasting changes in the reward systems of the brain, with the mesolimbic system being of considerable importance for development of addiction related behaviors (Chen et al., 2010; Robinson and Kolb, 2004). Impulse control dysfunction is yet another important feature in patients with compulsive overeating as well as drug dependence (Volkow and Fowler, 2000; Volkow and Li, 2004).

Alcohol (Ethanol) addiction

Alcohol addiction is a chronic relapsing brain disease with major costs for individuals, families as well as society (Garbutt et al., 1999). The alcohol attributed deaths are according to WHO more than two millions each year, 3.8% of global deaths in 2004. The cause of alcohol addiction is a complex multifactorial one with environmental as well as genetic base, where the genetics seem to be responsible for around 40-60 % (Prescott and Kendler, 1999). Different kinds of alcoholism has been proposed, such as the differentiation between Type 1 – late onset environmentally driven alcoholism and Type 2 – early onset more genetically dependent alcoholism (Cloninger et al., 1981). Neural changes occurring after longer times of alcohol overconsumption are for instance brain atrophy with enlarged ventricles and sulci (Ding et al., 2004), and a diminished dopamine (DA) release combined with a reduced number of DA-receptors (Volkow et al., 2002; Volkow et al., 2003). As alcohol is a small molecule with widespread pharmacodynamics and a versatile solvent, passing through cell membranes and diffusing through all tissues the understanding of the mechanisms are incomplete. Some of the receptors affected and potentially involved in the effects are the strychnine-sensitive glycine receptor (GlyR), the gamma-aminobutyric acid A receptor ($GABA_AR$), the nicotinic acetylcholine receptors (nAchRs), the $5HT_3$ receptor, the N-Methyl-D-aspartic acid receptor (NMDAR), the glutamate receptor, the opioid receptor as well as Ca2+ and K+ channels (Herz, 1997; Lewohl et al., 1999; Lovinger and White, 1991; Lovinger et al., 1989; Lovinger and Zhou, 1994; Mascia et al., 1996; Narahashi et al., 1999; Suzdak et al., 1986; Wang et al., 1994) The currently available treatments of alcohol addiction (Acamprosate – among other effects an NMDAR-modulator(Spanagel et al., 1998), Naltrexone – a competitive opioid receptor-antagonist and Disulfiram – an aldehyde

dehydrogenase inhibitor, have clinically been proven insufficient and therefore the development of new treatment strategies would be desirable (Franck and Jayaram-Lindstrom, 2013). The neural mechanisms preceding addiction are still being researched and a better understanding could lead to future pharmacological treatments of alcohol addiction.

The reward systems

During the evolution of animals and the human race, areas of the brain activated by natural rewards have developed. Rewards positive for the survival of our species such as food and sex causes feelings of pleasure, reward and euphoria, an effect of the reward systems' activation (Hansen et al., 1991; Wise and Rompre, 1989). Additional triggers of these systems such as substances of abuse, and compulsive behaviors, for example overeating and gambling, can lead to a more potent activation. Continued activation via these triggers can reinforce behaviors and through plastic neural adaptations lead to addiction (Kelley and Berridge, 2002). Parts of the brain making up these reward systems are located in the midbrain, medial forebrain as well as parts of cortical structures and the limbic system. A part shown to be of utmost importance is the mesocorticolimbic DA system (Wise and Rompre, 1989). It can be further fractioned into the mesocortical and the mesolimbic DA systems where the latter is the dominant part (Koob, 1992a; Koob, 1992b)

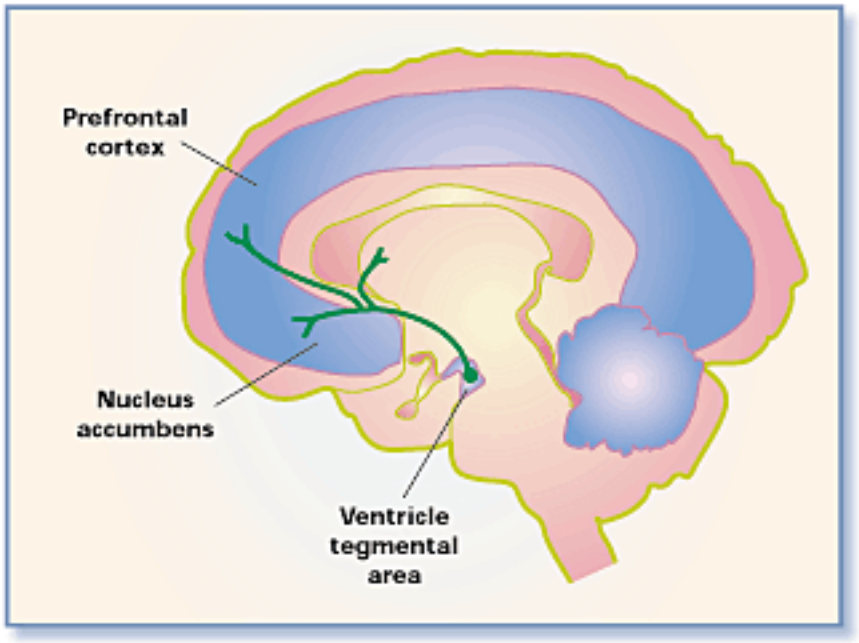

Figure 1 – an overview of the mesocorticolimbic DA-system, formed by DA neurons in the ventral tegmental area (VTA), projecting to nucleus accumbens (N.Acc.) as well as the prefrontal cortex (Piomelli, 2001)

