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Ghrelin and aggressive behaviours—Evidence from preclinical and human genetic studies

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ABSTRACT

Aggressive behaviour is of crucial importance in the defence for limited resources including food and mates and involves central serotonin as well as dopamine signalling. As ghrelin modulates food intake and sexual behaviour we initially investigated the hypothesis that central ghrelin signalling regulates aggressive behaviour in the resident intruder paradigm in male mice. Moreover, interaction between ghrelin signalling and serotonergic, noradrenergic as well as dopaminergic neurotransmission in aggression was investigated. The relevance of ghrelin for human aggression *per se* as well as for aggression induced by alcohol was evaluated in a human genetic association study comprising young men (n = 784) from the normal population assessed for anti-social behaviours. The present study demonstrates that central ghrelin infusion, but not ghrelin administered systemically, increases aggression. Moreover aggressive behaviour is decreased by pharmacological suppression of the growth hormone secretagogue receptor-1 A (GHSR-1A) by JMV2959. As indicated by the *ex vivo* biochemical data serotonin, rather than dopamine or noradrenaline, in amygdala may have central roles for the ability of JMV2959 to reduce aggression. This link between central serotonin, GHSR-1A and aggression is further substantiated by the behavioural data showing that JMV2959 cannot decrease aggression following depletion of central serotonin signalling. The genetic association study demonstrates that males carrying the Leu72Leu genotype of the pre-pro-ghrelin gene and displaying hazardous alcohol use are more aggressive when compared to the group carrying the Met-allele. Collectively, this contributes to the identification of central ghrelin pathway as an important modulator in the onset of aggressive behaviours in male mice.

1. Introduction

Ghrelin is released from the stomach into the circulation at hunger (Cummings et al., 2001) and it acts, at least in part, on hypothalamic growth hormone secretagogue receptors-1 A (GHSR-1A; ghrelin receptors) to increase food intake and appetite (Nakazato et al., 2001). During recent years a modulatory role of ghrelin in reward processing, via activation of reward related areas, has been established in both rodents and humans (Abizaid et al., 2006; Jerlhag, 2008; Jerlhag et al., 2006; Malik et al., 2008; Muller et al., 2015; Quarta et al., 2009; Wellman et al., 2012). Moreover preclinical, human genetical as well as clinical findings show that ghrelin signalling is crucial for alcohol reinforcement and development of alcohol addiction (for review see (Engel and Jerlhag, 2014; Zallar et al., 2017)). In addition to its established ability to increase appetite and regulate reinforcement ghrelin has been attributed pleiotropic physiological roles including sexual behaviour (Egecioglu et al., 2016; Prieto-Garcia et al., 2015).

As violent aggression, anger and irritability contribute considerably to the burden of various psychiatric disorders and as available treatments thereof are associated with insufficient efficacy and substantial side effects (Posternak and Zimmerman, 2002) the importance of further neurochemical understanding of aggression is highlighted. It has

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been established that both serotonin (Ambar and Chiavegatto, 2009; de Boer et al., 2009) and dopamine (Zai et al., 2012) are important central regulators of aggression. Aggressive behaviour is complex but is of crucial importance in the defence and competition for limited resources including food and mates. Therefore, in the first part of the present study we therefore sought to investigate the possible influence of ghrelin signalling on aggressive behaviours in rodents. Using an animal model of overt aggression, we sought to investigate whether i) ghrelin injected peripherally, ii) ghrelin infused into the third ventricle or iii) GHSR-1A antagonist (JMV2959) administered peripherally affects overt aggressive behaviours. Secondly, we examined the effects of acute JMV2959 administration on the *ex vivo levels* of serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), noradrenaline as well as dopamine and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in regions regulating aggression i.e. amygdala, ventromedial preoptic area, ventral tegmental area and nucleus accumbens. These biochemical data intrigued us to further explore if serotonin and JMV2959 regulate aggression by actions in the same or in parallel circuits. In order to investigate this relationship we evaluated the effects of JMV2959 on aggression in serotonin-depleted mice in the resident intruder paradigm. Thirdly we explored a possible interaction between acute treatment with JMV2959 and alcohol on aggressive behaviours in male mice.

In the final part of this study, the relevance of ghrelin signalling for human aggression was investigated in a human genetic association study examining polymorphisms in ghrelin-related genes in young-men from the Child and Adolescent Twin Study in Sweden (CATSS) assessed for aggressive behaviours directed directly towards others (overt aggression). Some patients with alcohol dependence display pathological anger, which contributes considerably to the burden of disease (Colman et al., 2009). Therefore, as a forth part of the present study, we examined the potential association between genotypes in ghrelin-related genes and overt aggression or hazardous alcohol use. We thereafter investigated whether there was an interaction between genotype and risk-drinking on overt aggression.

2. Material and methods

2.1. Preclinical studies

2.1.1. Animals

Adult post-pubertal and sexual naïve male C57Bl/6 N mice (resident mice: 8–10 weeks old, 25–30 g body weight; Taconic, Ejby, Denmark) and 129/SvEv mice (intruder mice: 8–10 weeks, 20–25 g body weight; Taconic) were used. Using two different strains, with a smaller and submissive intruder with similar age, creates a robust aggressive behaviour of the dominant resident mouse. They were group-housed and allowed to acclimatize to the animal facility at least one week before the start of the experiments. Following the resident mice were housed in individual cages to build up territories. The mice were maintained at a 12/12 h light/dark cycle and at 20 °C with 50% humidity and tap water and food (normal chow; Harlan Teklad, Norfolk, England) were supplied ad libitum. Experiments were approved by the Swedish Ethical Committee on Animal Research in Gothenburg. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.1.2. Drugs

