

Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and acetylcholine receptor gene expression.

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ABSTRACT

The decision to eat is strongly influenced by non-homeostatic factors such as food palatability. Indeed, the rewarding and motivational value of food can override homeostatic signals, leading to increased consumption and hence, obesity. Ghrelin, a gut-derived orexigenic hormone, has a prominent role in homeostatic feeding. Recently, however, it has emerged as a potent modulator of the mesolimbic dopaminergic reward pathway, suggesting a role for ghrelin in food reward. Here we sought to determine whether ghrelin and its receptors are important for reinforcing motivation for natural sugar reward, by examining the role of ghrelin receptor (GHS-R1A) stimulation and blockade for sucrose progressive ratio operant conditioning, a procedure used to measure motivational drive to obtain a reward. Peripherally and centrally administered ghrelin significantly increased operant responding and therefore incentive motivation for sucrose. Utilizing the GHS-R1A antagonist, JMV2959, we demonstrated that blockade of GHS-R1A signaling significantly decreased operant responding for sucrose. We further investigated ghrelin's effects on key mesolimbic reward nodes, the ventral tegmental area (VTA) and nucleus accumbens (NAcc), by evaluating the effects of chronic central ghrelin treatment on the expression of genes encoding major reward neurotransmitter receptors, namely dopamine and acetylcholine. Ghrelin treatment was associated with an increased dopamine receptor D5 and acetylcholine receptor nAChR β 2 gene expression in the VTA and decreased expression of D1, D3, D5 and nAChR α 3 in the NAcc. Our data indicate that ghrelin plays an important role in motivation and reinforcement for sucrose and impacts on the expression of dopamine and acetylcholine encoding genes in mesolimbic reward circuitry. These findings suggest that ghrelin antagonists have

therapeutic potential for the treatment of obesity and to suppress the over-consumption of sweet food.

Keywords: food motivation, food reward, ghrelin, operant conditioning, obesity, GHS-R1A, dopamine, acetylcholine

INTRODUCTION

It is well established that the circulating hormone, ghrelin, plays an important role in the regulation of energy balance (Kojima et al., 1999; Nogueiras et al., 2008). Released primarily by the stomach (Dornonville de la Cour et al., 2001), ghrelin elicits potent orexigenic effects both in rodents and in man (Wren et al., 2001; Wren et al., 2000) via its central nervous system receptor (Salomé et al., 2009a), the growth hormone secretagogue receptor (GHS-R1A) (Howard et al., 1996). Indeed, ghrelin targets hypothalamic and brain stem circuits involved in feeding and energy homeostasis (Bailey

et al., 2000; Dickson et al., 1993; Faulconbridge et al., 2003; Faulconbridge et al., 2008; Hewson and Dickson, 2000). Feeding behavior, however, is not only motivated by the need for nutrient repletion (i.e. the need to restore homeostasis); palatable high fat and/or sugar foods can motivate intake despite a state of satiety (Zheng et al., 2009). The over-consumption of palatable natural reinforcers such as sugar is a major factor driving the current obesity epidemic. It remains to be determined whether the central ghrelin signaling system is important for non-homeostatic sugar consumption, thereby providing a potentially important therapeutic target to suppress the intake of caloric, palatable and rewarding sweet foods.

Inspired by recent findings that ghrelin interacts with mesolimbic areas involved in non-homeostatic/reward feeding (Jerlhag et al., 2007) we sought to assess the role of ghrelin and its receptor in food motivation and goal directed behavior for sucrose reward. These mesolimbic areas have long been the focus of drug addiction research as they are a major target for most drugs of abuse (Engel, 1977; Koob, 1992). The target mesolimbic pathway for ghrelin includes the dopamine projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc)(Jerlhag et al., 2006; Jerlhag et al., 2007) a pathway conferring reward from both addictive chemical drugs and natural rewards, including food (Koob, 1992). Interestingly, GHS-R1A is expressed on dopaminergic neurons (Abizaid et al., 2006), implicating possible direct effects of ghrelin on the VTA dopamine system. These immunohistochemical data are complemented by the accumulating behavioral and electrophysiological evidence of ghrelin's effect in the VTA. For example, intra-VTA administration of ghrelin increases the activity of VTA

dopamine neurons (Abizaid et al., 2006) and increases the release of dopamine into the NAcc (Jerlhag et al., 2007). Ghrelin also increases the activity of the cholinergic-dopaminergic link, an important reward pathway, and at least part of ghrelin's effects on dopamine seem to be mediated by the cholinergic system (Jerlhag et al., 2007).

While established that ghrelin has a potent orexigenic effect when food is readily available, it is not yet known whether the orexigenic effects of ghrelin can be extended to include changing motivation and reinforcing aspects of natural reinforcers such as palatable sweet food (i.e. increasing wanting, and the effort/work one is willing to put into obtaining a sweet treat). The motivation and reward efficacy of drugs of addiction can be evaluated in the self-administration, operant conditioning model. Operant conditioning is a principal procedure for the analysis of motivated behavior that assesses acquired and voluntary behavior directed towards obtaining a reward. By measuring the amount of work a subject is willing to expend to obtain the reward it offers an objective measure of reward value (Hodos, 1961). Mesolimbic regions are crucial for motivational aspects of behavior including feeding and it is clear that ghrelin affects neuronal activity in relevant mesolimbic regions. What has not yet been shown is the direct effect of ghrelin on the motivation for high sugar food. The primary aim of our study was to investigate whether the central ghrelin signaling system plays a role in the hedonic/motivational or positive reinforcing properties of high sugar food reward and whether suppression of this system, utilizing a novel selective GHS-R1A antagonist JMV2959 (Salomé et al., 2009a), can suppress motivation to obtain sweets. GHS-R1A antagonists are currently being evaluated therapeutically in type 2 diabetic patients as suppression of ghrelin signalling has beneficial effects on glucose homeostasis (Sun et

al., 2006), effects that would also benefit from reduced intake of sweet foods. Several lines of evidence suggest that dopaminergic and cholinergic neurotransmission play an important role in motivated reward behavior. Therefore, to further characterize the effects of ghrelin on central reward circuitry we evaluated the impact of ghrelin treatment on dopamine and acetylcholine receptor gene expression changes in key reward nodes, the VTA and NAcc, after ghrelin treatment.

METHODS

Animals

Adult male Sprague-Dawley rats (200-250 g, Charles River, Germany) were housed in a 12-hour light/dark cycle with regular chow and water available *ad libitum*, except when indicated otherwise. All animal procedures were carried out with ethical permission and in accordance with the University of Gothenburg Institutional Animal Care and Use Committee guidelines.