The mesolimbic dopamine system

The mesolimbic DA system has pathways projecting from the VTA, via the medial forebrain bundle to N.Acc. where DA is released to limbic structures such as hippocampus and amygdala. The two parts (inner core and surrounding shell) of N.Acc. have different functions where the one important for reward systems is the shell(Graybiel and Ragsdale, 1978; Heimer et al., 1991; Voorn et al., 1989). Afferent signaling to the VTA is received from the laterodorsal tegmental area (LDTg) where the acetylcholine (ACh) neurons originate (Blaha et al., 1996). The so called cholinergic-dopaminergic reward link consists of these ACh neurons and the mesolimbic DA system(Larsson and Engel, 2004). The activation of this link through the excitation of ACh neurons on LDTg that in turn activate nAChRs and muscarinic acetylcholine receptors (mAChRs) on the surface of DA neurons in VTA leading to an activation of the mesolimbic DA system and the following DA release in N.Acc. (Forster and Blaha, 2000). This activation can

through microdialysis be measured as substantially increased accumbal DA levels, following morphine, alcohol as well as barbiturate administration. Furthermore, these increased DA levels are related to stimulatory behavioral effects, correlating on a time as well as dose basis (Di Chiara and Imperato, 1986). This also hold true for cocaine, amphetamine and nicotine (Di Chiara and Imperato, 1988), strengthening the link between addicting drugs and raised accumbal DA levels. Accumbal DA release is also involved in the hedonic feelings elicited by natural rewards (Robinson and Berridge, 1993; Wise and Bozarth, 1987; Yoshida et al., 1992). Additionally, alcohol and food enhances acetylcholine levels of the VTA, implying that that the cholinergic-dopaminergic link, involving accumbal DA signaling, is important for reinforcing aspects of natural as well as drug induced reward, in turn important for the development of addiction (Lanca et al., 2000; Larsson et al., 2005). In addition to reward mediating mechanisms, the mesolimbic DA system is also important for the motivation to seek out rewards {Lex, 2008 #159; Rada et al., 2000; Saunders et al., 2013; Wassum et al., 2013; Yeomans et al., 1993)

Ghrelin

Ghrelin is a 28-amino acid orexigenic gut brain peptide discovered in 1999 as the first endogenous ligand for the growth hormone secretagogue receptor 1A (GHS-R1A) (Kojima et al., 1999). It is produced in the gastrointestinal tract (mainly in the stomach)(Kojima et al., 1999)) but may also be produced in the brain (Cowley et al., 2003; Lu et al., 2002; Mondal et al., 2005). It has the ability to pass the blood brain barrier and has a diverse physiological profile such as regulation of appetite, body weight and energy homeostasis (Nakazato et al., 2001; Tschop et al., 2000; Wren et al., 2000).

Raised ghrelin levels correlate closely with an increased food intake in humans (Cummings et al., 2001; Cummings and Schwartz, 2003; Cummings et al., 2002; Wren et al., 2001a). Ghrelin stimulates food intake, boosts weight gain as well as augments adiposity in rodents (Beck et al., 2002; Wren et al., 2001b)

The existence of GHS-R1A was first discovered in 1996 (Howard et al., 1996). In addition to the first found expression sites, hypothalamus and pituitary gland(Howard et al., 1996), it has later been observed in hippocampus, nucleus accumbens as well as on dopaminergic neurons in VTA and cholinergic neurons in LDTg (Dickson et al., 2010; Guan et al., 1997; Jerlhag et al., 2006; Landgren et al., 2011a).

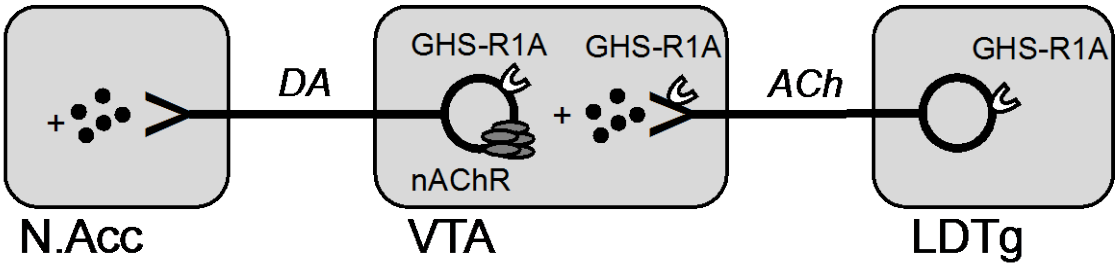

Figure 2 – A schematic overview of ghrelin receptors in the cholinergic-dopaminergic reward link (Dickson et al., 2010; Guan et al., 1997; Jerlhag et al., 2006; Landgren et al., 2011a)

