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Midbrain raphe 5-HT$_{1A}$ receptor activation alters the effects of ghrelin on appetite and performance in the elevated plus maze

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Abstract

Prior research suggests that midbrain serotonergic signaling and hypothalamic ghrelinergic signaling both play critical roles in appetitive and emotional behaviors. In the present study, we investigated the effects of median raphe nucleus (MRN) somatodentritic 5-HT$_{1A}$ receptor activation on the feeding-stimulant and anxiogenic action of paraventricular nucleus (PVN) ghrelin. In an initial experiment, adult male Sprague-Dawley rats were injected with either ghrelin (200–800 pmol) into the PVN or 8-OH-DPAT (2.5–10 nmol), a 5-HT$_{1A}$ receptor agonist, into the MRN. Performance on the elevated plus maze (EPM) was then assessed. In separate rats, MRN 8-OH-DPAT (2.5–5 nmol) was administered 5 min prior to PVN injection of ghrelin (400 pmol) followed by EPM testing. The orexigenic effects of MRN 8-OH-DPAT (0.1–1.6 nmol) paired with PVN ghrelin (50 pmol) were also examined. When administered alone into the PVN, ghrelin significantly decreased the number of entries and time spent in the open arms of the EPM. This anxiogenic effect was blocked if rats were allowed to eat immediately after ghrelin administration and then tested in the plus maze. MRN injections of 8-OH-DPAT were anxiolytic, and when rats were pretreated with 8-OH-DPAT prior to ghrelin, the anxiogenic action of the peptide was attenuated. In contrast, MRN administration of 8-OH-DPAT potentiated the eating-stimulant effect of PVN ghrelin. Overall, our findings demonstrate that ghrelinergic and serotonergic circuits interact in the neural control of eating and anxiety-like behaviors, with 5-HT$_{1A}$ receptor mechanisms potentiating the orexigenic action of ghrelin while inhibiting ghrelin-induced anxiogenesis as measured via the EPM.

Keywords

8-OH-DPAT, anxiety, food intake, ghrelin, hypothalamus, median raphe nucleus, paraventricular nucleus, 5-HT$_{1A}$

Introduction

We have recently reported that hypothalamic administration of ghrelin, including direct injection into the paraventricular nucleus (PVN), elicits anxiety-like behaviors in the rat (Currie et al., 2012). This is expressed as an avoidance of the open arms of the elevated plus maze (EPM) as well as an increase in stereotypic behaviors. The anxiogenic action of ghrelin, mediated within the PVN, is evident at feeding-effective doses (Currie et al., 2005, 2011) and is consistent with emerging physiological and behavioral evidence implicating the peptide in stress and anxiety mechanisms. For example, ghrelin has a stimulatory effect on the hypothalamic–pituitary–adrenal (HPA) axis (Broglio et al., 2003; Muccioli et al., 2002). In rats, acute ventricular ghrelin administration increases anxiety-like behavior as measured using open field and EPM paradigms, and direct injections into the amygdala, dorsal raphe nucleus, and hippocampus have been reported to alter anxiety (Alvarez-Crespo et al., 2012; Carlini et al., 2002, 2004; Schuette et al., 2012). Further, chronic ventricular ghrelin treatment evokes pro-anxiety and pro-depressive behavioral responses (Hansson et al., 2011), while ghrelin antisense oligonucleotide administration elicits anxiolytic-like effects (Kanehisa et al., 2006).

Alterations in serotonin (5-hydroxytryptamine; 5-HT) transmission have also been implicated in anxiety, depression, and symptoms of stress (Guilloux et al., 2011; Haleem, 2011; Lalonde and Strazielle, 2010). Indeed, the regulatory effect of 5-HT on emotional behavior is widely recognized. With at least 15 5-HT receptor types and subtypes, particular attention has focused on the role of central 5-HT$_{1A}$ receptors. In the mammalian brain, the 5-HT$_{1A}$ receptor is expressed presynaptically on the cell bodies and dendrites of neurons in the midbrain raphe nuclei and post-synaptically on neurons of forebrain terminal areas, including limbic structures (Hannon and Hoyer, 2008). Numerous studies have reported on the role of amygdaloid 5-HT$_{1A}$ receptors in the regulation of anxiety-like behaviors and depression (Bonn et al., 2013; Leite-Panissi et al., 2006; Savitz and Drevets, 2013; Villela de Andrade Strauss et al., 2013). In a recent report (Li et al., 2012), recombinant adenovirus containing 5-HT$_{1A}$ promoter-controlled 5-HT$_{1A}$ antisense sequence was injected into the amygdala of rats prior to conducting EPM and open field tests used to determine the impact on anxiety behavior. Reduction in the expression of 5-HT$_{1A}$ receptors significantly attenuated the time spent in the open arms and time spent in the center of the

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open field. The reduction in the percentage of time spent in the open arms of the EPM was negatively correlated with 5-HT_{1A} receptor density in this region.