Surgery

For behavioral experiments targeting the central nervous system, a third ventricular guide cannula (26 gauge; Plastics One, Roanoke, VA; coordinates: on the midline, 2 mm posterior to bregma, and 5.5 mm ventral to dura mater, with injector aimed 7.5 mm ventral to the dura) was implanted under isoflurane anesthesia. Cannulae were attached to the skull with dental acrylic and jeweler's screws and closed with an obturator, as described previously (Skibicka et al., 2009). Placement of the cannula in the third

ventricle was verified 1 week after surgery, by measurement of the sympathoadrenal-mediated glycemic response to central injection of 5-thio-D-glucose [210 µg in 2 µl of vehicle (saline)] (Ritter et al., 1981). In this placement verification protocol, a post-injection elevation of at least 100% of baseline plasma glucose level was required for subject inclusion. For the gene expression experiment rats were anaesthetized (60-75 mg/kg Ketalar® and 0.5 mg/kg Domitor® i.p.; Pfizer, Sweden; Orion Co, Finland) and a chronic ICV cannula (Alzet Brain Infusion Kit II, DURECT Corp, Cupertino, CA, USA) was inserted into the lateral ventricle using the following coordinates from: 0.6mm posterior from bregma, 1.4 mm lateral from midline, 2.3 ventral from skull. The cannula was connected via a polyethylene catheter to an osmotic minipump (Alzet Mini-Osmotic Pump Model 2002, Durect, Cupertino, flow rate, 0.5 µl/h for 14 days) implanted subcutaneously in the back of the animals.

Operant conditioning model

Apparatus: Operant conditioning experiments took place in eight operant conditioning chambers designed for rats (30.5×24.1×21.0 cm; Med-Associates, Georgia, VT, USA), which were placed in a sound-attenuated, dimly lit cabinet. Each chamber had a metal grid floor, two retractable levers with white light bulbs above them and a food pellet dispenser that can deliver 45 mg sucrose pellets (GlaxoSmithKline, Test Diet, Richmond, IN, USA) to the food tray. Data collection and processing were controlled by MED-PC software.

Training: The procedure used for operant conditioning was adapted from (la Fleur et al., 2007) and (Tracy et al., 2008). All rats were subjected to a mild food restriction paradigm

during which their initial body weight was gradually reduced to 90% over a period of one week. For the ICV cannulated rats the training commenced one week after the surgery. Prior to placement in the operant boxes, rats were exposed to the sucrose pellets in the home cage environment on at least two occasions. Next, rats learned to lever press for sucrose pellets under a fixed ratio FR1 schedule, with 2 sessions/day. In FR1, a single press on the active lever resulted in the delivery of one sucrose pellet. All FR sessions lasted 30 min or until the rats earned 100 pellets, whichever occurred first. Most rats achieved the 100 pellets per session criterion after 10 to 15 sessions. Presses on the inactive lever were recorded, but had no programmed consequence. FR1 schedule sessions were followed by FR3 and FR5 (i.e. 3 and 5 presses per pellet respectively). Again, a minimum of 100 responses per session on the active lever was required for the advancement to the next schedule; most rats required only one to two FR3 and FR5 schedule(s) to achieve this level. The FR5 schedule was followed by the progressive ratio (PR) schedule during which the cost of a reward is progressively increased for each following reward, in order to determine the amount of work the rat is willing to put into obtaining the reward. The response requirement increased according to the following equation: response ratio = $(5e^{(0.2 \times \text{infusion number})}) - 5$ through the following series: 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328. The PR session ended when the rat had failed to earn a reward within 60 min. The break point was defined as the final completed ratio before the session ended. Responding was considered stable when the number of food pellets earned per session did not differ more than 15% for three consecutive sessions. In most cases, responding stabilized within 5–7 sessions. PR test was carried out 1 session/day. Sessions lasted on average 75 min

although all rats stayed in the operant boxes until 120 min to allow for all sessions to end. Rats were subsequently transferred to home cages for a 1 hr free-feeding chow intake measurement. At the end of training and prior to testing rats were returned to an *ad libitum* feeding schedule.

Experimental Design: All rats received intraperitoneal (IP) or, in a separate group of rats, third ventricle (3rd ICV) injections early in the light cycle (for ghrelin tests) and late in the light cycle for ghrelin antagonist experiments, 20 min prior to the start of operant testing. All conditions were separated by a minimum of 48 hr and run in a counterbalanced manner (each rat received all conditions on separate testing days).

Experiment 1: Impact of peripheral or central ghrelin administration on progressive ratio operant responding for sucrose in rats. For all rats, lever-pressing responses were examined after two conditions: IP treatment with saline or acylated rat ghrelin (Tocris, Bristol, UK; 0.33 mg/kg body weight at 1 ml/kg). The selected IP ghrelin dose has been shown previously to induce a feeding response in rats (Wren et al., 2000) and also to induce accumbal dopamine release and locomotor activity in mice (Jerlhag, 2008). Subsequently to operant testing, rats were allowed free access to chow and chow intake was measured after a 1 hr period. Next, in a separate group of rats, we examined responses after targeted CNS drug delivery in a separate group of rats after three conditions as follows: control condition with third ventricle saline, 0.5 µg or 1.0 µg of acylated rat ghrelin (Tocris) in a 1 µl volume. The selected doses of ghrelin have previously been shown to elicit feeding responses (Nakazato et al., 2001). For both the

ICV and the IP ghrelin studies, lever-pressing experiments were performed in the satiated state (ie when food intake would be driven by the rewarding properties of the food rather than homeostatic drives). Also, in both studies, subsequent to operant testing, rats were allowed free access to chow and chow intake was measured after a 1 hr period.

Experiment 2: Impact of peripheral or central treatment with a ghrelin receptor (GHS-R1A) antagonist (JMV2959) on incentive motivation for a sucrose reward in rats.

Progressive ratio operant responses were examined after three conditions as follows: control condition with IP saline, 1 mg/kg or 3 mg/kg of JMV2959 (AEZS-123, AeternaZentaris GmbH, Frankfurt, Germany). The JMV2959 doses were selected based on (Egecioglu et al., 2010; Jerlhag et al., 2009) and preliminary data, previously shown to decrease conditioned place preference behavior but not have an independent effect on locomotor activity. Subsequent to operant testing, rats were allowed free access to chow. To assess the effects of direct acute central antagonist action, in a separate group of rats, operant behavior was examined after the following three conditions: control condition with third ventricle saline injection, 5 µg or 10 µg of JMV2959 in a 1 µl volume. The selected ICV doses of JMV2959 dose was based on (Salomé et al., 2009a) in which the orexigenic action of 1 µg ghrelin administered ICV was blocked. Subsequent to operant testing, rats were allowed free access to chow and chow intake was measured after a 1 hr period and also at 24 hr after the initial injection. Studies with the GHS-R1A antagonist, in contrast to those performed with ghrelin (see above) were performed in rats after a 16 hr food restriction prior to the injections in order to ensure high levels of endogenous circulating ghrelin (Cummings et al., 2001).

