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Sub-chronic Ghrelin Receptor Blockade Attenuates Alcohol- and Amphetamine-Induced Locomotor Stimulation in Mice

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Abstract

Aims: Ghrelin initially emerged as a gut-brain hormone controlling food intake, meal initiation and appetite mainly via hypothalamic circuits in both rodents and humans. The findings that ghrelin receptors (GHS-R1A) are expressed in reward-related areas, including the nucleus accumbens (NAc) and ventral tegmental area (VTA), suggest that ghrelin is a novel reward regulator. Indeed, ghrelin signalling mediates the rewarding and motivational properties of addictive drugs. In addition, daily co-administration of a GHS-R1A antagonist and various addictive drugs prevents the drug-induced locomotor sensitization in rats.

Methods: The present series of experiment were designed to evaluate the effect of repeated pharmacological GHS-R1A suppression on drug-induced locomotor stimulation in more detail.

Results: We showed that sub-chronic pre-treatment of the GHS-R1A antagonist, JMV2959, attenuated the ability of acute administration of alcohol as well as of amphetamine to stimulate locomotion. However, there was no effect of sub-chronic JMV2959 treatment on locomotor activity *per se* or on the expression of the GHS-R1A gene (*Ghsr*) in the VTA or the NAc compared with vehicle treatment. In addition, sub-chronic ghrelin treatment caused a locomotor sensitization.

Conclusions: While previous research has pinpointed ghrelin as an appetite regulator the present study together with previous studies suggest that ghrelin signalling modulates various reward-mediated behaviours in rodents. Collectively, this suggests that the GHS-R1A could be a key target for novel treatment strategies for addiction.

INTRODUCTION

Ghrelin, a 28 amino acid gut-brain peptide hormone, is predominantly produced and secreted from a specific type of endocrine cells in the stomach (Rindi *et al.*, 2002). The peptide is transported passively across the blood–brain barrier (Banks *et al.*, 2002) and acts through its unique ghrelin receptor (GHS-R1A) that is expressed throughout the brain (Kojima *et al.*, 2001). Ghrelin has emerged as a peptide orchestrating a variety of behaviours, but was initially discovered as a regulator of growth hormone release in rodents and in humans (Kojima *et al.*, 1999; Schmid *et al.*, 2005). Evidence has since then demonstrated that ghrelin controls food intake, meal initiation as well as appetite via a complex network of neuronal circuits including hypothalamic GHS-R1A [for review see (Egecioglu *et al.*, 2011)]. GHS-R1A are expressed in reward-related areas including the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Guan *et al.*, 1997; Abizaid *et al.*, 2006; Landgren *et al.*, 2011b) which strongly suggest that ghrelin signalling regulate reward processing via

modulation of brain dopamine. Initially it was shown that central or intra-VTA administration of ghrelin activates the mesolimbic dopamine reward system as measured by locomotor stimulation, conditioned place preference, accumbal dopamine releases and NAc-dopamine turn over in rodents (Abizaid *et al.*, 2006; Jerlhag *et al.*, 2006, 2007; Engel and Jerlhag, 2014). In addition, a peripheral ghrelin injection causes dopamine release in NAc (Jerlhag, 2008), specifically in the shell region (Quarta *et al.*, 2009), and induces a hyperlocomotion and conditions a place preference (Jerlhag, 2008) in rodents. Moreover, ghrelin administered peripherally causes a concomitant release of VTA-acetylcholine and NAc-dopamine (Jerlhag *et al.*, 2012). Circulating ghrelin is believed to target the dopamine system directly via ventral tegmental GHS-R1A as intra-VTA infusion of a GHS-R1A antagonist attenuates not only ghrelin-induced synchronous acetylcholine-dopamine release, but also elevated food intake and reward normally seen following ghrelin administration (Abizaid *et al.*, 2006; Dickson *et al.*, 2011; Jerlhag *et al.*, 2012; Engel and Jerlhag, 2014). This is further substantiated by imaging data showing that peripheral ghrelin causes a focal activation of brain structures including the VTA and NAc in rats (Wellman *et al.*, 2012) and alters the brain response to visual food cues in NAc in healthy volunteers (Malik *et al.*, 2008). Subsequently studies showed that ghrelin increases whereas pharmacological or genetic GHS-R1A suppression decreases the reinforcing properties of addictive drugs in rodents (Wellman *et al.*, 2005, 2008, 2011; Davis *et al.*, 2007; Tessari *et al.*, 2007; Jerlhag *et al.*, 2009, 2010, 2011b; Abizaid *et al.*, 2011; Jerlhag and Engel, 2011; Clifford *et al.*, 2012; Bahi *et al.*, 2013). In the present series of experiments we sought to determine whether injection of a GHS-R1A antagonist, JMV2959, each day for five subsequent days (i.e. sub-chronic treatment), could attenuate acute alcohol- or amphetamine-induced locomotor stimulation in mice. In addition, the possibility that sub-chronic treatment of ghrelin causes locomotor sensitization *per se* was investigated. Finally, the effect of sub-chronic JMV2959 treatment on locomotor activity as well as GHS-R1A gene (*Ghsr*) expression in the VTA and NAc was studied.

