Ghrelin activates the mesolimbic dopamine system via nitric oxide dependent mechanisms in the ventral tegmental area

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Article

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Abstract

Besides enhanced feeding, the orexigenic peptide ghrelin activates the mesolimbic dopamine system to cause reward as measured by locomotor stimulation, dopamine release in nucleus accumbens shell (NAcS), and conditioned place preference.

Although the ventral tegmental area (VTA) appears to be a central brain region for this ghrelin-reward, the underlying mechanisms within this area are unknown. The findings that the gaseous neurotransmitter nitric oxide (NO) modulate the ghrelin enhanced feeding, led us to hypothesize that ghrelin increases NO levels in the VTA, and thereby stimulates reward-related behaviors. We initially demonstrated that inhibition of NO synthesis the ghrelin-induced activation of the mesolimbic dopamine system We then established that antagonism of downstream signaling of NO in the VTA, namely cGMP, prevents the ability of ghrelin to stimulate the mesolimbic dopamine system. The association of ghrelin to NO was further strengthened by *in vivo* electrophysiological recordings showing that ghrelin enhances the NO release in the VTA. Besides a $GABA_B$-receptor agonist, known to reduce NO and cGMP, blocks the stimulatory properties of ghrelin. The present series of experiment reveal that ablated NO signaling, through reduced production of NO and/or cGMP, prevents the ability of ghrelin to induced reward-related behaviors.

Introduction

The gut produced hormone ghrelin acts centrally to increase both the homeostatic and hedonic aspects of feeding (for review [1]). Before a meal the circulating levels of ghrelin are enhanced, causing hunger and appetite [2]. Although ghrelin initially was considered an appetite regulatory peptide, additional physiological properties including reward regulation have been attributed this peptide (for review [3]). Notably, physiologically relevant doses of ghrelin given systemically enhances reward-related behaviors including locomotor stimulation, conditioned place preference (CPP) and dopamine release in nucleus accumbens shell (NAcS) [4–10]. Besides, the systemic administration of ghrelin or an antagonist of its receptor (growth hormone secretagogue receptors; GHSR-1A) modulates reward induced by addictive drugs such as alcohol and natural rewards like sexual behaviors (for review [1]).

The ventral tegmental area (VTA), a key structure of the mesolimbic dopamine system, is involved in ghrelin's ability to cause reward via local GHSR-1A [11,12]. The direct effects of ghrelin within the VTA include an enhanced consumption of chow, peanut butter or alcohol, hyperlocomotion and dopamine release in NAcS [5,7,8,13–21]. Besides, GHSR-1A within the VTA modulates cocaine reward, sexual behaviors, heroin seeking and the motivation to eat [16,22–31].

Although administration of ghrelin into the VTA enhances reward-related behaviors, the underlying mechanisms remains to be fully explored. The gaseous neurotransmitter nitric oxide (NO) has been linked to the reward-related effects of ghrelin. Specifically, inhibition of the enzyme synthesizing NO (NO synthetase; NOS) attenuates ghrelin's ability to either increase feeding [32–35] or activate neurons [36].

Similarly, reduced NO production prevents various reward-related behaviors induced by addictive drugs [37–47]. Besides, NO enhances the activity of dopaminergic neurons within the VTA [48,49]. These findings led us to hypothesize that ghrelin increases NO levels in the VTA, and thereby stimulates reward-related behaviors; the focus of the present studies of male rodents. First, we explored if ghrelin-induced reward involves NO. We explored the effect of inhibition of NOS on the ghrelin-induced locomotor stimulation, dopamine release in NAcS, reward and memory of reward in the conditioned place preference (CPP) paradigms (Fig. 1A-B). Then, the role of NO in the VTA for the behavioral outcome of ghrelin was studied. We blocked the downstream signaling of NO in the VTA, by attenuating the NO-sensitive soluble guanylyl cyclase (sGC), and investigated the outcome on ghrelin-induced locomotor stimulation and dopamine release in NAcS (Fig. 2A-B). In a further attempt to understand the association of ghrelin to NO and cGMP we attempted to block the locomotor stimulatory properties of ghrelin with the $GABA_B$ -receptor agonist baclofen, known to reduce NO and cGMP [50,51] (Fig. 3A). Finally, *in vivo* electrophysiological recordings were conducted to define the effect of ghrelin on NO release in the VTA (Fig. 4A). The present series of experiments collectively reveals that NO signaling within the VTA is linked to the ability of ghrelin to activate the mesolimbic dopamine system in male rodents.

Results

L-NAME attenuates the ghrelin-induced locomotor stimulation, dopamine release in NAcS and reward in the CPP paradigm in mice

An overall main effect of treatment on locomotor activity was found in mice following systemic administration of L-name and ghrelin ($F(3,28)=28.13$, $P<0.0001$; n=8 per group). As shown in Figure 1C, post-hoc analysis revealed that ghrelin caused a locomotor stimulation compared to vehicle ($P<0.0001$). This ghrelin-hypermotion was significantly reduced by pre-treatment with an injection of L-NAME ($P<0.0001$), at a dose that alone had no significant effect on locomotor activity compared to vehicle treatment ($P>0.9999$). There was no difference in locomotor activity response in vehicle treated mice and L-NAME-ghrelin treated mice ($P>0.9999$).

The ghrelin response was lower in L-NAME compared to vehicle treated mice when evaluating the effect of ghrelin-induced reward in the CPP paradigm ($P=0.0158$, n=14 in each group; Figure 1D). Additional control experiments showed that L-NAME treatment (0±4%) during conditioning had no effect *per se* compared to vehicle treatment (4±3%) on conditioned place preference in vehicle-conditioned mice ($P=0.3906$, n=8 for both groups). There was no difference in ghrelin response between vehicle and L-NAME treated mice when evaluating the reward-dependent memory retrieval effect of ghrelin ($P=0.6573$, n=16 for Ghr-Veh and n=15 for Ghr-L-NAME; Figure 1E). Control experiments show that acute L-NAME treatment (5±4%) had no effect per se compared to vehicle treatment (14±5%) on CPP in vehicle-conditioned mice ($P=0.1910$, n=8 for both groups). In total, 5 mice were excluded following repeated escapes out of the CPP box.