Experiment 3: Ghrelin-induced changes in expression of dopamine- and acetylcholine-genes in the VTA and NAcc. Here we determined the effects of chronic ICV ghrelin infusion for two weeks on the expression of selected genes involved in dopaminergic and cholinergic transmission, in two key mesolimbic reward pathway nodes, the VTA and NAcc. The selected dopamine-related genes were genes encoding the dopamine receptors (D1A, D2, D3, D5), catechol-O-methyltransferase, tyrosine hydroxylase (TH; in VTA only) and monoamine oxidase A. The acetylcholine-related genes were: nicotinic receptor subunits ($\alpha 3$, $\alpha 6$, $\beta 2$, $\beta 3$). The genes we chose to evaluate have previously been implicated in ghrelin's effects and/or to reward/motivation behavior (Dalley et al., 2007; Figlewicz et al., 2006; Jerlhag et al., 2006; Jerlhag et al., 2007; Kelley et al., 2002; Kuzmin et al., 2009; Lee et al., 2009; Nimitvilai and Brodie, 2010; Perello et al., 2010; Sibilia et al., 2006). A chronic ghrelin/saline infusion protocol was used in preference to acute injection in order to increase chances of seeing an effect on gene expression; moreover, if ghrelin is an important regulator of the reward system in the longterm, promoting over-eating and obesity, its chronic effects to alter key reward mechanisms are likely to be of considerable importance.

Drug administration and tissue dissection: The catheter and the osmotic pump were filled with acetylated human ghrelin (gift from Rose Pharma, Copenhagen, Denmark) solution (8.3 μg /rat/day), or saline vehicle solution (0.9 % NaCl); this dose and length of treatment has previously been show to affect gene expression in the hypothalamus (Salomé et al., 2009b). Fourteen days after implantation of the minipumps, rats were

killed by decapitation. Brains were rapidly removed and the VTA and the NAcc were dissected using a brain matrix (borders of each regions were determined based on Paxinos and Watson, 1983), frozen in liquid nitrogen and stored at -80° C for later determination of mRNA expression.

RNA isolation and mRNA expression: Individual brain samples were homogenized in Qiazol (Qiagen, Hilden, Germany) using a TissueLyzer (Qiagen). Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) or RNeasy Micro Kit (Qiagen), both with additional DNase treatment (Qiagen). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis, total RNA was reversed transcribed using random hexamers (Applied Biosystems, Sundbyberg, Sweden), and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK), according to the manufacturer's description. Recombinant RNaseout® Ribonuclease Inhibitor (Invitrogen) was added to prevent RNase-mediated degradation. All the cDNA-reactions were run in triplicate. Real-time RT PCR was performed using TaqMan® Custom Array assays. They were designed with TaqMan probe and primer sets for target genes chosen from an on-line catalogue (Applied Biosystems). Each port on the TaqMan® Array platforms was loaded with cDNA corresponding to 100 ng total RNA, combined with nuclease free water and 50 µl TaqMan® Gene Expression Master Mix (Applied Biosystems) to a final volume of 100 µl. The TaqMan® Arrays were analyzed using the 7900HT system with a TaqMan Array Upgrade (Applied Biosystems). Thermal cycling conditions were: 50°C for 2 min, 94.5°C for 10 min, followed by 40 cycles of 97°C for 30 s, and 59.7°C for 1 min.

Gene expression values were calculated based on the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), where the saline-treated group was designated the calibrator. Briefly, ΔC_t represents the threshold cycle (C_t) of the target gene minus that of the reference gene and $\Delta\Delta C_t$ represents the ΔC_t of the target gene minus that of the calibrator. Relative quantities were determined using the equation; relative quantity = $2^{-\Delta\Delta C_t}$. For the calibrator sample, the equation is relative quantity = 2^{-0} , which is 1; therefore, every other sample is expressed relative to this. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene.

Statistics

All behavioral parameters were analyzed by analysis of variance (ANOVA) followed by *post hoc* Tukey test or t tests as appropriate. Statistical analyses were conducted using Statistica software (Tulsa, Oklahoma). In order to analyse the effect of chronic central ghrelin treatment on gene expression, t-test was used, with P-values calculated using the ΔC_t - values. Differences were considered significant at $P < 0.05$. Data are expressed as mean \pm SEM.

RESULTS

Experiment 1: Impact of peripheral or central ghrelin administration on progressive ratio operant responding for sucrose in rats.

Here we employ a paradigm utilized in addiction research to assess the role of ghrelin in natural sweet food motivation and reinforcing properties of sugar. Specifically, to determine the role of peripheral ghrelin administration on sucrose reward efficacy, we examined sucrose self-administration in a progressive response schedule in rats 20 min after IP injection of vehicle or ghrelin. All measures of operant behavior were significantly increased in rats after acute peripheral ghrelin injection: active lever pressing ($P < 0.05$ for all time points), number of sugar pellets earned ($P < 0.005$ for all time points) and 120 min break point ($P < 0.005$, 32.53 ± 3.4 and 41 ± 4.3 for vehicle and ghrelin respectively; Figure 1 A-B). The literature primarily supports a central site of action for ghrelin's orexigenic effect. However GHS-R1A is also expressed outside of the CNS in sites relevant for food intake control, for example on the vagus nerve; therefore it cannot be ruled out that part of the observed effects of IP ghrelin are mediated by those peripheral receptors. Central injection of low volume and dose of ghrelin however stimulates only the CNS GHS-R1A. Therefore, in order to determine a direct CNS effect of ghrelin on sucrose reward efficacy we performed a parallel study in which vehicle or ghrelin were administered by third ventricle injection, also 20 min prior to the operant paradigm. Consistent with a central site of effect hypothesis, acute ICV ghrelin injection to rats (both 0.5 μg and 1.0 μg doses) significantly increased all of the aforementioned measures of operant behavior (Figure 2 A-B). The time course of the active lever