MATERIAL AND METHODS

Animals

Adult post-pubertal age-matched male NMRI mice (8–12 weeks old and 25–40 g body weight, Charles River, Sulzfeld, Germany) were used. All mice were group-housed ($n = 8$) during the entire experiment and maintained at a 12/12 h light/dark cycle (lights on at seven am). Tap water and chow (Harlan Teklad, Norfolk, England) were supplied *ad libitum* except during the experimental setups. The mice were allowed to acclimatize at least one week before the start of the experiments. The experiments were approved by the Swedish Ethical Committee on Animal Research in Gothenburg (ethical permission no: 67-2077, 195-2014).

Drugs

The selected dose of JMV2959 (provided by Æterna Zentaris GbmH, Frankfurt am Main, Germany), a GHS-R1A antagonist, was determined previously [6 mg/kg, intra peritoneal (IP)] (Jerlhag *et al.*, 2009). Acute administration of the selected dose, calculated as the salt, has no effect *per se* on locomotor activity, accumbal dopamine release and conditioned place preference in mice (Jerlhag *et al.*, 2009). In addition, sub-chronic pre-treatment of JMV2959 does not alter locomotor activity compared with vehicle treatment in rats (Wellman *et al.*, 2011; Clifford *et al.*, 2012). Radioligand binding studies have established that JMV2959 is a selective competitive GHS-R1A antagonist (Moulin *et al.*, 2007) and that it does not bind the dopamine receptors (D1, D2L and D2S receptors) (Jerlhag *et al.*, 2010). JMV2959 was dissolved in vehicle (saline, 0.9% sodium chloride solution) and was always administered 20 minutes prior to the initiation of the experiment. Alcohol (ethanol, Finsprit 95% Kemetyl; VWR International AB, Stockholm, Sweden) was diluted in vehicle (saline) to 15% v/v and a dose of 1.75 g/kg (IP), administered 10 minutes prior to experiment, was used. Dex-amphetamine sulphate (RBI; Natick, USA) was dissolved in vehicle (saline) and was administered IP at a dose of 2 mg/kg 10 min prior to initiation of the experiment. The doses of alcohol and amphetamine were chosen on the basis of previous studies in which they were found to activate the mesolimbic dopamine system as measured by locomotor activity, conditioned place preference and accumbal dopamine release in mice (Jerlhag *et al.*, 2009, 2010). Acylated rat ghrelin (Bionuclear; Bromma, Sweden) was dissolved in vehicle (saline). The dose of 0.33 mg/kg (100 nmol/kg, IP) was selected since it previously has been shown to increase locomotor activity and accumbal dopamine release as well as to condition a place preference in mice (Jerlhag, 2008) as well as food intake in rats (Wren *et al.*, 2001). Ghrelin was always administered 10 min prior to the initiation of the experiment.