Following L-NAME and ghrelin administration there was an overall main effect of treatment ($F(1,84)=54.46$, $P<0.0001$) and treatment x time interaction ($F(11,84)=2.04$, $P=0.0344$), but not of time $F(11,84)=0.31$, $P=0.9823$) (Figure 1F; n=8 in each group) on dopamine release in NAcS. L-NAME pre-treatment (40 mg/kg, sc) attenuated the ghrelin-induced dopamine release in NAcS at time interval 40 ($P=0.02095$), 60 ($P=0.0008$), 80 ($P=0.0188$) and 120 minutes ($P=0.00403$).

Control experiments showed that there was no overall main effect of treatment ($F(1,84)=3.05$, $P=0.0846$), of time $F(11,84)=0.31$, $P=0.9922$) or of treatment x time interaction ($F(11,84)=1.59$, $P=0.1182$) (Figure 1G; n=8 in each group). This demonstrates that the selected dose of L-NAME had no significant effect on dopamine release in NAcS compared to vehicle treatment. A total of 4 mice were excluded due to misplaced probe placements in the NAcS.

ODQ into the VTA attenuates the ghrelin-induced locomotor stimulation and dopamine release in NAcS in mice

An overall main effect of treatment on locomotor activity was found in mice following infusion of ODQ into the VTA, and ghrelin systemically ($F(3,28)=9.25$, $P=0.0002$; n=8 per group). As shown in Figure 2C, post-hoc analysis revealed that ghrelin induced a locomotor stimulation compared to vehicle ($P=0.0004$). This ghrelin-induced locomotor stimulation was reduced by pre-treatment by ODQ ($P=0.0048$), at a dose that alone had no significant effect on locomotor activity compared to vehicle treatment ($P>0.9999$). There was no difference in locomotor activity response in vehicle treated mice and ODQ-ghrelin treated mice ($P>0.9999$).

In mice treated with ODQ into the VTA, and ghrelin systemically there was an overall main effect of treatment ($F(1,96)=45.45$, $P<0.0001$), but no time effect ($F(11,96)=1.34$, $P=0.2199$), and a trend of an effect of treatment x time interaction ($F(11,96)=1.84$, $P=0.0573$) (Figure 2F; n=9 in each group) on dopamine release in NAcS. Local administration of ODQ attenuated the ability of ghrelin to increase accumbal dopamine at time interval 40 minutes ($P=0.0091$), 80 ($P=0.0220$), 100 ($P=0.0106$) and 120 minutes ($P=0.0495$), as well as tendency to a difference at 60 minutes ($P=0515$). Separate control experiments showed that there was no overall main effect of treatment ($F(1,96)=0.02$, $P=0.8962$), of treatment x time interaction ($F(11,96)=0.47$, $P=0.9202$), but of time $F(11,96)=3.04$, $P=0.0016$) (Figure 2G; n=9 in each group), demonstrating that the selected dose of ODQ had no significant effect on dopamine release in NAcS compared to vehicle treatment. A total of 6 mice were excluded due to misplaced probe placements in the NAcS.

Baclofen attenuates the ghrelin-induced locomotor stimulation in mice

Following systemic administration of baclofen and ghrelin, there was an overall main effect of treatment on locomotor activity ($F(3,27)=9.18$, $P=0.0002$). Post-hoc analysis revealed that ghrelin (n=8) significantly increased the locomotor activity compared to vehicle ($P=0.0015$, n=7; Figure 3B). A single injection of baclofen (n=8) blocked this ghrelin-induced locomotor stimulation ($P=0.0003$), at a dose that had no significant effect on locomotor activity compared to vehicle treatment ($P>0.9999$; n=8). There was no

difference in locomotor activity response in vehicle treated mice and baclofen-ghrelin treated mice (P>0.9999). One mouse was excluded due technical problems with the activity box.

Systemic ghrelin administration increases the nitric oxide levels in the VTA in rats

In rats treated with ghrelin or vehicle (n=8, per group) there was an overall effect of treatment ($F(1,119)=13.92$, P=0.0003) and time $F(16,119)=2.16$, P=0.0.0097), but no treatment x time interaction ($F(16,119)=0.48$, P=0.9529), demonstrating that ghrelin increases NO in the VTA (Figure 4C). In total, four rats were excluded due to misplaced sensor placements.

Discussion

The present series of experiment reveal that ablated NO signaling, through reduced production of NO and/or cGMP, prevents the ability of ghrelin to induced reward-related behaviors. In all probability the mechanism underlying this interaction resides in the VTA.

The present findings reveal that an overall reduction of NO production through inhibition of NOS prevents the ability of ghrelin to cause a locomotor stimulation, to enhance dopamine release in NAcS, and to induce reward in the CPP paradigm. Supportively, the locomotor stimulatory properties of ghrelin are attenuated by baclofen, which via activation of $GABA_B$ receptors on dopaminergic neurons reduces NO and cGMP synthesis as well as the dopaminergic activity possibly in the VTA [50–53]. Recent advances show that NO mediates other physiological properties of ghrelin, like feeding [32–35], neuronal activation [36], respiratory exchange ratio and growth hormone release [32,54]. It should be noted that L-NAME does not alter the memory of ghrelin-induced reward in the present experiments. This implies that NO is an important mediator of some, but not all, ghrelin-induced behaviors. Besides ghrelin, NO appear to mediate reward from addictive drugs as pharmacological or genetical suppression of NOS prevents the behavioral responses of nicotine, alcohol or cocaine [37–47].

