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Neuroscience, 2010 Dec 29;171(4):1180-6

URL: http://dx.doi.org/10.1016/j.neuroscience.2010.10.005

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Running title: nAChR and ghrelin-induced feeding

Key words: GHS-R1A, nAChR, dopamine, appetite, hunger, ventral tegmental area, laterodorsal tegmental area, reward-seeking, CPP, palatable food

Abstract

Here we sought to determine whether ghrelin's central effects on food intake can be interrupted by nicotinic cholinergic receptor (nAChR) blockade. Ghrelin regulates mesolimbic dopamine neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens, partly via cholinergic VTA afferents originating in the laterodorsal tegmental area (LDTg). Given that these cholinergic projections to the VTA have been implicated in natural as well as drug-induced reinforcement, we sought to investigate the role of cholinergic signaling in ghrelin-induced food intake as well as fasting-induced food intake, for which endogenous ghrelin has been implicated. We found that intraperitoneal (i.p.) treatment with the non-selective centrally active nAChR antagonist, mecamylamine decreased fasting-induced food intake in both mice and rats. Moreover, central administration of mecamylamine decreased fasting-induced food intake in rats. I.c.v. ghrelin-induced food intake was suppressed by mecamylamine but not by hexamethonium, a peripheral nAChR antagonist. Furthermore, mecamylamine (i.p.) blocked food intake following ghrelin injection into the VTA. Expression of the ghrelin receptor, the growth hormone secretagogue receptor 1A (GHS-R1A), was found to co-localise with ChAT, a marker of cholinergic neurons, in the LDTg. Finally, mecamylamine (i.p.) treatment decreased the ability of palatable food to condition a place preference. These data suggest that ghrelin-induced food intake is partly mediated via nAChRs and that nicotinic blockade decreases the rewarding properties of food.

Introduction

It now seems clear that the orexigenic gastric-derived hormone ghrelin targets CNS circuits involved in both energy balance (Tschop et al., 2000) and reward (Jerlhag et al., 2006, Malik et al., 2008). Indeed, ghrelin appears to activate the midbrain dopamine neurons (Abizaid et al., 2006, Jerlhag et al., 2006) that form a key component of the reward system and include a prominent dopaminergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (N.Acc.). Intracerebroventricular (i.c.v.) injections of ghrelin increase accumbal dopamine levels, measured by microdialysis (Jerlhag et al., 2006) and also dopamine turnover in this area (Abizaid et al., 2006). These effects appear to be exerted, at least in part, at the level of the VTA as direct VTA injection of ghrelin also increases accumbal dopamine release (Jerlhag et al., 2007). Consistent with this, the ghrelin receptor, GHS-R1A (growth hormone secretagogue receptor 1A) has been identified on VTA dopamine neurons and ghrelin has been shown to increase the electrical activity of VTA dopamine neurons recorded *in vitro* (Abizaid et al., 2006).

Ghrelin's effects on the VTA dopamine system may also include indirect effects. Within the VTA GHS-R1A is also present on pre-synaptic afferents (Abizaid et al., 2006). Pharmacological studies investigating the identity of such afferents using selective blockers highlight especially the importance of cholinergic and glutamatergic signaling within the VTA for ghrelin's dopamine-releasing effects (Jerlhag et al., 2006, Jerlhag et al., 2008, Jerlhag et al., 2010). Indeed, ghrelin-induced accumbal dopamine release was completely blocked by mecamylamine a non-selective nicotine acetylcholine receptor (nAChR) antagonist administered peripherally or directly into the VTA (Jerlhag et al., 2006, Jerlhag et al., 2008). Taken together with a further study using a more selective nAChR antagonist, alpha conotoxin MII, to suppress ghrelin-induced dopamine release (Jerlhag et al., 2008), it

seems clear that nicotinic cholinergic signaling plays an important role in ghrelin's effects on the midbrain dopamine system. The precise origin of the ghrelin-responsive cholinergic afferents remains to be elucidated although it seems likely that these include the cholinergic projection from the laterodorsal tegmental area (LDTg) as intra-LDTg injection of ghrelin to mice also increases accumbal dopamine release (Jerlhag et al., 2007). Moreover, the ability of ghrelin administered into the LDTg to increase accumbal dopamine is attenuated by blockade of nAChRs in the VTA (Jerlhag et al., 2008). Collectively these data indicate that the so-called "cholinergic-dopaminergic reward link", a circuit conferring the incentive value of natural and artificial rewards (Yeomans et al., 1993, Lanca et al., 2000, Rada et al., 2000, Larsson and Engel, 2004), is a potential target for ghrelin's effects on the reward system.

Recently, we provided evidence that the central ghrelin signaling system is important for reward from both artificial drugs such as alcohol (Jerlhag et al., 2009), cocaine and amphetamine (Jerlhag et al., 2010) and from rewarding/palatable food (Egecioglu et al., 2010). In the latter study, intra-VTA ghrelin injection increased the intake of palatable/rewarding food whereas chronic treatment with a GHS-R1A antagonist reduced preference for such foods. Taken together with the aforementioned effects of ghrelin on the midbrain dopamine system, it seems likely that ghrelin increases the incentive value of rewards such as food by activating the VTA dopamine system, however the role of cholinergic afferents in the ghrelin-dopamine interaction remains to be elucidated.