Locomotor activity experiments

Most drugs of abuse cause locomotor stimulation, an effect, at least in part, mediated by their ability to enhance the extracellular concentration of accumbal dopamine (Di Chiara and Imperato, 1986; Wise and Bozarth, 1987). Locomotor activity was recorded as described previously (Jerlhag *et al.*, 2006). Locomotor activity was registered in eight sound attenuated, ventilated and dim lit locomotor boxes (420 × 420 × 200 mm, Kungsbacka mät- och reglerteknik AB, Fjärås, Sweden). Five by five rows of photocell beams, at the floor level of the box, creating photocell detection allowed a computer-based system to register the activity of the mice. The mice were always allowed to habituate to the locomotor activity box 1 h prior to activity recording. The activity registration started 10 min after the last injection and locomotor activity was defined as the accumulated number of new photocell beams interrupted during a 60-minute period. The design of the present experiments stems from our previous studies which show that five sub-chronic treatment-days with nicotine induced a robust locomotor sensitization 72 h following the last injection (Jerlhag and Engel, 2011; Egecioglu *et al.*, 2013).

First, the effects of sub-chronic administration of JMV2959 or ghrelin *per se* on locomotor activity were investigated. JMV2959 (6 mg/kg, IP, $n = 8$) or an equal volume of vehicle ($n = 8$) was administered each day for five subsequent days. Following each drug administration (day one to five) the mice were exposed to the locomotor activity box. Seventy-two hours following the last JMV2959 or vehicle injection, the activity of the mice was investigated for 60 min, i.e. the mice were untreated this day.

In a separate set of mice, ghrelin (0.33 mg/kg, IP, $n = 8$) or an equal volume of vehicle ($n = 8$) was administered each day for five subsequent days. Following each drug administration (day one to five) the mice were exposed to the locomotor activity box. Seventy-two hours following the last ghrelin/vehicle injection the activity of the mice was investigated for 60 min, i.e. the mice were untreated on this day.

Second, the ability of sub-chronic JMV2959 pre-treatment to attenuate the acute alcohol- or amphetamine-induced locomotor stimulation was investigated.

In a separate set of mice, JMV2959 (6 mg/kg, IP) or an equal volume of vehicle was administered each day for five subsequent days.

Following each JMV2959/vehicle administration (day one to five) the mice were exposed to the locomotor activity box. Seventy-two hours following the last pre-treatment session alcohol (1.75 g/kg, IP) or an equal volume of vehicle was administered and the activity of the mice was investigated for 60 min. Each mouse received only one treatment combination (Veh-Veh, Veh-Alc, JMV-Veh or JMV-Alc, $n = 8$ per treatment combination).

In other mice, JMV2959 (6 mg/kg, IP) or an equal volume of vehicle was administered each day for five subsequent days. Following each JMV2959/vehicle administration (day one to five) the mice were exposed to the locomotor activity box. Seventy-twoh following the last pre-treatment session of amphetamine (2 mg/kg, IP) or an equal volume of vehicle was administered and the activity of the mice was investigated for 60 min. Each mouse received only one treatment combination (Veh-Veh, Veh-Amph, JMV-Veh or JMV-Amph, $n = 8$ per treatment combination).

RNA isolation

The locomotor activity studies revealed that sub-chronic JMV2959 pre-treatment attenuated the alcohol- and amphetamine-induced locomotor stimulation. Therefore, we investigated the effects of sub-chronic JMV2959 treatment on *Ghsr* expression in NAc and VTA. Mice were injected with either JMV2959 (6 mg/kg, IP, $n = 8$) or vehicle ($n = 8$) for five subsequent days. Seventy-two hours after the last drug/vehicle administration the mice were sacrificed and the brains collected. Two sagittal cuts were made (posterior slice: −3.4 mm until −3.6 mm, and anterior slice: +1.2 mm until +1.5 mm) with a brain-slicing matrix. The VTA as well as NAc were rapidly punched out from the posterior and anterior slice respectively (Franklin and Paxinos, 1997), immediately put on dry ice and then stored at −80°C until further processing. Frozen tissue samples were placed in an eppendorf tube and disrupted using a bead mill (TissueLyser; Qiagen, Hilden, Germany) with stainless steel beads (5 mm, Qiagen). Total RNA was extracted in an automated sample preparation robot (QIAcube; Qiagen) using the **RNeasy Lipid Tissue Mini Kit** (Qiagen). The quality and concentration of the RNA samples were assessed using a NanoDrop (Thermal Scientific, Odessa, TX, USA).