In addition to forebrain limbic function, other work has implicated midbrain median raphe nucleus (MRN) 5-HT_{1A} receptors in anxiety. Direct MRN administration of the 5-HT_{1A} agonist 8-OH-DPAT elicits significant anxiolytic effects measured using social interaction and plus maze protocols (File et al., 1996) and impairs the acquisition of inhibitory avoidance in the elevated T-maze (Vicente et al., 2008). Local infusion of the 5-HT_{1A} antagonist WAY-100635 blocks this anxiolytic effect (Vicente et al., 2008). Presumably, activation of 5-HT_{1A} somatodendritic autoreceptors decreases 5-HT release at terminal areas of the forebrain, thereby resulting in a suppression of anxiogenic behavior. Finally, consistent with a role of altered 5-HT signaling, antidepressant and anxiolytic behavioral responses have been reported in 5-HT transport-deficient mice (5-HTT −/−) in a variety of test paradigms including the EPM, tail suspension, and forced swim test (Holmes et al., 2003; Renoir et al., 2008).

The primary aim of the present study was to investigate the potential interaction of ghrelin and 5-HT mechanisms in the control of anxiogenic behavior. Since the individual orexigenic effects of PVN ghrelin (Currie et al, 2005, 2012; Wren et al, 2001) and MRN 8-OH-DPAT (Currie et al., 1994; Fletcher and Coscina, 1993) are well established, we also investigated the potential interaction of ghrelinergic and serotonergic transmission on appetitive behavior. We hypothesized that MRN somatodendritic 5-HT_{1A} receptor activation would attenuate PVN ghrelin-induced anxiogenesis but potentiate the feeding-stimulant action of the peptide. Again, this hypothesis is consistent with evidence cited above implicating both transmitter systems in stress and anxiety, as well as work demonstrating that hypothalamic 5-HT and ghrelin may interact in the neural control of eating and energy metabolism.

Materials and methods

Animals

Adult male Sprague-Dawley rats (*N*=200; Harlan, Kent, WA) weighed 275–300 g at the time of surgery. Rats were housed individually in polypropylene cages with free access to rodent chow pellets (LabDiet, Brentwood, MO) and water. The animal colony room was maintained on a 12 h light/dark cycle (lights off at 18.00) and at a temperature of 22 ± 2°C. Experimental, surgical, and anesthetic protocols were approved by the Institutional Animal Care and Use Committee of Reed College as described in the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Experimental Animals. All testing was conducted during the dark cycle (18.00–20.00).

Surgery

Rats were anesthetized with pentobarbital sodium (50 mg/kg IP) and placed in a Kopf stereotaxic frame with the incisor bar set 3.5 mm below the interaural line. Coordinates (Paxinos and Watson, 2007) for PVN guide cannulae relative to bregma were posterior 1.8 mm, lateral ±0.3 mm, and ventral 3.9 mm. Median raphe cannulae were implanted on an angle of 20° to the vertical, and coordinates, anterior 1.2 mm, lateral +1.2 mm, and ventral +5.5 mm, were relative to interaural zero. Unilateral guide cannulae (26-gauge; Plastics One, Roanoke, VA) were implanted 4 mm dorsal to target sites. Implants were secured with acrylic cement and a stainless steel inner stylet maintained cannula patency. Rats were administered 0.4 mg/kg SC of the analgesic buprenorphine at the time of surgery and once again 12 h later. Behavioral testing began after a postoperative recovery period of 2 weeks.

Apparatus

The EPM apparatus (Med Associates, St Albans, VT) consisted of two open arms (10 × 50 cm) and two closed arms (10 × 50 cm with walls 40 cm in height) that intersected at a central neutral zone (10 × 10 cm). The floor of the maze consisted of a near-infrared backlight runway, and the apparatus itself was elevated 50 cm from the ground. A digital camera and video tracking software (Version 1.14; Med Associates) recorded behavioral activity.

Experimental procedure

Rats (*n*=10–12/condition) were injected with sterile saline vehicle or acylated rat ghrelin (200–800 pmol in 0.2 µL) into the PVN and then after 5 min were placed in the EPM for 10 min. Measurements of the percentage of time spent in the open arms, the percentage of open arm entries, and total open and closed arm entries combined were assessed. In separate groups of rats (*n*=12/group), ghrelin was administered into the PVN at the above doses and rats were returned to their home cages with free access to rodent chow for 45 min. Food intake was measured 45 min postinjection. Rats were then immediately placed in the EPM, where open and closed arm exploration was assessed.