In the present study we sought evidence that cholinergic signaling, likely involving cholinergic afferents to the mesolimbic VTA dopamine system, is important both for ghrelin's effects on food intake and for food reward. We therefore tested the effects of nAChR blockade on (1) ghrelin-induced food intake, (2) fasting-induced food intake (that appears to

involve increased endogenous ghrelin signaling) and (3) the ability of palatable food to condition a place preference, as measure of food reward. Finally, we sough morphological evidence that cholinergic neurons originating in the LDTg express GHS-R1A.

Materials

Animals. Adult male NMRI mice (25-30 g BWt, B&K, Sollentuna, Sweden) were used for fasting and i.c.v. ghrelin-induced feeding experiments. Adult male Sprague-Dawley rats (~200 g BWt, Charles River, Germany or Taconic, Denmark) were used for fasting-induced, i.c.v. and intra-VTA ghrelin-induced feeding experiments and for food-induced conditioned place preference (CPP) studies. Standard chow (Harlan Teklad; Norfolk, England) and water were available *ad libitum* unless otherwise stated. The animal room was maintained on a 12/12 hour light/dark cycle (with lights on at 0600), at 20°C and 50% humidity. All animals were singly housed following i.c.v. surgery and during the food intake and CPP testing. The local Ethics Committee for Animal Experiments (Gothenburg, Sweden) approved all procedures involving food intake and CPP experiments. Histological procedures to study GHS-R1A mRNA expression were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Drugs and administration. Acetylated rat ghrelin (Bionuclear, Bromma, Sweden) was dissolved in vehicle solution (Ringer, NaCl 140 mM, $CaCl_2$ 1.2 mM, KCl 3.0 mM and $MgCl_2$ 1.0 mM; Merck KGaA, Darmstadt, Germany) and administered into the lateral ventricle at dose of 1 µg/animal in 1 µL. Injections into the VTA were performed unilaterally or bilaterally at a dose of 1 µg/animal in a volume of 500 nL. The doses of ghrelin chosen have previously been shown to affect feeding in rodents and activate the mesolimbic dopamine system (Egecioglu et al., 2010, Naleid et al., 2005, Jerlhag et al., 2006). Mecamylamine (Sigma-Aldrich, Schnelldorf, Germany) was dissolved in normal saline and administered by intraperitoneal (i.p.) injection at 0.5, 1.0, 2.0 and 4.0 mg/kg in a volume of 1 mL/kg. The dose interval of mecamylamine used was selected to cover a range previously shown to affect ghrelin-induced locomotor activity and dopamine release in the N. Acc. (Jerlhag et al., 2006). For i.c.v. injections of mecamylamine a comparatively low dose of 3 µg was used since

higher doses have been reported to have unspecific effects, for example, on the NMDA receptor (O'Dell and Christensen, 1988, Papke et al., 2001). Hexamethonium (Sigma-Aldrich, Munich, Germany) was dissolved in normal saline and administered at 2 mg/kg i.p, in a volume of 1 mL/kg, a dose used previously in comparative studies with mecamylamine of central and peripheral effects of nAChR signaling (Tani et al., 1997, Kamens and Phillips, 2008). All central injections were performed using a microinfusion pump (CMA 400 syringe pump, Solna, Sweden).

Surgery. Mice were implanted with guide cannulae into the dorsal third ventricle and rats into the lateral ventricle using stereotaxic co-ordinates as described previously (Jerlhag et al., 2007, Salomé et al., 2009). In rats, the coordinates used for cannulae directed at the VTA were 6.0 mm posterior, ± 0.6 mm lateral and 8.4 mm deep relative to bregma according to (Paxinos and Watson, 1986). All surgery was preformed at least 4 days prior to the experiments.

Studies investigating the effects of nAChR blockade on i.c.v. ghrelin-induced food intake in mice and rats.

All feeding experiments commenced in the morning. Satiated mice were i.p. injected with mecamylamine (2 mg/kg) or saline vehicle at 10 min prior to i.c.v injection of ghrelin (1 μg) or an equal volume (1 μL) of vehicle. Food intake was measured at 4 hr following central ghrelin or vehicle administration. Likewise, satiated rats were i.p.-injected with either mecamylamine (2 mg/kg), hexamethonium (2 mg/kg, a peripheral nicotinic antagonist) or saline vehicle at 10 min prior to i.c.v. injection of ghrelin (1 μg) or an equal volume (1 μL) of vehicle solution. Food intake was measured at 4 hr following central injections.

Studies investigating the effects of nAChR blockade on intra-VTA ghrelin-induced food intake in rats.

Satiated rats were i.p. injected with mecamylamine (2 mg/kg) or saline at 10 min prior to injections of ghrelin (1 μg) or an equal volume (0.5 μL) of vehicle solution into the VTA. Food intake was measured at 4 hr following the intra-VTA treatment.

Studies investigating the effects of nAChR blockade on re-feeding following an overnight fast in mice and rats.