Gene expression analysis

The preparation of cDNA was done using the QuantiTect Reverse Trascription Kit (Qiagen). TaqMan Custom Array micro fluidic cards were configured and ordered at Applied Biosystems (Foster City, CA, USA). The target gene as *Ghsr* (assay number: Rn00821417_m1) and two endogenous controls were selected (*Gapdh*: Rn01775763_g1, *Actb*: Rn00667869_m1). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in a quantitative real time polymerase chain reaction (qRT-PCR) performed on an ABI 7900HT (Applied Biosystems). The data obtained was analysed using the comparative C_T method, described in detail previously (Livak and Schmittgen, 2001), where the group of low alcohol consuming mice was set as the calibrator. ΔC_T values were obtained by subtracting the threshold cycle (C_T) of the endogenous control gene from that of the target gene. $\Delta\Delta C_T$ were then calculated by subtracting the mean ΔC_T of the calibrator from the ΔC_T of the target gene for each subject. Relative quantities to the calibrator were calculated as Fold = $2_T^{-\Delta\Delta C}$.

Statistical analysis

The locomotor activity experiments were analysed by either a one-way ANOVA followed by Bonferroni post-hoc tests or by an unpaired t-test. An independent sample t-test was applied to the $2_T^{-\Delta\Delta C}$ values to explore the impact of JMV2959 on *Ghsr* expression in rats. The estimated effects of JMV2959 treatment from the independent sample t-test were expressed as up or down folds. A fold change reduction (i.e. down-fold and a fold change <1) was calculated by taking the inverse negative inverse of $2_T^{-\Delta\Delta C}$.

RESULTS

Effects of sub-chronic JMV2959 or ghrelin treatment on locomotor activity in mice

Sub-chronic JMV2959 pre-treatment did not affect the locomotor activity compared with sub-chronic vehicle pre-treatment ($P = 0.5472$, $n = 8$ in each group, Fig. 1A). Sub-chronic ghrelin pre-treatment significantly increased the locomotor activity compared with sub-chronic vehicle pre-treatment ($P = 0.0210$, $n = 8$ in each group, Fig. 1B).

