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The alcohol-induced locomotor stimulation and accumbal dopamine release is suppressed in ghrelin knockout mice

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Short title: Ghrelin knockout mice and alcohol

Abstract

Ghrelin, the first endogenous ligand for the growth hormone secretagogue receptor (GHS-R1A), plays a role in energy balance, feeding behaviour, as well as reward. Previously, we showed that pharmacological and genetic suppression of the GHS-R1A attenuates the alcohol-induced stimulation, accumbal dopamine release and conditioned place preference as well as alcohol consumption in mice, implying that the GHS-R1A is required for alcohol reward. The present study further elucidates the role of ghrelin for alcohol-induced dopamine release in nucleus accumbens and locomotor stimulation by means of ghrelin knockout mice. We found that the ability of alcohol to increase accumbal dopamine release in wild type mice is not observed in ghrelin knockout mice. Furthermore, alcohol induced a locomotor stimulation in the wild type mice as well as ghrelin knockout mice, however the locomotor stimulation in homozygote mice was significantly lower than in the wild type mice. The present series of experiments suggests that endogenous ghrelin may be required for the ability of alcohol to activate the mesolimbic dopamine system.

Key words: ghrelin, dopamine, appetite, hedonic, reward, ethanol

Introduction

Besides growth hormone release, ghrelin controls food intake, energy homeostasis as well as appetite (Kojima et al., 1999; Tschöp et al., 2000; Cummings et al., 2001; Wren et al., 2001). This gut-brain hormone also plays a role in brain reward and food-seeking behaviour (Naleid et al., 2005; Abizaid et al., 2006; Jerlhag et al., 2006; Jerlhag et al., 2007; Jerlhag et al., 2008; Jerlhag, 2008; Egecioglu et al., 2010). Moreover, hyperghrelinemia is associated with certain forms of compulsive over eating (Cummings et al., 2002). Interestingly, human imaging studies reveal an underlying disruption in the reward systems in addictive behaviours including binge eating and alcohol dependence (Volkow et al., 2003; Volkow and Li, 2004; Reuter et al., 2005). Moreover, a co-morbidity of addictive behaviours is well documented, where alcohol-use disorder is frequent in the anorexia nervosa binge eating subtype and in individuals with bulimia nervosa (Henzel, 1984; Bulik et al., 1992). Common neurobiological mechanisms underlying such addictive behaviours have been implicated. The mechanisms involved in alcohol dependence are complex and are not fully elucidated, but recently central ghrelin signalling was suggested as such a candidate (Jerlhag et al., 2009). Specifically, central ghrelin administration increased alcohol intake in mice, while ghrelin receptor (GHS-R1A) knockout mice as well as mice treated with two different GHS-R1A antagonists show attenuated alcohol reward and reduced alcohol consumption (Jerlhag et al., 2009). This implies that central ghrelin signalling, in particular the GHS-R1A, constitutes a novel potential target for treatment of alcohol-related disorders. However, the role of endogenous ghrelin for alcohol-induced reward needs to be further elucidated and the present study examines this in studies incorporating ghrelin knockout mice.

Materials and Methods

Animals

All experiments were performed in adult post-pubertal age-matched male mice (8-12 weeks old; 25-35 g body weight). Ghrelin knockout mice on a mixed 129 Sv/Evbrd(LEX1)/C57BL/6 background (backcrossed 3 times and on average 87.5% C57BL/6 mice (De Smet et al., 2006)) and their corresponding wild type littermates, generated through heterozygous breeding, were used in all experimental protocols (locomotor activity, microdialysis and alcohol consumption experiments). All mice were maintained at 20°C with 50% humidity and a 12/12 hour light/dark cycle (lights on at 7 am) unless otherwise stated. Tap water and food (normal chow; Harlan Teklad, Norfolk, England) were supplied *ad libitum*, except following drug/vehicle administration. Studies were approved by the Ethics Committee for Animal Experiments in Gothenburg, Sweden.