First, we investigated the effects of peripheral mecamylamine (0.5 and 4 mg/kg) or vehicle solution administered by i.p. injection on re-feeding in overnight fasted mice (16 hr) and rats (20 hr). In all experiments the injections of mecamylamine took place between 0900 and 0930 and food intake was followed during the first 4 hours-post-injection in mice and first 3 hours-post-injection in rats. In the mouse study, body weight regain was also measured at 4 hr. Finally, we investigated the effects of central mecamylamine injection (3 μg) versus an equal volume (3 μL) of vehicle solution on food intake and body weight following an overnight (20 hr) fast in rats.

Co-expression of GHS-R1A and ChAT (a marker of acetylcholine containing neurons)

To generate a cRNA probe against GHS-R1A, a 837-bp cDNA fragment was amplified with PCR from mouse hypothalamic cDNA. The amplicon (corresponding to bases 237–1073 of GHS-R1A mRNA; NM_177330) was inserted into plasmid vector using the PGEM T cloning kit from Promega (Madison, WI). A 2.3 Kb rat choline acetyltransferase (ChAT) cDNA, inserted into the pSPT18 plasmid vector, was kindly made available for these studies by Dr. S. Berrard (Hôpital de la Pitié-Salpêtrière, Paris, France). Both plasmids were grown in DH5α cells (Invitrogen, Carlsbad, CA, USA), isolated with the QIAfilter Plasmid Maxi kit (Qiagen;

Valencia, CA, USA), linearized, purified with phenol/chloroform/isoamyl alcohol (PCI), followed by chloroform/isoamyl alcohol (CI) extractions, and then, precipitation with NaCl and ethanol. The plasmid templates were transcribed *in vitro* with appropriate RNA polymerases to yield antisense probes, and for control purposes, sense transcripts. The ChAT template was labeled with digoxigenin-11-UTP (Roche Diagnostics Co., Indianapolis, IN, USA), and the GHS-R1A template with ^{35}S-UTP (NEN Life Science Products, Boston, MA, USA), using reaction conditions detailed elsewhere (Hrabovszky et al., 2004). In dual-labeling studies, the two probes were combined in the hybridization buffer.

Twelve-µm thick coronal sections were prepared through the N.Acc. and the LDTg of four adult male rats. Prehybridization hybridization and posthybridization procedures have been carried out on slide-mounted sections (Hrabovszky et al., 2004). In dual-labeling experiments, the ChAT probe was visualized first with alkaline phosphatase conjugated anti-digoxigenin antibodies and the BCIP/NBT chromogen system (Roche). Then the slides were coated with 1% Parlodion (SPI-Chem, West Chester, PA, USA), air-dried, dipped into Kodak NTB nuclear track emulsion (Kodak; Rochester, NY; diluted 1:1 with MQ water) and exposed for 4 weeks. The autoradiographic images were developed with Kodak processing chemicals. Dark-field images of emulsion autoradiographs from single-labeling experiments, bright-field images of the cresyl violet-stained neighboring sections and sections dual-labeled for the simultaneous detection of ChAT and GHS-R1A mRNAs were scanned with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope, using a 10X objective lens and the Mozaics modul of the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). The digital images were processed with the Adobe Photoshop 7.1 software at 300dpi resolution.

Conditioned place preference (CPP) for palatable food. CPP testing for palatable food was preformed as previously described (Egecioglu et al., 2010). In this test, the rats spend more

time in a rewarding food paired environment. Briefly rats were free to explore the entire two compartment apparatus for 20 min without food present and initial preference was scored. During the conditioning phase (day 2-10) the rats were confined for 20 min to one of the two compartments in the morning and to the other compartment in the afternoon. The least preferred compartment (determined from the pretest) was always paired with rewarding food (5 pellets of Non-stop chocolate, Marabou, Kraft Foods, Upplands Väsby, Sweden), the other side was paired to standard chow and the conditioning phase was balanced so that the conditioned stimulus was alternated between morning and afternoon sessions. On day 12 rats were i.p. injected with either mecamylamine (1 or 2 mg/kg) or saline 10 min before being placed in the CPP apparatus for 20 min. On day 14 the rats received the opposite treatment in a counterbalanced design at 10 min prior to being placed in the CPP apparatus for 20 min. In this paradigm in which the antagonist was administered prior to the acute CPP test, we test for expression of a CPP response, rather than aquisition, consolidation or extinction of the CPP response, parameters that would require further testing. The animals had *ad libitum* access to food between sessions. During the tests on the two testing days (day 12 and 14) the animals were video recorded and the amount of time spent in each compartment was scored by visual analysis. All rats were habituated to the palatable food for 5 days in their home cages prior to the CPP testing in order to avoid neophobia during the testing. During the conditioning phase, rats were habituated to i.p. injections of saline five times.

Statistics

Data were analyzed using Student's *t*-tests, sign-rank test, one-way, two-way or repeated measures analysis of variance (ANOVA) followed by Bonferroni *post hoc* test or the Fisher's protected least significant difference (PLSD) test. The significance level was $P < 0.05$ for all experiments. All data are presented as means ± SEM.

Results

nAChR blockade: impact on i.c.v. ghrelin-induced feeding in rats and mice.