responses in the ICV ghrelin study revealed that while the effect emerged slowly during the 10 and 30 min time points, it reached significance at 60 min [active lever: 10 min $F(2, 24) = 0.94, P=0.41$, 30 min $F(2, 24) = 3.13, P = 0.06$, 60 min $F(2, 24) = 5.86, P<0.01$, 90 min $F(2, 24) = 6.42, P<0.01$, 120 min $F(2, 24) = 6.03, P<0.01$; rewards earned: 10 min $F(2, 24) = 0.26, P=0.78$, 30 min $F(2, 24) = 2.76, P = 0.08$, 60 min $F(2, 24) = 8.31, P<0.005$, 90 min $F(2, 24) = 10.16, P<0.001$, 120 min $F(2, 24) = 11.93, P<0.001$; and break point: $F(2,24) = 7.22, P<0.005$ ($17.31\pm1.53, 33.15\pm5.52, 36\pm6.95$ for vehicle, 0.5 μg and 1.0 μg ghrelin respectively)], a time course consistent with other reports of ghrelin-induced feeding latency when delivered by this route (Faulconbridge et al., 2003). In both experiments, activity at the inactive lever was minor and did not differ significantly between the different treatment groups (IP $4.1\pm1.1, 4.1\pm1.1$ for vehicle and ghrelin respectively; ICV $3.9\pm1.1, 2.1\pm0.7, 3.5\pm1.6$ for vehicle, 0.5 μg and 1.0 μg ghrelin respectively), suggesting that the treatment does not produce unspecific non-goal directed changes in activity. Immediately after operant testing, rats were returned to their home cages and allowed free access to chow; rats injected with ghrelin, whether given peripherally ($P<0.05$) or centrally ($F(2, 24) = 12.64, P<0.001$), nearly doubled their chow intake during the first hour as compared to the vehicle-treated groups (Figure 1C, 2C). In line with previous data (Faulconbridge et al., 2003) indicating that most of the hyperphagic effect of acute central ghrelin injection takes place within 3 hr after the injection, no effect on chow intake was noted in our study at 3-24 hr after ICV administration of either dose of ghrelin ($17.4\pm1.12, 18.42\pm1.34, 19.12\pm1.43$ vehicle, 0.5 μg and 1.0 μg ghrelin respectively, $F(2, 24) = 2.27, P=0.13$).

Experiment 2: Impact of peripheral or central treatment with a ghrelin receptor (GHS-R1A) antagonist (JMV2959) on incentive motivation for sucrose reward in rats.

Next we explored the effects of pharmacological blockade of GHS-R1A on sucrose reward efficacy. Thus, sucrose self-administration in a progressive response schedule was examined in overnight food-restricted rats, to ensure high levels of endogenous circulating ghrelin, 20 min after IP injection of vehicle or 1 mg/kg or 3 mg/kg of JMV2959, a GHS-R1A antagonist. All of the measures of operant behavior were significantly decreased in rats after peripheral injection of JMV2959 [active lever: 5 min $F(2, 24)=11.53$ $P<0.0005$, 120 min $F(2, 24)=11.27$, $P <0.001$; rewards earned: 5 min $F(2, 24)=23.39$ $P<0.0005$, 120 min $F(2, 24)=9.26$, $P <0.001$ and break point at 120: $F(2,24) = 5.98$, $P<0.01$ (45.31 ± 6.45 , 42.08 ± 5.80 , 30.0 ± 5.89 for vehicle, 1 mg/kg and 3mg/kg JMV2959 respectively)]. Post hoc analysis revealed that the main effect was driven by the 3 mg/kg dose (Figure 3A-B). To determine the role of the central ghrelin receptor in sucrose reward efficacy, a similar study was performed in which vehicle or JMV2959 (5 μ g or 10 μ g) was administered to the third ventricle 20 min before the operant measurements. All of the aforementioned measures of operant behavior were significantly decreased in rats after acute third ventricle infusion of both doses of JMV2959 (Figure 4 A-B). The observed effect was immediate as post hoc analysis revealed significant differences among the treatment groups only after 10 min of activity in the operant chamber that were maintained throughout the testing period [active lever: 10 min $F(2, 24)=10.16$, $P<0.0005$, 30 min $F(2, 24)=11.48$, $P<0.0005$, 60 min $F(2, 24)=9.11$, $P<0.001$, 90 min $F(2, 24)=8.30$, $P<0.001$, 120 min $F(2, 24)=4.95$, $P<0.05$; rewards earned: 10 min $F(2, 24)=21.23$, $P<0.0001$, 30 min $F(2, 24)=25.08$, $P<0.0001$, 60

min $F(2, 24)=19.24, P<0.0001$, 90 min $F(2, 24)=20.04, P<0.0001$, 120 min $F(2, 24)=5.44, P<0.01$; and break point: $F(2,24) = 3.78, P<0.05$ ($51.4\pm 8.58, 38.13\pm 5.07, 33.67\pm 5.21$ for vehicle, 5 μg and 10 μg JMV2959 respectively)].

As expected (Hodos, 1961; Jewett et al., 1995), in all treatment groups, including both IP and ICV administration routes, the effect of food deprivation on the operant response for sucrose was evident (Figure 3A, 4A) and contrasts with that observed in the satiated state (Figure 1A, 2A). Activity on the inactive lever was minor (IP $9.6\pm 3.0, 6.8\pm 2.2, 5.6\pm 1.9$ for vehicle and 1 mg/kg or 3 mg/kg JMV2959; ICV $6.4\pm 1.3, 4.6\pm 1.3, 4.4\pm 1.7$ for vehicle, 5 μg and 10 μg of JMV2959 respectively) and, whether administered peripherally or centrally, JMV2959 did not have any significant effect on that activity (this activity did not differ significantly between the different treatment groups). For the ICV study, immediately after the operant testing, rats were returned to their home cages and allowed free access to chow; interestingly, no effect on chow intake was noted at either the 1 hr (Figure 4C) or 24 hr time point (data not shown). This could indicate that while ghrelin signaling is required for the deprivation-induced food motivation it is not essential for the free feeding induced by 16 hr food deprivation, likely due to other redundant mechanisms activated during a deprivation period. All of the free feeding measurements took place 140 min post injection of the drug and so we cannot exclude that the lack of effect is partially due to wash out of the drug.

Experiment 3: Ghrelin-induced changes in expression of dopamine- and acetylcholine-genes in the VTA and NAcc. In the present study we also explored whether the dopamine and acetylcholine related genes are altered by ghrelin in key mesolimbic nodes, the VTA

and NAcc, by examining the effects of chronic central ghrelin treatment on the expression of selected dopamine receptors and enzymes involved in dopamine production and metabolism, in a paradigm already established to produce ghrelin associated changes in gene expression in the hypothalamus (Salomé et al., 2009b). In the VTA dopamine receptor D5 and nicotinic acetylcholine receptor (nAChR β 2) had an increased mRNA expression in the ghrelin-treated rats compared to the saline-treated group (Figure 5A). In the NAcc, there was a decreased mRNA expression of the genes encoding dopamine receptors D1A, D3, and D5 and also the nicotinic acetylcholine receptor nAChR α 3 in the ghrelin-treated rats compared to the saline-treated group (Figure 5B).

DISCUSSION

Here we reveal a role for the central ghrelin signaling system in the modulation of incentive motivation and reinforcing properties of sucrose reward and indicate an impact of chronic central ghrelin treatment on gene expression of dopaminergic and cholinergic receptors in key mesolimbic reward nodes. The results demonstrate that both central and peripheral delivery of ghrelin significantly increases the amount of work an animal is willing to do to obtain sucrose reward. Furthermore, systemic or central blockade of the ghrelin receptor suppressed operant responding for sucrose. Thus, we may infer that endogenous ghrelin signaling is of importance for the incentive motivation for a sucrose reward. Our findings are in line with the hypothesis that an important role of the central ghrelin signaling system is to increase the incentive value of rewards, including food. Given that food restriction increases the rewarding value of sucrose (Hodos, 1961; Jewett et al., 1995) and that ghrelin levels are elevated during short term food restriction

(Gualillo et al., 2002) it is possible that during a state of food restriction/deprivation ghrelin is one of the contributing factors that increases the rewarding value of food/food motivation. Indeed, peripheral ghrelin exposure increased operant behavior to levels similar to those observed in food deprived rats and conversely, blockade of ghrelin signaling decreased operant behavior to levels noted in non-deprived rats.