The findings that central ghrelin administration displays robust effects on food intake and activation of the mesolimbic dopamine system and that systemic ghrelin administration does not reach deeper brain areas (Furness et al., 2011; Pirnik et al., 2011; Sakata et al., 2009), intrigued us to use both intracerebroventricular (icv) infusion into the third ventricle and intraperitoneally (ip) administrations of ghrelin. Acylated rat ghrelin (Bionuclear; Bromma, Sweden) was diluted in vehicle (Ringer solution for icv and 0.9% NaCl for ip). The selected ghrelin doses for central (1 μg in 1 μl) and systemic (0.33 mg/kg) injections, were administered 10 min prior to test based on earlier studies showing that these doses increase locomotor activity and accumbal dopamine release (Jerlhag, 2008; Jerlhag et al., 2006). To conform a role of GHSR-1A, JMV2959 (Moulin et al., 2007) (6 mg/kg, ip; Æterna Zentaris GbmH, Frankfurt am Main, Germany) a GHSR-1A antagonist, with no effect on locomotor activity or gross behaviour, but attenuates alcohol reinforcement (Jerlhag et al., 2009), was used. Notably, JMV2959 does not bind to the dopamine D1 receptor, short or long dopamine D2 receptor (Jerlhag et al., 2010). JMV2959, administered 20 min prior to test, was dissolved in vehicle (0.9% NaCl). The tryptophan-5-hydroxylase inhibitor, pCPA methyl ester hydrocholoride (pCPA; 300 mg/kg, ip), eliminates central serotonin (Pettersson et al., 2016) and thereby increases aggressive behaviours by decreasing the latency to aggression (Chiavegatto et al., 2001; Mosienko et al., 2012). pCPA was diluted in vehicle (0.9% NaCl buffered to pH 6), and was administered once daily for three consecutive days (last injection 24 h before test). A low dose of alcohol (0.5 g/kg, ip; 96%; VWR International AB, Stockholm, Sweden) dissolved in vehicle (0.9% NaCl) was injected acutely 30 min prior to test (Mamiya et al., 2017). Albeit this acute alcohol injection might cause some initial peritoneal irritation this administration rout produces a robust behavioural response (Jerlhag et al., 2009).

2.1.3. Guide cannula implantation

For central administration of ghrelin or vehicle solution a guide cannulae was surgically implanted as previously described in detail (Jerlhag et al., 2006). The mouse was anaesthetized with isofluran (Isofluran Baxter; Univentor 400 Anaesthesia Unit, Univentor Ldt., Zejtun, Malta), placed in a stereotaxic apparatus (David Kopf Instruments; Tujunga, CA, USA) and kept on a heating pad to prevent hypothermia. Two drops of Xylocain (10 mg/ml) adrenalin (5 μg/ml) (Pfizer Inc, Apoteket AB, Gothenburg, Sweden) were used for local anaesthesia. The skull bone was exposed after an incision and two holes were drilled, one for the guide cannulae and one for the anchoring screw. The coordinates relative to bregma for the third ventricle were: 0.9 mm AP and ± 0.0 mm ML (Franklin and Paxinos, 1997). The guide cannula was placed 1 mm below the surface of the brain and was anchored to the screw and the skull bone with dental cement (DENTALON® plus; Agntho's AB, Lidingö, Sweden). After surgery the mice were injected with carprofen (Rimadyl®, 5 mg/kg subcutaneously; Astra Zeneca, Apoteket AB) to relieve pain and were kept in individual cages. One hour prior to drug administration a dummy cannula was carefully inserted and retreated into the guide cannula to remove clotted blood and hamper spreading depression. The cannula for drug administration was inserted and extended another 1.1 mm ventrally beyond the tip of the guide cannulae aiming for the third ventricle (Franklin and Paxinos, 1997). The drug was administered over one minute; the cannula was left in place for another minute and it was then retracted (5 μl Kloehn, microsyringe; Skandinaviska Genetec AB, V. Frölunda, Sweden). Verification of cannula placement was performed after termination of the experiment and only animals with correct placement were included in the statistical analysis (Supplementary material 1).

2.1.4. Resident intruder test

The resident intruder test is a valid model of overt aggression, in which a male mouse tends to defend his territory against a conspecific intruder (Parmigiani et al., 1998). In all tests, the resident male mice were housed individually, with no change of bedding material, allowing formation of territorial behaviour during a total of nine days. Thereafter the resident mice were exposed to six training days where the bedding material as well as food was removed from the home cage ten minutes prior to interaction. During this interaction a novel male intruder mouse, weighing at least 10% less than the resident, was introduced in the opposite corner relative to that of the resident mouse. It was removed following the first attack or a maximum of a ten-minute interaction. The purpose of this initial six-day training was to discard mice

not displaying aggression from further experiments; however, no non-aggressive animals were identified. In addition, this allows mice to be randomized to treatment based on stratification of attack latency during the six initial encounters. At the following test day the resident mice with similar baseline of aggression during training days 1–6 receive pharmacological manipulations before being introduced to a novel intruder for 10 min in it's home cage.

In the first series of experiments, the resident mice were treated acutely with i) ghrelin (icv 10 min prior to test, experiment 1), ii) ghrelin (ip 10 min prior to test, experiment 2) or iii) JMV2959 (ip 10 min prior to test, experiment 3) or relevant vehicle. An observer blinded to treatment of the animals scored the duration, frequency and latency of aggressive (*i.e.* attack bites, threat postures, chasing (part of aggression sequence), tumbling and mounting of the intruder), social (*i.e.* sniffing, following (part of social sequence), attending and grooming of the intruder) as well as non-social (i.e. all behaviours where residents do not interact with the intruder including running, walking, sitting still, self-grooming and digging) behaviours of all mice.

The second run of behavioural experiment investigates the effects of JMV2959 on the elevated aggressive behaviour, as measured by latency to attack, in serotonin-depleted mice (Pettersson et al., 2016) that previously have been established to display a decreased latency to aggressive behaviours (Chiavegatto et al., 2001; Mosienko et al., 2012). Thus, following three days of pCPA or vehicle treatment the resident mice were treated acutely with either JMV2959 or an equal volume of vehicle creating the following treatment groups: vehicle-vehicle, vehicle-JMV, pCPA-vehicle and pCPA-JMV.