Although previous studies have shown that NO signaling within the hypothalamus is important for the feeding aspects of ghrelin [32], we here show that NO in the VTA modulates the ability of ghrelin to activate the mesolimbic dopamine system. Indeed, selective reduction of cGMP, which is the downstream mediator of NO, blocks ghrelin-induced hyperlocomotion and NAcS dopamine release. In addition, we demonstrate that ghrelin increases NO levels in the VTA. The importance of NO within the VTA for reward is further supported by the findings that local VTA infusion of L-NAME prevents the nicotine mediated reversal of morphine's effect on memory [40] or attenuates cocaine induced locomotor sensitization [55]. This is further evident in electrophysiological recordings of freely moving rats where L-NAME blocks the nicotine induced burst firing of dopamine neurons in the VTA [56]. Other studies have shown NO to activate the dopaminergic neurons within the VTA [48,49] and causes reward in the CPP test [57]. In contrast, L-NAME inhibits burst firing of VTA dopamine neurons in rat brain slices [58]. Moreover, the tyrosine hydroxylase expression in the VTA is lower in NOS knockout mice compared to their wild type controls [39]. Summarized, we and others have shown that ghrelin increases NO levels and subsequently cGMP levels

in the VTA, and thereby stimulates the dopaminergic neurons. This signaling cascade is linked to some of ghrelin's behavioral effects on the reward system.

The circuitry within the VTA through which ghrelin increases NO remains to be determined. It may involve activation of GHSR-1A on GABAergic interneurons as they profoundly express GHSR-1A [8,59–61] and are crucial for the stimulatory properties of ghrelin [61]. We therefore suggest that ghrelin decreases GABA in the VTA [7] which is followed by a reduced GABAergic tone on $GABA_B$-receptors located on dopamine neurons [52]. This in turn, would cause a release of NO and subsequent cGMP (for review see [62]), which then enhance the activity of dopaminergic neurons [63] (Fig. 5). Other potential mechanisms such as NO release from GABAergic interneurons that either reduce GABA production and enhance glutamate synthesis should also be considered (for review see [62]).

There are limitations associated with the present study. For instance, it is difficult to elucidate the synaptic NO response by ghrelin as the sensor most likely measures the extrasynaptic levels of NO [64]. Moreover, other areas than the VTA, where ODQ was infused, might be important for the ghrelin-NO association since ghrelin, L-NAME and baclofen was administered systemically. The hypothalamus is one area of interest as ghrelin increases NOS gene expression in this region [65], and ghrelin-enhanced food intake involves NO in parts of the hypothalamus [32]. Although, ODQ was locally infused into the VTA in a low volume, with previous studies reporting low diffusion of the drug to surrounding areas, such effects cannot be excluded. Moreover, tissue damage following local infusions can influence the results. However, tissue damage would also be present in vehicle controls, making it a less likely source of bias. The lack of female mice is another limitation, and therefore future studies should explore whether female and male rodents respond similarly to ghrelin and NO or not. In the present study a non-selective NOS inhibitor was used, and therefore the subtype of NOS responsible for the ghrelin-induced release of NO cannot be determined. Further studies should thus explore the subtype of NOS involved, and also give more details into the cellular types responsible for the ghrelin-induced NO increase and define the up-/down-stream mechanisms.

In summary, suppression of the NO pathway attenuates ghrelin-induced locomotor stimulation, dopamine release in NAcS and the rewarding effects of ghrelin in the CPP test in mice. VTA is central for the ghrelin-NO link as ghrelin increases NO in this area, and local infusion of a drug that prevents NO signaling, blocks the hyperlocomotion and DA increase in NAcS following ghrelin administration. Collectively, this indicates that NO signaling within the VTA is required for ghrelin's ability to activate the mesolimbic system.

Methods

Animals

Herein, adult post-pubertal male mice and rats were used. NMRI mice (25–35 g body weight; Charles River, Sulzfeld, Germany) were used for the behavioral tests whereas Wistar rats (250–300 g body weight; Charles River) were selected for the nitric oxide measurements. These strains were selected as they display a robust activation of the mesolimbic dopamine system after ghrelin [4–10, 19,66]. The group housed rodents acclimatized at the animal facilities (a room temperature of 20°C, a 12/12 hour light/dark cycle and a humidity of 50%) at least one week prior to test, which were conducted during the light phase. Tap water and food (normal chow; Teklad Rodent Diet; Envigo, Madison, WI, USA) were supplied *ad libitum* in the rodents' home cages, but not during testing. The Swedish Ethical Committee on Animal Research in Gothenburg approved the experiments. All experiments were performed in accordance with the relevant guidelines and regulations. Reporting in the manuscript follows the recommendations in the ARRIVE guidelines. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Drugs

Acylated rat ghrelin (Bionuclear, Bromma, Sweden) was diluted in vehicle solution (saline, 0.9% NaCl) for subcutaneous (sc) injection. The selected dose of ghrelin (0.33 mg/kg) activates the mesolimbic dopamine system in male rodents [4,19,66]. The non-selective inhibitor of neuronal and endothelial NOS, N^G-nitro-l-arginine methyl ester (L-NAME; RBI, Natick, USA), was diluted in vehicle solution (saline, 0.9% NaCl). The selected dose of L-NAME (40 mg/kg, sc) blocks the behavioral responses to phencyclidine (PCP) without altering the gross behavior [67]. 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; SigmaAldrich; Stockholm, Sweden), an inhibitor of sGC, was diluted in Ringer solution (NaCl 140 mM, Ca Cl_2 1.2mM, KCl 3.0mM and $MgCl_2$ 1.0mM; Merck KGaA, Darmstadt, Germany). ODQ (0.1 mM at a volume of 0.5 µl/side) was infused locally and bilaterally into the VTA. This dose has previously been found to inhibit the behavioral response to PCP without altering the gross behavior in mice [67]. Baclofen (SigmaAldrich), a $GABA_B$ receptor agonist known to reduce NO [50] and cGMP [51], was diluted in vehicle solution (saline, 0.9% NaCl). The selected dose (2.5 mg/kg; intraperitoneal, ip) restores the behavioral deficits induced by PCP without altering behavior *per se*[50]. Vehicle was always administered at an equal volume as the pharmacologically active substance.