It now seems clear that problematically increased food intake likely reflects a dysregulation of the central mechanisms of food reward, involving both hedonic and motivational aspects. As free feeding and reward motivated feeding appear to be two separable phenomena with differential controlling neuroanatomical substrates (Salamone et al., 1991), it is important to examine both when assessing a role of agents involved in feeding behavior. Ghrelin's potent orexigenic effects have largely been studied in free feeding access models, in which it would be difficult to distinguish between its role in nutrient repletion versus reward motivated feeding. In the present study, we found that GHS-R1A ligands interfere with the motivation for sucrose reward, using an experimental model that has been used in other contexts to show wanting and motivation for addictive drugs of abuse. An increase in motivated behavior is common to both chemical drug addiction and caloric restriction and likely involves overlapping neurobiological mechanisms. In the present study, we also detected a ghrelin-induced increase in free feeding of normal chow food, in the same animals that expended significantly more work for food in the operant chamber. Therefore our data, taken together with earlier reports of ghrelin effects in free feeding models (Wren et al., 2000),

indicate that ghrelin has the ability to modulate both free feeding as well as feeding motivation.

Given that the ghrelin receptor GHS-R1A is present in key hypothalamic, hindbrain and mesolimbic areas involved in energy balance and reward (Zigman et al., 2006) and that central ventricular injection of GHS-R1A ligands likely gain widespread access to these CNS areas, there could be several relevant neuroanatomical substrates for the sucrose reward motivation effect of ghrelin shown here. It seems likely that ghrelin acts directly on key mesolimbic areas as ghrelin activates VTA dopamine neurons (Abizaid et al., 2006) and direct administration of ghrelin to the VTA increases accumbal dopamine release (Jerlhag et al., 2007). Consistent with this, we have previously reported effects of intra-VTA ghrelin to increase the consumption of rewarding/palatable food in free-choice feeding paradigms and also that lesions of the VTA blunt ghrelin-induced exploratory behavior of palatable food (Egecioglu et al., 2010). The NAcc may also be a direct target for ghrelin in modulating motivational aspects of food intake; when injected directly into this area, ghrelin induces a feeding response (Naleid et al., 2005), although the presence of GHS-R1A in this area in rodents was not described by other investigators (Zigman et al., 2006) and hence, requires further clarification.

Consistently with its essential role in motivated behaviors, several genes within the dopamine system were altered by central ghrelin treatment. These data raise the possibility that regulation of dopamine receptor expression is a long-term mechanism via which ghrelin impacts on the reward-related function and signaling. Evaluation of

dopamine receptors is not only important at the site of release like the NAcc but also in the VTA, as due to dendritic dopamine release (Cragg and Greenfield, 1997), it is likely that it acts locally to influence reward motivated behaviors. Here we found an increased expression of D5 in the VTA after ghrelin treatment. Dopamine D5 receptors are present on the cell bodies of dopaminergic VTA neurons (Ciliax et al., 2000) and their activity is required to restore the VTA dopamine neuron activity after a period of desensitization (Nimitvilai and Brodie, 2010). In the NAcc we noted a decreased expression of D1. In fact reduced expression of this receptor has been recently shown in the NAcc of obesity prone but not obesity resistant rats on high fat diet indicating its potential role in NAcc in obesity and overconsumption (Alsio et al., 2010). Also the expression of genes encoding D3 was reduced by ghrelin, a finding of particular interest, given the decreased availability of D2/D3 receptors in both rat and human drug users correlates with increased impulsivity (Dalley et al., 2007; Lee et al., 2009). Interestingly we did not see any significant changes in the enzymes involved in dopamine synthesis or production.

The important role of the acetylcholine system for drug and food rewards is well documented; here we show that ghrelin treatment was associated with changes in expression of genes encoding several acetylcholine nicotinic receptors subunits, providing another route by which ghrelin can potentially alter reward function. Ghrelin can regulate VTA dopaminergic neurons indirectly, via its action on the cholinergic neurons in the laterodorsal tegmental area (LDTg), an area rich in GHS-R1A, that is important for alcohol reward involving a cholinergic projection to the VTA dopamine system. In fact, previously we showed that bilateral ghrelin injection into the LDTg in mice stimulates dopamine release in a cholinergic-dependent manner (Jerlhag et al.,

2007; Jerlhag et al., 2008) and increases consumption of alcohol in a free choice (alcohol/water) drinking paradigm (Jerlhag et al., 2009). Indeed, recent studies have implicated the cholinergic-dopaminergic reward link in food reward (Dickson et al (in press). Another interesting possibility is that ghrelin can enhance cholinergic signaling in the VTA via upregulation of cholinergic receptors. Indeed our current gene expression data seems to support that mechanism as VTA nAChR β 2 mRNA levels were increased in the ghrelin-treated rats.

The function of NAcc cholinergic neurons and acetylcholine in the NAcc on the other hand have been more controversial with some reports indicating a role of acetylcholine in increasing reward oriented behavior (Pratt and Kelley, 2005, Pratt and Blackstone, 2009), but other indicating that Ach in NAcc may act to inhibit feeding and play a role in satiety mechanism (Helm et al., 2003, Hoebel et al., 2007). Indeed our results seem to be consistent with the latter as ghrelin treatment was associated with a decreased expression of one of the nicotinic receptor subunits, the nAChR α 3. It is important to note that the gene expression studies while very valuable in indicating potential downstream targets of ghrelin only suggest the type of relationship (upregulation or downregulation) needed for expression of the orexigenic/reward oriented response, but do not define it, as it would be difficult to dissociate direct from compensatory changes. Therefore our gene expression studies indicate a connection and provide a platform for future genetic and pharmacological studies determining the role of those genes in ghrelin's effects on free and reward motivated feeding.

Although hypothalamic and brainstem areas most likely contribute to homeostatic feeding, we cannot exclude an indirect role for hypothalamic and/or brainstem afferent systems in ghrelin-induced food reward motivation. Indeed the orexinergic neurons project from the lateral hypothalamus to mesolimbic reward circuitry including the VTA and NAcc (Toshinai et al., 2003, Harris et al., 2005, Perello et al., 2010). The NPY/AgRP neurons of the arcuate nucleus, another target for GHS-R1A ligands (Dickson and Luckman, 1997, Keen-Rhinehart and Bartness, 2007), may also play an important role. NPY has been shown to increase reward efficacy of chow as well as sucrose (Brown et al., 1998) whereas AgRP appears to increase reward efficacy of high fat food only (Tracy et al., 2008). Ghrelin appears to have a role in both sucrose reward (present study) and in high-fat reward (Perello et al., 2010), however the relative importance of the NPY/AgRP neurons for these effects of ghrelin remains to be elucidated. In summary, ghrelin has food motivational properties spanning across nutritional components and most likely affects several brain areas to synchronize a coordinated behavioral response to promote feeding.