In a subsequent experiment, rats (*n*=11–12/group), we investigated the anxiogenic effects of 8-OH-DPAT alone administered directly into the MRN (2.5–10 nmol in 0.2 µL). In order to examine a potential interaction between serotonergic and ghrelinergic mechanisms, additional rats (*n*=10–12) were implanted with two guide cannulae, one aimed at the MRN and another directed at the PVN. Animals were injected with 8-OH-DPAT (2.5–5 nmol in 0.2 µL) or sterile saline vehicle into the MRN 5 min prior to PVN injection of ghrelin (400 pmol in 0.2 µL) or vehicle. After PVN treatment, rats were placed in the EPM. In all EPM testing, a between subjects design was employed and each test was performed as a single trial per animal to avoid the anti-anxiety effect of familiarity with the paradigm.

In a separate group of rodents (*n*=8), the feeding effects of 8-OH-DPAT, ghrelin, and co-administration of 8-OH-DPAT paired with ghrelin were examined. 8-OH-DPAT (0.1–1.6 nmol) or vehicle was injected into the MRN 5 min prior to PVN administration of ghrelin (50 pmol) or vehicle. After microinjections, rats were returned to their home cages, in which preweighed amounts of standard rodent chow had been placed. Food intake, corrected for spillage, was measured 2 h later. A within subjects design was used, such that rats received all treatment conditions administered in randomized order. At least three non-injection days were required between successive testing conditions. Doses of drug and peptide used in the present study were based on