Neurochemical analogies between alcohol and ghrelin

Recent findings indicate that ghrelin in addition to hunger-regulation is involved in the reward regulation (Dickson et al.). Indeed, administration of ghrelin into the third ventricle, the VTA or LDTg respectively resulted in an increased DA release in nucleus accumbens as well as significantly raised locomotor activity (Jerlhag et al., 2006; Jerlhag et al., 2007). The similarity with alcohol is evident with raised accumbal DA release as well as an increased locomotor activity, following a lower dose of alcohol (Imperato and

Di Chiara, 1986; Waller et al., 1986). By unselectively blocking nAchRs with mecamylamine, the rewarding effects of ghrelin were blocked, suggesting a role for central cholinergic transmission in these events (Jerlhag et al., 2006). Yet another nAChR-antagonist, alpha-conotoxin MII (targeting $\alpha_3\beta_2$, β_3 and/or α_6 receptor subunits), inhibited the rewarding properties of ghrelin (Jerlhag et al., 2008). Interestingly, injecting alpha-conotoxin MII also significantly negated locomotor activity increase as well as accumbal DA overflow caused by alcohol administration (Blomqvist et al., 1997; Larsson and Engel, 2004), Another feature indicating nAchRs' importance for the reward of alcohol is the injection of mecamylamine into the VTA. This measure effectively lowered alcohol consumption among high-alcohol preferring rats as well as negated the placebo-treated rats' increased accumbal DA release (Blomqvist et al., 1996; Ericson et al., 1998). Taken together, these facts acts as evidence for ghrelin displaying a similar activation pattern of the mesolimbic dopamine system to that of alcohol (Soderpalm et al., 2000; Soderpalm et al., 2009). As injecting an NMDA receptor antagonist (AP5) into the VTA negates the accumbal dopamine release and the locomotor stimulation of ghrelin, another activation mechanism of the cholinergic-dopaminergic, namely the glutamatergic, could prove to be of importance (Jerlhag et al., 2011a). Indeed, the ability of alcohol as an acute inhibitor of the NMDA receptor (Hoffman et al., 1989; Lovinger et al., 1989) further endorses the theory of neurochemical similarities between alcohol and ghrelin. These finding suggest, together with the presence of GHS-R1A in LDTg as well as VTA (Dickson et al., 2010; Guan et al., 1997; Jerlhag et al., 2006; Landgren et al., 2011a), support the involvement of ghrelin in the cholinergic-dopaminergic reward link. Further supporting this hypothesis, ghrelin by peripheral or

local administration into the LDTg causes a concomitant release of Ach-VTA and DA-NAcc. (Jerlhag et al., 2012)

A role for ghrelin signaling in addiction

In addition to activation of the cholinergic-dopaminergic reward link and thereby increasing the incentive salience of motivated behaviors, a role of central ghrelin signaling in alcohol and drug-induced reward has been shown. Indeed, centrally or peripherally administrating GHS-R1A antagonists suppressed the alcohol intake in a two bottle (alcohol/water) free choice limited access paradigm in mice. This while ghrelin in the same model increased alcohol intake (Jerlhag et al., 2009). Furthermore, alcohol-induced conditioned place preference (CPP), accumbal DA release and locomotor stimulation were all negated when central ghrelin signaling were suppressed, either via GHS-R1A, ghrelin knockout or GHS-R1A antagonism (Jerlhag et al., 2010; Jerlhag et al., 2009; Jerlhag et al., 2011b). Supportingly, another GHS-R1A antagonist, JMV2959, was found reducing high alcohol consumption among high-alcohol consuming Wistar as well as alcohol-preferring (AA) (Landgren et al., 2012, attenuate alcohol preference and voluntary intake in mice {Bahi, 2013 #176). The administration of yet another GHS-R1A antagonist, D-Lys3-GHRP-6, decreases alcohol consumption in rats (Kaur and Ryabinin, 2010). Furthermore, rats voluntarily consuming alcohol for two, five and ten months had their alcohol intake reduced after the administration of JMV2959 (Suchankova et al., 2013). After repeated JMV2959 treatment, mice with attenuated alcohol intake failed to show enhanced tolerance or an alcohol rebound effect (Suchankova et al., 2013). Moreover, JMV2959 attenuates the motivation for alcohol consumption, measured with the operant lever pressing model (Landgren et al., 2012), in addition to preventing rebound drinking in rats, evaluated from it's effects on negated alcohol deprivation effect.

This indicates that the central ghrelin signaling system is required for the stimulatory effects of alcohol, and could be a candidate for future pharmacological treatment of alcohol addiction. The very same system has proven to be involved in mediating the rewarding effects of amphetamine, nicotine, cocaine and palatable food (Egecioglu et al., 2010; Jerlhag et al., 2010; Jerlhag and Engel, 2011; Landgren et al., 2011b).

