Ghrelin directly targets the ventral tegmental area to increase food motivation

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ABSTRACT

Ghrelin, a circulating orexigenic stomach-derived hormone, has recently been implicated in extra-homeostatic feeding, increasing food reward and food-motivated behavior. The precise target site(s) of ghrelin's effects on food reward have yet to be elucidated. The neurocircuitry underpinning food-motivated behavior involves, in particular, the dopamine cells of the ventral tegmental area (VTA) that project to the nucleus accumbens (NAcc). Ghrelin stimulation in both of these mesolimbic reward areas increases chow intake. Here we sought to determine if ghrelin acts directly within these mesolimbic reward areas to increase food reward/motivation in studies that combine feeding behavior, pharmacology and neuroanatomy. We found that motivated behavior for a sucrose reward, assessed in an operant conditioning paradigm in rats, was increased when ghrelin was microinjected directly into the VTA but not into the NAcc. By contrast ghrelin administration to both areas increased the free feeding of chow. Importantly, in a state of overnight food restriction, where endogenous levels of ghrelin are increased, ghrelin receptor (GHS-R1A) blockade in the VTA was sufficient to decrease the motivation to work for a sugar reward. Blockade of the GHS-R1A in VTA or NAcc was not sufficient to reduce fasting-induced chow hyperphagia. Taken together our data identify the VTA but not the NAcc as a direct, necessary and sufficient, target site for ghrelin's action on food motivation.

1. INTRODUCTION

Rates of obesity and overweight continue to grow at an alarming rate. There is therefore an escalating and urgent need to better understand the underlying pathophysiology of problematic over-eating with a view to identify novel therapeutic targets for this disease area. Homeostatic signals determine food intake that is dictated by the need for nutrient repletion (metabolic hunger) (Saper et al., 2002). It seems clear, however, that a considerable amount of food intake escapes homeostatic control and occurs despite a state of satiation. Moreover, both rewarding and environmental factors likely play a pivotal role for this non-homeostatic food intake. Ghrelin, a circulating hormone produced primarily in the stomach (Kojima et al., 1999, Date et al., 2000), is a potent orexigenic agent with a well-established role in homeostatic feeding (Kojima et al., 1999, Wren et al., 2000). Ghrelin levels are highly correlated with meal initiation and increase during fasting (Cummings et al., 2001). Conversely, blockade of ghrelin receptors (growth hormone secretagogue receptor, GHS-R1A) decreases food intake (Salome et al., 2009). Ghrelin receptors are abundantly expressed in CNS areas associated with homeostatic feeding, including the hypothalamus and brainstem (Guan et al., 1997, Katayama et al., 2000) and direct ghrelin microinjection in these areas increases food intake (Wren et al., 2001, Faulconbridge et al., 2003b). Interestingly, however, ghrelin has recently emerged as one of the major contributing factors to reward-driven feeding that can override the state of satiation (Egecioglu et al., 2010, Perello et al., 2010, Skibicka et al., 2010). The underlying neuroanatomical targets for this novel role of ghrelin in reward-motivated feeding remain unexplored and provide a basis for the present study.

Substances that affect reward-driven behaviors, e.g. alcohol, cocaine or food, do so by complex neurobiological mechanisms that result in an altered incentive motivational value of the conditioned reward-predictors in the environment (Wise, 2002) and the reward reinforcer. Operant conditioning is a foremost procedure utilized in addiction research to evaluate the addictive/motivational properties of such agents in animal models (Hodos, 1961). A core element of the underlying neurobiology of the motivated behaviors for reward reinforcers is the mesolimbic reward system, especially the dopamine cells of the ventral tegmental area (VTA) that project to the nucleus accumbens (NAcc). Consistent with a role of the ghrelin system in motivated behavior/food reward, both systemic and central (ventricular) ghrelin injection increases operant behavior for a food reward (Skibicka et al., 2010). Conversely, suppression of central ghrelin signaling by peripheral administration of a GHS-R1A antagonist decreased operant responses for a food reward (Skibicka et al., 2010). Preference for a food reward-paired environment in the conditioned place preference test was reduced by a GHS-R1A antagonist and also in GHS-R1A knockout mice, further evidencing a role for the central ghrelin signaling system in food reward. (Egecioglu et al., 2010, Perello et al., 2010). These behavioral expressions of reward that are dependent on central ghrelin signaling are accompanied by molecular and electrophysiological evidence: ghrelin increases dopamine neuron activity in the VTA (Abizaid et al., 2006) and also increases accumbal dopamine release with an associated locomotor response (Jerlhag et al., 2007). Relevance of these data to food reward mechanisms in man is highlighted by the finding that acute ghrelin injection alters the brain response to visual food cues, notably in corresponding reward areas such as the ventral striatum (Malik et al., 2008).

While the importance of the central ghrelin signaling system to reward-motivated feeding is now supported, the ghrelin-responsive neuroanatomical substrates underpinning these effects remain to be elucidated. Ghrelin receptors are expressed in several nuclei with direct or indirect connections to the mesolimbic reward system (Zigman et al., 2006). Strong association of ghrelin's feeding effects with the hypothalamic nuclei and an abundant expression of the GHS-R1A in the hypothalamic nuclei enforced the view that ghrelin might exert its effect on food motivation via its action on the arcuate nucleus or lateral hypothalamus. However, ghrelin microinjection directly into key mesolimbic areas, the VTA and the NAcc, has been shown to increase food intake (Naleid et al., 2005) and also, in the VTA, to increase preference for high calorie preferred food (Egecioglu et al., 2010). Consistent with these findings, GHS-R1A is known to be expressed in the VTA, notably on both dopaminergic and GABAergic neurons (Abizaid et al., 2006). However, GHS-R1A expression in NAcc remains controversial and is evaluated in the current publication (Guan et al., 1997, Naleid et al., 2005, Zigman et al., 2006).