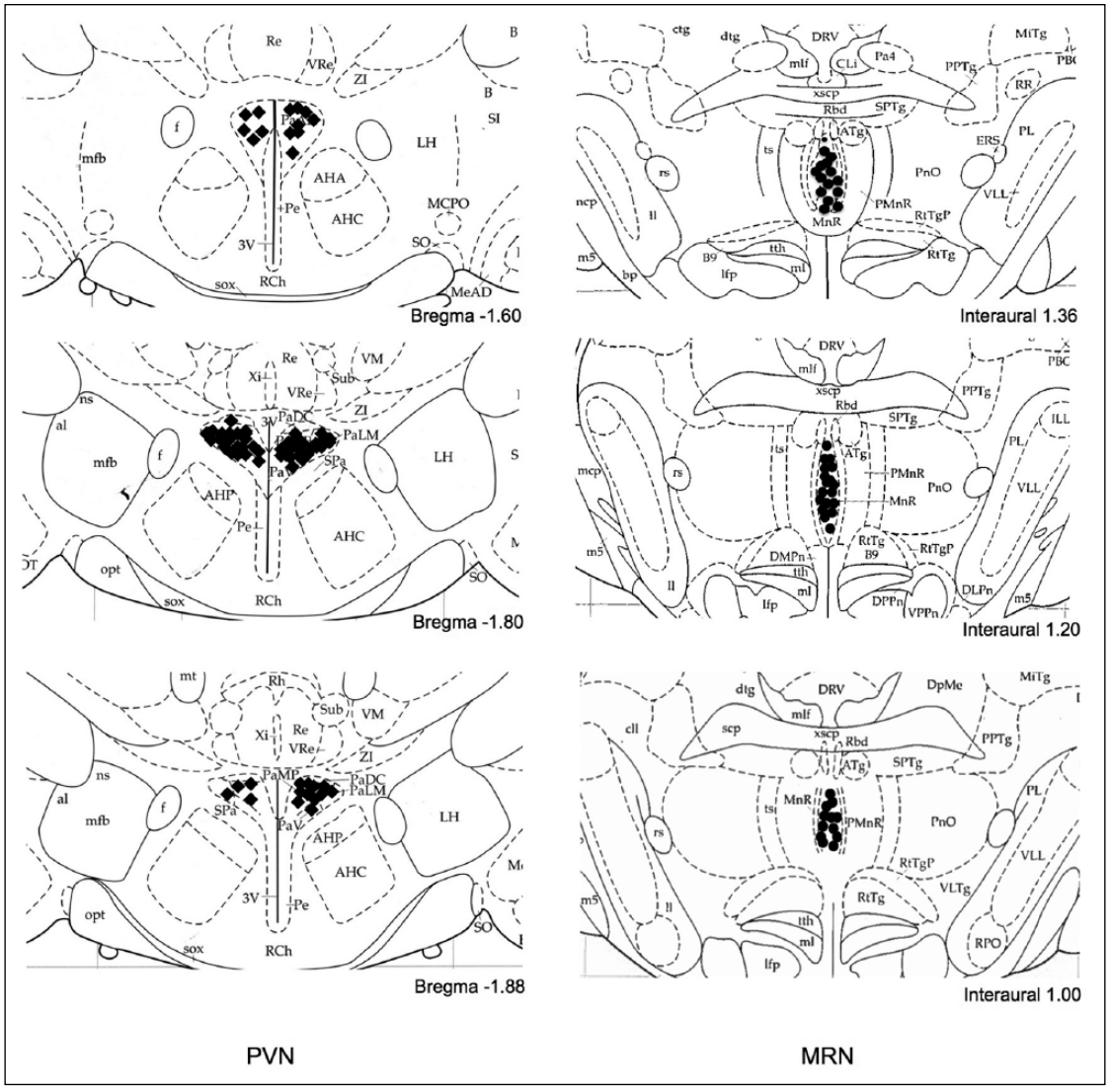

Figure 1. Schematic of coronal sections indicating representative PVN and MRN cannula placements.

previously published reports (Currie et al., 2004, 2012; Vicente et al., 2008) and preliminary testing in our lab.

Histological and statistical analyses

Cannulae placements were confirmed via histological analysis. Immediately prior to brain extractions, black ink was injected in a volume equal to that administered in test microinjections. The black ink assisted in localizing the site of injection as well as its dispersal pattern. Coronal sections (40 μm) were cut through the hypothalamus and midbrain using a cryostat and then stained with Cresyl violet. Sections were examined using light microscopy and viewed relative to the stereotaxic atlas of Paxinos and Watson (2007). Only data from rats found to have injector tracks extending into appropriate target sites were used in the analyses. As an example, a schematic of coronal sections of the rat brain illustrating representative placements of guide cannula in rats with both PVN and MRN implants, and tested for anxiety, is shown in Figure 1. Overall, a total of 10 rats were deemed to have inaccurate placements and were deleted from the data sets. For statistical evaluations, data were analyzed using one-way or two-way analyses of variance (ANOVA). Specific comparisons between means were evaluated using post hoc Tukey tests. The criterion for statistical significance was $p<0.05$.

Results

The anxiogenic effects of PVN ghrelin as measured using the EPM are shown in Figure 2. A one-way between groups ANOVA indicated that ghrelin elicited an increased avoidance of the open arms of the EMP. This was reflected in a reduction in the percentage of time spent in the open arms ($F(3,40)=5.56$, $p<0.003$) as well as in the percentage of open arm entries ($F(3,40)=7.25$, $p<0.006$). We did not observe any alterations in total number of entries ($F(3,40)=0.59$, $p>0.05$). When separate rats were injected with ghrelin and then given access to food, intakes were increased across all doses ($F(3,44)=5.23$, $p<0.004$; Figure 3). However, when these same rats were examined in the EPM immediately