The final resident intruder test evaluates the interaction between acute alcohol, JMV2959 and aggressive behaviour (duration, frequency and latency). Therefore the test mice were treated acutely with either alcohol or vehicle prior to treatment with JMV2959 or vehicle, creating the following treatment groups: vehicle-vehicle, alcohol-vehicle, vehicle-JMV2959 and alcohol-JMV2959.

2.1.5. Biochemical analysis of brain areas

As previous studies have established that central ghrelin infusion increases 5-HIAA levels in amygdala (Hansson et al., 2014), the effect of peripheral acute JMV2959 treatment on *ex vivo* 5-HIAA, serotonin, noradrenaline as well as HVA, DOPAC and dopamine in the amygdala, ventromedial preoptic area, ventral tegmental area and nucleus accumbens was investigated in resident-intruder naïve male mice.

The mice were decapitated 20 min after the drug injection, the brains were removed following decapitation, relevant brain areas were rapidly dissected out on a cold glass plate, and kept frozen at −80 °C until analysis which was conducted identical to an established procedure described previously (Prieto-Garcia et al., 2015). In brief, the dissected brain tissue samples were homogenized by ultrasound homogenization (Sonifier Cell Disruptor B30; Branson Sonic Power Co. Danbury, CT, USA) in a solution of 0.1 M perchloric acid, 5.37 mM EDTA and 0.65 mM glutathione. Following centrifugation (10,000 rpm, 5 °C, 10 min) the supernatant was collected and analysed for neurotransmitters and the metabolites using a split fraction HPLC-ED system. Serotonin, noradrenaline and dopamine were analysed on an ion-exchange column (Nucleosil, 5 µ SA 100 A, 150 x 2 mm, Phenomenex; Torrance, CA, USA) with a mobile phase consisting of 13.3 g citric acid, 5.84 g NaOH, 40 mg EDTA and 200 ml methanol in distilled water to a total volume of 1000 ml. 5-HIAA, DOPAC and HVA were analysed on a reverse phase column (Nucleosil, 3 µ, C18, 100 A, 50 x 2 mm, Phenomenex) with a mobile phase consisting of 11.22 g citric acid, 3.02 g dipotassium phosphate, 40 mg EDTA and 60 ml methanol in distilled water to a volume of 1000 ml. The electrochemical detections were performed by two amperometric detectors (Waters 460) and the currents were recorded with the Dionex Chromeleon software package (Dionex; Sunnyvale, CA, USA).

2.1.6. Statistical analysis of preclinical data

The effects of ghrelin/JMV2959 on aggressive behaviours and JMV2959 on biochemistry were analysed by an unpaired t-test. The remaining resident intruder tests were analysed with a one-way ANOVA followed by Bonferroni post-hoc test for comparisons between different treatments. All data are presented as mean ± SEM. A probability value of $P < 0.05$ was considered as statistically significant.

2.2. Human genetic study

2.2.1. Participants

Participants was a subsample from CATSS an on-going nation-wide study targeting all twins born in Sweden since July 1992 (Anckarsater et al., 2011). Aggressive and non-aggressive antisocial behaviour and alcohol use of these twins was assessed using various instruments (see below) at age 18 (response rate = 50%) when the twins were contacted again. We included twins aged 18 who had completed these instruments and provided a DNA sample. Given that the preclinical study of the present investigation included only male animals, we focused our human genetic study on the males (n = 788, including 111 monozygotic twin pairs, 88 dizygotic twin pairs, 386 individuals without their co-twin). Zygosity was determined using a panel of 47 SNPs (Hannelius et al., 2007). 2 individuals with documented brain damage and 2 non-Caucasian individuals were excluded from the analyses, resulting in a final sample of n = 784. CATSS was approved by the Ethics Committee at Karolinska Institutet and participants were protected by the informed consent procedure.

2.2.2. Self-reported delinquency questionnaire

A modified version of the self-reported delinquency questionnaire (SRD) consisting of 25 items was used (Junger-Tas et al., 1994; Ring, 1999). SRD measures the frequency of three kinds of law-breaking behaviour over the past 12 months: property offenses (*e.g.* shoplifting, breaking and entering, vandalism), violent offenses (*e.g.* assault, robbery, sexual violence) and drug-related offenses (use and distribution). Each item can be scored from 0 (never) to 4 (> 10 times). The items can be subdivided into two scales, Overt Aggression (targeting another individual directly; 9 items) and Covert Aggression (not targeting another individual directly; 16 items). The Overt Aggression subscale included solely aggressive actions, and was thus used as our outcome measure (see Supplementary Material 2). The total score of the Overt Aggression ranged from 0 to 36, and extreme outliers were winsorized to the mean plus two standard deviations in order not to unduly influence the results. Cronbach's alpha for the Overt Aggression scale was 0.72.

2.2.3. Alcohol use disorder identification test

The participants of CATTS were asked to fill out the alcohol use disorder identification test (AUDIT), a self-report questionnaire used in the health care to identify individuals with hazardous patterns of alcohol consumption (Babor et al., 2001). It consists of 10 questions with a five- and three-point response format, giving a total AUDIT score from 0-40. According to the World Health Organization, AUDIT scores of ≥8 for men indicate hazardous alcohol use (Babor et al., 2001), and this was coded as a dichotomous variable for each individual (see Supplementary Material 3).

2.2.4. DNA extraction, SNP selection and genotyping

DNA was extracted from saliva samples using OraGene DNA self-collection kit (DNA Genotek, Inc, Ottawa, Canada). Two missense SNPs in the *pre-pro-ghrelin gene (GHRL)* (rs696217/Leu72Met and rs4684677/Gln90Leu) and one SNPs in the GHS-R1 A gene (*GHSR*; rs2948694) were selected based on recent studies (Landgren et al., 2011, 2010; Landgren et al., 2008; Suchankova et al., 2017). SNPs with a previously reported minor allele frequency of < 5% were not included in the present study. The SNPs were genotyped by LGC Genomics (http://www.lgcgenomics.com) using the KASPar chemistry - a

competitive allele specific PCR SNP genotyping system performed with FRET quencher cassette oligos. The genotyping success rate was > 95% (see Supplementary Material 4). The SNPs did not differ significantly from Hardy-Weinberg Equilibrium (HWE) as assessed using PLINK (Purcell et al., 2007) (see Supplementary material 4).