The role of Nitric oxide (NO) in ghrelin-induced food intake

NO is a gaseous short lived signaling molecule produced by nitric oxide synthase (NOS) from arginine in many mammalian cells, involved in the nervous system as well as having numerous diverse functions such as blood vessel dilatation and homeostasis, inflammation and immune response. One pathway including NO, namely the neuronal nitric oxide synthase (nNOS)/NO/soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) signaling pathway (Figure 3) has been proposed to be of importance for reward mediation (Itzhak, 1996; Kim and Park, 1995) The ghrelin-induced food intake augmentation involves NO (Gaskin et al., 2003). Ghrelin supposedly operates through neuropeptide Y (NPY) (Bagnasco et al., 2002; Morley et al., 1999), increasing food intake through a NO pathway (Morley et al., 1999; Small et al., 2002). By blocking NOS with the NOS inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME)(Mulsch and Busse, 1990), the feeding increasing mechanisms of ghrelin were significantly negated while ghrelin administration per se increased the nNOS levels in the hypothalamus (Gaskin et al., 2003). Additionally, intra-ventral tegmental injections, as well as intrahippocampal CA1 injections of L-NAME have attenuated the morphine-induced conditioned place preference (Gholami et al., 2003; Karami et al., 2002), suggesting a role of NO in the signaling pathways involved in mediating the rewarding effects of morphine. Recently, inhibition of nNOS was shown to reduce the number of

mice sensitized from cocaine exposure (Gabach et al., 2013). Therefore, NO may be a potential mediator of reward in general, such as ghrelin as well as alcohol induced reward.

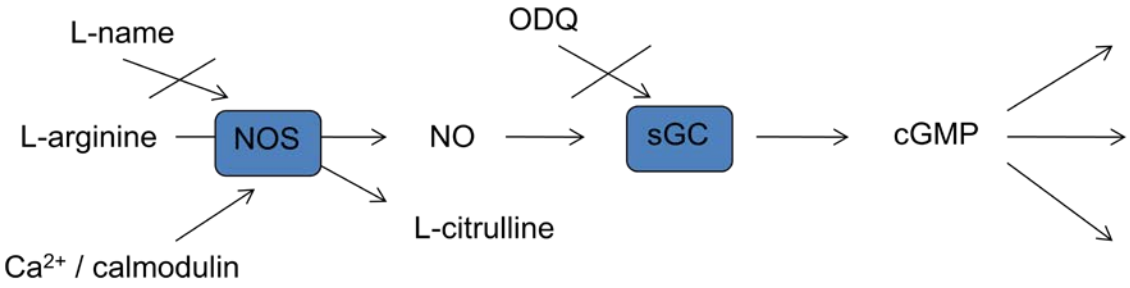

Figure 3 – the nNOS/NO/ sGC/cGMP signaling pathway, where L-NAME inhibits NOS

The Aim

The purpose of this project is to elucidate and give further insight into the mechanisms, specifically NO signaling, mediating the rewarding properties of alcohol and ghrelin measured with CPP. This is done in order to find possible pharmacological targets suitable for the treatment of alcohol addiction as well as binge eating.

Material and Methods

Mice

The mice used in the conditioned place preference experiments were NMRI mice (8-12 weeks old and 25-40 g body weight; B&K Universal AB, Sollentuna, Sweden). The cages used (Macrolon III: 400 x 250 x 150 covered with filter tops (Tecniplast, Italy)) each gave housing for eight mice, and a 12/12 hour light/ dark cycle (lights turned on at 7 am) were maintained. Prior to experiment initiation the mice were allowed one week of environment adaptation, kept in 20°C with 50 % humidity. Unlimited availability of tap water and food (Normal chow; Harlan Teklad, Norfolk, England) were provided.

Drugs

The alcohol injected (95 % ethanol, Kemetyl AB, Haninge, Sweden) was diluted in 0.9% sodium chloride to 15 % v/v, which was then administered intraperitoneally (ip) 5 minutes before experiment initiation at a dose of 1.75g/kg. The dose was chosen since it induces increased locomotor activity, increased accumbal dopamine release as well as an induced CPP in NMRI mice (Jerlhag et al., 2009). L-NAME (Sigma Chemical Co. St. Louis, MO, USA) was diluted in 0.9% sodium chloride and administered subcutaneously (sc) 15 minutes before experiment initiation at a concentration of 40 mg/kg. Higher doses of 60-100 mg/kg has previously shown no effect on CPP (Kiyani et al., 2011), locomotor

activity (Ulusu et al., 2005), and the dose of 40 mg/kg has shown no effect on DA release in N.Acc. (unpublished data). Moreover, it has previously been shown that this dose have no effect per se on prepulse inhibition and attenuates PCP disrupted prepulse inhibition in mice (Klamer et al., 2001)). Ghrelin (Bionuclear; Bromma, Sweden) was diluted in 0.9% sodium chloride and administered (sc) 5 minutes before experiment initiation at a concentration of 0.33 mg/kg. This dose has previously been shown to stimulate the reward system, measured through an increased locomotor activity, accumbal dopamine release as well as an induced CPP when administered ip (Jerlhag, 2008) as well as sc (unpublished data).