Here we combine behavioral studies, pharmacology and neuroanatomy to investigate ghrelin's potential targets in the mesolimbic pathway. We sought to determine the effects of ghrelin or a GHS-R1A antagonist, applied directly into the VTA or NAcc, on the operant response for sugar pellets and on the free feeding of normal chow.

2. EXPERIMENTAL PROCEDURES

2.1 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.

2.2 Surgery*:* All rats in the behavioral studies were implanted with a guide cannula targeting the VTA or the NAcc shell, (26 gauge; Plastics One, Roanoke, VA) under isofluorane anesthesia (2.2% isofluorane content in the air flow into the face mask, placed in the stereotaxic frame for 30 min). Cannulae were placed 1.5 mm above the target site, and an injector extending 1.5 mm from guide cannulae was used for microinjections. To target the VTA, the following coordinates were chosen modified from (Egecioglu et al., 2010): ±0.75 from the midline, 5.7 mm posterior to bregma, and 6.5 mm ventral from the surface of the skull, with injector aimed 8.0 mm ventral to skull. For the NAcc shell, the following coordinates were used (modified from (Quarta et al., 2009): ±0.75 from the midline, 1.7 mm anterior to bregma, and 6.0 mm ventral to skull, with injector aimed 7.5 mm ventral). Cannulae were attached to the skull with dental acrylic cement and jeweler's screws and closed with an obturator, as described previously (Skibicka et al., 2009). In all rats, the microinjection site for both VTA and NAcc was verified post mortem, by microinjection of India ink at the same microinjection volume (0.5 µl) used throughout the study. Only subjects with the correct placement were included in the data analysis.

2.3 Operant conditioning procedure

2.3.1 Instrumental conditioning apparatus: Operant conditioning experiments took place in eight rat operant conditioning chambers (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 delivers 45 mg sucrose pellets (Test Diet, Richmond, IN, USA) to the food tray. Data were collected and processed by MED-PC software.

2.3.2 Training: The procedure used for operant conditioning was adapted from (la Fleur et al., 2007, Tracy et al., 2008b, Skibicka et al., 2010). 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. 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 5 to 7 days. 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 was 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×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. 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 sessions. Those rats that did not reach the required criteria in that amount of time were trained in additional sessions. The PR test was carried out on 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 their home cages for 1 hr chow intake measurement. At the end of training and prior to testing, rats were returned to an *ad libitum* feeding schedule.

2.4 Experimental Design

All rats received intra-parenchymal (VTA or NAcc) microinjections early in the light cycle 10 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 three conditions (vehicle, dose 1 or dose 2 of drug) on separate testing days. On each day each condition was represented equally. All injections were unilateral. Residual effects of acute ghrelin injection past 24 hr were unlikely, based on (Faulconbridge et al., 2003a) however 24 hr food intake was measured to make sure ghrelin does not have longer term effects that would interfere with the current counterbalanced design. After collection of data from all 3 conditions data were also examined for an interaction of day with treatment, to further eliminate the possibility of repeated injections to interfere with the results.

2.4.1 Effect of VTA and NAcc ghrelin stimulation on operant lever pressing for sucrose in rats. Responses were examined after targeted VTA (n=12) or, in a separate group of rats, NAcc shell (n=16) drug delivery after three conditions as follows: control condition (saline microinjection), 0.33 µg or 1.0 µg of acylated rat ghrelin (Tocris, Bristol, UK) in a 0.5 µl volume. The 1.0 µg dose of ghrelin used was previously shown to induce an orexigenic response when injected into the VTA and NAcc, while the 0.33 µg dose was subthreshold (Naleid et al., 2005). For both the VTA and the NAcc ghrelin studies, lever-pressing experiments were performed in the satiated state. Also, in both studies, immediately subsequent to operant testing, rats were allowed free access to chow. On experimental days rats were returned to their home cages after 120 min of operant testing and chow intake was measured after a 1 hr and again after a 21 hr period in the home cage environment.

2.4.2 Impact of blockade of VTA and NAcc ghrelin receptors (GHS-R1A) with JMV2959 on operant lever pressing for sucrose in rats. Responses were examined after targeted VTA (n=12) or, in a separate group of rats, NAcc shell (n=8) drug delivery after three conditions as follows: control condition with 0.5 µl of saline, 2.0 µg or 10 µg of JMV2959 (AEZS-123, AeternaZentaris GmBH, Frankfurt, Germany). The JMV2959 dose was selected based on (Salome et al., 2009, Skibicka et al., 2010) and preliminary data. Subsequent to operant testing rats were allowed free access to chow and chow intake was measured after a 1 hr period and also at 21 hr after the initial microinjection. 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 microinjections in order to ensure high baseline motivation for

food along with increased levels of endogenous circulating ghrelin, the function of which we sought to block with the antagonist during the experiment.

All behavioral parameters were analyzed by repeated measures analysis of variance (ANOVA) followed by *post hoc* Tukey HSD test as appropriate. All statistical analyses were conducted using Statistica software (Tulsa, Oklahoma). Differences were considered significant at $P < 0.05$.

2.5 VTA and NAcc GHS-R1A mRNA expression

Expression of GHS-R1A in VTA is well established; however GHS-R1A has not been clearly detected in the NAcc. Expression of GHS-R1A mRNA using real-time PCR was evaluated here in the NAcc (n=7) and compared with that in the VTA (n=6). While this method does not provide spatial resolution within each nucleus, its high sensitivity allows for detection of very low levels of mRNA. Briefly brains were rapidly removed after decapitation and the VTA and the NAcc were dissected using a brain matrix according to coordinates from the Paxinos and Watson 1998 rat brain atlas, frozen in liquid nitrogen and stored at -80° C for later determination of mRNA expression. mRNA was subsequently extracted and reversed transcribed. Real-time PCR was performed using TaqMan® assay, designed with TaqMan probe and primer set for rat GHS-R1A (Applied Biosystems, Sundbyberg Sweden). In detail: ***RNA isolation and mRNA expression:*** Individual brain samples were homogenized and total RNA was extracted. RNA quality and quantity were assessed by spectrophotometry (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis, total RNA was reversed transcribed using random hexamers, 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 duplicate. Real-time PCR was performed using TaqMan® assay, designed with TaqMan probe and primer set for GHS-R1A (Applied Biosystems). Gene expression values were calculated based on the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene. In order to analyse the difference in GHS-R1A expression between the VTA and NAcc, a t-test was used, with P-values calculated using the ΔCt- values. Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1 Effect of GHS-R1A stimulation on operant lever pressing for sucrose in rats.