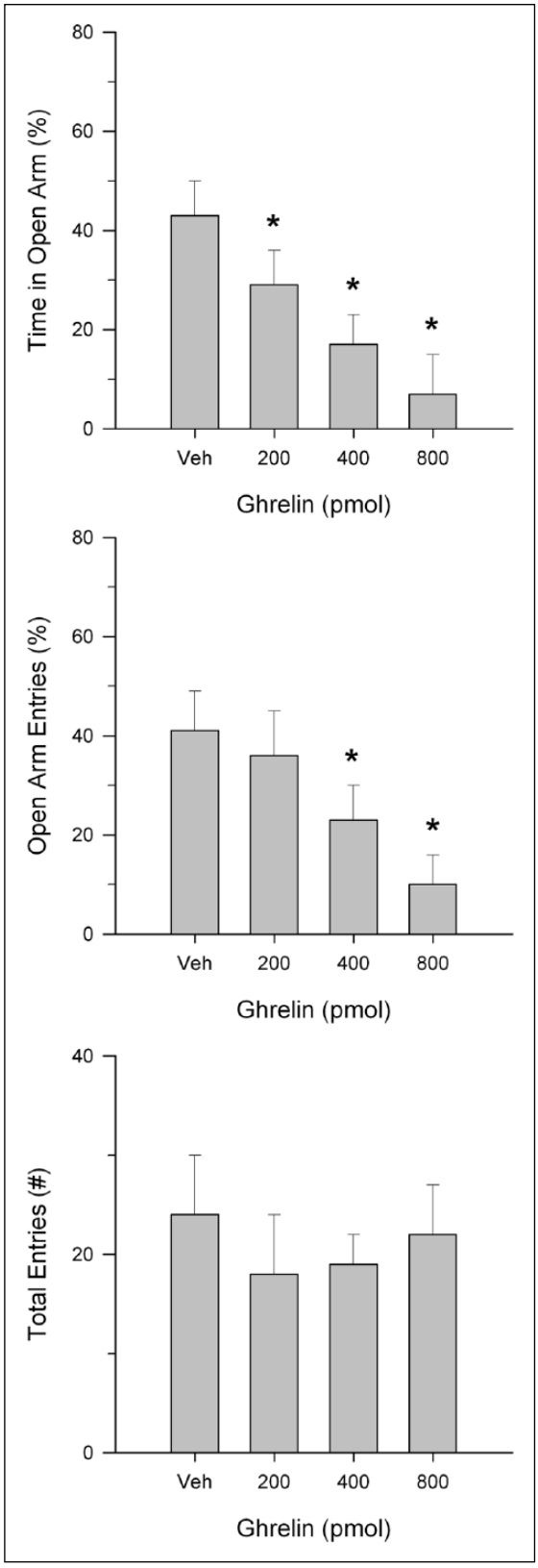

Figure 2. Effect of ghrelin microinjections into the PVN on the percentage of time spent in the open arms, open arm entries, and combined total open and closed arm entries of the elevated plus maze. Values represent mean ± SEM. *p<0.05 compared with vehicle (Veh); n=10–12/group.

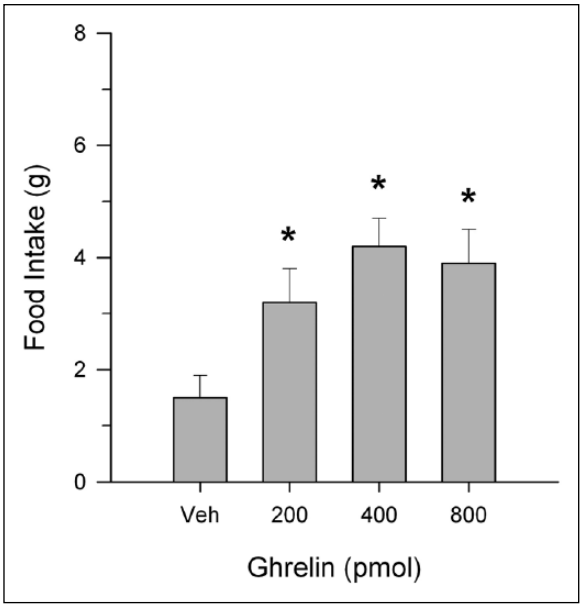

Figure 3. Effect of PVN ghrelin on food consumption. Intakes are mean ± SEM. *p<0.05 compared with vehicle (Veh); n=12/group.

after the 45-min feeding test, no alterations in open arm activity were observed in terms of time spent in the open arms (F(3,44)=0.98, p>0.05; Figure 4) or in open arm entries (F(3,44)=1.43, p>0.05) as compared to the control group. No impact on the total number of entries was observed (F(3,44)=0.79, p>0.05).

In contrast, as illustrated in Figure 5, MRN injections of 8-OH-DPAT, at doses of 5–10 nmol, increased the percentage of time spent in the open arms (F(3,42)=6.15, p<0.002) and the percentage of open arm entries (F(3,42)=7.31, p<0.005) compared with vehicle-treated rats. There was no overall impact on the total number of combined open and closed arm entries (F(3,42)=1.10, p>0.05).

In a separate study, when rats were pretreated with a subthreshold (2.5 nmol) or a threshold (5 nmol) dose of 8-OH-DPAT into the MRN followed by injection of ghrelin (400 pmol) into the PVN, the anxiogenic effects of ghrelin were attenuated. These behavioral responses are shown in Figure 6 in relation to the percentage of time spent in the open arms of the EPM (F(3,40)=4.71, p<0.007) and the percentage of open arm entries (F(3,40)=8.37, p<0.0002). No reliable effect on the total number of entries was observed (F(3,40)=1.52, p>0.05).

In feeding tests (Figure 7), repeated measures ANOVA revealed a significant two-way interaction (F(3,21)=18.07, p<0.0001). Both PVN ghrelin (50 pmol) and MRN 8-OH-DPAT (0.4–1.6 nmol) significantly increased food intake, and 8-OH-DPAT pretreatment reliably potentiated ghrelin-stimulated eating. The potentiation of the orexigenic action of ghrelin was observed at threshold (0.4–1.6 nmol) and subthreshold (0.1 nmol) doses of the 5-HT_{1A} agonist.