Conditioned place preference - CPP

To evaluate the effect of L-NAME conditioning in the different models, a two chambered CPP apparatus (Tzschentke, 2007) with distinguishable tactile as well as visual cues were utilized. Procedures consisted of pre-conditioning (day 1), conditioning (days 2-5) and post-conditioning (day 6). On the first day initial place preference was defined during 20 minutes, when mice were allowed to move freely between the two chambers. A biased procedure was used selecting mice for the conditioning (sessions of 20 minutes each). Four rounds of experiments were undertaken where mice received L-NAME/vehicle + vehicle/alcohol/ghrelin, four injections daily. L-NAME/vehicle (15 minutes prior to conditioning) + vehicle/alcohol/ghrelin (5 minutes prior to conditioning) conditioning was done in the least preferred chamber, whereas vehicle (15 minutes prior to conditioning) + vehicle (5 minutes prior to conditioning) conditioning was done in the most preferred chamber. Mice undergoing conditioning with vehicle/alcohol received two injections daily. Two rounds of experiments were performed where mice were conditioned with a single substance, and instead received L-NAME on the post-

conditioning day. Vehicle/alcohol (5 minutes prior to conditioning) conditioning was done in the least preferred chamber, and vehicle/vehicle (5 minutes prior to conditioning) conditioning was done in the most preferred chamber. Between the two daily conditioning rounds, all mice had at least two hours of undisturbed time in their home cages for rest. Mice were conditioned with active substance during morning every other day, and during afternoon the other conditioning days.

On post-conditioning day, mice conditioned with L-NAME + vehicle/alcohol/ghrelin had their place preference examined and compared to their initial place preference without receiving any prior injection. These are the subchronic L-NAME groups.

The mice conditioned with alcohol/vehicle received an injection of L-NAME/vehicle 15 minutes before the post-conditioning examination of place preference, the acute L-NAME groups

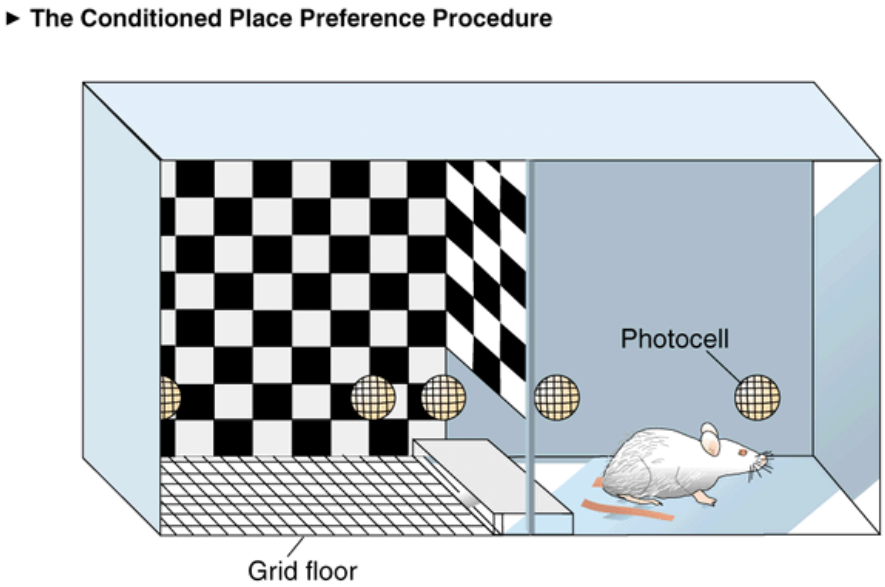

Figure 4 – a model of the CPP apparatus, with distinct visual as well as tactile cues, clearly differentiating the two chambers.

Data collection procedures / Variable analyses / Statistical methods

The videotapes were analyzed visually with a timer for 20 minutes in a blinded manner (the treatment to mice was unknown). Conditioned place preference was defined as the percentage of changed preference, measured with the formula (time in unpreferred chamber pre-conditioning / time in this unpreferred chamber also at post-conditioning) / 1200 * 100. For comparison of conditioned place preference data an unpaired t-test was used. A probability value of $P<0.05$ was considered as statistically significant.

Ethics

All experiments were authorized by The Ethics Committee for Animal Experiments in Gothenburg, Sweden, and performed according to the recommendations in the Swedish Animal Welfare Act.

Results

Acute L-NAME treatment did not effect CPP per se compared to vehicle conditioned mice (P=0.1910, n=8 in each group)

The results of acute L-NAME injection (40mg/kg, sc) on vehicle (0.9% natrium chloride, ip) conditioned mice compared to those receiving acute vehicle injection (equal amount of 0.9% natrium chloride, sc) showed no significant difference (P=0.1910) (Figure 5).

Subchronic L-NAME treatment during conditioning did not effect CPP per se compared to vehicle treatment (P=0.3906, n=8 in each group)

The results of L-NAME (40mg/kg, sc) and vehicle (equal amount of 0.9% natrium chloride) conditioned mice compared to those receiving vehicle (equal amount of 0.9% natrium chloride, sc) showed no significant difference (P=0.3906), indicating that L-NAME has no intrinsic effect (Figure 6).