Surgeries

In the present project surgeries were conducted to enable i) local infusions into the VTA, ii) measurements of dopamine in NAcS or iii) nitric oxide recordings in VTA. As described in detail before [5,42], the rodents were following anesthesia (Isofluran Baxter; Kronans Apotek, Gothenburg, Sweden) placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA). Analgesia was obtained by locally adding xylocaine/adrenaline (10 mg/ml, 5 µg/ml, AstraZeneca; Kronans Apotek), followed by a carprofen injection (sc, Rimadyl®, 5 mg/kg, Zoetis; Kronans Apotek). To counter hypothermia and dehydration, the rodents were placed on a heating pad and injected with saline (0.9% NaCl). Holes for guides (stainless steel, length 10 mm, o.d./i.d. of 0.6/0.45 mm), microdialysis probe (20 kDa cut off with an o.d./i.d. of 310/220 µm, HOSPAL, Gambro, Lund, Sweden), sensor, the reference electrode (8T Ag wire, 200-µm bare

diameter; Advent Research Materials, UK) and anchoring screws were drilled. For the rats, the auxiliary (8T Ag wire) electrode was attached to one of the anchoring screws. The coordinates used are presented in Supplementary table 1 [68,69]. The guides/probe/sensor and screws were stabilized with dental cement (DENTALON® plus; Agntho's AB, Lidingö, Sweden). After surgery the rodents were kept in individual standard plastic cages for two days before the experiment.

Locomotor activity experiments

At each test day the mice habituated to the behavioral room, and to the locomotor activity boxes for one hour. As previously described [18], the activity of each mouse was registered in a sound attenuated, ventilated and dim lit locomotor box (420 x 420 x 200 mm, Kungsbacka mät- och reglerteknik AB, Fjärås, Sweden). Photocell beams (5 x 5 rows) register the number of newly interrupted photocell beams during a 60-minute period. Three different locomotor activity experiments were conducted to establish the role of NO and/or cGMP, specifically in the VTA, for the ghrelin-induced locomotor stimulation.

First (Fig. 1A-B), the effects of L-NAME (40 mg/kg, sc) on the ghrelin-induced (0.33 mg/mg, sc) locomotor stimulation were investigated. L-NAME or vehicle was injected ten minutes prior to ghrelin or vehicle. Ten minutes later the recording of activity started. Each mouse received one of the following treatment combinations: vehicle/vehicle, vehicle/ghrelin, L-NAME/vehicle or L-NAME/ghrelin (n = 8 per treatment combination).

Secondly (Fig. 2A-B), the effects of infusion of ODQ (0.1 mM) into the VTA on the ghrelin-induced (0.33 mg/mg, sc) locomotor stimulation were investigated. In these experiments, a cannula (3.8 mm ventrally beyond the tip of the guide) was carefully inserted and retracted into the VTA 60 minutes prior to test in attempt to remove clotted blood and hamper spreading depression. During 60 seconds, ODQ or vehicle (0.5 µl) was infused into the VTA (Kloehn5 µl microsyringe; Skandinaviska Genetec AB, V. Frölunda, Sweden). One minute later the cannula was retracted. After five minutes ghrelin or vehicle was injected and the activity registration started after an additional ten minutes. Each mouse received one treatment combination: vehicle/vehicle, vehicle/ghrelin, ODQ/vehicle or ODQ/ghrelin (n = 8 per treatment combination).

Thirdly (Fig. 3A-B), the effects of baclofen (2.5 mg/kg, ip) on the ghrelin-induced (0.33 mg/mg, sc) locomotor stimulation were evaluated. Baclofen or vehicle was administrated ten minutes prior to ghrelin or vehicle. Ten minutes later the recording of activity started. Each mouse received one of the following treatment combinations: vehicle/vehicle, vehicle/ghrelin, baclofen/vehicle or baclofen/ghrelin (n = 8 per treatment combination).

In vivo microdialysis and dopamine release measurements

In vivo microdialysis experiments in freely moving mice were conducted to establish the role of the NO pathway, specifically in the VTA, for the ghrelin-induced dopamine release in NAcS. In all of these, the probe was perfused with Ringer solution (1.5 µl/minute) using a pump (U-864 Syringe Pump; AgnThós AB). After one hour of habituation, perfusion samples were collected every 20 minutes. The baseline

dopamine level was defined as the average of two consecutive samples before the first drug challenge (Time − 10). Following the second drug administration nine additional samples were collected, which defines the drug effect on dopamine release in NAcS.

Firstly (Fig. 1A-B), L-NAME (40 mg/kg, sc) or vehicle was administered 10 minutes prior to ghrelin (0.33 mg/kg, sc), which was administered at 0 minutes (n = 9 in each group). Secondly (Fig. 2A-B), ODQ (0.1 mM per side) or vehicle was infused into the VTA 10 minutes prior to ghrelin (0.33 mg/kg, sc), which was administered at 0 minutes (n = 9 in each group). Thereafter, two control experiments were conducted to define the effect of L-NAME or ODQ on dopamine per se. In these, either L-NAME/vehicle or ODQ/vehicle was administered 10 minutes prior to vehicle (n = 9 in each group). As described before [70], the extracellular dopamine levels in NAcS were measured with two different high-performance liquid chromatography systems with electrochemical detection.

Conditioned place preference

The effects of L-NAME (40 mg/kg, sc) on the ghrelin-induced (0.33 mg/mg, sc) reward (Test 1, n = 16 per treatment group) or reward-dependent memory retrieval of ghrelin (Test 2, n = 16 per treatment group) were evaluated in the CPP paradigm (Fig. 1A-B). Besides, the effect on L-NAME *per se* on such behaviors was defined (n = 8 per treatment group).