3.1.1 VTA ghrelin microinjection

To determine whether ghrelin receptors in the VTA are relevant and directly engaged in changing the motivational value of palatable food, specifically sucrose, we examined sucrose self-administration in a progressive ratio response schedule in rats 10 min after VTA vehicle or ghrelin microinjection. Operant behavior (expressed as number of sugar rewards earned) was significantly increased in rats after ghrelin microinjection into the VTA (Figure 1A), with nearly a 50% increase in rewards earned at the end of session. In accordance with results reported for operant behavior after central ventricular ghrelin application (Skibicka et al., 2010), significant responses emerged after 1 hr of activity in the operant chambers for the 1.0 µg dose, and a trend (p=0.06) at 1.5 hr for the lower 0.33 µg dose that became significant at 110 min.

The volume used for ghrelin injection into the VTA was based on previous studies (Abizaid et al., 2006). Spread from the site of injection is always a possibility although our attempts to assess this (by reproducing the injection, this time with a water-soluble dye) suggested that this is unlikely to be a concern. One further consideration is whether more rostral injections could even reach the lateral hypothalamus, a site where GHS-R1A is expressed and that projects to the VTA. Further examination of the tissue damage at the site of cannula placement showed that none of the rats included in the analysis had any damage near the lateral hypothalamus. Furthermore we reanalyzed the data after dividing them into two groups one consisting of the more rostral VTA (n=7) and the second with more caudal placements (n=5). If the leakage to lateral hypothalamus had contributed to the ghrelin response we would expect an enhanced effect of ghrelin in the rostral group. Reanalysis indicated no differences in the effect size in the two groups; ANOVA indicated significant effect of the drug in both groups ($p=0.016$ and $p=0.026$; rostral and caudal respectively), and tukey post-hoc tests indicated that neither the vehicle nor the ghrelin injected groups were differentiated based on the placements. Furthermore the only two subjects out of 12 that did not increase their responses to the higher dose of ghrelin were in the rostral group, all caudal VTA injected rats responded to this treatment. The lack of significant difference between the rostral and caudal VTA placements contrasts with other substances showing rostro-caudal differences in their ability to change reward behaviors for e.g cholinergic agonists (Ikemoto and Wise, 2002). Immediately after operant testing, rats were returned to their home cages and allowed free access to chow for 1 hr. Consistent with previous reports (Naleid et al., 2005), rats injected with 1.0 µg dose of ghrelin nearly doubled their chow intake during the first hour of chow consumption as compared to the

vehicle-treated group (Figure 1B). In line with previous data indicating that most of the hyperphagic effect of acute central ghrelin microinjection takes place within 3 hr after microinjection (Faulconbridge et al., 2003a), no effect on chow intake was noted in our study at 24 hr (chow intake from 3-24 hr) after VTA administration of either dose of ghrelin (Figure 1C).

3.1.2 NAcc ghrelin microinjection

Given the aforementioned controversies regarding the NAcc as a potential target of ghrelin's orexigenic effects we sought to (i) confirm previous reports describing the orexigenic response induced by direct administration of ghrelin into the NAcc and (ii) assess whether ghrelin signaling at the level of the NAcc alters the motivational value of palatable food. In contrast to the response obtained from the VTA, ghrelin microinjection into NAcc did not alter operant behavior (Figure 2A). However, consistent with previous reports (Naleid et al., 2005), intra-NAcc ghrelin increased 1 hr intake of freely available chow (Figure 2B). In addition to the orexigenic effect of the 1.0 μg dose of ghrelin, the lower dose also significantly increased the intake of chow. In longer latency measurements, 24 hr chow intake (Figure 2C) was not altered by intra-NAcc treatment. Given the small difference (not significant but slightly higher (+1.2 pellets) responding in NAcc rats) in operant responding in the basal (vehicle-treated) condition, one potential concern is that we were already approaching a maximal (ceiling) response that would make it harder to expose effects of ghrelin in the NAcc-injected group. Therefore we reanalyzed the NAcc data after taking out of the study the six highest responding rats (on vehicle), making the average response on vehicle identical to that of the VTA (VTA n=12, 4.5±0.5, 6.3±0.6, 7.1±0.7; NAcc n=10, 4.5±0.6, 5.4±0.7, 5.5±0.7 pellets earned at the end of the session for vehicle,

the 0.33 µg and 1.0 µg dose of ghrelin respectively). This procedure did not change the results; there were still no significant effects of intra-NAcc ghrelin treatment. Therefore it seems unlikely that the lack of effect in NAcc is due to a higher vehicle-baseline.

3.2.1 Effect of GHS-R1A blockade on operant lever pressing for sucrose in rats.

3.1.1 VTA JMV2959 microinjection

To assess the role of GHS-R1A blockade in the VTA in a physiological situation of elevated food motivation and also increased levels of endogenous ghrelin, we injected a GHS-R1A antagonist JMV2959 into the VTA in overnight food restriction rats. As expected (Hodos, 1961, Jewett et al., 1995) food restriction prominently increased operant responses for sucrose (Figure 3A vs. Figure 1A for satiated responses). This effect was ablated by administration of JMV2959. The 10 µg dose significantly reduced the amount of pellets earned (~30% decrease) with short latency starting at the first (10 min) measurement through the 120 min of the operant test. That the unilateral GHS-R1A antagonist injections into the VTA were sufficient to decrease operant responding for sucrose in a rat food restricted, therefore highly motivated to obtain food, highlights the importance of GHS-R1A receptors in this area for food motivation. In contrast to the prominent effect of VTA ghrelin on chow intake, blockade of VTA GHS-R1A was not sufficient to reduce the 1 hr chow intake in food-restricted rats (Figure 3B). It is possible that the lack of effect is partially due to the length of time between the JMV2959 injection and the chow test (2 hr) such that the effects of the drug dissipate with time, especially given that ventricular administration of JMV2959 was most effective in reducing intake at 1-2 hr post-

injection. Twenty-four hour food intake (Figure 3C) was not altered by the JMV2959 treatment in food restricted rats.