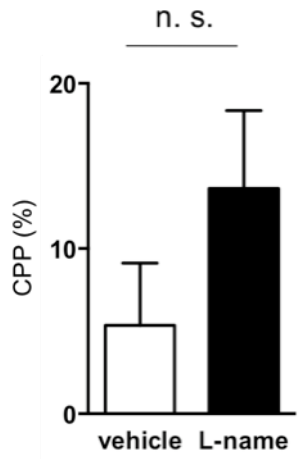

Figure 5 – effects of acute L-NAME treatment on vehicle conditioned mice

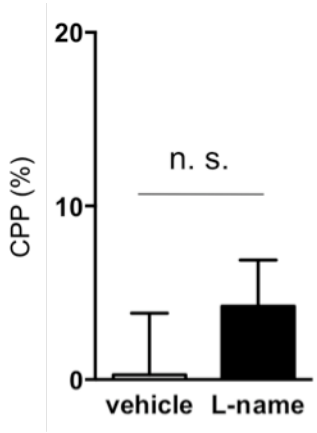

Figure 6 – effects of chronic L-NAME treatment compared to vehicle conditioning

Acute L-NAME treatment did not effect alcohol induced CPP compared to vehicle treatment in mice (P=0.2270, n=8 in each group)

The results of acute L-NAME injection (40mg/kg, sc) on alcohol (1.75g/kg, ip) conditioned mice compared to those receiving acute vehicle injection (equal amount of 0.9% natrium chloride, sc) showed no significant difference (P=0.2270) (Figure 7).

Acute L-NAME treatment did not effect ghrelin induced CPP compared to vehicle treatment in mice (P=0.6573, n=8 in each group)

The results of acute L-NAME injection (40mg/kg, sc) on ghrelin (0.33mg/kg, sc) conditioned mice compared to those receiving acute vehicle injection (equal amount of 0.9% natrium chloride, sc) showed no significant difference (P=0.6573) (Figure 8)

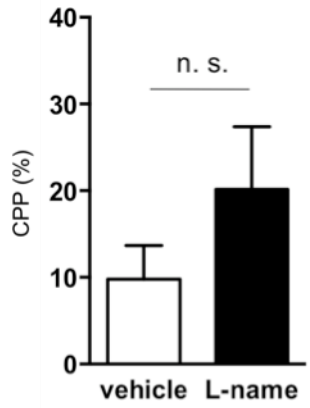

Figure 7 – effects of acute L-NAME treatment on alcohol conditioned mice

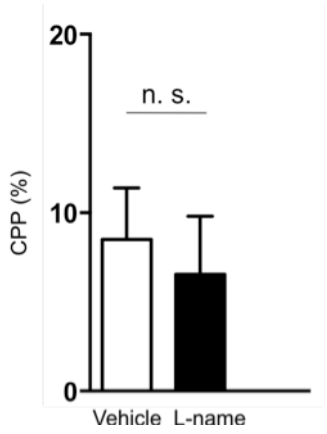

Figure 8 – effects of acute L-NAME treatment on ghrelin conditioned mice

Subchronic L-name treatment during conditioning did not alter the alcohol-induced CPP in mice (P=0.5041, n=8 in each group)

The results of L-NAME (40mg/kg, sc) and alcohol (1.75g/kg, ip) conditioned mice compared to those receiving vehicle (equal amount of 0.9% natrium chloride, sc) and alcohol (1.75g/kg, ip) showed no significant difference (P=0.5041) (Figure 9).

Subchronic L-NAME treatment during conditioning attenuated the ghrelin-induced CPP in mice (P=0.0158, n=14 in each group)

The results of L-NAME (40mg/kg, sc) and ghrelin (0.33mg/kg, sc) conditioned mice compared to those receiving vehicle (equal amount of 0.9% natrium chloride, sc) and ghrelin (0.33 mg/kg, sc) showed a significant difference (P=0.0158) (Figure 10).