A two-chambered CPP box (45 lux) with distinct tactile and visual cues was used. The paradigm consists of three parts; pre-conditioning (day 1), conditioning (days 2 to 5), and post-conditioning (day 6). The mouse is at day 1 placed at the midline allowing exploration of both compartments during 20 minutes. This determines the least preferred compartment. During day 2–5, the CPP box is divided into the two chambers, and the mouse is only to one compartment at time. During day 2–5, ghrelin (0.33 mg/kg, sc) is injected and the mouse is then exposed to the least preferred compartment for 20 minutes per day. During day 2–5, each mouse also received one vehicle injection paired with exposure to the preferred compartment for 20 minutes. The injections are altered between morning and afternoon in a balanced design (*i.e.* biased design). The mouse is at day 6 placed at the midline allowing exploration of both compartments during 20 minutes. CPP was calculated as the difference in % of total time spent in the ghrelin-paired (*i.e.*, least preferred) compartment during the postconditioning compared the preconditioning session.

In test 1, L-NAME (40 mg/kg, sc) or vehicle was administered 10 minutes prior to the ghrelin injection on each of the four conditioning days. The mice were untreated at pre- and post-conditioning. In a control experiment, mice were subjected to the same procedure but received vehicle injections instead of ghrelin throughout the conditioning.

In test 2, only ghrelin was injected on each of the four conditioning days. In addition, all mice received a vehicle injection at pre-conditioning and either a L-NAME (40 mg/kg, sc) or vehicle injection at post-conditioning. A control group of animals were subjected to the same procedure but received only vehicle injections throughout the conditioning.

Experiment with local infusion of ODQ into the VTA was not conducted as local infusions only allows investigations of the memory of ghrelin-reward, which was unaltered by L-NAME.

Electrochemical detection of nitric oxide

Electrochemical recordings were conducted to evaluate the effects of ghrelin (0.33 mg/mg, sc) or vehicle on NO levels in VTA in freely moving rats (Fig. 4A). Brain nitric oxide levels were monitored in real-time using a nitric oxide selective amperometric microsensor (BioAnalytics Laboratory, Chemistry Department, Maynooth University). The microsensor is a Nafion-modified Pt disk electrode which has been extensively validated for in vitro and in vivo nitric oxide sensitivity [71,72] and in vitro selectivity against ascorbic acid, uric acid and dopamine [73]. The nitric oxide oxidation current (electrode potential of + 0.90 V against an Ag reference electrode) was detected using a low-noise potentiostat (ACM Instruments, United Kingdom) and converted using an A/D converter (PowerLab, ADInstruments, United Kingdom). The digital signal was then recorded using Chart software (v5, ADInstruments) running on an Apple computer.

A rat in their home cage was connected to the in vivo voltammetry equipment on the day before the experiment to allow the nitric oxide oxidation current to reach a stable baseline. The baseline nitric oxide levels were detected during 30 minutes. Thereafter, ghrelin (0.33 mg/kg, sc) or vehicle (n = 8, per group) was administered. The first 15 minutes following injection was not analyzed due to movement artifacts in the current. Then the levels of NO in the VTA were measured for an additional 45 minutes, which corresponds to the locomotor stimulatory effects obtained by ghrelin. The effect on NO levels was calculated as percent of baseline (average of the first 30-minute detections).

Statistical analysis

The placements of guides, probes and sensors were verified following the termination of the experiment, and only animals with correct placements were included in the statistical analysis (Fig. 1H, Fig. 2D, Fig. 2E, Fig. 4B). The data from the locomotor activity experiments were analyzed with a one-way ANOVA, and a Bonferroni's post hoc test was used for comparisons between treatments. The microdialysis experiments and electrophysiological recordings were analyzed with a two-way repeated measures ANOVA, followed by Tukey's post-hoc test for multiple comparisons between treatments and across time points. The CPP data were analyzed with an un-paired t-test. All data are presented as mean ± SEM. A probability value of $P < 0.05$ was considered statistically significant.

Declarations

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involvement in data collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Author contribution

JAE designed experiment; DV performed the pre-clinical experiments. EJH and EP analyzed pre-clinical data; EJ wrote the first draft of the paper. All authors contributed to and approved the final manuscript.

Data availability statement

All data are in repositories and are available on request.

Additional information

The authors declare no conflict of interest.