2.2.5. Statistical analysis of the human genetic study

Associations between SNPs and the behavioural measures were estimated using a linear mixed effect model in the MIXED procedure of SAS 9.3 (SAS Institute, Inc, Cary, NC, USA), which allowed us to adjust for the dependent nature of twin observations as well as the dependence of individuals from the same family (that is, scores from all genotyped subjects were included in the analyses). Given that monozygotic twins, on average, share 100% of their genome, whereas dizygotic twins share approximately 50%, we modelled two separate variance–covariance matrices: (1) for monozygotic twins and (2) for dizygotic twins. By using R-side random effects with an unstructured variance–covariance matrix, correlations between individuals in groups (1) and (2) were calculated. As genetic variation partly explains alcohol-heightened aggression in males (Cloninger, 1987; Grove et al., 1990) and as a number of studies have reported associations between alcohol dependence and AUDIT scores as well as SNPs located in ghrelin-related genes we also investigated the interaction between each SNP and hazardous alcohol use, determined as either having (1) or not having (0) an AUDIT score of ≥8, against overt aggression. Given the very low frequency of uncommon homozygotes for all three SNPs they were grouped with their respective heterozygotes in the analyses. To control for multiple testing, Bonferroni correction was used: primary interaction analyses of 3 SNPs and two analyses (i.e. one primary outcome and one interaction analysis) yielded a corrected alpha of 0.008.

3. Results

3.1. Central, but not systemic, ghrelin administration increases aggressive behaviours in male mice

Central ghrelin (n = 10) treatment significantly increased the duration ($P < 0.05$, Fig. 1A) and frequency ($P < 0.05$, Fig. 1B) of aggressive behaviours compared with vehicle (n = 14). There was a tendency towards a decreased latency ($P = 0.0877$, Fig. 1C) to engage in aggressive behaviours. There was no effect of central ghrelin treatment on social behaviours (duration $P > 0.05$, Fig. 1D; frequency, $P > 0.05$, Fig. 1E; latency $P > 0.05$, Fig. 1F) or on non-social behaviours (duration, $P > 0.05$, Fig. 1G).

On the other hand systemic administration of ghrelin (n = 12) had no effect on the duration of ($P > 0.05$, Fig. 1H), frequency of ($P > 0.05$, Fig. 1I) or latency to ($P > 0.05$, Fig. 1J) aggressive behaviours compared to vehicle (n = 13). There was no effect of systemic ghrelin treatment on social behaviours (duration $P > 0.05$, Fig. 1K; frequency, $P > 0.05$, Fig. 1L; latency $P > 0.05$, Fig. 1M) or on non-social behaviours (duration, $P > 0.05$, Fig. 1N).

3.2. The GHSR-1A antagonist JMV2959 reduces aggressive behaviours in male mice

Systemic JMV2959 significantly decreased the duration of ($P < 0.05$, Fig. 2A) as well as the frequency of ($P < 0.01$, Fig. 2B), and increased the latency to ($P < 0.05$, Fig. 2C) aggressive behaviours (n = 8 per treatment group). Systemic JMV2959 significantly reduced the duration of ($P < 0.05$, Fig. 2D), frequency of ($P < 0.0001$, Fig. 2E) and had a tendency in increasing the latency to ($P = 0.0885$, Fig. 2F) social behaviours. Systemic JMV2959 increased the duration of ($P < 0.001$, Fig. 2G) non-social behaviours.

3.3. GHSR-1A antagonist treatment decreases the 5-HIAA/serotonin ratio in amygdala

Compared to vehicle, systemic administration of JMV2959 decreases the 5-HIAA/serotonin ratio ($P < 0.05$) in amygdala (Table 1). As further shown in Table 1 no other significant effects were obtained. There were no effects of JMV2959 on noradrenaline, dopamine, dopamine metabolites nor dopamine turnover in brain regions investigated (Supplementary material 5).

3.4. There was no difference in latency to aggressive behaviours between vehicle and JMV2959 in mice treated with pCPA

There was an overall main effect of treatment on the latency to aggressive behaviours ($F(3,37) = 7.91$, $P = 0.0003$; one-way ANOVA followed by Bonferroni post-hoc test; Fig. 3). Post hoc analysis revealed that JMV2959 (veh-JMV, n = 12) increased the latency to engage in aggressive behaviour compared to vehicle treatment (veh-veh, $P < 0.05$, n = 13). The latency was lower in both pCPA-vehicle ($P < 0.001$, n = 9) as well as pCPA-JMV2959 ($P < 0.01$, n = 7) treated mice compared to vehicle-JMV2959 treated mice. However, there was no difference in latency to aggression between vehicle and JMV2959 in mice treated with pCPA ($P > 0.05$).

As shown in Supplementary material 6, JMV2959 reduces aggressive behaviour in both vehicle and alcohol treated mice.

3.5. Risk-drinking males carrying the Leu72Leu genotype score significantly higher in overt aggression

In the human genetic study none of the studied SNPs (all $P > 0.08$) were by itself associated with overt aggression or AUDIT scores. The rs696217 polymorphism located in the *GHRL* did however show a significant interaction with risk-drinking on overt aggression ($F(1,717) = 7.99$, $P = 0.00482$). None of the other SNP interaction tests survived correction for multiple testing.

Upon closer inspection of the interaction between rs696217 and risk-drinking (Fig. 4), it was apparent that male carrying the Met allele (Leu72Met + Met72Met) and having a hazardous alcohol use displayed lower levels of aggression than risk-drinkers who did not have the Met allele (Leu72Leu; $P = 0.011$). In males with low alcohol use (AUDIT < 8) there was however no difference in overt aggression between the genotype groups, confirming previous studies reporting that males with low alcohol consumption display lower overt aggression scores. There was no protective effect of the Met-allele against aggression genotype in these individuals with low intake and aggression.