3.2.2 NAcc JMV2959 microinjection

As for the VTA study we assessed the role of GHS-R1A blockade in the NAcc in overnight food-restricted rats. Neither the operant behavior nor the chow intake was altered by GHS-R1A blockade in the NAcc (Figure 4A-C).

3.3 VTA and NAcc GHS-R1A mRNA expression

Since some controversy remains over expression of GHS-R1A in NAcc, while not the primary aim of our study, we set out to determine if NAcc contains GHS-R1A mRNA and compare the expression levels of this gene with that in the VTA, an area with prominent and confirmed GHS-R1A expression. Low but consistently detectable levels of GHS-R1A were found in the NAcc. Here we confirmed GHS-R1A mRNA expression in both VTA and NAcc, albeit with mRNA levels that were over twelve-fold higher in VTA compared with those in the NAcc ($p<0.0005$; Figure 5). It is possible that the low expression of GHS-R1A in NAcc might be increased during food restriction, making this nucleus more responsive to ghrelin during times of energy shortage. There is indeed some literature showing that a long (48h) deprivation increases hypothalamic GHS-R1A (Kim et al., 2003), although there are other reports indicating that the levels of ghrelin receptor do not change in response to the same 48h fast (Harrold et al., 2008) at least in the hypothalamus. The ghrelin tests in our study were performed in sated rats, similarly to those used in expression study. That we were able to show an effect of ghrelin in both VTA and NAcc on chow

intake in a sated state could indicate that this low level of receptors in NAcc detected in our study seems to be still sufficient to drive an orexigenic response to ghrelin.

4. DISCUSSION

In the present study, we identify the VTA, a key structure in the mesolimbic reward system, as a primary target for ghrelin's effects to increase incentive motivated behavior for a sweet food reward. Specifically, we used an operant responding paradigm to show that motivated behavior for a sucrose reward (reflected by increased performance in a progressive ratio operant conditioning paradigm) was increased by direct VTA microinjection of ghrelin and, conversely, was decreased by direct VTA microinjection of a GHS-R1A antagonist. By contrast, ghrelin and GHS-R1A antagonists did not alter operant responding for a sucrose reward when injected directly into another key reward node, the NAcc. Collectively our data suggest that ghrelin signaling at the level of the VTA provides a primary target for incentive motivated behavior for a food reward. These data demonstrate that the central ghrelin signaling system is a key target in the control of the food reward mechanism, impacting directly on the mesolimbic circuitry.

Our data provide direct evidence that central ghrelin signaling at the level of the VTA is required for incentive motivated behavior for a sweet food reward (and its conditioned predictors). The importance of the VTA GHS-R1A is further highlighted by the finding that selective and only unilateral GHS-R1A blockade in VTA was sufficient to decrease food motivated behavior in rats otherwise highly motivated to obtain food due to an overnight food restriction. The crucial role of ghrelin acting

directly on the VTA neurons is highlighted by the fact that the magnitude of the effect on sucrose self-administration by VTA-administered ghrelin nearly accounts quantitatively for the response obtained from the previously reported ventricular ghrelin administration (Skibicka et al., 2010). This stimulatory effect of ghrelin in the VTA is consistent with an emerging role of the central ghrelin signaling system in the integration of food reward signals and reward expectation and in line with previous studies indicating that VTA dopaminergic cells that project to NAcc are activated by ghrelin. Activation of this pathway by ghrelin is of importance for food intake and food preference (Abizaid et al., 2006, Egecioglu et al., 2010), however we cannot exclude the possibility that VTA projections to other areas than NAcc including the dorsal striatum or the lateral hypothalamus may be involved in the responses studied here. Both the peripheral circulating ghrelin that can cross the BBB into brain parenchyma (Diano et al., 2006) or potentially the, not well characterized, hypothalamic ghrelin (Cowley et al., 2003) expressing neurons could be the endogenous source of ligand for the VTA GHS-R1A.

Interestingly, the increase in operant behavior induced by intra-VTA ghrelin microinjection seems to be more sensitive than the orexigenic effect on free-feeding of normal chow since, in the current study, a lower dose (0.33µg) of intra-VTA ghrelin increased operant responding without altering chow intake (for chow see also (Naleid et al., 2005)). That the primary role of the VTA is in motivated behavior rather than free-feeding is also highlighted by the lack of effect of VTA-directed JMV2959 on food restriction-induced feeding. This result combined with the prominent effect of VTA-JMV2959 on motivated behavior could suggests that while other ghrelin sensitive sites (eg arcuate or NTS) or other systems rescue the

restriction-induced chow intake, GHS-R1A in the VTA is indispensible for restriction-induced motivated behavior.

In the present study we confirmed the mRNA expression of GHS-R1A in the NAcc, thereby providing molecular evidence supporting the NAcc as a potential target for ghrelin, although the level of expression was clearly substantially lower than that detected in the VTA, which might have contributed to the lack of detection by other methods for e.g. in situ hybridization histochemistry (Zigman et al., 2006). Moreover, we were able to reproduce the findings of Naleid and colleagues (Naleid et al., 2005) that ghrelin increases intake of regular chow when injected into the NAcc as well as the VTA. These results confirm that stimulation of the small GHS-R1A population in the NAcc, presence of which we have confirmed here, can indeed drive an orexigenic response. An unexpected but interesting aspect of the present work is our observation that motivated behavior for food was unaltered by NAcc shell microinjection of ghrelin or the GHS-R1A antagonist. We may infer, from the lack of effect on motivated behavior from direct NAcc ghrelin application that whereas the VTA provides a direct target for ghrelin's effects on several motivated behaviors, the NAcc appears to be an indirect target for these effects. Indeed, given the pivotal role of the VTA-NAcc dopamine neurons in motivated behavior for food reward, it seems likely that ghrelin increases the incentive value of food reward by targeting the VTA aspect of this projection. The NAcc ghrelin-driven response on free-feeding combined with no effect on motivated behavior contrasts with results obtained from the VTA, where both responses were enhanced and suggests a potential dissociation of neuroanatomical underpinnings of different aspects of feeding behavior.