Genotyping procedure

Genotyping of all mice were performed to confirm ghrelin knockout, wild type and heterozygous mice. Deoxyribonucleic acid (DNA) was extracted from tail samples using Qiagen DNeasy® Tissue Kit (Qiagen, Hilden, Germany). The DNA was diluted 1:5 and then amplified by polymerase chain reaction (PCR) on a PTC-200 Thermal Cycler (Biorad, Hercules, CA, USA) using the following conditions: 95°C 15min, 95°C 30s, 62°C 45s and 72°C 60s for 33 cycles followed by 72°C for 10min. The primer sequences used were: Ghrl1 '5-CCT GGC AGA CAG AGC ACC TG-3', Ghrl2 '5-CAG GTC AGT CAA GTC TGT CTC-3' and GhrlNeo2 '5-GCA GCG CAT CGC CTT CTA TC-3'. The PCR product was then analysed on a 1.5% agarose gel. The bands representing the ghrelin knockout is produced by primers Ghrl1 and

GhrlNeo2 and is of 791 base pair in length and the band representing the ghrelin wild type is produced by Ghrl1 and Ghrl2 and is of 842 base pair in length.

Drugs

For studies investigating alcohol-induced locomotor stimulation and dopamine release alcohol (VWR International AB, Stockholm, Sweden) was diluted in saline (0.9% NaCl) to 15 % v/v for i.p. injections and was administered 5 minutes prior to initiation of the experiments. The dose of alcohol injected i.p. was pre-determined to 1.75 g/kg from dose-response studies in wild type littermates, which also correlates with doses in previous studies shown to activate the mesolimbic dopamine system (Larsson et al, 2002). For alcohol consumption experiments the % alcohol (diluted in tap water) tested was predetermined in an established protocol, as described below (Larsson et al., 2004). Acylated ghrelin (Bionuclear, Bromma, Sweden) was administered i.c.v. at a dose of 2 µg/mouse. This dose has previously been shown to increase the alcohol intake in mice (Jerlhag et al., 2009). Acylated ghrelin was diluted in Ringer vehicle (Merck KgaA, Darmstadt, Germany) and was administered in a volume of 1 µl via chronically implanted catheters, over 60 s, 10 minutes prior to alcohol/vehicle injection. The cannula was left in place for a further 60 s to facilitate diffusion.

Locomotor activity experiments

We measured alcohol-induced locomotor activity as most drugs of abuse (including alcohol) cause locomotor stimulation, an effect mediated, at least in part, by their ability to enhance the extracellular concentration of accumbal dopamine (Engel et al., 1988; Imperato and DiChiara, 1986; Wise and Bozarth, 1987). It should however be emphasized that other neurotransmitters mediate alcohol-induced locomotor

stimulation (Engel et al., 1992). Locomotor stimulation provides a more indirect yet supportive measure. Locomotor activity was recorded as described previously (Jerlhag et al., 2006). Mice were allowed to habituate to the locomotor activity box one hour prior to drug challenge. Alcohol-induced locomotor stimulation was investigated in ghrelin knockout mice, heterozygote and in their littermate controls following i.p. injection of alcohol or an equal volume of vehicle.

In vivo microdialysis and dopamine release measurements

For measurements of extracellular dopamine levels (that reflect dopamine release), ghrelin knockout mice were implanted unilaterally with a microdialysis probe positioned in the nucleus accumbens. The surgery was performed as previously described (Jerlhag et al., 2006). The effects of i.p. administered alcohol on accumbal dopamine release using microdialysis was investigated in freely moving ghrelin knockout mice, heterozygote and their littermate controls. After one hour of habituation to the microdialysis set-up, perfusion samples were collected every 20 minutes. The baseline dopamine level was defined as the average of three consecutive samples before the first drug/vehicle challenge. Baseline samples were followed by an i.p. injection of vehicle and thereafter alcohol (i.p.) was administered and nine consecutive samples were collected. The dopamine levels in the dialysates were determined by HPLC with electrochemical detection as described previously (Jerlhag et al., 2006).

Alcohol consumption measurements using a limited access paradigm in ghrelin knockout mice.