4. Discussion

We demonstrate that ghrelin regulates aggression and thereby adds another physiological function to the pleiotropic responses of the orexigenic peptide ghrelin. Indeed, central ghrelin infusion into the third ventricle increases the duration and frequency of aggression, and that there is a tendency of an effect on reducing the latency to engage in this behaviour. In line are the data displaying that systemic administration of the GHSR-1A antagonist, JMV2959, reduces aggressive behaviour as measured by a decreased duration, frequency as well as an increased latency to engage in this behaviour. The present findings implying that central ghrelin signalling regulate aggressive behaviour are supported by previous studies showing that mice with high plasma levels of butyrylcholineesterase, which among other things decreases ghrelin, display less spontaneous and intruder-induced fighting (Chen et al., 2015). In addition, indirect support are provided from the preclinical studies showing that ghrelin signalling is associated with the risk of various psychiatric disorders displaying aggression as a cardinal feature (Engel and Jerlhag, 2014; Meyer et al., 2014; Wittekind and Kluge, 2015;

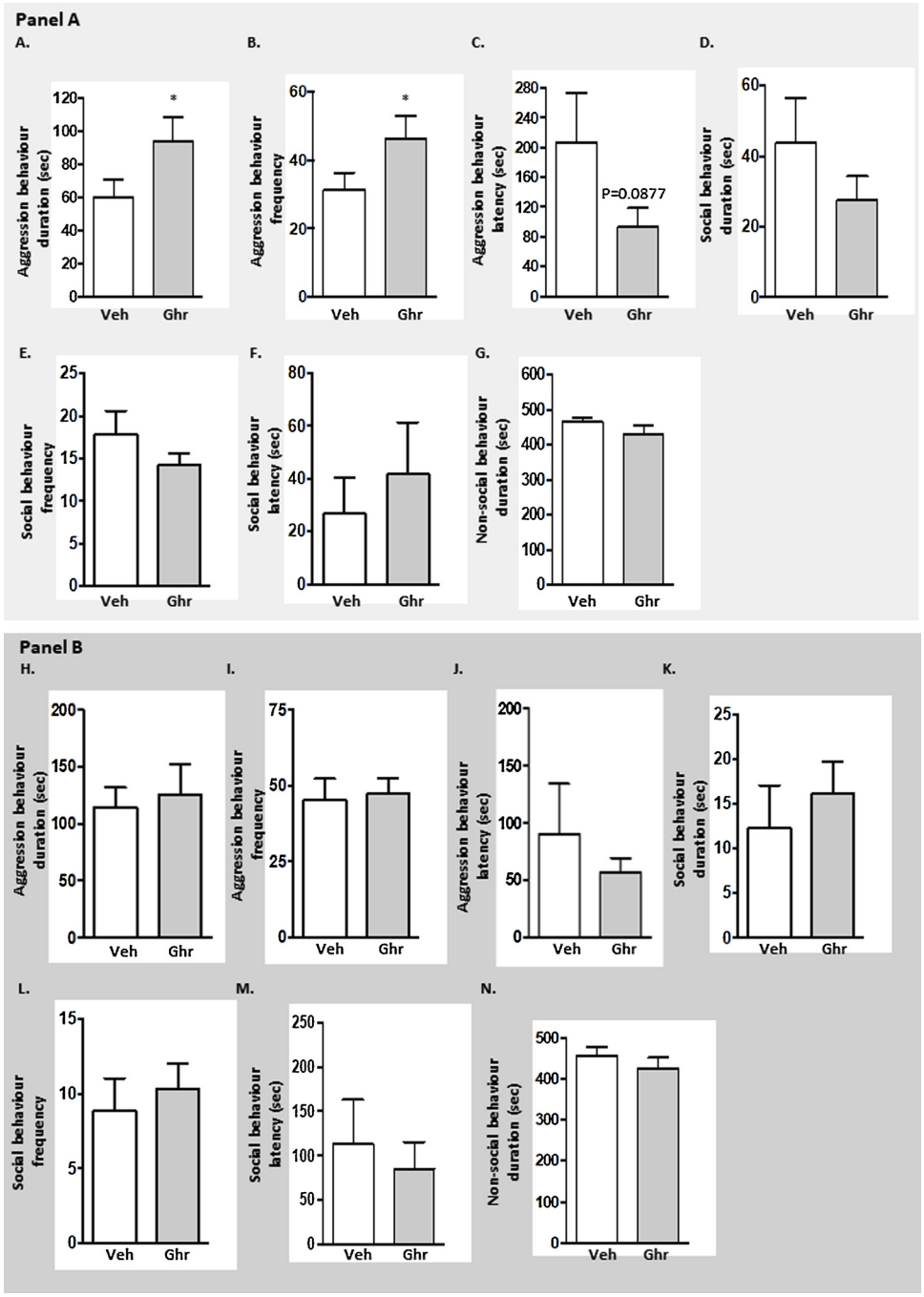

Fig. 1. Central, but not systemic, administration of ghrelin increases aggressive behaviours. Panel A display that central (icv) ghrelin (Ghr, n = 10) significantly increased the duration (A) as well as frequency (B) of aggressive behaviours compared to vehicle (Veh, n = 14). There was a tendency towards a decreased latency C) to engage in aggressive behaviours. There was no effect of ghrelin infusion into the third ventricle on the duration of (D), frequency of (E) and latency to (F) social behaviours. There was no effect of central ghrelin treatment on the duration (G) of non-social behaviours. As revealed in panel B, systemic administration (ip) of ghrelin (n = 12) had on the other hand no effect on the duration of (H), frequency of (I) or latency to (J) aggressive behaviours compared to vehicle (n = 13). Neither was there an effect of systemic ghrelin on the duration of (K), frequency of (L) and latency to (M) social behaviours nor on the duration of (N) non-socials behaviours. Data are presented as mean ± SEM; *P < 0.05, unpaired t-test.

Yoshimoto et al., 2017). However, we did not find an association between any of the SNPs and our main outcome variable overt aggression, indicating that these preclinical data cannot be linked to genetic alterations in humans.