While the importance of ghrelin in reward-motivated feeding is now strongly supported, and here we have indicated the VTA as a ghrelin-responsive neuroanatomical substrate underpinning motivated food reward behavior, it is possible that there are additional anatomical loci underlying these responses. In the arcuate nucleus ghrelin signaling stimulates the activity of NPY/AgRP neurons (Dickson et al., 1993, Kamegai et al., 2001) and, in lateral hypothalamus, the orexin neurons (Toshinai et al., 2003); orexin, NPY and AgRP have some role in reward behavior (Jewett et al., 1995, Tracy et al., 2008a, Cason et al., 2010) and therefore these cannot be excluded as an additional target site(s) of ghrelin that mediate some effects of ghrelin on the mesolimbic circuitry in addition to the direct effect of ghrelin on the mesolimbic circuit shown here.

Our study identifies the VTA GHS-R1As as a primary necessary and sufficient target for ghrelin's effect on food reward motivation. Although GHS-R1A is also present in the NAcc, an important element of the mesolimbic reward circuit, ghrelin action directly at this site does not appear to be important for food motivation. Here we lay the groundwork for future studies identifying molecular targets of ghrelin's actions in the VTA and the downstream circuitry that exerts a coordinated behavioral response on food motivation. An interesting question worth taking up in future studies would be the relationship of ghrelin to other neuropeptide signals known to regulate VTA dopamine projection and motivated behavior- are they working independently, in concert or serially? Given the contribution of reward feeding to over-eating, ghrelin system can potentially be a target for development of future therapies that address problematic over-eating that leads to obesity.

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Figure 1: Ghrelin injection into the VTA increases motivated as well as free feeding. Intra-VTA ghrelin increases motivation to work for sugar as expressed by increased number of rewards earned in a progressive ratio schedule (A). 1 hr free feeding of chow is also increased by intra-VTA ghrelin (B). 24 hr chow intake remains unchanged (C). Only data from rats with verified VTA injection placement were included in the analysis, included placements are indicated here on coronal rat brain sections (D). Histograms represent means + SEM. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Figure 2: Nucleus accumbens ghrelin injection increases free feeding but does not change motivated behavior. Intra-NAcc ghrelin failed to increase the motivation to work for sugar in a progressive ratio schedule (A). In contrast 1 hr free feeding of chow is significantly increased (B). 24 hr chow intake remains unchanged (C). Only rats with verified NAcc injection placement were included in the study, and indicated here on coronal rat brain sections (D). Histograms represent means + SEM. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Figure 3: GHS-R1A antagonist injection into the VTA decreases motivated but not free feeding in food-restricted rats. Intra-VTA JMV2959 decreases the motivation to work for food, as expressed by the decreased number of sugar pellets earned in a progressive ratio schedule in rats receiving the antagonist (A). In contrast, compensatory free feeding on chow was not altered (B and C). Only rats with verified VTA injection placement were included in the study, and indicated here on coronal rat brain sections (D). Histograms represent means + SEM. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Figure 4: GHS-R1A antagonist microinjection into NAcc does not alter either motivated or free feeding in food-restricted rats. Intra-NAcc JMV2959 does not change the motivation to work for food in a progressive ratio schedule (A) or chow free feeding (B and C). Only rats with verified NAcc injection placement were included in the study, and indicated here on coronal rat brain sections (D). Histograms represent means + SEM.

Figure 5: Comparison of ghrelin receptor (GHS-R1A) gene expression in VTA and NAcc. Histograms represent means + SEM. ***, $P < 0.0005$