During the entire drinking procedure the mice were housed in reversed dark-light cycle (lights on at 9 am). Initially, all mice were group housed and had continuous access to both tap water and increasing concentration of alcohol (2, 4, 6, 8, 10%) over a two week period (approximately 3 days at each percentage alcohol). Thereafter, they were housed individually for nine weeks with continuous access to tap water and alcohol solution (10%). Following this free choice continuous access paradigm, alcohol intake was limited to the first 90 min of the dark period (*i.e.* a limited access paradigm) (Rhodes et al., 2005). These two-bottle (alcohol/water) free choice limited access paradigms were maintained for two weeks prior to ghrelin/vehicle testing. Four days preceding treatment mice were inserted with an i.c.v. guide cannula for central administration of ghrelin, as previously described (Jerlhag et al., 2006). The mice received either ghrelin or vehicle on day 1 and the reverse treatment on day 2, according to a balanced design. Drugs were administered prior to lights off. The same experimental setups were used both days (*vide infra*). In all experiments the intake of alcohol, water and food were measured throughout the 90 minute drinking session. The measurements of alcohol consumption are expressed per gram body weight and 90 minutes.

Verification of probe and/or guide cannula placement

After completion of locomotor activity measurements, microdialysis as well as alcohol consumption experiments, the locations of the probe and/or cannulae were verified as previously described (Jerlhag et al., 2006). Only mice with guide cannula

placement in the third ventricle and/or probe placement in the nucleus accumbens were included in the statistical analysis (Figure 1) (Franklin and Paxinos, 1996).

Statistical analyses

The locomotor activity data was evaluated by a two-way ANOVA followed by Tukey/Kramer post-hoc tests comparing treatments. Dixon's Q-test identified two outliers in the heterozygote group treated with alcohol. These were removed prior to further analysis. The microdialysis experiments were evaluated by a two-way ANOVA followed by Bonferroni post-hoc test for comparisons between different treatments at given time points. The limited access drinking data were evaluated by paired *t*-test (effect of ghrelin vs. vehicle) as the individual mice serve as their own controls. An unpaired *t*-test was used when comparing, in different genotypes, the effect of treatment on alcohol or food consumption as well as spontaneous alcohol or food intake prior to treatment. Data are presented as mean \pm SEM. A probability value of $p < 0.05$ was considered as statistically significant.

Results

The alcohol-induced locomotor stimulation observed in wild type mice is reduced in ghrelin knockout mice

Results from the dose response study show that alcohol at a dose of 1.0 g/kg, i.p. ($F(5,35)=0.308$, $p>0.05$, $n=4-13$) or 2.0 g/kg, i.p., ($F(5,31)=2.083$, $p>0.05$, $n=5-9$) does not affect locomotor activity in wild type, heterozygote nor homozygote ghrelin knockout mice (data not shown). This biphasic effect of alcohol, in which higher doses do not induce a locomotor stimulation due to sedation, is commonly observed (Engel and Liljequist, 1983). Alcohol (1.75 g/k, i.p.) caused a locomotor stimulation in wild type mice ($p=0.0001$) as well as in heterozygote ($p=0.0001$) and in

homozygote ($p=0.0002$) ghrelin knockout mice ($F(5,50)=19.98$, $p<0.001$, $n=5-15$) (Figure 2A). However, the alcohol-induced locomotor stimulation was significantly lower in the heterozygote ($p=0.0001$) and homozygote ($p=0.0004$) ghrelin knockout mice compared wild type mice. There was no difference in response between heterozygote or homozygote mice ($p=0.1732$); thus the alcohol-induced locomotor stimulation observed in wild type mice is reduced in heterozygote and homozygote ghrelin knockout mice.