First we tested whether nAChRs are involved in ghrelin-induced food intake, in studies involving peripheral pretreatment with the centrally active non-selective nAChR antagonist, mecamylamine. Mecamylamine pretreatment (i.p.) attenuated 4 hr i.c.v. ghrelin-induced food intake by ~50% in both *ad lib* fed mice and rats (treatment interaction, two way ANOVA in mice ($F(1,30)=5.1$ $P < 0.05$), in rat ($F(1,42)=10.4$ $P < 0.01$), Figure 1A and 1B). In contrast to the results obtained for mecamylamine, rats pretreated with hexamethonium, a non-selective peripheral nAChR antagonist, did not display an attenuated 4 hr feeding response to i.c.v. ghrelin (sal/veh: 0.73 ± 0.28 gram, sal/ghrelin: 4.34 ± 0.35 gram, hex/veh: 0.59 ± 0.22 gram, hex/ghrelin: 3.44 ± 0.42 gram, no significant treatment interaction, two way ANOVA ($F(1,42)=1.32$ $P = 0.25$), n=11-12 per group). Neither of the cholinergic blockers affected food intake *per se*.

nAChR blockade: impact on intra-VTA ghrelin-induced feeding in rats

Given that intra-VTA ghrelin injection increases food intake and dopamine release in the N.Acc. and that the latter effect on dopamine is completely blocked by pretreatment with mecamylamine, we sought to determine whether mecamylamine pretreatment suppresses the effects of intra-VTA ghrelin on food intake. Injections of ghrelin into the VTA increased 4 hr food intake in rats; this effect was completely blocked by mecamylamine pretreatment (treatment interaction, two way ANOVA ($F(1,69)=4.731$ $P < 0.05$), Figure 2).

nAChR blockade: impact on fasting-induced food intake in rats and mice.

We also explored the effects of nAChR blockade on food intake that follows an overnight fast, that appears to be dependent upon increased endogenous ghrelin signaling (Salomé et al.,

2009). Both doses of mecamylamine tested decreased food intake during the period immediately after the fast (one way ANOVA, mice ($F(1,22)=11.4$ $P < 0.001$), rat ($F(1,14)=4.7$ $P < 0.05$) Figure 3A and 3B). Furthermore, body weight regain in mice was decreased by both doses of mecamylamine at the 4 hr time point (saline: 0.77 ± 0.15 gram, mec 0.5 mg/kg: 0.31 ± 0.13 gram, mec 4 mg/kg: 0.23 ± 0.11 gram, oneway ANOVA followed by Bonferroni *post hoc* test ($F(2,22)=5.60$ $P < 0.05$), n=6-9). Body weights were not recorded in the fasting-induced feeding experiment in rats. Central (i.c.v.) administration of mecamylamine (3 µg/rat) inhibited fasting-induced food intake over time, compared to vehicle treatment (veh 1 hr: 17.28 ± 0.91, mec 1 hr: 14.43 ± 1.87, veh 4 hr: 21.70 ± 1.45, mec 4 hr: 16.21 ± 1.15, veh 24 hr: 201.31 ± 6.21, mec 24 hr: 167.43 ± 12.78, values presented are food (gram)/body weight (kg), repeated measures two way ANOVA ($F(2,26)=3.76$ $P < 0.05$), n=7-8).

Colocalization of GHS-R1A on cholinergic neurons in the LDTg

In order to identify possible neuronal pathways through which ghrelin might affect food intake in an acetylcholine-dependent manner, we examined the expression of GHS-R1A mRNA using in situ hybridization techniques. While no GHS-R1A signal was found in the N.Acc, it was expressed abundantly in the LDTg of rats (Figure 4B). Dual-label in situ hybridization experiments identified GHS-R1A mRNA signal in ChAT mRNA expressing cholinergic neurons of the LDTg (Figure 4C). Nissl-stained reference images of neighboring sections are shown for orientation (Figure 4A).

nACHR blockade: condition place preference testing for palatable food.

Given that acetylcholine signaling is implicated in motivational and rewarding aspects of food intake we tested if mecamylamine treatment could affect the ability of palatable food to condition a place preference. Expression of the CPP response to chocolate pellets was decreased by treatment with mecamylamine (2 mg/kg) administered immediately prior to CPP testing (Figure 5A). There was no difference between vehicle and mecamylamine (2 mg/kg) treatment in the number of crossings between the two compartments (Figure 5B). However, a decrease in the number of crossings was noted between the pretest and the two test days. The lower dose of mecamylamine used (1 mg/kg) did not effect the time spent in the rewarding food-paired compartment.

Discussion

The present series of experiments are built around our working hypothesis that the cholinergic-dopaminergic reward link, a circuit that increases the incentive value of natural and artificial rewards, mediates, at least in part, ghrelin's orexigenic effects. This hypothesis was inspired both by neuroanatomical studies of the distribution of GHS-R1A, that include both the VTA and the LDTg (Guan et al., 1997) and by our previous finding that ghrelin's dopamine-releasing effects can be blocked by suppressing nAChR signaling (Jerlhag et al., 2006, Jerlhag et al., 2007, Jerlhag et al., 2008). Consistent with our hypothesis, the main finding in the present study is that nAChR signaling is required for the orexigenic effects of ghrelin exerted at the level of the VTA.