Although ghrelin transport into the brain is limited (Banks et al., 2002), peripheral ghrelin appears to access and target areas such as the hippocampus (Diano et al., 2006) and VTA (Jerlhag, 2008). Although there remains some debate over the relevance of the vagus nerve as an indirect route for ghrelin's central effects (Date et al., 2002, Dornonville de la Cour et al., 2005, Arnold et al., 2006), a direct action within the CNS seems likely as the effects of peripheral ghrelin on food intake can be suppressed by intra-VTA administration of ghrelin antagonists (Abizaid et al., 2006). Ghrelin is produced within

the brain (Cowley et al., 2003), although it remains to be determined how this is regulated and whether brain-derived ghrelin provides an important centrally generated signal for food intake and for the motivation to eat. Taken together with the fact that the ghrelin receptor GHS-R1A is constitutively active (i.e. has activity in the absence of ghrelin ligand)(Holst et al., 2003), the question arises as to whether circulating ghrelin provides a physiologically relevant gut-brain signal for incentive motivation for food reward. The results of the present study, showing similar effects on sucrose reward work can be obtained via central and peripheral administration of GHS-R1A ligands, could indicate that both centrally released as well as peripherally released ghrelin can potentially affect food motivation.

In conclusion, our new data provide new evidence that ghrelin signaling is important for the motivation to obtain sucrose reward and impact on dopaminergic and cholinergic gene expression in mesolimbic reward pathway. Our findings inspire important questions regarding the role of the endogenous ghrelin in determining the incentive value for natural rewards such as sugar, in normal appetitive behavior and in the pathophysiology of eating disorders and obesity. Although significant work remains to relate causally the molecular changes in the dopamine and acetylcholine system to impact of ghrelin on reward, our data potentially indicate a novel mechanism by which ghrelin impacts on the reward behavior. Understanding ghrelin's role in reward processes is important for the understanding of the overlapping neurobiology of eating disorders and chemical drug addiction and provides a potential avenue for understanding the etiology of these diseases and for the development of novel therapies. Finally, the possibility to suppress

problematic over-eating of palatable sweet foods using GHS-R1A antagonists may have clinical and therapeutic relevance for the emerging beneficial effects of such compounds for blood glucose control (Sun et al., 2006) in type 2 diabetic patients (Esler et al., 2007).

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Acknowledgements

Research supported by the Swedish Research Council for Medicine (VR 2006-5663; 2009-S266), European Union 7th Framework (FP7-HEALTH-2009-241592; FP7-KBBE-2009-3-245009), ALF Göteborg (SU7601), the Swedish Institute and the Swedish Foundation for Strategic Research to Sahlgrenska Center for Cardiovascular and Metabolic Research (A305-188). We would also like to thank Dr Daniel Perrissoud (AeternaZentaris, GmbH, Germany) for providing the GHS-R1A antagonist JMV2959 and Anders Friberg for assistance with submission.

Figure legends

Figure 1. Peripheral ghrelin injection increases the motivation to obtain palatable food in a PR ratio operant conditioning model. The number of responses on the active lever (A) and the number of 45 mg sucrose rewards obtained (B) are significantly increased by 0.33 mg/kg IP ghrelin injection for a 120 min period of operant testing. Intake of freely available chow is also increased by IP ghrelin injection (C). Data represent the mean \pm SEM., n=15, *p<0.05, **p<0.005 from vehicle.

Figure 2. CNS (3rd ICV) ghrelin delivery increases the rewarding value of sucrose in a PR ratio operant conditioning model. The number of responses on the active lever (A) and the number of 45 mg sucrose rewards obtained (B) are significantly increased by 3rd icv ghrelin injection for the 120 min period of operant testing. Short term intake of freely available chow is also increased by IP ghrelin injection (C). Data represent the mean \pm SEM., n=13, *p<0.05, **p<0.005 from vehicle, post hoc Tukey analysis.

Figure 3. Peripheral delivery of a ghrelin receptor antagonist, JMV2959. decreases the motivation to obtain palatable food in a PR ratio operant conditioning model. The number of responses on the active lever (A) and the number of 45 mg sucrose rewards obtained (B) are significantly decreased by IP JMV2959 injection for the 120 min period of operant testing. Data represent the mean \pm SEM, n=13, *p<0.05, **p<0.005 from vehicle, post hoc Tukey analysis.

Figure 4. Central blockade of GHS-R1A with JMV2959 decreases the motivation to obtain food reward in a PR ratio operant conditioning model. The number of responses on the active lever (A) and the number of 45 mg sucrose rewards obtained (B) are significantly decreased by 3rd ICV delivery of the GHS-R1A antagonist for the 120 min period of operant testing. Short term intake of freely available chow was not altered by central JMV2959 treatment in this paradigm (C). Data represent the mean \pm SEM, n=15, *p<0.05, **p<0.005, ***p<0.0005 from vehicle, post hoc Tukey analysis.

Figure 5. Dopamine and acetylcholine associated gene expression in VTA (A) and NAcc (B) after chronic ICV ghrelin or vehicle treatment. Data represent the mean of fold change relative to saline treatment. D1, dopamine D1 receptor; D2 dopamine D2 receptor; D3, dopamine D3 receptor; D5, dopamine D5 receptor; COMT, catechol-O-methyltransferase, TH tyrosine hydroxylase; and MAOA, monoamine oxidase A; nAChR, nicotinic acetylcholine receptor subunits α 3 α 6, β 2, β 3 *p<0.05, **p<0.005, ***p<0.0005 from vehicle.