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Figures

Figure 1

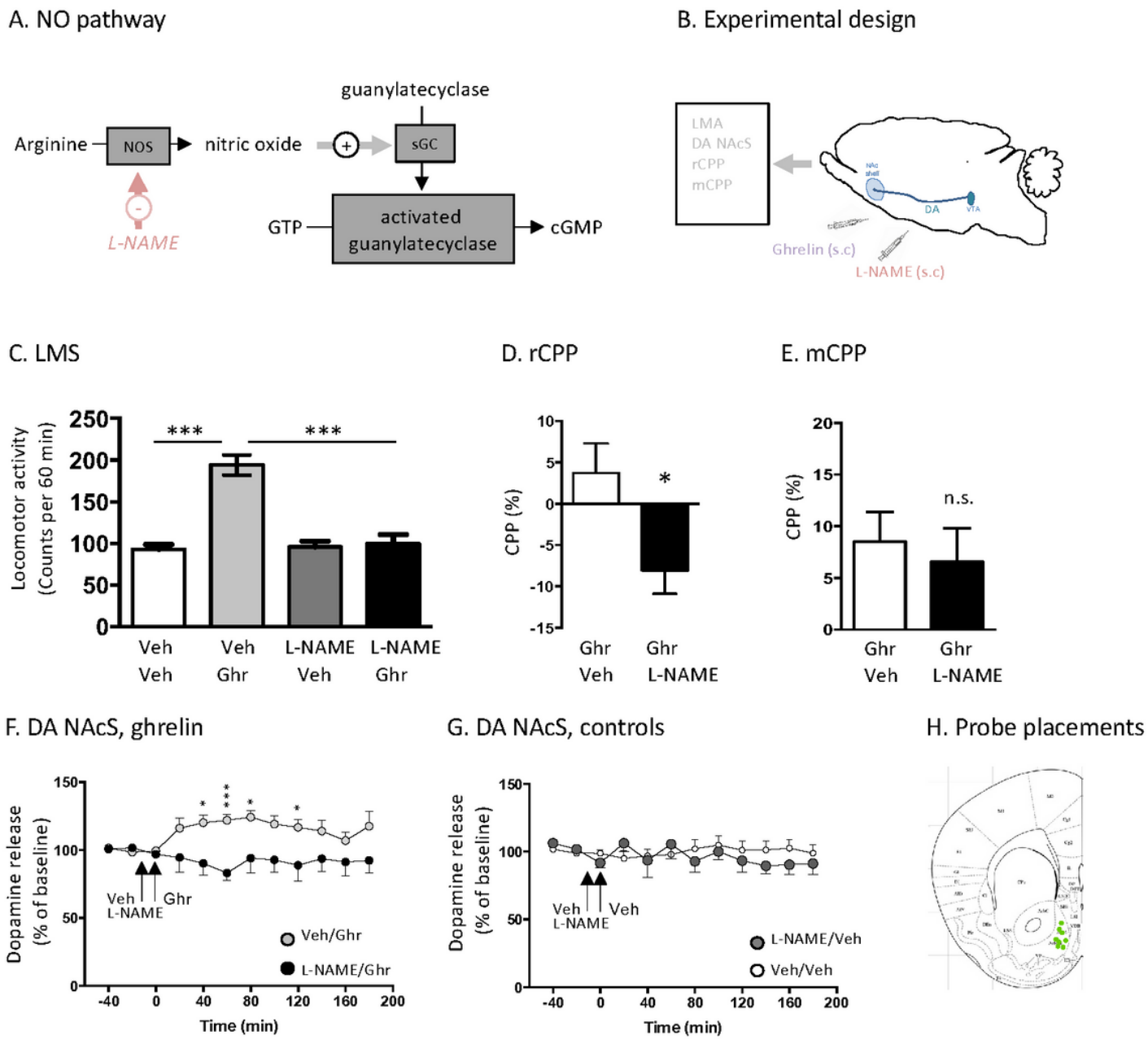

Figure 1

L-NAME, a non-selective inhibitor of neuronal and endothelial NOS, attenuates the ghrelin-induced locomotor stimulation, dopamine release in NAcS and reward in the CPP paradigm in mice

Schematic illustration of **(A)** nitric oxide (NO) pathway, and **(B)** the experimental design. Systemic administration of L-NAME (40 mg/kg, sc) blocked the ghrelin (Ghr;0.33 mg/kg, sc)-induced **(C)** locomotor

stimulation (LMS), and **(D)** reward in the conditioned place preference paradigm (rCPP). On the contrary, **(E)** L-NAME did not alter the memory of ghrelin-induced reward (mCPP). **(F)** The ability of ghrelin to increases dopamine in nucleus accumbens shell (DA NAcS) was blocked by L-NAME. **(G)** Compared to vehicle (Veh), L-NAME did not alter dopamine release in NAcS. (H) schematic illustration of probe placements within the NAcS. Each data set is presented as mean ± SEM. (*P<0.05, ***P<0.001, n.s. = not significant).