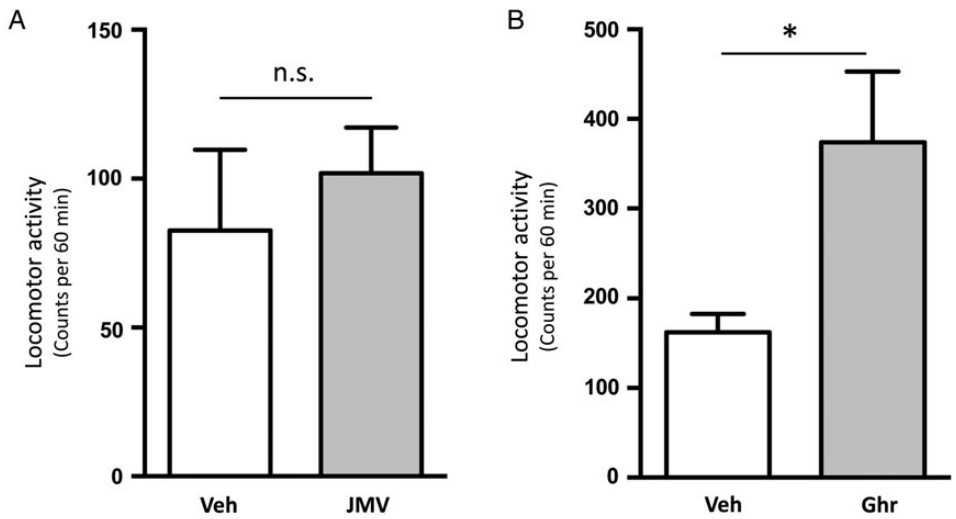

Fig. 1. Effects of sub-chronic ghrelin treatment and GHS-R1A antagonist treatment on locomotor activity in mice. (**A**) Sub-chronic GHR-R1A antagonist, JMV2959 (JMV), treatment (6 mg/kg iIP) has no effect on locomotor activity compared with vehicle treatment. (**B**) Sub-chronic ghrelin (Ghr) treatment (0.33 mg/kg IP) causes a locomotor sensitization compared with vehicle treatment. Data are presented as mean ± SEM (*$P < 0.05$, n.s. $P > 0.05$).

Effects of sub-chronic JMV2959 pre-treatment on locomotor stimulation induced by acute administration of alcohol or amphetamine in mice

An overall main effect of treatment was found on locomotor activity in mice following sub-chronic JMV2959 (6 mg/kg) pre-treatment and acute systemic administration of alcohol (1.75 g/kg) ($F(3,27) = 4.27$, $P = 0.0137$; $n = 8$ for vehicle-vehicle, vehicle-alcohol and JMV2959-vehicle and $n = 7$ for JMV2959-alcohol). As shown in Fig. 2A, post-hoc analysis revealed acute alcohol administration significantly increased the locomotor activity compared with vehicle ($P < 0.05$), and that sub-chronic JMV2959 (6 mg/kg) pre-treatment attenuated the alcohol-induced locomotor stimulation (JMV2959-alcohol versus vehicle-vehicle). Sub-chronic JMV2959 had no effect *per se* on locomotor activity compared with vehicle treatment.

An overall main effect of treatment was found on locomotor activity in mice following sub-chronic JMV2959 (6 mg/kg) pre-treatment and acute systemic administration of amphetamine (2 mg/kg) ($F(3,23) = 3.71$, $P = 0.0259$; $n = 8$ for JMV2959-vehicle, $n = 7$ for vehicle-vehicle and $n = 6$ for JMV2959-aphetamine and vehicle-amphetamine). As shown in Fig. 2B, post-hoc analysis revealed that acute amphetamine administration significantly increased the locomotor activity compared with vehicle ($P < 0.05$) and that sub-chronic JMV2959 (6 mg/kg) pre-treatment attenuated the amphetamine-induced locomotor stimulation (JMV2959-amphetamine versus vehicle-vehicle). Sub-chronic JMV2959 had no effect *per se* on locomotor activity compared with vehicle treatment ($P > 0.05$).

Effects of sub-chonic JMV2959 treatment on *Ghsr* expression

All but two NAc samples (one in each group) were successfully analysed in the qRT-PCR experiment. No significant effect was observed on *Ghsr* expression in neither the VTA nor the NAc following sub-chronic JMV2959 administration in mice (Table 1).

DISCUSSION

In the present study we show that sub-chronic pre-treatment with a GHS-R1A antagonist attenuates the acute locomotor stimulatory effects of alcohol and of amphetamine in mice. These data are in accordance with previous studies showing that pharmacological (acute) as well as genetic suppression of the GHS-R1A attenuates alcohol-induced locomotor stimulation, conditioned place preference and dopamine release in NAc (Jerlhag *et al.*, 2009). Moreover, the rewarding properties of alcohol are attenuated in ghrelin knockout mice compared with wild type mice (Jerlhag *et al.*, 2011b; Bahi *et al.*, 2013). In addition to alcohol, JMV2959 inhibits the ability of nicotine, cocaine as well as amphetamine to active the mesolimbic dopamine system in mice (Jerlhag *et al.*, 2010; Jerlhag and Engel, 2011). Consistent with these effects are reports in which systemic administration of ghrelin enhanced cocaine-induced hyperlocomotion (Wellman *et al.*, 2005) as well as conditioned place preference (Davis *et al.*, 2007). The plasma levels of ghrelin are furthermore positively correlated to cue-induced reinstatement of intravenous cocaine-seeking behaviour in rats (Tessari *et al.*, 2007) and GHS-R1A knockout rats display an attenuated locomotor sensitization of cocaine compared with wild type mice (Clifford *et al.*, 2012). Supportively, daily co-administration of a GHS-R1A antagonist together with either nicotine or cocaine prevents the nicotine- or cocaine-induced sensitization in rats (Wellman *et al.*, 2011; Clifford *et al.*, 2012). Sub-chronic ghrelin administration augments the acute stimulatory properties of cocaine in rats (Wellman *et al.*, 2008) and ghrelin knockout mice exhibit diminished locomotor sensitization to cocaine (Abizaid *et al.*, 2011). Human genetic findings support a role of ghrelin in reinforcement since variations in the genes encoding ghrelin or GHS-R1A are associated with alcohol intake, smoking and amphetamine dependence (Landgren *et al.*, 2008, 2010, 2011a; Suchankova *et al.*, 2013, 2015).