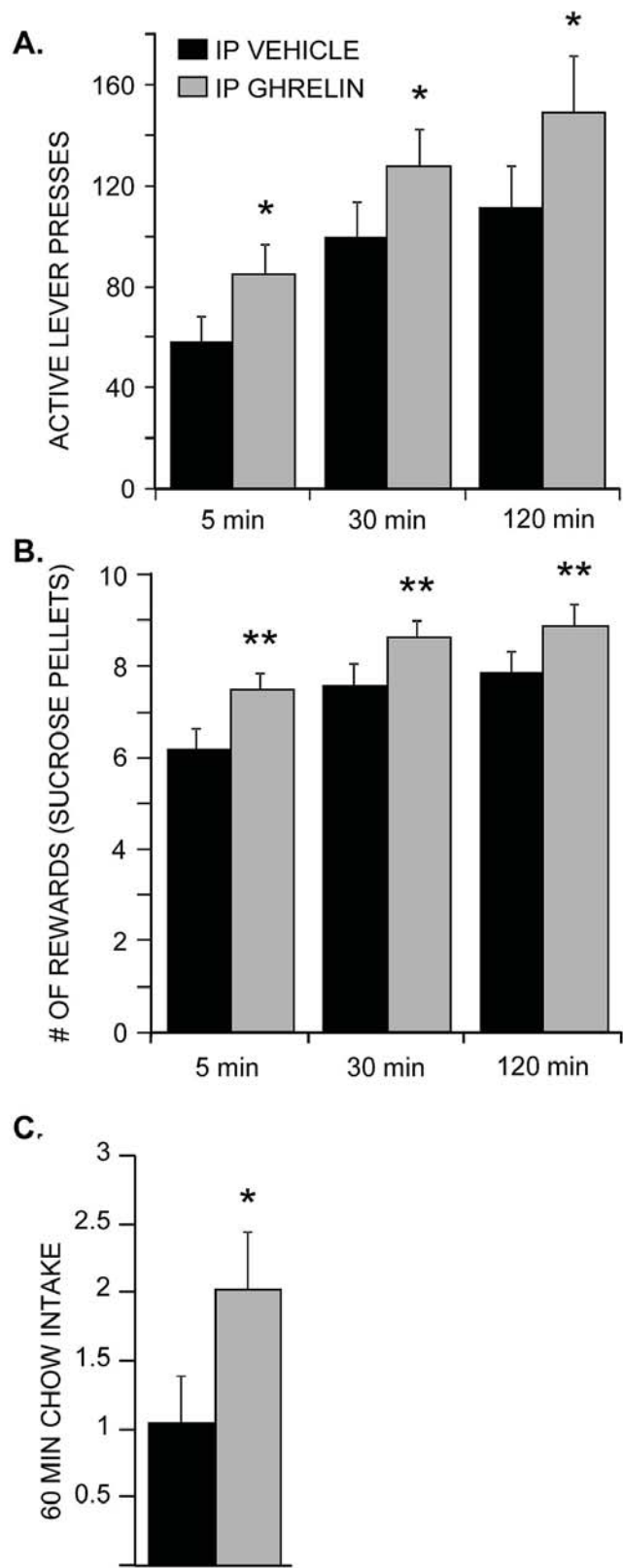
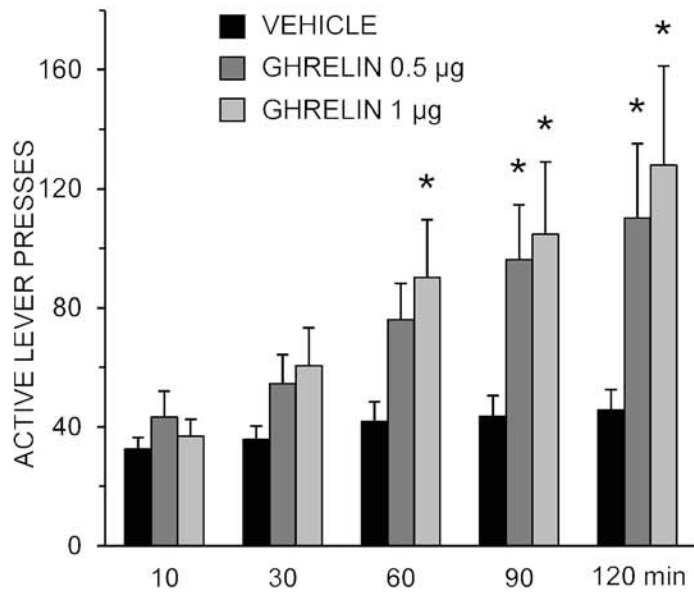
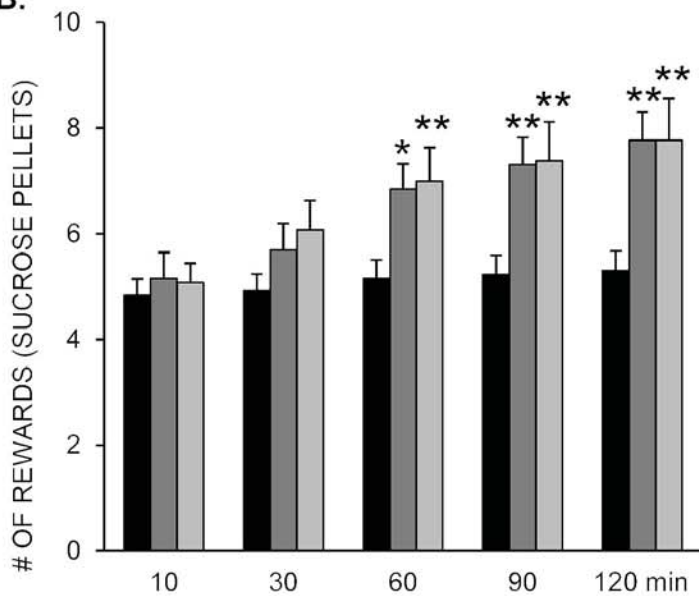


Figure 1, Skibicka et al

A.



B.



C.

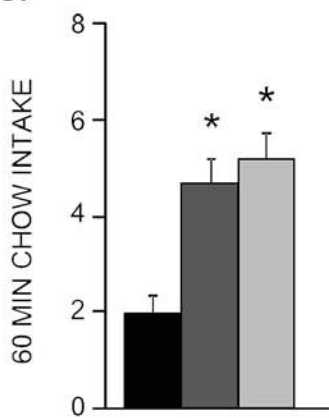


Figure 2. Skibicka et al.