Discussion

As described above, previous research implicates central ghrelin and serotonin (5-HT) transmission in the neural control of appetitive, anxiety, and stress-related behaviors. In the present study,

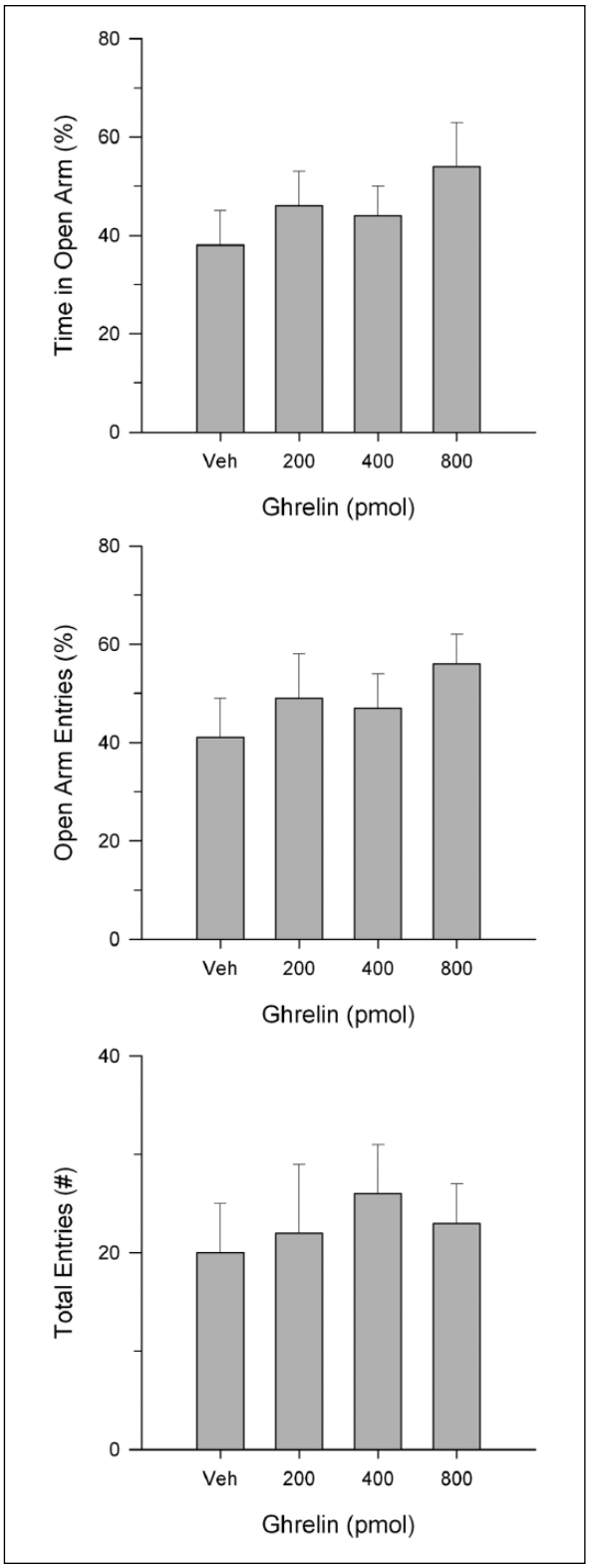

Figure 4. Effect of PVN ghrelin administration on the percentage of time spent in the open arms, open arm entries, and combined total open and closed arm entries of the elevated plus maze. EPM testing occurred immediately after food exposure. Values represent mean ± SEM; *n*=12/group.