The present study confirms previous reports (Wellman *et al.*, 2011; Clifford *et al.*, 2012), which show that sub-chronic pre-treatment of JMV2959 does not alter locomotor activity compared with vehicle treatment. We also demonstrated that there was no effect observed on *Ghsr* expression in neither the VTA nor the NAc following sub-chronic JMV2959 administration in mice. We therefore suggest that the ability of sub-chronic JMV2959 treatment to attenuate the acute stimulatory effects of alcohol and amphetamine is not due to an altered number of GHS-R1A receptors. A tentative explanation may be that sub-chronic JMV2959 treatment either diminishes the ability of GHS-R1A to heterodimerize with dopamine D1 and D2 receptors (Jiang *et al.*, 2006; Kern *et al.*, 2012) or decreases the constitutive activity of the GHS-R1A (Holst *et al.*, 2003). In support for a role of ventral tegmental as well as accumbal GHS-R1A in drug-reward are the findings showing that the stimulatory effects of addictive drugs involve

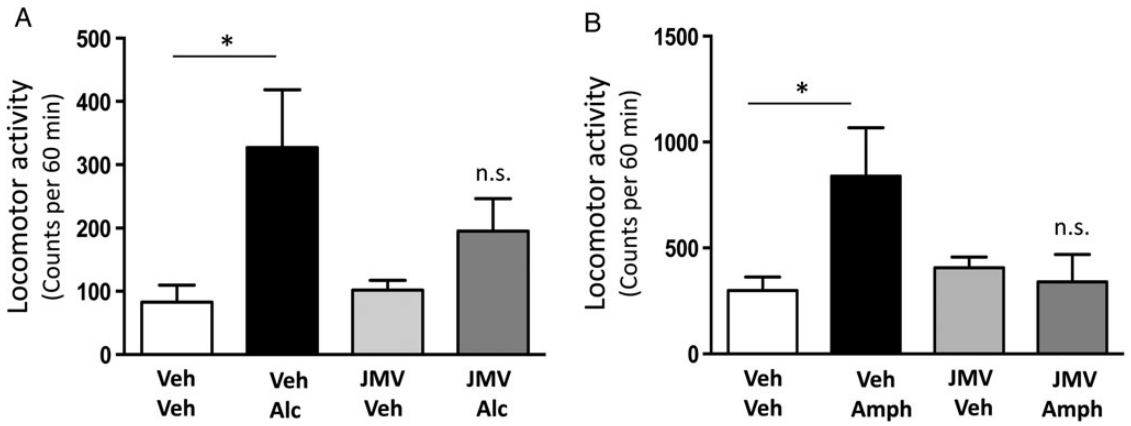

Fig. 2. Effects of sub-chronic GHS-R1A antagonist treatment on acute alcohol- and amphetamine-induced locomotor stimulation in mice. (**A**) Sub-chronic GHR-R1A antagonist, JMV2959 (JMV), treatment (6 mg/kg IP) attenuates the ability of acute alcohol administration (Alc, 1.75 g/kg IP) to cause a locomotor stimulation in mice. (**B**) Sub-chronic GHR-R1A antagonist, JMV2959 (JMV), treatment (6 mg/kg IP) attenuates the ability of acute amphetamine administration (Amph, 2 mg/kg IP) to cause a locomotor stimulation in mice. Data are presented as mean ± SEM (*$P < 0.05$, n.s. $P > 0.05$).