The alcohol-induced accumbal dopamine release observed in wild type mice is attenuated in ghrelin knockout mice

The alcohol-induced increase in accumbal dopamine release observed in wild type mice was attenuated in both heterozygote ($p<0.05$) and homozygote ($p<0.01$) ghrelin knockout mice (Figure 2B) (treatment $F(2,18)=7.506$, $p<0.01$; time $F(14,252)=2.897$, $p<0.001$; treatment x time interaction $F(28,252)=2.397$, $p<0.001$; $n=6-8$). This difference was observed at the time intervals 120-180 and 220 minutes ($p<0.01$). Vehicle solution did not affect the accumbal dopamine levels in either group of mice ($p>0.05$), indicating that injection or volume given *per se* does not affect accumbal dopamine release.

Alcohol, water, total fluid and food intake in ghrelin knockout mice following central ghrelin treatment

Ghrelin significantly increased alcohol intake in wild type, heterozygote as well as in homozygote ghrelin knockout mice ($p<0.05$) ($n=5-7$), and the magnitude of the response did not differ between genotype ($p>0.05$). No difference in the response to vehicle treatment was observed between the genotypes ($p>0.05$). Likewise there was

no difference in spontaneous alcohol intake between the genotypes in untreated mice measured during the weeks before treatment (average alcohol intake in the limited access paradigm: wild type 1.24 ± 0.13 g/kg/90 min; heterozygote 1.25 ± 0.09 g/kg/90 min; homozygote 1.10 ± 0.12 g/kg/90 min). Ghrelin did not affect water intake ($p>0.05$). Ghrelin increased total fluid intake as well as food intake in wild type, heterozygote and homozygote ghrelin knockout mice ($p<0.05$), and the magnitude of the response did not differ between the genotypes ($p>0.05$). In untreated mice, there was no difference in spontaneous food intake measured during the weeks before treatment between genotypes (wild type 1.14 ± 0.26 g/90 min; heterozygote 0.66 ± 0.22 g/90 min; homozygote 0.86 ± 0.26 g/90 min).

Discussion

Here we show for the first time that the ability of alcohol to increase accumbal dopamine release is absent in ghrelin knockout mice, in contrast to their wild type littermates. Moreover, the alcohol-induced locomotor stimulation observed in wild type mice was significantly lower in ghrelin knockout mice. Alcohol-induced locomotor stimulation is regulated, at least in part, by its ability to enhance dopamine in the nucleus accumbens (Engel et al., 1988; Imperato and DiChiara, 1986; Wise and Bozarth, 1987). Thus whereas accumbal dopamine release provides a direct measure of activation of the mesolimbic dopamine system by alcohol, the locomotor stimulatory response provides a more indirect yet supportive measure of this activation. Neurotransmitter systems other than dopamine may also be of importance for the alcohol-induced locomotor stimulation (Engel et al., 1992) and likely explain why the alcohol-induced (in this study) and the cocaine-induced (Jerlhag et al.,

2010b) accumbal dopamine release appears to be suppressed by disrupted ghrelin signalling to a greater extent than the locomotor stimulation.

Previously, it was shown that GHS-R1A antagonists suppressed alcohol intake and the alcohol-induced locomotor stimulation, accumbal dopamine release and conditioned place preference were abolished in GHS-R1A knockout mice as well as in mice treated with two different GHS-R1A antagonists (Jerlhag et al., 2009). Here we show that endogenous ghrelin regulate the ability of alcohol to activate the mesolimbic dopamine system. Moreover, SNPs and haplotypes in the GHS-R1A as well as pro-ghrelin genes have been associated with heavy alcohol consumption and increased body mass (Landgren et al., 2008). Strengthening a role for ghrelin in the heritability for alcohol dependence is the association between a GHS-R1 or pro-ghrelin haplotype with type 2 alcohol dependence or paternal history of alcohol dependence in alcohol dependent females (Landgren et al., 2010).