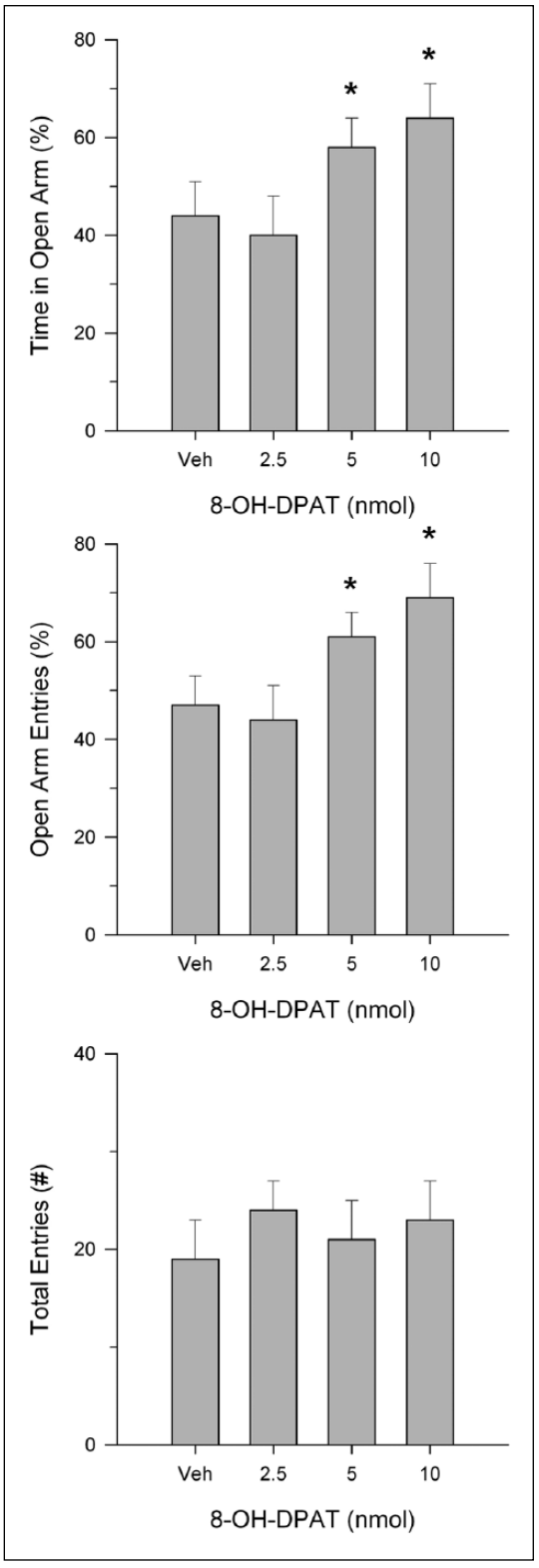

Figure 5. Effect of MRN administration of 8-OH-DPAT on performance in the elevated plus maze as measured by the percentage of time spent in the open arms, open arm entries, and total entries. Values are mean ± SEM. *p<0.05 compared with vehicle (Veh); *n*=11–12/group.