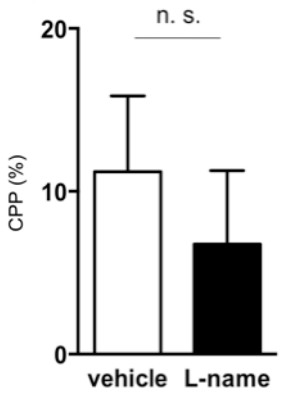

Figure 9 – effects of chronic L-NAME treatment on alcohol conditioned mice

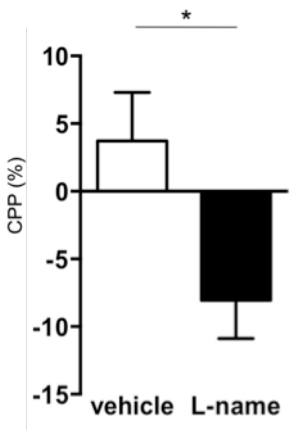

Figure 10 – effects of chronic L-NAME treatment on ghrelin conditioned mice

Discussion with conclusions and implications

Results

In the results of acute L-NAME treatment on vehicle conditioned mice a trend towards a stimulated reward memory could be seen (p=0.1910). However the substance injected during conditioning in the least preferred compartment was merely NaCl, the very same substance as was received by animals prior to conditioning in the most preferred compartment. This suggests that these data can give no support to L-name having any stimulatory effect. It was further shown in the present series of experiment that subchronic l-name treatment during conditioning did not have an effect on CPP per se as compared to vehicle treatment, a result earlier reproduced for doses of L-NAME up to 100 mg/kg (Kiyani et al., 2011). In addition, other studies have shown that acute treatment of L-name has no effect on accumbal dopamine release (unpublished data) or on locomotor activity in mice in doses up to 60 mg/kg (Ulusu et al., 2005). In other behavioral studies it has been shown that treatment with the same dose of L-name has no effects on prepulse inhibition in mice (Klamer et al., 2001). Treating alcohol/ghrelin conditioned mice acutely with L-NAME did not show any significant CPP induction either. A tendency towards increased stimulatory effects (p=0.2270) could be seen among mice given L-name after alcohol conditioning. This can alone however not imply that L-NAME has a stimulatory effect. Moreover, subchronic treatment of L-NAME did not effect alcohol-induced CPP in mice.

Finally, a significant CPP was induced when conditioning with L-NAME/ghrelin compared to a vehicle/ghrelin control treatment group. This is supporting the theory that the nNOS/NO/ sGC/cGMP signaling pathway is of importance for mediating the rewarding properties of ghrelin.

Collectively, these data seem to speak against an effect of systemic administration of L-NAME on the rewarding properties of alcohol. NOS enzymes other than those in VTA, either peripheral or central, could have been affected and negated the reward blocking effects previously noticed (Gholami et al., 2003; Karami et al., 2002). The effect of L-NAME on the rewarding properties of alcohol and other drugs (for example cocaine) (Bozarth et al., 1994) might be non-existent.

Important reward mechanisms

The rewarding properties of alcohol might mainly be mediated through other means than the nNOS/NO/ sGC/cGMP signaling pathway, as previously suggested receptors potentially involved and more important in the effects are the strychnine-sensitive glycine receptor (GlyR), the gamma-aminobutyric acid A receptor ($GABA_AR$), the nicotinic acetylcholine receptors (nAchRs), the $5HT_3$ receptor, the N-Methyl-D-aspartic acid receptor (NMDAR), the glutamate receptor, the opioid receptor as well as Ca2+ and K+ channels (Herz, 1997; Lewohl et al., 1999; Lovinger and White, 1991; Lovinger et al., 1989; Lovinger and Zhou, 1994; Mascia et al., 1996; Narahashi et al., 1999; Suzdak et al., 1986; Wang et al., 1994). The ability of alcohol to activate the mesolimbic dopamine system involves both the VTA and N.Acc. (Hauser et al., 2011; Lof et al., 2007). The mechanisms behind the rewarding properties of cocaine as well as morphine has previously been shown to involve NO-signaling (Gabach et al., 2013; Gholami et al., 2003; Karami et al., 2002). It must however be mentioned that they target the mesolimbic dopamine system in a different way than alcohol. Morphine act mainly as a agonist on the μ-opioid receptor in the VTA (Kieffer, 1995; Mansour et al., 1988), whereas cocaine acts as a serotonin–norepinephrine–dopamine reuptake inhibitor in N.Acc.(Carrera et al.,

2004; Galli et al., 1995). While cocaine and morphine share mechanisms of action with alcohol like raised mesolimbic DA levels (Di Chiara and Imperato, 1988), they differ in others as mentioned above, providing a possible explanation for differences following L-NAME administration. Supportively, local VTA administration of L-NAME reduces morphine induced reward (Gholami et al., 2003).

In the present experiment ghrelin-induced CPP was attenuated by subchronic, but not by acute, treatment with L-name. Acute treatment with L-NAME on post-conditioning day is supposed to measure the ability of the substance to block previously conditioned memory of a reward. Thus affecting the memory consolidation. Subchronic L-NAME treatment during conditioning is however measuring L-NAME's ability to directly attenuating the rewarding properties of the substance. This suggests that L-NAME affects the rewarding properties of ghrelin (Sanchis-Segura and Spanagel, 2006). In support are the recent findings from our research group showing that acute L-NAME treatment attenuates the ability of ghrelin-induced locomotor stimulation and accumbal DA release (unpublished data). In support for a role of NO signaling for ghrelin-induced reward are the findings that ghrelin-induced food intake is reduced by L-NAME administration (Gaskin et al., 2003). Collectively, NO appears to be an important player for ghrelin-, but not alcohol-induced reward.

The possibility that areas such as LDTg, VTA and N.Acc. are involved in the ability of NO to mediate ghrelin-induced reward should be considered. Indeed, local administration of ghrelin into the LDTg or VTA, areas known to express GHS-R1A, increase accumbal DA release, stimulates the locomotor activity and induces a CPP in mice as well as increases alcohol intake in mice (Jerlhag et al., 2009). In addition, accumbal ghrelin

administration increases the locomotor activity, induced a CPP, increase the intake of palatable foods (unpublished data) as well as increases chow intake (Naleid et al., 2005), suggesting that NO within N.Acc. may be important for ghrelin-induced reward. In support for a role of VTA in NO-mediated ghrelin reward are the findings showing that local administration of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), which prevents NO signaling, into the VTA attenuates ghrelin-induced locomotor stimulation as well as accumbal dopamine release in mice (unpublished data).