Ghrelin's orexigenic effects appear to be mediated by pathways involved in both non-homeostatic feeding (eg mesolimbic reward pathways) as well as those involved in energy balance (eg the hypothalamus and brainstem) (Wren et al., 2001, Faulconbridge et al., 2003, Naleid et al., 2005). For this reason, our studies investigating the effects of nAChR blockade

on ghrelin-induced food intake incorporate both i.c.v. delivery of ghrelin as well as ghrelin injection directly into the VTA, a key node in the mesolimbic reward circuits. We found that peripheral administration of a centrally active nAChR antagonist (mecamylamine) but not a peripherally active nAChR antagonist (hexamethonium) suppresses i.c.v. ghrelin-induced food intake by as much as 50%. As mecamylamine did not fully block the effect of i.c.v. ghrelin we may infer that there also exist ghrelin-sensitive orexigenic pathways that are independent of nAChR signaling. When we focused specifically on ghrelin's actions at the level of the VTA, we found that i.p. mecamylamine completely blocked intra-VTA ghrelin-induced feeding. Thus both the orexigenic and accumbal dopamine-releasing effects of ghrelin exerted at the level of the VTA (Naleid et al., 2005, Jerlhag et al., 2007) appear to be dependent on nAChR signaling, implicating a role for the cholinergic-dopaminergic reward link in ghrelin-induced food intake. Our findings suggest that nAChR blockade provides a novel therapeutic route to interrupt ghrelin's effects on food intake that are exerted at the level of the VTA.

Our hypothesis that ghrelin activates the cholinergic-dopaminergic reward link is also supported by novel neuroanatomical data. It is well established that GHS-R1A, the ghrelin receptor, is present in the VTA (Guan et al., 1997, Zigman et al., 2006) where it has been shown to be colocalised on a sub-population of dopamine cells and also on other cells in this region (Abizaid et al., 2006). GHS-R1A is also present in the LDTg (Guan et al., 1997) and, in the present study, we demonstrate its presence on cholinergic neurons in this area (by colocalization between GHS-R1A and ChAT mRNAs). Again, these data follow the aforementioned dopamine measurements in which ghrelin injection into the LDTg stimulates accumbal dopamine release in a cholinergic dependent manner (Jerlhag et al., 2007, Jerlhag et al., 2008). The LDTg appears to provide the major afferent cholinergic projection to the VTA (Butcher and Woolf, 2003). The possibility remains that additional cholinergic afferents play

a role in ghrelin's orexigenic effects; cholinergic neurons with in the pedunculopontine tegmental area (PPTg) are activated by ghrelin *in vitro* (Kim et al., 2009) and constitute an additional source of cholinergic input to the VTA, although these neurons mainly project to the substantia nigra (Beninato and Spencer, 1987).

It may seem rather paradoxical to propose that nAChR antagonism to suppress food intake and food reward, when nicotine itself has powerful anorexigenic properties, with weight gain being a common occurrence after smoking cessation (Klesges et al., 1989, Jo et al., 2002). However, there are indications that nicotine addiction causes desensitization and a subsequent up regulation of nAChRs (Mansvelder et al., 2002, Wooltorton et al., 2003, Tapper et al., 2006) and that the hyperphagia following smoking cessation could very likely be due to increased expression of and hypersensitivity of nAChRs (Jo et al., 2002), thereby increasing the output of the endogenous cholinergic pathways involved in reinforcement and reward seeking. In line with a role for nAChRs in weight gain following smoking cessation long term treatment with mecamylamine decreases body weight in genetic and hypothalamic lesion models of obesity, effects attributed entirely to a decrease in food intake (Dulloo and Miller, 1986). Interestingly, feeding is associated with increased extracellular acetylcholine in the VTA (Rada et al., 2000). The orexigenic overlap between ghrelin and cholinergic signaling via nAChRs may converge within the VTA, however possible effects in other brain areas implicated in the regulation of food intake, such as the arcuate nucleus and the lateral hypothalamus or the N.Acc. which contain cholinergic neurons or receive cholinergic innervations, can not be ruled out.

In the present study, we tested the effects of nAChR antagonism on two different physiological models in which endogenous central ghrelin signaling has been implicated, namely in the CPP test of food reward (Egecioglu et al., 2010) and fasting-induced re-feeding (Salomé et al., 2009). Thus, as shown previously for GHS-R1A antagonists, expression of the

CPP response for palatable food was suppressed by the nAChR antagonist mecamylamine. In accord with our findings on palatable food-induced CPP other studies have shown that peripheral mecamylamine treatment also decreases drug-induced expression of CPP (Sershen et al., 2010, Zarrindast et al., 2005). The re-feeding after an overnight fast is suppressed both by GHS-R1A (Salomé et al., 2009) and by nAChR antagonists. Both peripheral and central delivery of mecamylamine at comparatively low doses block fasting-induced feeding, however neither central nor peripheral injections of mecamylamine had any effects on food intake or body weight in non-fasted animals. Collectively these data suggest that nAChR signaling may be of importance for the effects of endogenous ghrelin on food intake and food reward, although further experiments would be required to show interaction between nAChR signaling and ghrelin in these models and/or to exclude the role of ghrelin-independent mechanisms.