Ghrelin is mainly produced in peripheral organs raising the possibility that abolished peripheral ghrelin production in the ghrelin knockout mice may cause the reduced alcohol-induced locomotor stimulation and accumbal dopamine release observed in the present study. Even though the ability of ghrelin to pass the BBB is somewhat limited (Banks et al., 2002), peripheral ghrelin has been shown to target the CNS including dopaminergic cell group in the ventral tegmental area (Abizaid et al., 2006; Jerlhag et al., 2006; Jerlhag, 2008; Kawahara et al., 2009; Quarta et al., 2009; Jerlhag et al., 2010a). However, a role for centrally produced ghrelin should not be excluded since ghrelin containing cells have been identified in the hypothalamus (Lu et al., 2001) and ghrelin mRNA has been found in the hypothalamus, specifically adjacent

to the third ventricle in the brain and in the arcuate nucleus (Cowley et al., 2003; Mondal et al., 2005). It should also be taken into consideration that the ghrelin gene encodes pro-ghrelin, which is processed into acyl ghrelin, des-acyl ghrelin, and obestatin and that therefore our results may be influenced by knockdown of des-acyl ghrelin and obestatin, which may have different or opposite effects to the acylated-ghrelin (Asakawa et al., 2005; Chen et al., 2005a, Chen et al., 2005b; Ukkola, 2005; Ren et al., 2009). However, in contrast to ghrelin, there are no reports to date of an effect of des-acyl ghrelin or obestatin on chemical reward.

In the present series of experiments, exogenous ghrelin administration was found to increase alcohol as well as food intake independent of genotype, suggesting that the GHS-R1A pathway was still functional in the absence of endogenous ghrelin production. Given that the alcohol-induced accumbal dopamine release is attenuated in ghrelin knockout mice, these data suggest that the ghrelin knockout mice are responsive to ghrelin, but not to alcohol. No differences in basal drinking between genotypes in ghrelin knockout mice were found here based on previous data showing no difference in spontaneous alcohol intake between genotypes in the GHS-R1A knockout mice (Jerlhag et al., 2009). It seems reasonable to suggest that this reflects the recruitment of compensatory mechanisms in these knockout mice, as suggested previously to explain why these mice are not lean and have normal spontaneous food intake (Sun et al., 2003; Wortley et al., 2004; De Smet et al., 2006). To expose a modest energy balance phenotype in the ghrelin knockout mice, it was necessary to expose the mice to a high fat diet from an early age (Wortley et al., 2004) or, for the food intake phenotype, alter their light/dark cycle (De Smet et al., 2006). Likewise it may be necessary to introduce appropriate challenges to expose the " alcohol

drinking" phenotype. While compensatory mechanisms may be important in these chronic alcohol as well as food consumption protocols, they do not appear to influence the acute effects of alcohol. Supportively, unlike these genetic models, pharmacological suppression of the ghrelin system using GHS-R1A antagonists, reduced alcohol intake in actively drinking mice (Jerlhag et al., 2009; Kaur and Ryabinin, 2010). Additionally, in the present study we showed that acute alcohol-induced stimulatory effects on locomotor activity and dopamine release were attenuated in the ghrelin knockout mice. One final explanation for the lack of a basal alcohol-drinking phenotype in the ghrelin knockout mice addresses the possibility that alcohol intake may involve taste and homeostatic regulation rather than reward-related regulation. However this appears less likely since GHS-R1A knockout mice, despite having normal basal alcohol consumption (presumably reflecting a normal taste experience to alcohol), show reduced taste responsiveness to other (salty and sour) tastants (Shin et al., 2010).

Consistent with the animal studies, human studies have suggested a role of ghrelin in alcohol dependence. Plasma ghrelin levels have been reported as higher in alcoholics than in controls (Kim et al., 2005; Kraus et al., 2005) and have been positively correlated to the duration of abstinence (Kim et al., 2005) as well as to craving (Addolorato et al., 2006; Hillemecher et al., 2007a; Wurst et al., 2007). Interestingly, in active drinking alcoholics the ghrelin levels were reduced (Addolorato et al., 2006; Badaoui et al., 2008). Together, these studies suggest that ghrelin levels are decreased in active drinkers and are increase during abstinence. Consistently, acute alcohol consumption suppresses plasma ghrelin levels in healthy controls (Calissendorff et al., 2005; Zimmermann et al., 2007). This suggests a possible role of this gut-brain