Figure 1

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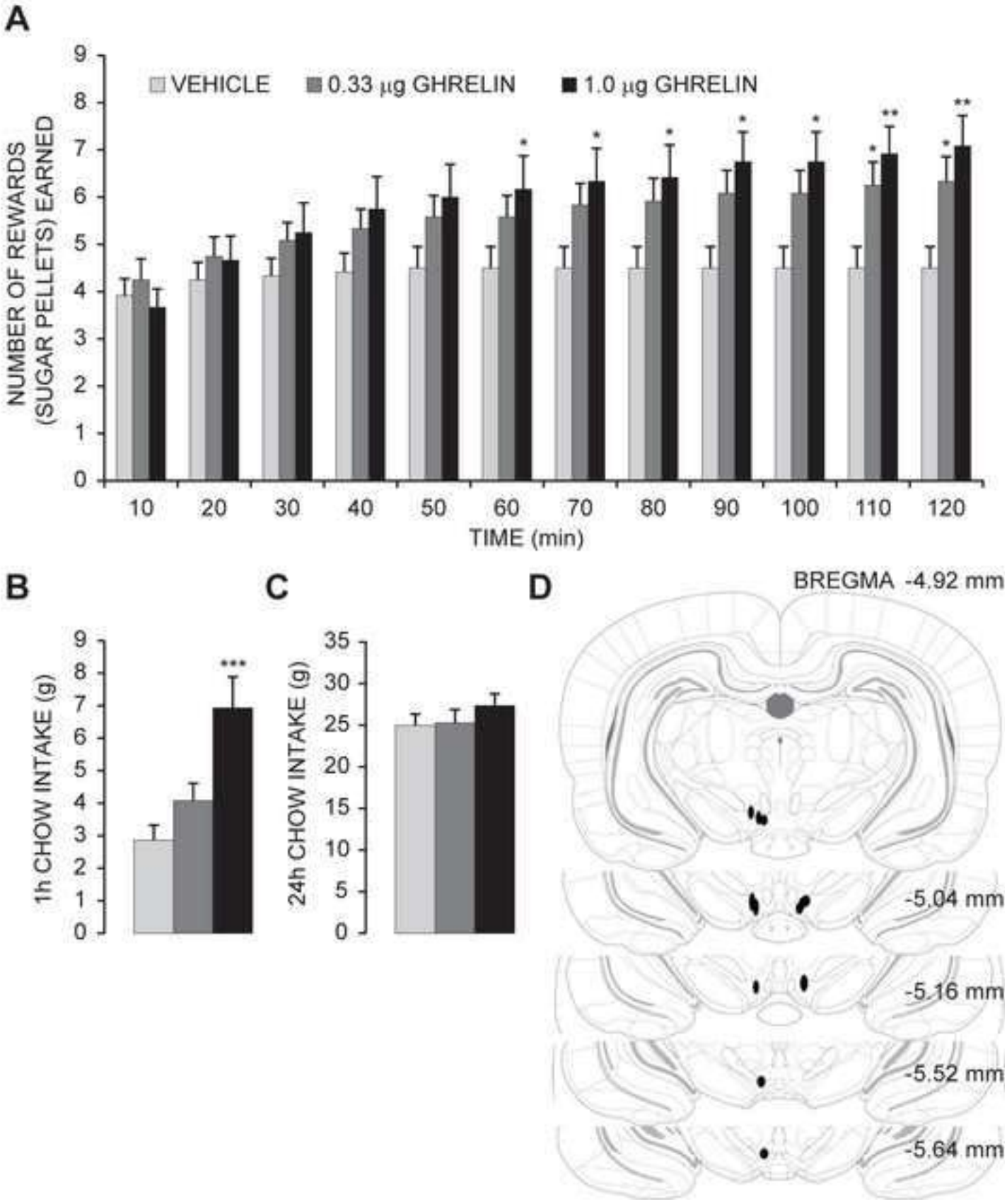

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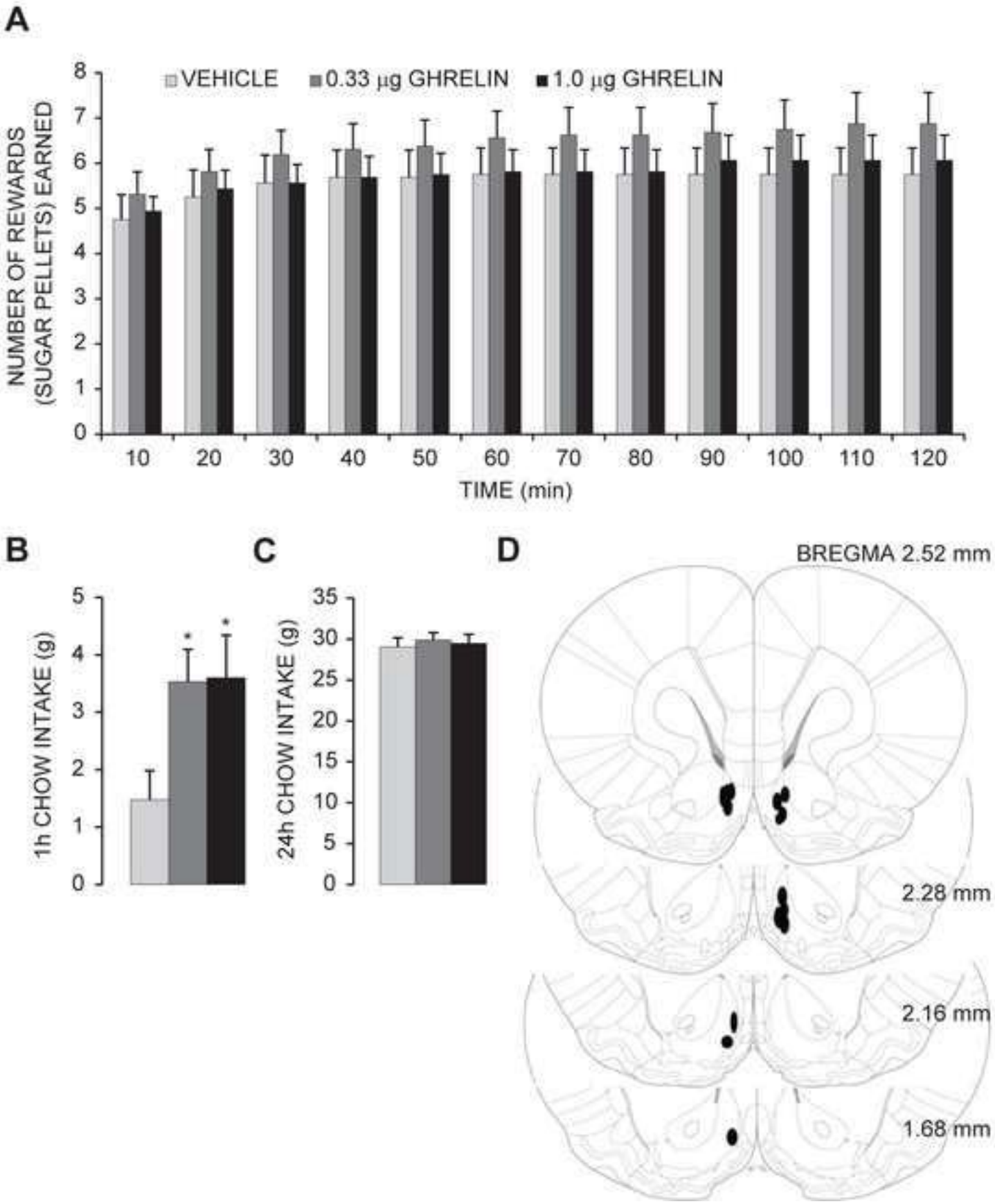