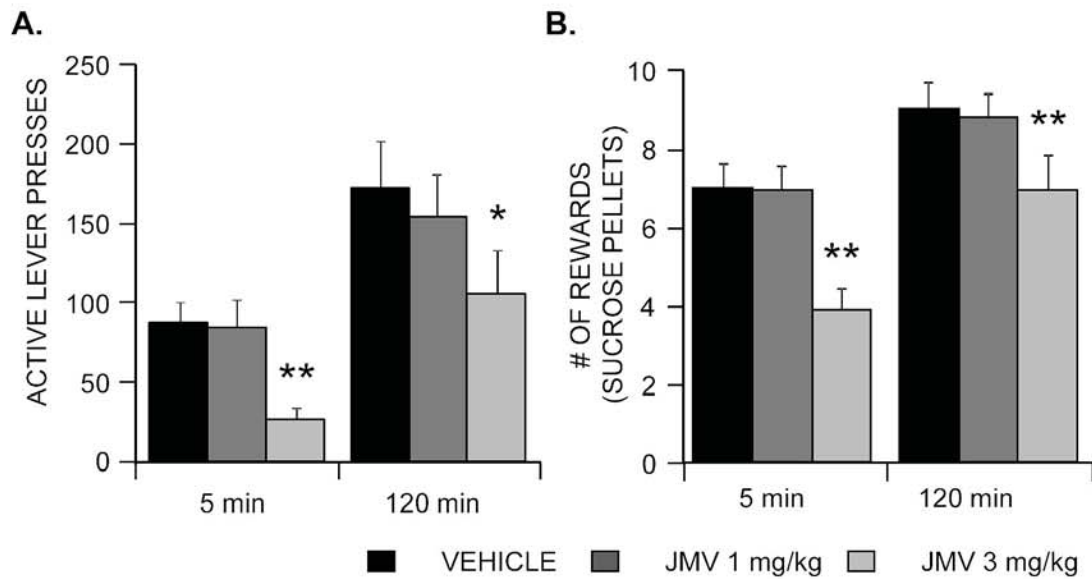


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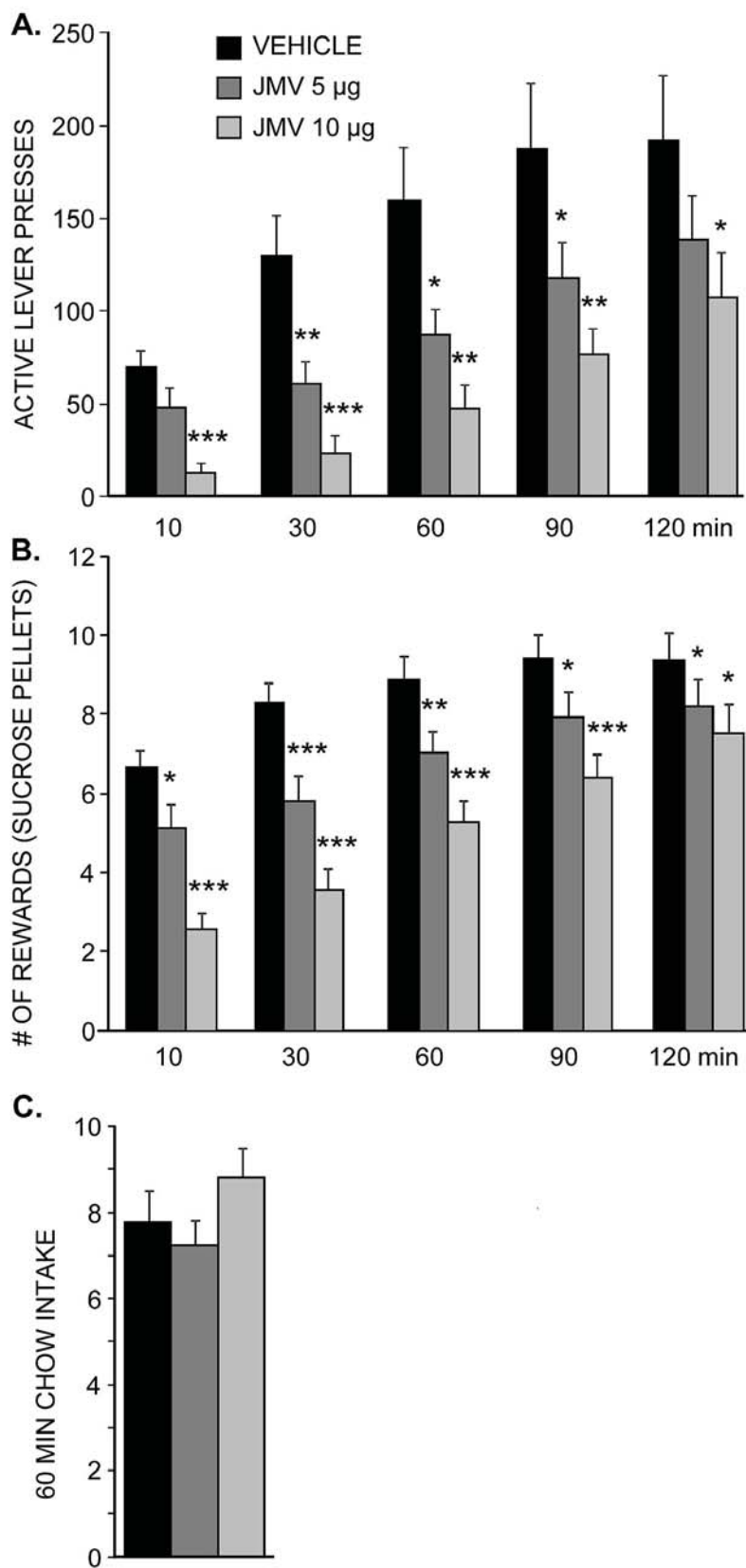


Figure 4, Skibicka et al

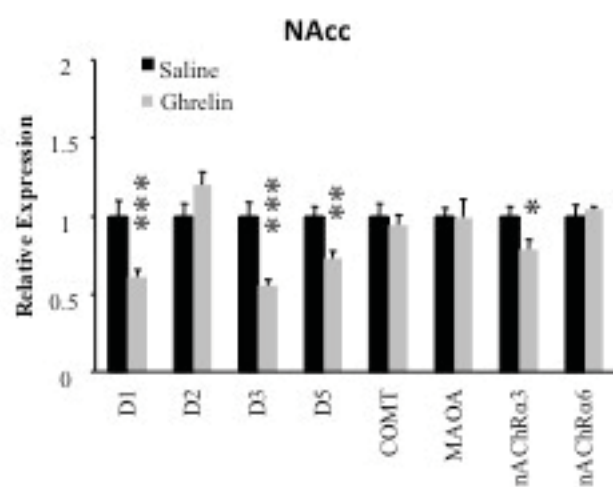
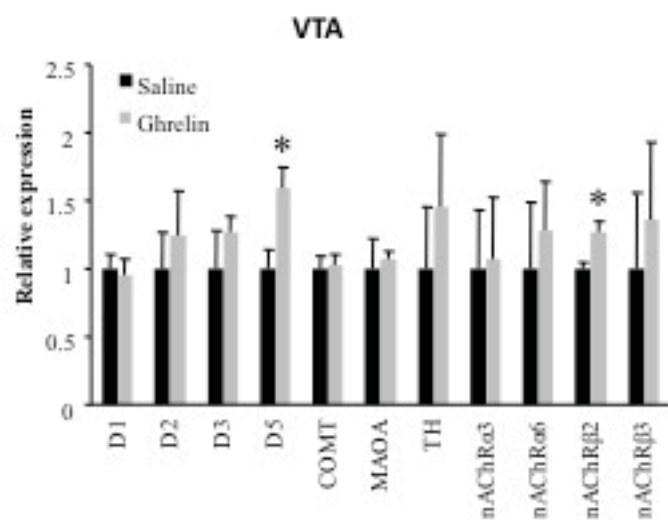


Figure 5. Skibicka et al