peptide in alcohol-seeking behavior. Studies on other feeding-related peptides show that the plasma levels of leptin, adiponectin and resistin are elevated in alcohol dependent humans as well as rodents (Hillemacher et al., 2007b; Hillemacher et al., 2009; Pradova et al., 2009). A role of ghrelin in reward induced by other addictive drugs has also been shown. Thus, systemic ghrelin enhances cocaine-induced locomotor stimulation as well as condition place preference in rats and high serum levels of ghrelin is associated with cocaine-seeking behaviour in rats (Wellman et al., 2005; Davis et al., 2007; Tessari et al., 2007). Moreover, GHS-1A antagonism attenuates the amphetamine- and cocaine-induced locomotor stimulation, accumbal dopamine release and condition place preference in mice (Jerlhag et al., 2010b). Conclusively, this implying that feeding-related peptides, including ghrelin, may represent a new target for the pharmacotherapy of alcohol dependence.

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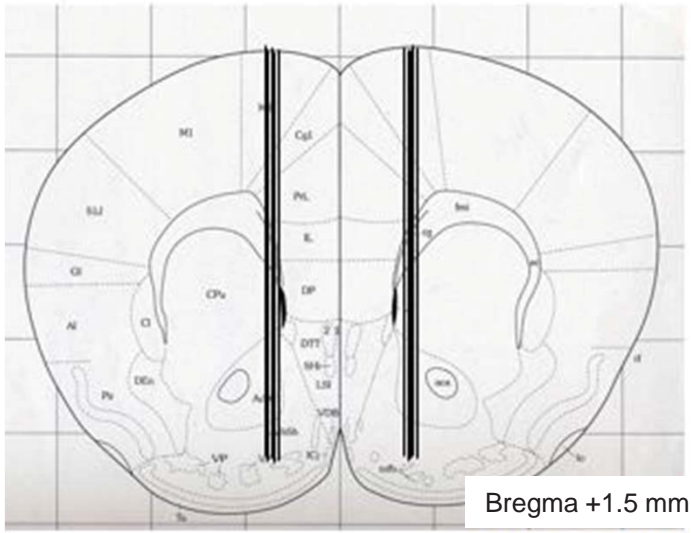
Figure 1.

A coronal mouse brain section showing ten representative probe placements (illustrated by vertical lines) in the nucleus accumbens (A) and i.c.v. (B) of mice used in the present study. The number given in each brain section indicates millimetres anterior (+) and posterior (-) from bregma.

Figure 2

(A) Injection of alcohol increased locomotor activity in wild type, heterozygote and homozygote ghrelin knockout (*ghr*^{-/-}) mice. The the alcohol-induced locomotor stimulation was significantly lower in heterozygote as well as homozygote ghrelin knockout compared to the alcohol response in wild type mice ($*** P < 0.001$, two way ANOVA followed by Tukey/Kramer *post hoc* test). (B) Alcohol increased accumbal dopamine release in wild type littermates but not in heterozygote or homozygote ghrelin knockout (*ghr*^{-/-}) mice (n=6-8; $**P < 0.01$, $*** P < 0.001$, two way ANOVA followed by Bonferroni *post hoc* test).

A.



B.

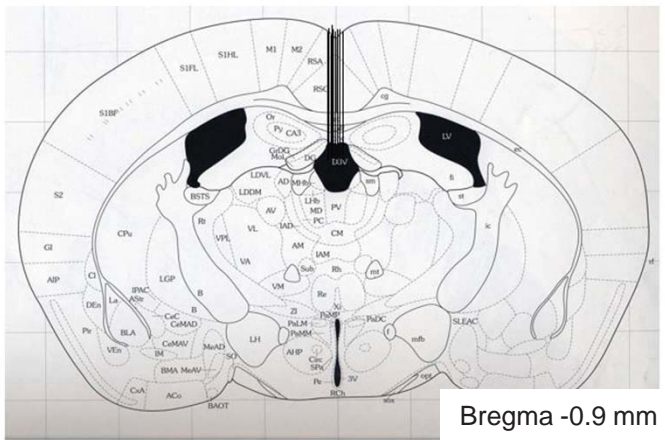


Figure 2