Acknowledgment

We thank Dr. S. Berrard at the Hôpital de la Pitié-Salpêtrière, Paris, France for providing the pSPT18 plasmid vector containing the choline acetyltransferase (ChAT) cDNA, Kosrat Latife for food intake mearuments. The research was supported by Konrad & Helgfrid Johanssons Fond, Fredrik & Ingrid Thurings Stiftelse, the EU (FP7- HEALTH- 2009- 241592, FP7-KBBE-2009-3-245009), Swedish Medical Research Council (K2007-54X-20328–013), ALF Göteborg (SU7601), the Swedish Foundation for Strategic Research to Sahlgrenska Center for Cardiovascular and Metabolic Research (A305-188) and the National Science Foundation of Hungary (OTKA K69127 and T73002).

Figure legends

Figure 1. I.c.v. Ghrelin-induced feeding is attenuated by peripheral administration of the nAChR antagonist, mecamylamine, in both mice and rats. The effects of i.c.v. injection of ghrelin (versus saline vehicle solution) on food intake were measured during the 4 hr period following injection in (A) mice and (B) rats. In both experiments mecamylamine (2 mg/kg) or vehicle was administered by i.p. injection at 10 min prior to the central treatments. (A) $^{**}P < 0.01$, $^{***}P < 0.001$, two-way ANOVA followed by PLSD test (treatment interaction ($F(1,30)=5.1$ $P < 0.05$), n=7-8). (B) $^{***}P < 0.001$, two-way ANOVA followed by Bonferroni *post hoc* test (treatment interaction ($F(1,42)=10.4$ $P < 0.01$), n=12).

Figure 2. Intra-VTA ghrelin-induced food intake is attenuated by peripheral administration of the nAChR antagonist, mecamylamine (2 mg/kg), in rats. Food intake was measured at 4 hr post ghrelin injection. ns= not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ two-way ANOVA followed by Bonferroni *post hoc* test (treatment interaction ($F(1,69)=4.731$ $P < 0.05$), n=12-22).

Figure 3. The re-feeding following an overnight fast is attenuated by peripheral administration of the nAChR antagonist, mecamylamine, in mice (A) and rats (B). Food intake was measured immediately after peripheral mecamylamine injection, during a 4 hr period in mice and a 3 hr period in rat following the fast (A) $^{**}P < 0.01$ one-way ANOVA followed by Bonferroni *post hoc* test ($F(1,22)=11.4$ $P < 0.001$), n=6-9. (B) $^{*}P < 0.05$ one-way ANOVA followed by PLSD *post hoc* test ($F(1,14)=4.7$ $P < 0.05$), n=5-6.

Figure 4. Acetylcholine positive neurons in the LDTg express GHS-R1A mRNA. The distribution of GHS-R1A mRNA was related to Nissl-stained (Cresyl) reference images of neighboring sections (A). Arrows point to the LDTg. In single-labeling experiments, neurons expressing GHS-R1A mRNA (clusters of silver grains) formed a conspicuous cell group in the LDTg (B). Dual-labeling studies, combining the non-isotopic in situ hybridization detection of ChAT mRNA (purple BCIP/NBT stain) with the isotopic detection of GHS-R1A mRNA (autoradiographic silver grains), demonstrated GHS-R1A expression in cholinergic neurons of the LDTg (C). Arrows point to dual-labeled neurons. Scale bars=500μm in A and 100 μm in B and C.

Figure 5. Effects of peripheral pretreatment (10 min prior to test) with the nAChR antagonist (mecamylamine, 2 mg/kg) on the ability of rewarding food to condition a place preference. (A) Pretreatment with mecamylamine decreased the time spent in the palatable food paired compartment. (B) The activity (crossings between compartments) was decrease by both vehicle and mecamylamine treatment compared to the pretest. n=18 $*P < 0.001$, $**P < 0.001$, $***P < 0.001$, Sign rank test.