Given that central as well as systemic administration of ghrelin increases food intake (for review see (Egecioglu et al., 2010), it is noteworthy that we in the present study show that ghrelin injected centrally, but not peripherally, increases the frequency as well as duration of aggressive behaviour, demonstrating a discrepancy in behaviour depending on administration route. In support for a divergence between ghrelin's effects, depending on administrations route, are the data showing that central ghrelin, as opposed to circulating, enhances reinforcement from alcohol (Jerlhag et al., 2009; Jerlhag et al., 2014). A tentative explanation to the different behavioural responses to central and peripheral ghrelin might lie in the possibility that peripheral ghrelin administration does not reach deeper brain areas (Furness et al., 2011; Pirnik et al., 2011; Sakata et al., 2009), which are central for aggressive behaviours.

We also provide data suggesting that GHSR-1A and serotonin regulate aggressive behaviour in the same neural circuits since we first show that JMV2959 reduces the 5-HIAA/serotonin ratio in amygdala. As there was no effect per se on either of these parameters of the ratio data these data indicate that JMV2959 collectively moderately increases serotonin and reduces 5-HIAA. This is substantiated by the previous findings reporting that central ghrelin infusion increases the levels of 5-HIAA in amygdala (Hansson et al., 2014). Further indirect support of this link are the data from a recent experimental study reporting that alcohol exposure potentiates ghrelin as well as serotonin signalling in amygdala (Yoshimoto et al., 2017). To further pinpoint the role of ghrelin signalling in amygdala for aggression, upcoming studies

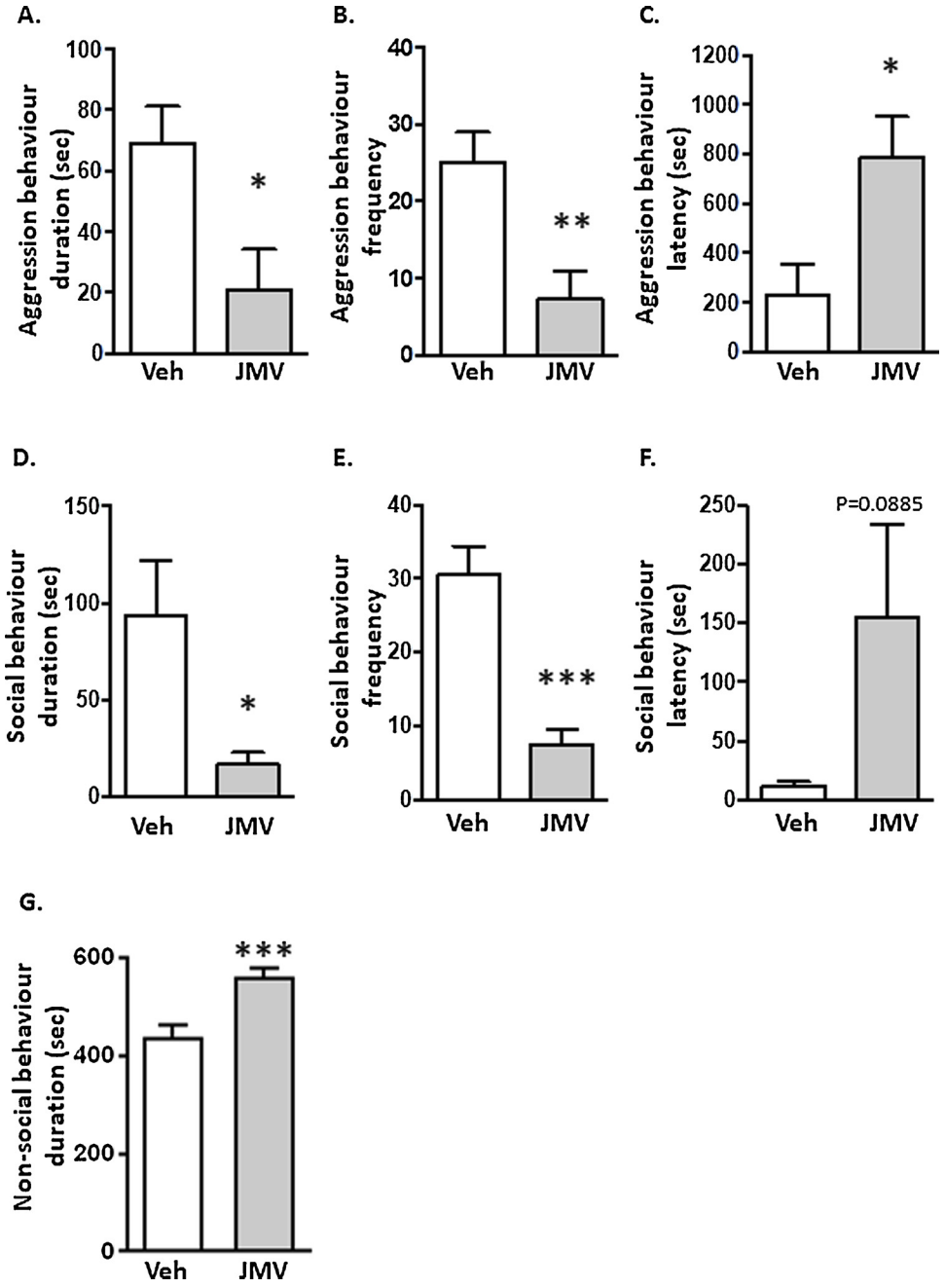

Fig. 2. Systemic administration of JMV2959 reduces aggressive behaviours. Treatment with JMV2959 (JMV, n = 8), the GHSR-1A antagonist, significantly decreased the duration of (A), the frequency of (B) as well as increased the latency to (C) aggressive behaviours compared to vehicle (Veh, n = 8). JMV2959 significantly reduced the duration of (D) and frequency of (E) social behaviours. There was a tendency towards increased latency to (F) social behaviours. JMV2959 increased the duration of (G) non-social behaviours. Data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t-test.