Figure 2

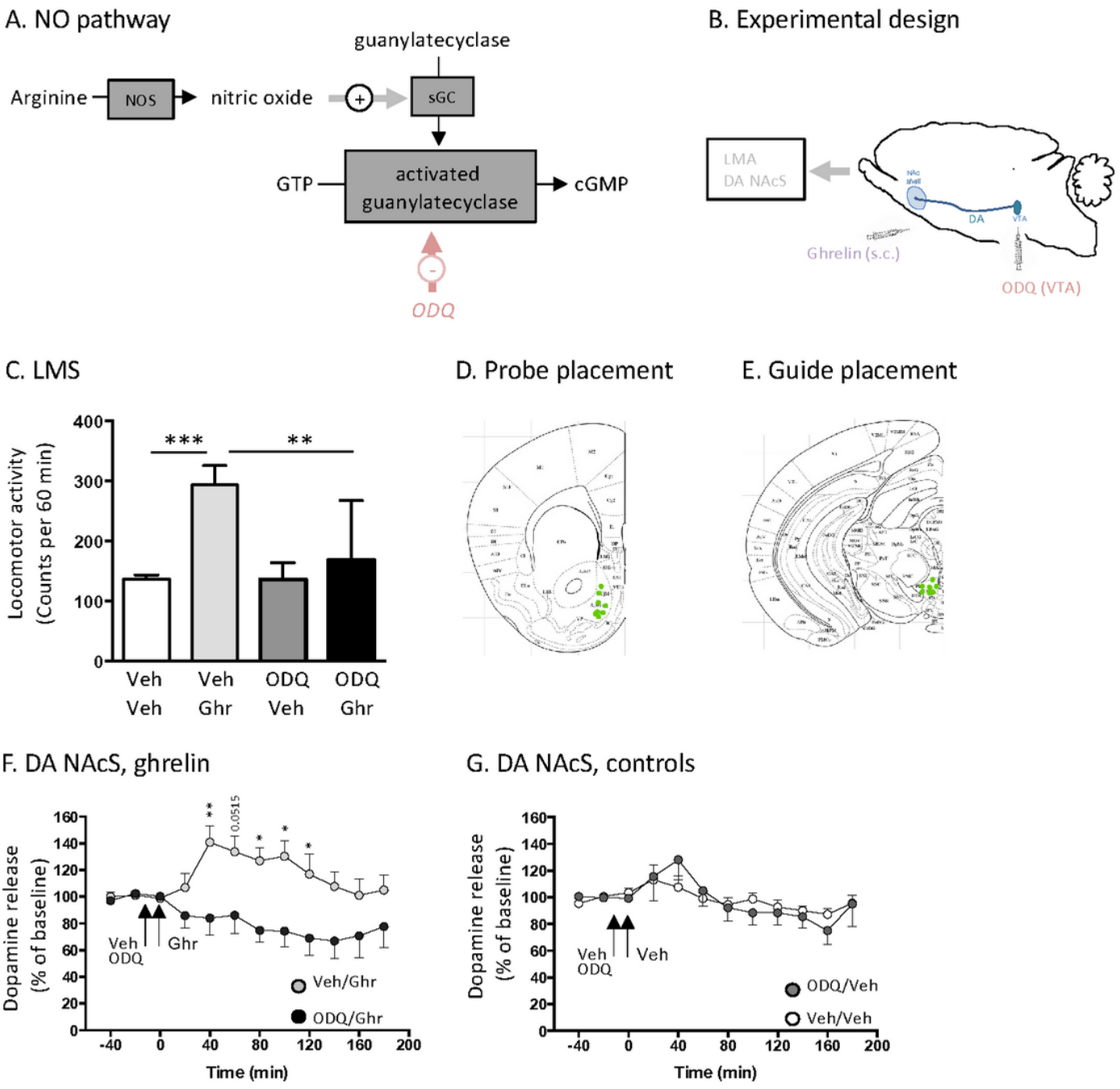

Figure 2

Local infusion of ODQ, an inhibitor of sGC, into the VTA attenuates the ghrelin-induced locomotor stimulation and dopamine release in NAcS in mice

Schematic illustration of **(A)** nitric oxide (NO) pathway, and **(B)** the experimental design. Local infusion of ODQ (0.1 mM at a volume of 0.5 µl/side) blocked the ghrelin (Ghr;0.33 mg/kg, sc)-induced **(C)** locomotor stimulation (LMS) in mice. Schematic illustration of **(D)** probe placement and **(E)** guide placement in the VTA. **(F)** The ability of ghrelin to increases dopamine in nucleus accumbens shell (DA NAcS) was blocked by ODQ. **(G)** In separate control experiments, ODQ did not alter dopamine release in NAcS compared to vehicle (Veh). (H) Schematic illustration of probe placements within the NAcS. Each data set is presented as mean ± SEM. (*P<0.05, **P<0.001,***P<0.001, n.s. = not significant).

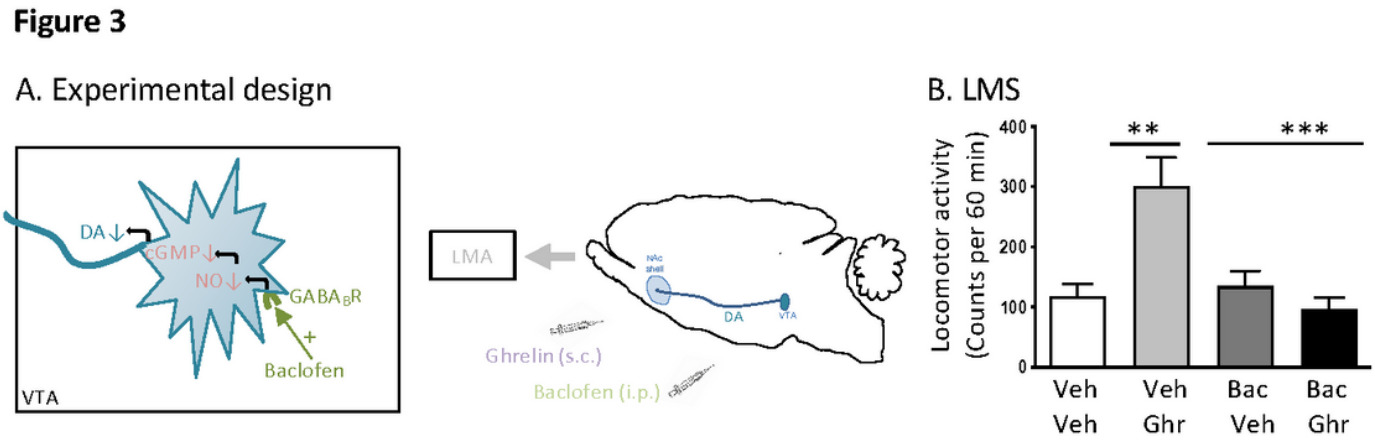

Figure 3

Baclofen a $GABA_B$ receptor agonist known to reduce NO and cGMP, attenuates the ghrelin-induced locomotor stimulation in mice

(A) Schematic illustration of the experimental design. **(B)** Systemic administration of baclofen (Bac; 2.5 mg/kg; intraperitoneal, ip), blocks the ghrelin-induced (0.33 mg/kg, sc) locomotor stimulation (LMS) in mice. Data are presented as mean ± SEM. (**P<0.001,***P<0.001, n.s. = not significant).