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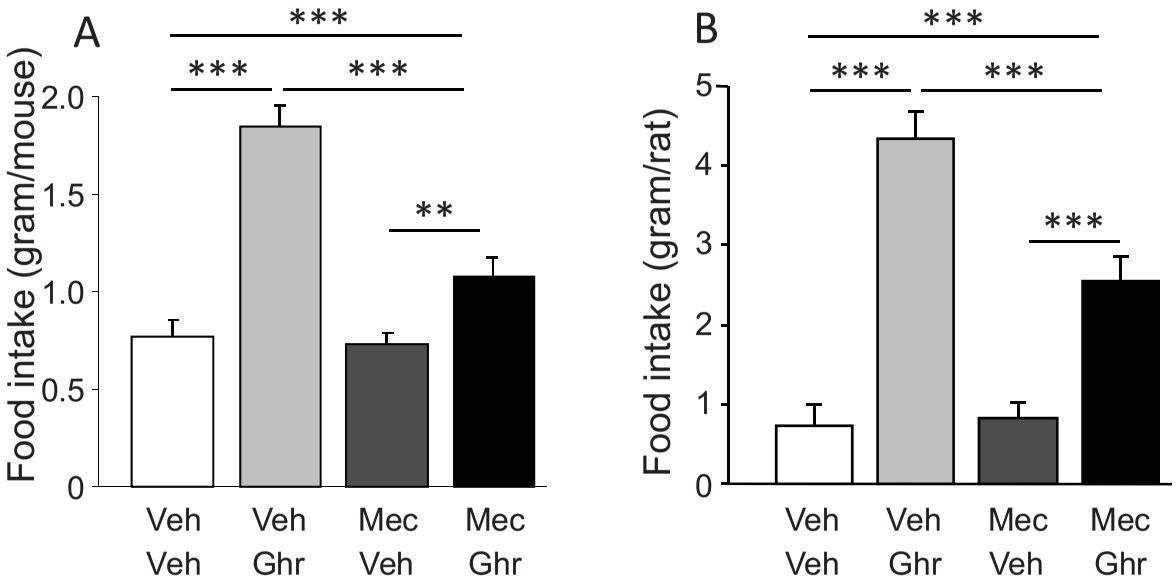
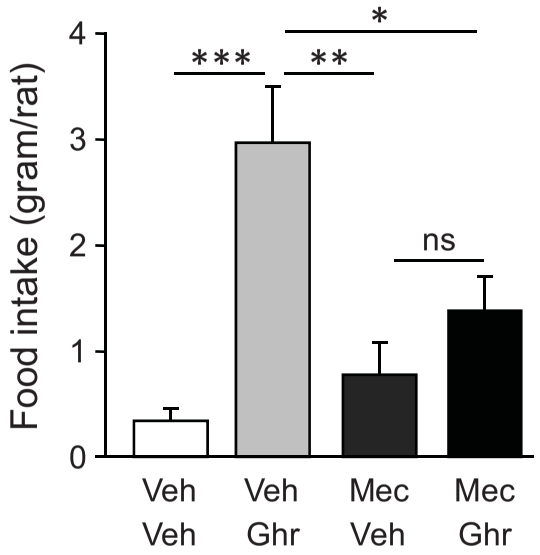


Figure 1, Dickson et al



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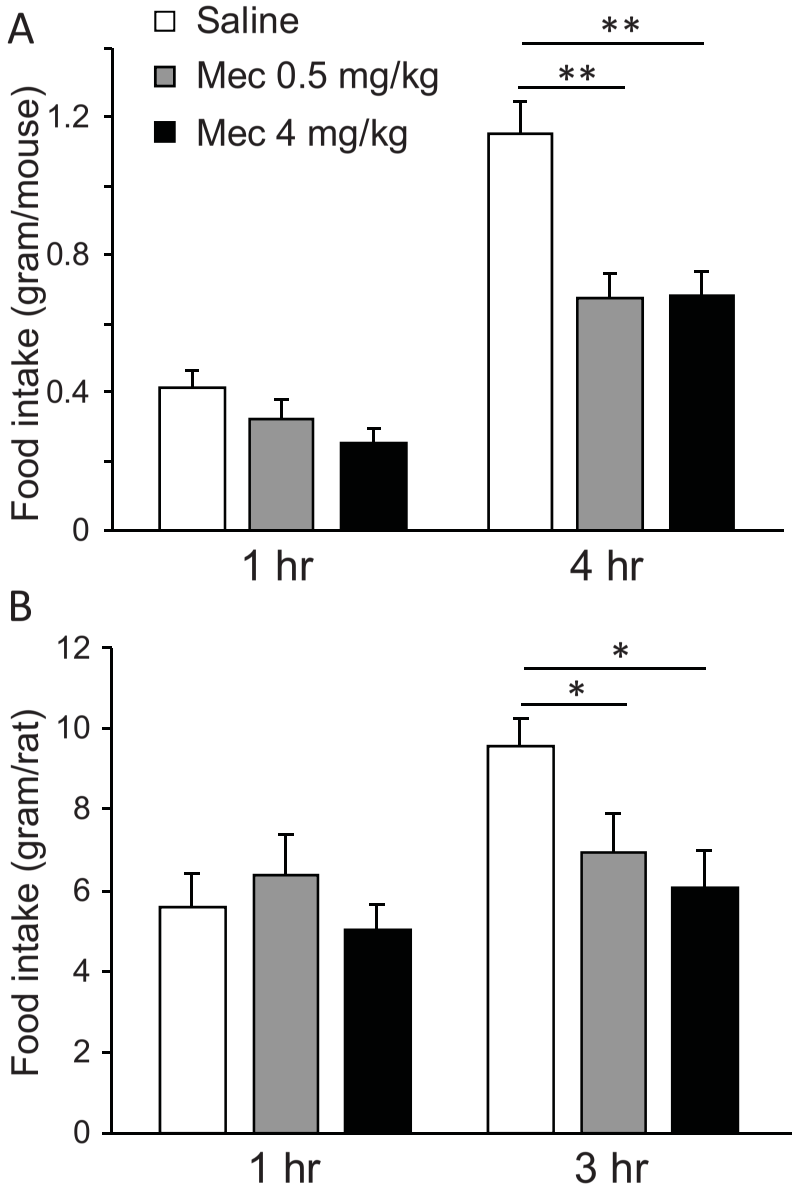