should evaluate the effects of local JMV2959 infusion into different parts of amygdala on behaviour in the resident intruder paradigm. The biochemical tissue analysis further demonstrates that serotonin signalling in other brain areas important for aggressive behaviours including the ventral tegmental area, the ventromedial preoptic area and nucleus accumbens does not participate in JMV2959's anti-aggressive properties. A link between the ghrelin signalling and serotonin systems is further supported by the behavioural data demonstrating that the elevated latency to aggression observed in the vehicle-JMV2959 group, is lower in PCPA-JMV2959 treated mice. This is further substantiated by the findings that the lower aggression latency is similar in PCPA-vehicle and PCPA-JMV2959 treated mice. As PCPA is known to delete central serotonin levels and enhance aggression (Chiavegatto et al., 2001; Mosienko et al., 2012; Pettersson et al., 2016), these data indicate that the ability of JMV2959 to reduce aggressive behaviour depends on intact central serotonergic neurotransmission in male mice. As the behavioural outcome of ghrelin as well as aggression involves various neurotransmitters the possibility should be considered that serotonin has a partial role in promoting ghrelin regulated aggression and that other signalling systems involved in aggressive behaviours such as melanocortin (Gonzalez et al., 1996; Morgan and Cone, 2006) which ghrelin pathway interact with (Huang et al., 2017a) also participate. Albeit it is well-known that serotonin is a potent regulator of aggression (Ambar and Chiavegatto, 2009; de Boer et al., 2009), and that previous studies have shown that ghrelin modulates serotonergic neurotransmission (Hansson et al., 2014; Wauson et al., 2015) this is the first

Table 1
Effects of acute JMV2959 treatment on serotonergic neurotransmission in brain areas.

	Nucleus accumbens (n = 8 and n = 7 for vehicle and JMV respectively)	Ventromedial preoptic area (n = 7 and n = 8 for vehicle and JMV respectively)	Ventral tegmental area (n = 6 for vehicle and JMV)	Amygdala (n = 8 and n = 7 for vehicle and JMV respectively)
5-HIAA	Veh: 1.5 ± 0.1 JMV: 1.3 ± 0.1 P = 0.3792	Veh: 2.4 ± 0.1 JMV: 2.3 ± 0.2 P = 0.7597	Veh: 4.4 ± 0.4 JMV: 5.1 ± 1.1 P = 0.6029	Veh: 3.4 ± 0.5 JMV: 2.0 ± 0.7 P = 0.1434
5-HT	Veh: 1.2 ± 0.2 JMV: 1.1 ± 0.1 P = 0.7555	Veh: 1.5 ± 0.1 JMV: 1.5 ± 0.1 P = 0.9981	Veh: 1.6 ± 0.3 JMV: 2.2 ± 0.8 P = 0.4434	Veh: 1.6 ± 0.2 JMV: 1.5 ± 0.2 P = 0.9381
5-HIAA/5-HT	Veh: 1.3 ± 0.1 JMV: 1.2 ± 0.2 P = 0.6861	Veh: 1.7 ± 0.2 JMV: 1.6 ± 0.1 P = 0.8030	Veh: 3.2 ± 0.5 JMV: 3.2 ± 0.8 P = 0.9881	Veh: 2.2 ± 0.2 JMV: 1.2 ± 0.4 P = 0.0396

JMV2959 (JMV), serotonin (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA).

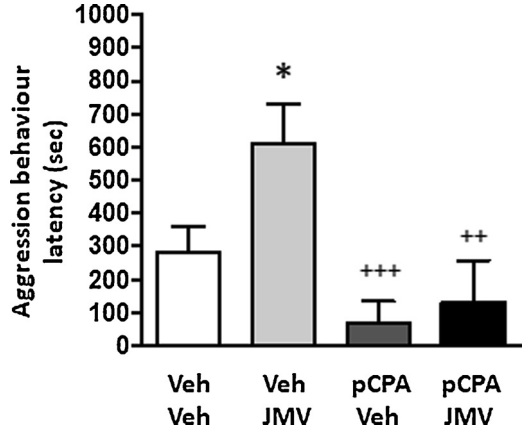

Fig. 3. The ability of GHSR-1A antagonist to reduce aggression involves central serotonin. Treatment with the GHSR-1A antagonist, JMV2959 (Veh/JMV, n = 12), increased the latency to engage in aggression behaviours compared to vehicle treatment (Veh/Veh, n = 13). In addition, this effect was ablated in mice pretreated with pCPA (pCPA/JMV, n = 7; pCPA/Veh, n = 9). Data are presented as mean ± SEM; *P < 0.05 versus Veh-Veh, ++P < 0.01, +++P < 0.001 versus Veh-JMV, one-way ANOVA followed by Bonferroni post-hoc test.

study providing evidence for a link between GHSR-1A, serotonin, and aggression. Given that ghrelin stimulates accumbal dopamine release (Jerlhag, 2008; Jerlhag et al., 2006) and that accumbal dopamine is elevated both during and in anticipation of scheduled aggressive behaviour (Ferrari et al., 2003) raises the possibility of an involvement of dopamine in the regulation of aggression by ghrelin. However, countering this suggestion is the fact that only ghrelin infused into the third ventricle, but not systemically, increased aggression in the current study, while both centrally and peripherally administered ghrelin increases accumbal dopamine levels and that JMV2959 has no effects on accumbal dopamine *per se* (Jerlhag, 2008; Jerlhag et al., 2006). In further support for this contention is our *ex vivo* data showing that JMV2959 does not influence on dopamine transmission in brain regions implicated in aggression behaviours. Moreover, JMV2959 does not modulate noradrenaline in neither investigated area. It should however be mentioned that the contribution of additional neuroanatomical regions and neurotransmitters remains to be investigated in upcoming studies.