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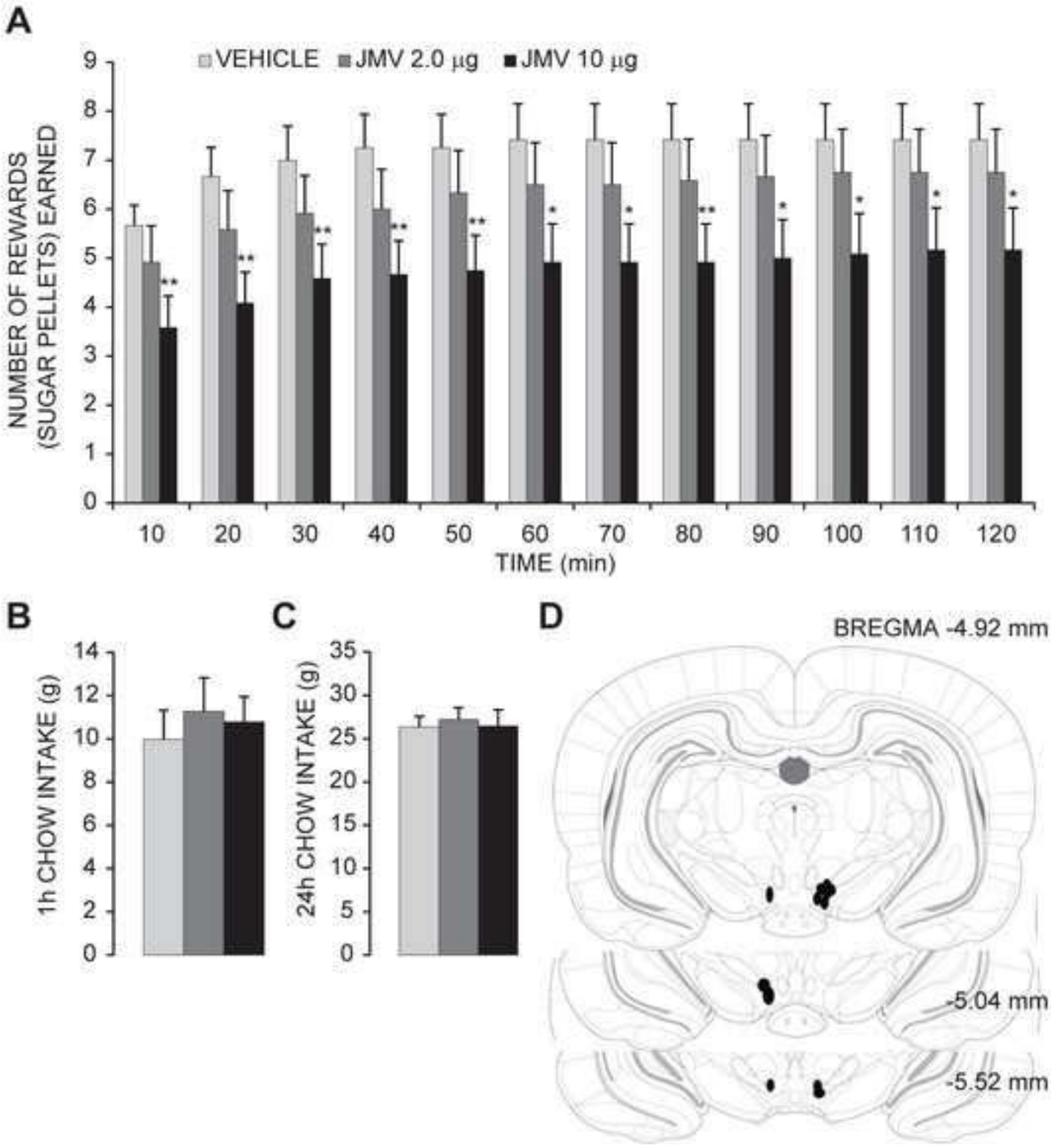

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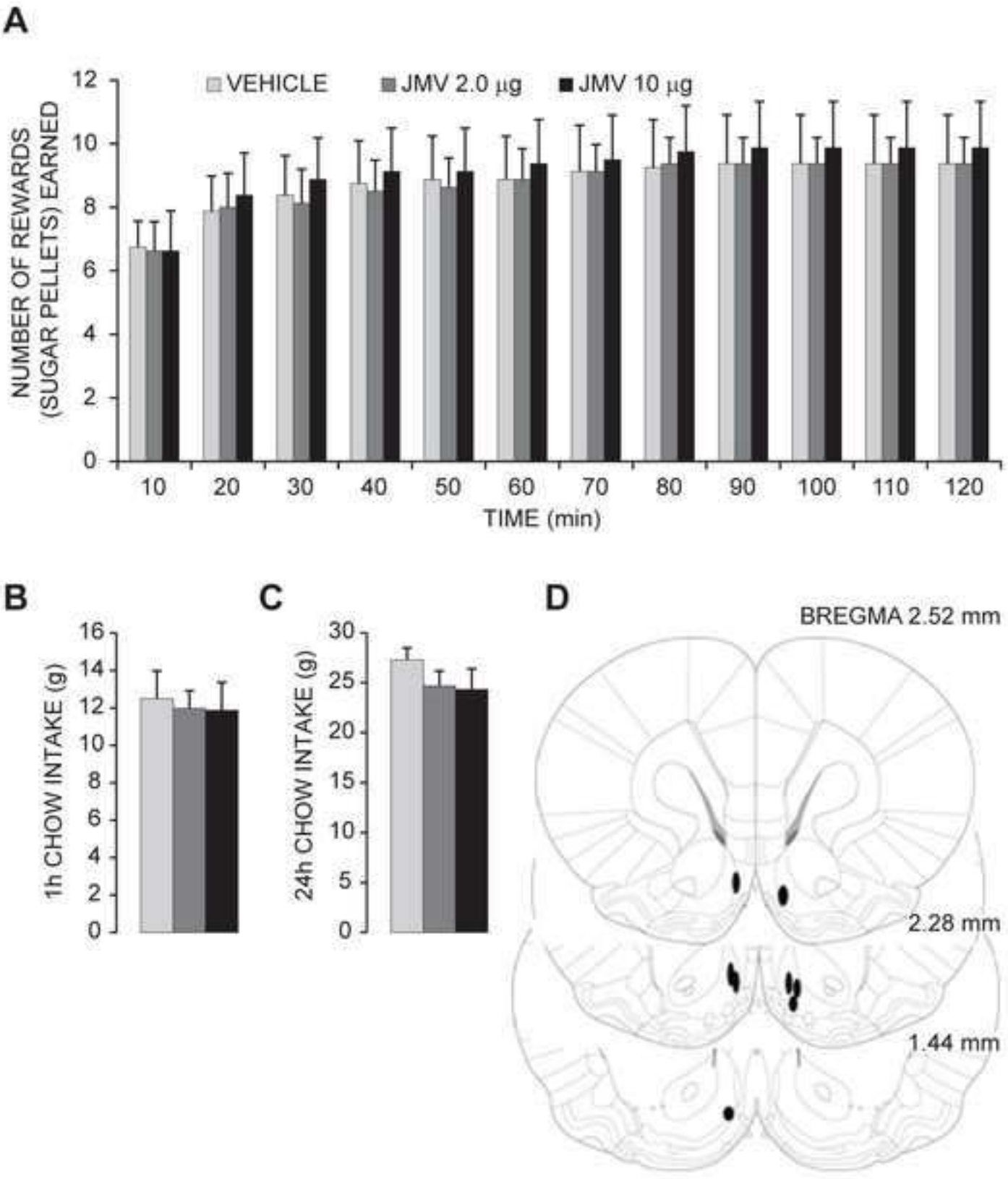