Table 1. Effects on *Ghsr* mRNA expression

Brain region	Control	JMV	*t*	d*f*	Effect[a]	*P*-value[b]
Ventral tegmental area (*n* = 8/8)	1.67 ± 1.39	2.85 ± 1.80	−1.463	14	1.66	0.17
Nucleus accumbens (*n* = 7/7)	1.25 ± 0.97	0.89 ± 0.26	0.960	12	−1.16	0.37

There are no effects on *Ghsr* mRNA expression in mice treated with vehicle or JMV2959 (6 mg/kg, IP) for five subsequent days. Data presented as mean arbitrary units $2^{-\Delta\Delta C}_{T}$ ± SD.

[a]Estimated effects of high alcohol consumption on *Ghsr* expression presented as up or down folds.

[b]Significant levels obtained by independent samples T-test.

the VTA-NAc mesolimbic dopamine projection and that intra-VTA ghrelin infusion causes a locomotor stimulation, accumbal dopamine release, increases the intake of alcohol, palatable food as well as sucrose in rodents (Jerlhag *et al.*, 2007, 2009, 2012; Egecioglu *et al.*, 2010; Skibicka *et al.*, 2011). Moreover, imaging studies show that intravenous ghrelin administration activates a network of brain areas including the VTA and NAc (Wellman *et al.*, 2012) and that intra-NAc administration of ghrelin increases whereas GHS-R1A suppression attenuates the cocaine-induced locomotor stimulation in rats (Jang *et al.*, 2013). We therefore hypothesize, albeit it should be investigated in detail, that sub-chronic JMV2959 pre-treatment via heterodimerization and/or the constitutive activity of the GHS-R1A in VTA and/or NAc may alter the sensitivity of the mesolimbic dopamine system and reduces the ability of addictive drugs to active this system.

The present study also reports that sub-chronic pre-treatment of ghrelin causes a locomotor sensitization. These results are in opposition to a previous study showing that repeated ghrelin treatment does not induce a locomotor sensitization (Wellman *et al.*, 2008). A tentative explanation for this discrepancy might be that a higher dose of ghrelin, known to increase food intake (Wren *et al.*, 2001), was used for the present study (100 compared with 5 nmol) (Wellman *et al.*, 2008).

The present preclinical ghrelin sensitization results may be coupled with certain clinical findings and together contribute towards understanding the pathophysiology of addictions. First, persistent elevated levels of ghrelin, e.g. seen at food restriction (Gualillo *et al.*, 2002), might sensitize the mesolimbic dopamine systems and could thereby alter the individual's response to reward. Supportively food restriction augments cocaine- as well as amphetamine-induced locomotor stimulation, conditioned place preference, enhances cocaine-seeking behaviour and increases the self-administration of cocaine or amphetamine in rats (Carroll *et al.*, 1979; Carroll and Meisch, 1980, 1981; Carroll, 1985; Bell *et al.*, 1997; Campbell and Carroll, 2001; Carr, 2002). On the contrary food satiation, which decreases ghrelin levels (Cummings *et al.*, 2001), delays the acquisition of cocaine self-administration (Carroll and Lac, 1998). Second, several studies have shown that elevated plasma levels of ghrelin are positively correlated to craving in abstinent patients with alcohol use disorder (Addolorato *et al.*, 2006; Koopmann *et al.*, 2012; Leggio *et al.*, 2012). It has been suggested that the long lasting sensitization effects may cause craving (Robinson and Berridge, 1993). The possibility should therefore be considered that persistent elevated ghrelin levels during withdrawal, may via sensitization of the reward systems, increase the incentive value of motivated behaviours and cause craving in alcohol-dependent individuals.