As our previous studies have shown that ghrelin increases alcohol consumption (Jerlhag et al., 2009) it is also interesting that our human genetic study demonstrates that young men carrying the Leu72Leu genotype of the *GHRL* and reporting hazardous alcohol use were significantly more aggressive when compared to the group carrying the Met-allele. These data may in part explain previous studies showing that genetic variation contributes to the alcohol-heightened aggression in humans (Cloninger, 1987; Grove et al., 1990). Further, the interaction analyses revealed a significant interaction effect between rs696217 in the *GHRL* and AUDIT in which male subjects with a hazardous alcohol use carrying the Met-allele had lower scores in overt aggression compared to those not carrying the allele. The present SNP results in an amino acid substitution (leucine to methionine) at residue 72, which is outside the mature ghrelin product of 28 amino acids. Although its function is yet to be established, the Met-allele (as SNP or part of haplotype) has in studies of alcohol dependent individuals been associated with fewer self-reported heavy drinking days (Suchankova et al., 2017), self-reported paternal alcohol dependence (Landgren et al., 2010) as well as decreased self-directedness (Landgren et al., 2011). However, no associations with AUD were reported in this normal population primarily assessed for anti-social behaviours. The genetic association study is faced with some limitations, the main being the small sample, the lack of a replication sample and that this SNP causes an amino acid substitution outside of the mature ghrelin product and therefore the physiological relevance of this remains to be elucidated. Despite the preliminary nature of these genetic studies, collectively with previous genetic findings they suggest that the Leu72Leu genotype may lead to an increased risk of aggressive behaviours at hazardous alcohol intake as well as increased risk of alcohol use disorder. However, the preclinical study does not display an interaction between alcohol and GHSR-1A in alcohol naïve mice since JMV2959 decreases aggressive behaviour with a similar magnitude in alcohol and vehicle

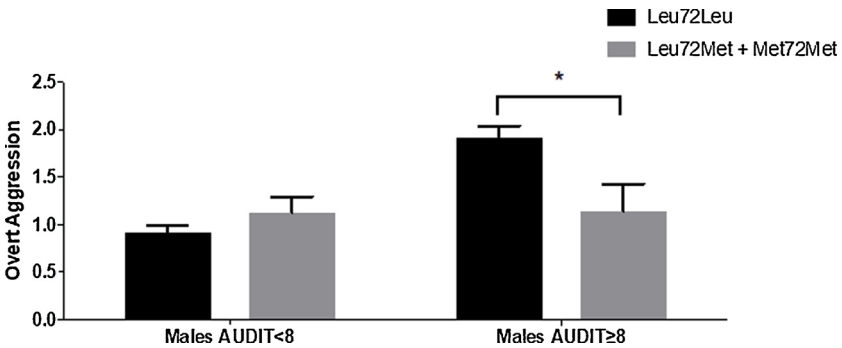

Fig. 4. Risk-drinking males carrying the Leu72Leu genotype score significantly higher in overt aggression. Interaction analysis between rs696217 and hazardous alcohol use, assessed as having hazardous alcohol use (score of ≥8) according to the alcohol use disorder identification test (AUDIT), against overt aggression. Data presented as estimated means ± SE generated from a model taking into account twinship; *P < 0.05.

exposed mice. As the behavioural outcome of acute and chronic alcohol involves different pathways, the interaction between chronic alcohol exposure, aggression and GHSR-1A might produce a different outcome. It is therefore possible that GHSR-1A only modulates the risk of aggressive behaviours at prolonged opposed to acute exposure to alcohol. There are comorbidities between aggression and other psychiatric conditions (Posternak and Zimmerman, 2002). We therefore suggest that future studies focus on the association between Leu72Leu genotype and other psychiatric conditions including drug abuse, anxiety and depressive mood disorders.

The present preclinical study provides compelling support for the role of central ghrelin signalling in aggression, however several limitations possibly influencing the obtained data should be taken into consideration. The behaviour of vehicle treated mice is lower following central (Fig. 1, Panel A) compared to peripheral (Fig. 1, Panel B) administration. This difference is commonly observed when investigating complex behaviours (Egecioglu et al., 2016; Prieto-Garcia et al., 2015).

Therefore inclusion of vehicle controls as well as separate analysis of experiment from central versus peripheral injections diminishes the possibility that intracranial injections damaging tissues could influence the obtained data. The behaviour of different batches of mice commonly vary and to avoid an influence of this confounder the mice were stratified into treatment groups based on their baseline aggression and vehicle controls are always included. Albeit the aggressive behaviours significantly are reduced after peripheral JMV2959 administration, this appears less likely to depend on sedative or exploratory effects on behaviour *per se* since previous studies have established that the selected dose of JMV2959 does not display an effect on locomotor activity nor gross behaviour in general (Jerlhag et al., 2009). The possibility that enhanced motor behaviour by ghrelin influence aggression appears less likely since both central and systemic ghrelin elevates the locomotor activity (Jerlhag, 2008; Jerlhag et al., 2006), whereas only central ghrelin infusion increases aggressive behaviour. The behaviour outcomes of ghrelin appear to be dependent on contextual factors as evident by the emotionality literature showing that ghrelin exhibit an antidepressant and anxiolytic response during pathological conditions (Carlini et al., 2012; Huang et al., 2017b; Lutter et al., 2008), while exhibit an anxiogenic response during normal condition (Asakawa et al., 2001; Carlini et al., 2002; Hansson et al., 2011). Given that acute antidepressant effects of drugs are associated with decreased aggression (for review see (Mitchell, 2005)), it is noteworthy that central ghrelin increases aggression but reduces depressive symptoms (Carlini et al., 2012; Huang et al., 2017b; Lutter et al., 2008). It is therefore likely that the aggression enhancing effects of central ghrelin are independent on its effect on depressive behaviour. Nevertheless should these complex links between contextual factors, emotional states and aggression behaviour which are mediated by ghrelin and serotonergic systems be further elucidated. Ghrelin is an orexigenic peptide, however food was removed prior to test, so the obtained data are independent of feeding behaviour.

In summary, the present result from experimental and human genetic studies reveal a functional role of ghrelin in aggressive behaviours. A vast emerging body of research linking ghrelin with alcohol-mediated behaviours is stemming from various directions including preclinical, clinical and human genetic studies (for review see (Engel and Jerlhag, 2014; Zallar et al., 2017)) aggression contributes significantly to the burden of disease in patients with alcohol dependence (Colman et al., 2009) the possible role of ghrelin in alcohol-induced aggression warrants further investigations.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.02.020.

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