Figure 4

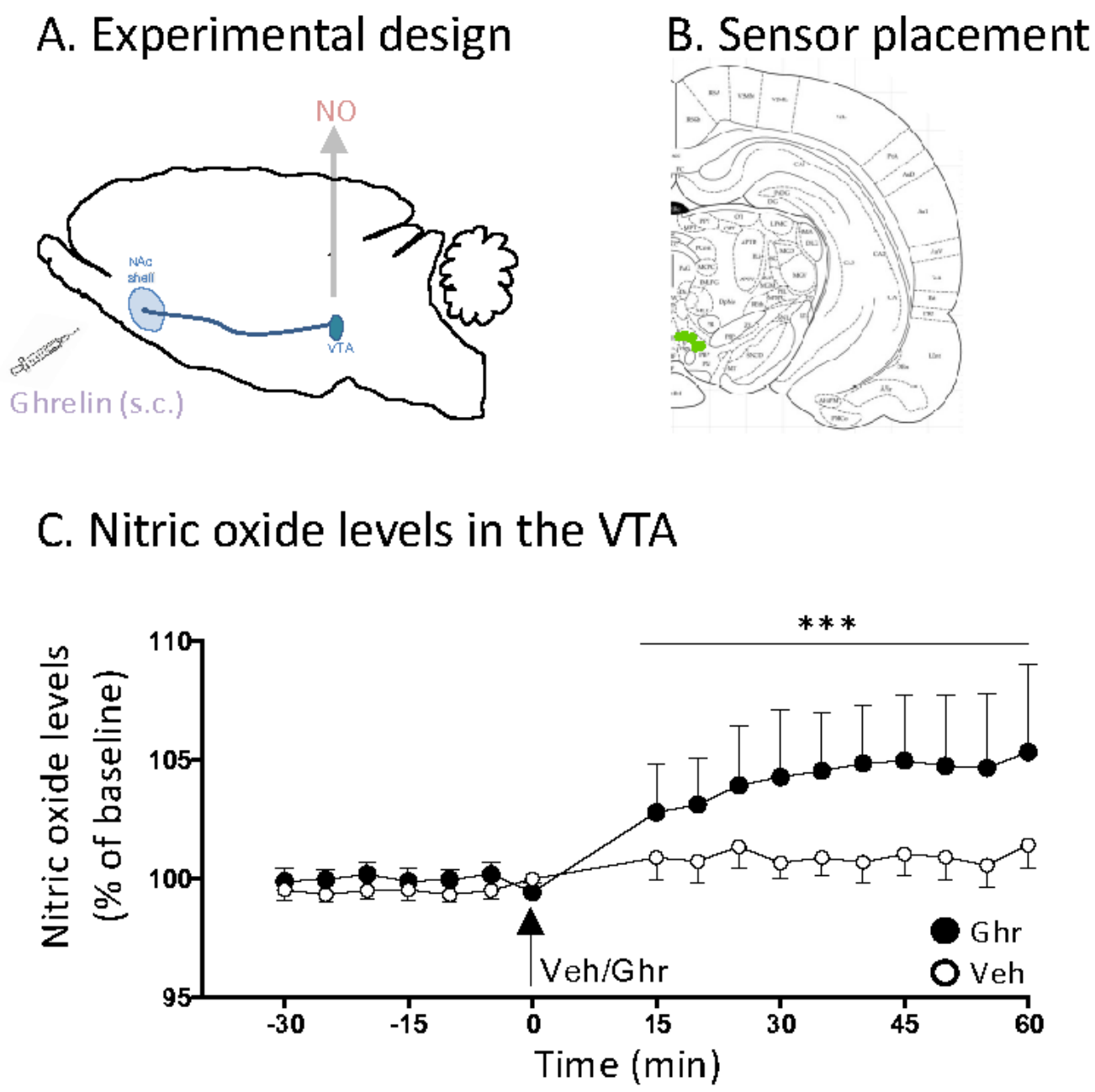

Figure 4

Systemic ghrelin administration increases the nitric oxide levels in the VTA in rats

Schematic illustration of **(A)** the experimental design and **(B)** the sensor placement in the VTA. **(C)** Compared to vehicle (Veh), ghrelin (Ghr; 0.33 mg/kg, sc) increases the nitric oxide levels in the VTA. Data are presented as mean ± SEM, ** P<0.01, treatment effect from repeated two-way ANOVA

Figure 5

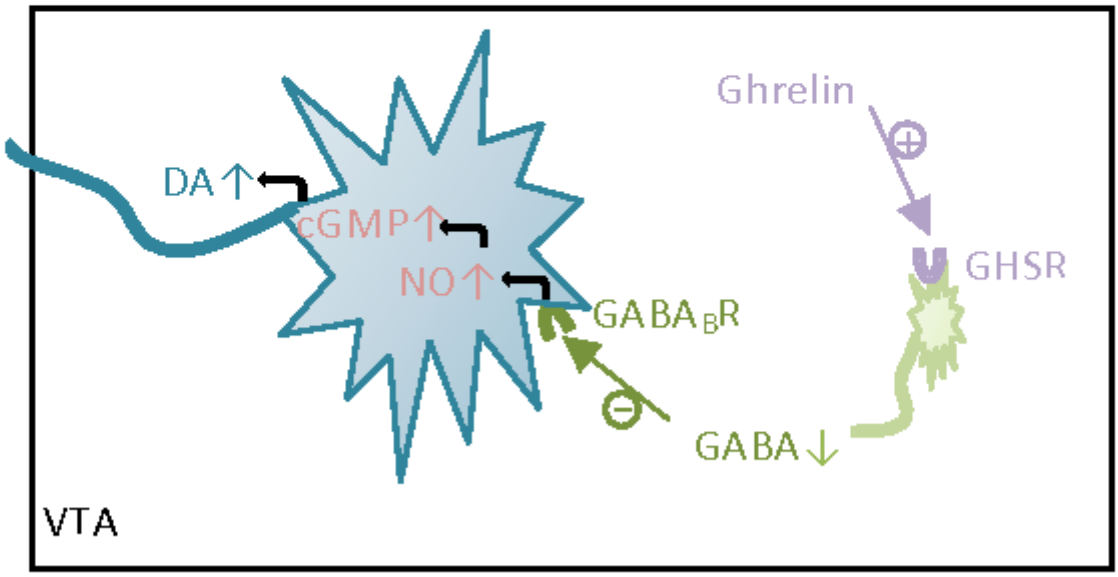

Figure 5

Illustration of tentative mechanism explaining the ghrelin-NO link

The circuitry within the VTA through which ghrelin increases nitric oxide NO remains to be determined. It may involve activation of GHSR-1A on GABAergic interneurons as they profoundly express GHSR-1A. Consequently, ghrelin decreases GABA in the VTA, followed by a reduced GABAergic tone on $GABA_B$-receptors located on dopamine neurons. This in turn, would cause a release of NO and subsequent cGMP, which then enhance the activity of dopaminergic neurons.