Methodology

Numerous methodological reasons for erroneous alterations of the results can be mentioned. Stress may very well have increased as a result of animal handlers being of the male sex (Sorge et al., 2014), leading to biased results. Humanely hearable noises originating from water conduits, fans as well as ultrasounds from numerous devices in the facility may have distorted the results. After prolonged use of the same CPP-boxes, impregnation of urine and excrements could skew the results. Another critique that can be applied to the CPP experiment is whether it at all measures the rewarding properties of certain conditioning. The mice seemingly preferring one compartment over the other during preconditioning, and then spending less time in the same compartment after conditioning may just have gotten weary from repeated exposure of the same dull environments. Aversive properties of the CPP chambers might be measured instead of rewarding properties. This is one of the reasons why numerous methodological studies must be conducted, in order to validate results.

Future experiments

Some studies that would further elucidate the mechanisms of the reward system and therefore would interesting to perform are local injection of L-NAME into specific cerebral parts. Nucleus Accumbens, the LateroDorsal Tegmental area as well as the Ventral Tegmental Area would be major candidates. Studying CPP, accumbal DA-release and locomotor activity after local injections would in addition to alcohol consumption be interesting studies to execute. Additionally, the effects of ODQ, another NO signaling constrainer could be intriguing to clarify, further improving our understanding of the role the nNOS/NO/ sGC/cGMP signaling pathway may have in the reward systems.

Conclusions and implications

The hypothesis that nNOS/NO/ sGC/cGMP signaling pathway is important for the rewarding properties of ghrelin is strengthened from these experiments. However, from the present series of experiments it cannot be suggested that NO is important for alcohol reinforcement, implying that nNOS-inhibitors cannot be used as a potential treatment regimen for alcohol addiction. Ghrelin plasma levels are higher in eating disorder patients that binge eat as compared to those that doesn't binge eat (Tanaka et al., 2004; Tanaka et al., 2003a; Tanaka et al., 2003b). Binge eating disorder, an addictive disorder mandated via disruption in the mesolimbic dopamine system, has many similarities to other addictive behaviors; for instance loss of control over intake, relapse and craving (Grant et al., 2006; Potenza et al., 2003; Volkow and Li, 2004; Wang et al., 2004). Given that NO signaling appears to be important for ghrelin-induced reward as well as for ghrelin-induced food intake it should be considered that NO signaling system instead be a potential candidate for treatment of binge eating disorders.

Populärvetenskaplig sammanfattning

I denna studie har jag forskat på en läkemedelskandidat för alkoholberoende. Ämnet vars effekt jag studerat heter L-NAME och dess effekt är att blockera ett enzym (aktivt protein) som är viktigt för signaleringsvägar i bland annat hjärnans belöningssystem. L-NAME visade sig inte i experimenten vi gjorde ha någon dämpande effekt på alkoholens belönande effekter. Konsekvenserna av detta blir att man får försöka hitta andra möjliga läkemedel för att kunna ge en bättre behandling av alkoholberoende. Däremot hade L-NAME effekt på hungerhormonet ghrelins belönande effekter. Detta gör att det skulle kunna vara ett möjligt läkemedel för att behandla hetsätning. Att hitta nya sätt att behandla alkoholberoende är väldigt viktigt då det är en sjukdom som ligger bakom miljontals dödsfall i världen varje år, och leder till en stor mängd sjukdomar. I Sverige beräknas antalet dödsfall direkt orsakade av alkohol vara ca 2000 per år. Problemen för individ, familjer och samhälle är väldigt stora. Idag finns i Sverige tre licensierade läkemedel för behandling av alkoholberoende och denna studie är viktig då den talar emot ett möjligt behandlingsalternativ och därmed gör att man kan fokusera forskningsresurserna på andra alternativ. Mössen som studien utfördes på fick under 20 minuter fritt gå runt i en låda med två stycken för dem klart avskiljbara halvor. Tiden de befann sig i respektive halva filmades och mättes sedan. Detta utgjorde underlag för vilken halva varje enskild mus föredrog. Nästa steg var att försöka ändra på denna preferens genom att spruta in alkohol/ghrelin och under 20 minuter placera mössen i halvorna de tidigare ej föredragit. Mössen fick också en spruta med vanlig koksaltslösning (placebo), varefter de under 20 minuter placerades i halvorna de tidigare föredragit. Efter att ha gjort denna konditionering under fyra dagar gav man återigen

mössen möjlighet att fritt välja mellan de två halvorna i lådan under 20 minuters filmande. Alkoholens och ghrelinets belönande effekter gjorde att mössen nu befann sig i tidigare ej föredragna halva under längre tid än de gjort under den första dagens filmande. Denna effekt var det som man försökte motverka genom att ge mössen en spruta med L-NAME. Antingen så fick de en spruta inför sista dagens filmande för att motverka minnet av den belönande effekten eller så fick de sprutor samtidigt som alkohol/ghrelin för att motverka den belönande effekten direkt. Alla grupper med möss hade även en kontrollgrupp för att öka tillförlitligheten i forskningen.

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