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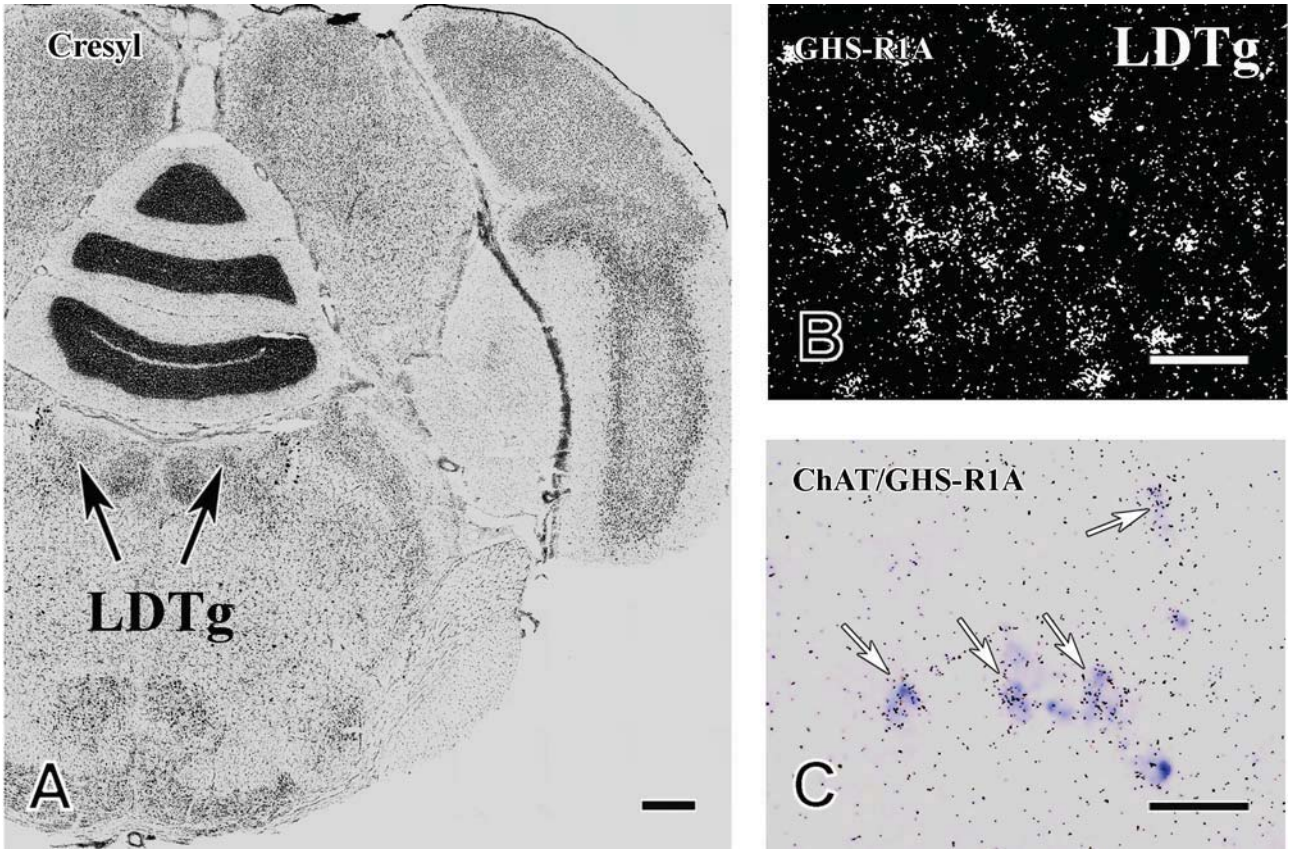

Figure 4, Dickson et al

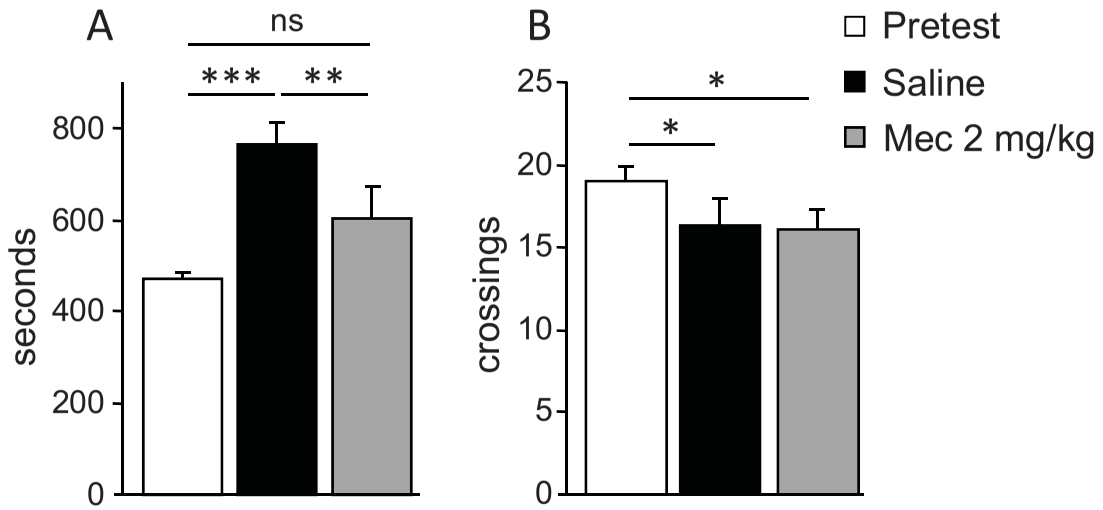

Figure 5, Dickson et al