Figure 5

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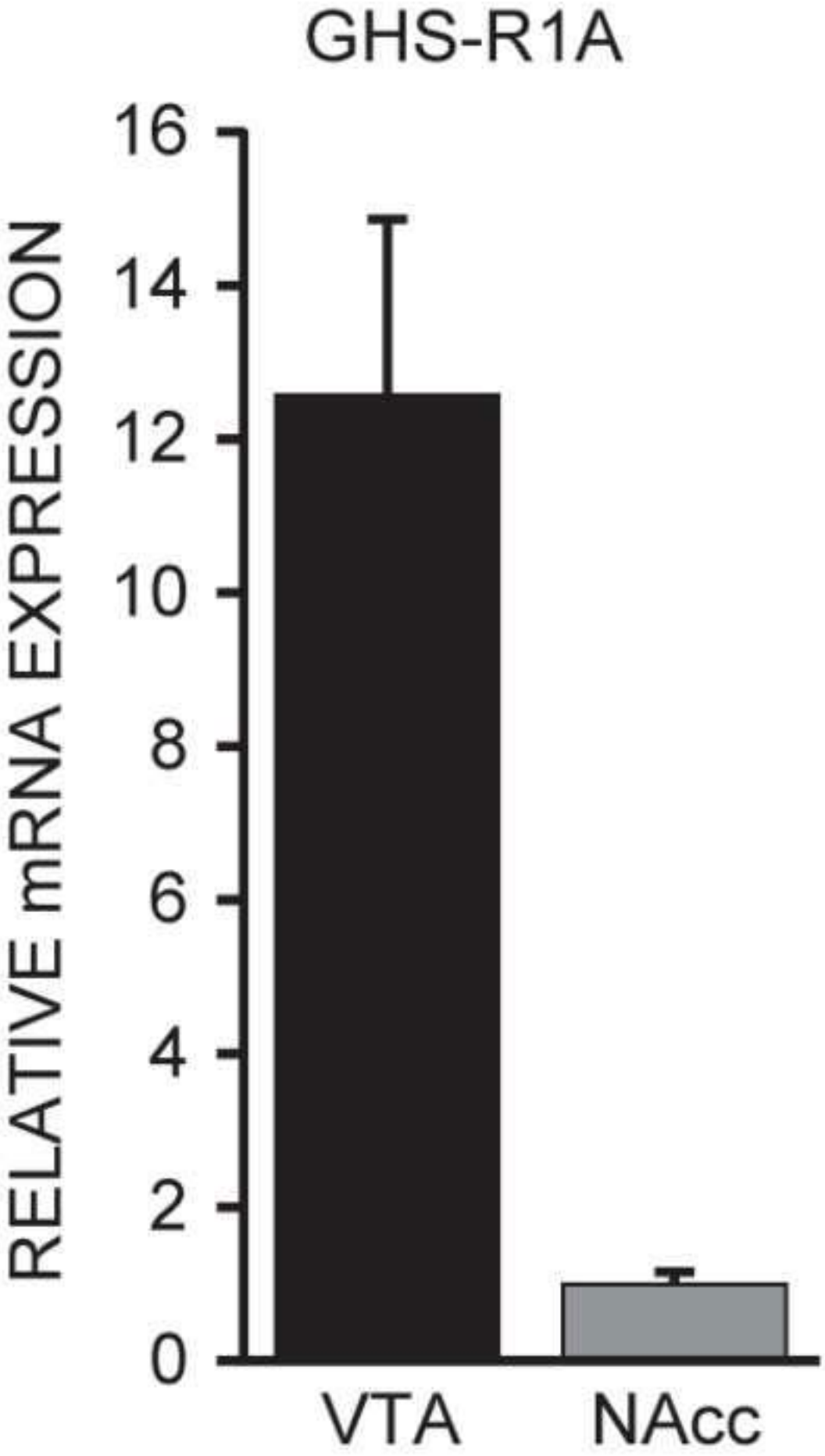

***Research Highlights**

Motivation for food increased after VTA, but not the NAcc, ghrelin stimulation
Ghrelin administration to both areas increased the free feeding of chow
GHS-R1A blockade in only the VTA was sufficient to decrease food motivation
VTA is a direct, necessary and sufficient target for ghrelin's food motivation action

Supplementary Material - Video(s)

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