The mechanism underlying the ability of ghrelin to induce a locomotor sensitization are not fully understood and needs to be investigated in detail. Albeit ghrelin was administered peripherally in the present study, we suggest that the locomotor sensitization is due to the effects of ghrelin at the mesolimbic dopamine system. Indeed, ghrelin is transported passively across the blood–brain barrier (Banks *et al.*, 2002) and systemic ghrelin administration activates the mesolimbic dopamine system (Jerlhag, 2008) via ventral tegmental GHS-R1A (Naleid *et al.*, 2005; Jerlhag *et al.*, 2011a). Furthermore, peripheral ghrelin induces the formation of c-Fos-like immunoreactivity within the paraventricular nucleus (Ruter *et al.*, 2003), excites dopamine neurons in the VTA (Abizaid *et al.*, 2006) and imaging studies reveal that intravenous ghrelin activates both the VTA and NAc (Wellman *et al.*, 2012). The possibility should be considered that circulating ghrelin might reach deeper brain areas, however such transport mechanisms are to our knowledge unknown. It is further possible that ghrelin activates the mesolimbic dopamine system via indirect mechanisms as fluorescently labelled ghrelin was found to bind exclusively to food regulatory neurons of the hypothalamus (Schaeffer *et al.*, 2013). In addition to peripheral ghrelin, some studies suggest that ghrelin could be produced in the brain, preferably in the hypothalamic nuclei (Cowley *et al.*, 2003; Mondal *et al.*, 2005; Sato *et al.*, 2005). Repeated drug administration, causing behavioural changes including locomotor sensitization, induces neuronal plasticity within several reward-related areas (Sanchis-Segura and Spanagel, 2006). In the present study GHS-R1A antagonism does not alter the *Ghsr* expression in the VTA and NAc. The possibility that ghrelin-induced sensitization involve other reward-related areas such as hippocampus should therefore be considered. Indeed, hippocampal ghrelin infusion enhances synaptic plasticity (Chen *et al.*, 2011).

The ability of addictive drugs, including alcohol and amphetamine, to induce a locomotor stimulation is, at least in part, mediated by accumbal dopamine release (Wise and Bozarth, 1987). Indeed, the behavioural effects of alcohol are closely time-locked with accumbal dopamine release in rats (Imperato and Di Chiara, 1986). In addition, lower doses of alcohol cause a locomotor stimulation in alcohol preferring rather than in non-alcohol preferring rats (Waller *et al.*, 1986). It has therefore been argued that the locomotor stimulatory properties of addictive drugs should be considered as putative endophenotype for drugs of abuse and addiction. With the present data in mind investigating locomotor activity and sensitization under different circumstances, we show that ghrelin signalling is a likely partaker in the complex and yet to be uncovered neurochemical pathways causing addiction.

CONCLUSION

The ghrelin signalling system has been a key target of anti-obesity drug development since ghrelin increases food intake while GHS-R1A antagonism reduces food intake, decreases the food preference for palatable food and reduces body weight [for review (Egecioglu *et al.*, 2011)]. In summary, the present study shows that sub-chronic pretreatment with JMV2959, with no effect *per se*, attenuates the ability of acute administration of alcohol as well as amphetamine to induce a

hyperlocomotion in mice. This effect was not due to an up or down regulation of ghrelin receptors as sub-chronic JMV2959 exposure did not alter the expression of *Ghsr* neither in the NAc nor in the VTA. In addition, sub-chronic pre-treatment with ghrelin induced a locomotor sensation. As ghrelin signalling regulates various drug-mediated behaviours [for review see (Engel and Jerlhag, 2014)] and that locomotor sensitization reflects persistent neurophysiological adaptations underlying compulsive drug intake as well as craving (Robinson and Berridge, 1993), we propose that pharmacological antagonism of GHS-R1A may diminish the development of drug dependence and believe that such agents deserve to be considered as novel treatment strategies for addiction.

AUTHORS CONTRIBUTION

J.A.E. contributed to the conception and interpretation, designed the study and wrote the manuscript, P.S. designed and performed the hands on work, analysed data, wrote the manuscript, E.J. designed the study, performed the hands on work, contributed to the conception and interpretation, managed literature search, analysed and undertook statistical analysis and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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