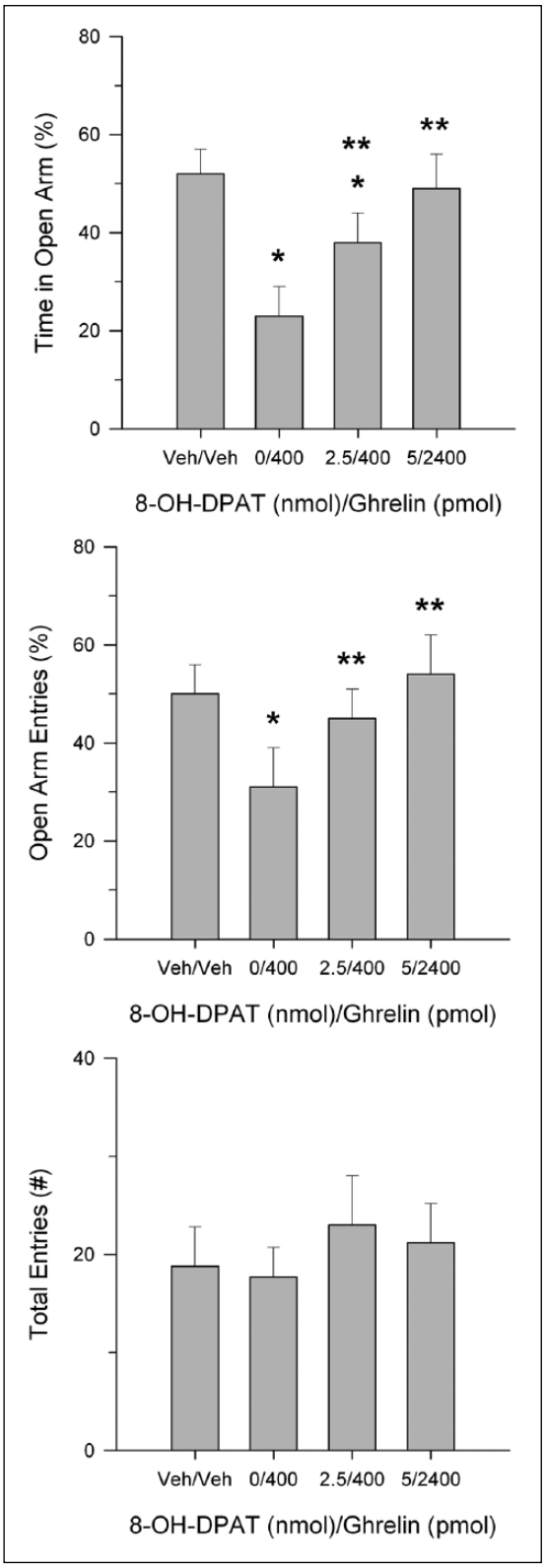

Figure 6. Effect of 8-OH-DPAT pretreatment on the anxiogenic action of ghrelin. 8-OH-DPAT was injected into the MRN while ghrelin was administered into the PVN. Performance in the elevated plus maze was measured as the percentage of time spent in the open arms, open arm entries, and total entries. Values are mean ± SEM. *p<0.05 compared with vehicle (Veh). **p<0.05 compared with ghrelin; n=10–12/group.

we found that PVN administration of ghrelin elicited an increase in anxiogenic behavior. Additionally, PVN ghrelin treatment evoked robust increases in food intake, and, in fact, if rats were first given access to food immediately after ghrelin treatment and then tested in the EPM, the induction of anxiogenic behavior was not observed. Therefore, ghrelin-stimulated eating prevents the subsequent expression of anxiety-like behavior. Since we have previously reported that ghrelin treatment into the PVN is effective in eliciting open arm avoidance when administered up to 60 min prior to EPM testing (Currie et al., 2008), it is arguable that the lack of effect of ghrelin we observed in the current study was not simply due to the time course of the peptide's effect. While MRN injection of 8-OH-DPAT also increased food intake, local infusion of the 5-HT_{1A} agonist elicited an anxiolytic response. Further, MRN 8-OH-DPAT pretreatment potentiated eating elicited by PVN ghrelin but antagonized ghrelin's anxiogenic action.

Ghrelin is a 28 amino acylated peptide originally identified in the rat stomach (Kojima et al., 1999). The peptide binds to the ghrelin 1a receptor, also referred to as the growth hormone secretagogue receptor (GHSR1a) (Kojima et al., 1999; Lim et al., 2010). Ghrelin binding to the GHSR1a results in the secretion of growth hormone by the pituitary (Kojima et al., 1999). The consequential action on downstream signaling cascades produces robust effects on food intake, energy homeostasis, and autonomic function (Briggs and Andrews, 2011; Currie et al., 2005; Freeman et al., 2013; Shrestha et al., 2009; Wren et al., 2001). Indeed, prior reports have shown that direct injections into hypothalamic nuclei, including the PVN and arcuate nucleus, elicit effects on energy intake and metabolism, such as alterations in carbohydrate oxidation and fat metabolism (Currie et al., 2005, 2012). Other work has shown that ghrelin-induced effects on eating and respiratory quotient (RQ) are attenuated by 5-HT pretreatment administered directly into the PVN (Currie et al., 2010). Ghrelin's effects are also blocked by PVN microinjection of the 5-$HT_{2A/2C}$ agonist DOI (Currie et al., 2010). Interestingly, similar serotonergic manipulations inhibit the orexigenic and metabolic effects of PVN neuropeptide Y (NPY) administration (Currie and Coscina, 1998; Currie et al., 1999).

The PVN plays a critical role in autonomic and metabolic regulation, including the integration of various neural and hormonal signals regulating food intake and energy metabolism as well as stress and anxiety (Herman et al., 2005; Onaka et al., 2012; Roubos et al., 2012; Zoli and Picciotto, 2012). While, admittedly, the expression of ghrelin in the rodent brain remains controversial (Furness et al., 2011), ghrelin receptors have been localized on presynaptic NPY terminals within the PVN (Shuto et al., 2002). Activation of these receptors stimulates NPY release (Cowley et al., 2003). While stimulation of 5-HT neurons within the PVN suppresses PVN ghrelin and NPY effects on energy homeostasis (Currie and Coscina, 1998; Currie et al., 2010), other evidence has demonstrated that systemic injections of 5-HT receptor agonists and 5-HT reuptake inhibitors induce c-Fos expression in hypothalamic PVN neurons at eating-inhibitory doses (Lee et al., 2004a, 2004b; Singewald et al., 2003). Collectively, these findings support an interaction of PVN neuropeptide and indoleamine signaling.

5-HT axonal terminals synapse with ghrelinergic neurons in the hypothalamus, and 5-HT receptor expression occurs within these PVN neurons (Heisler et al., 2006). Other work provides compelling support for an interaction between 5-HT and ghrelinergic systems. Hypothalamic 5-$HT_{1/2}$ receptor mRNA and plasma

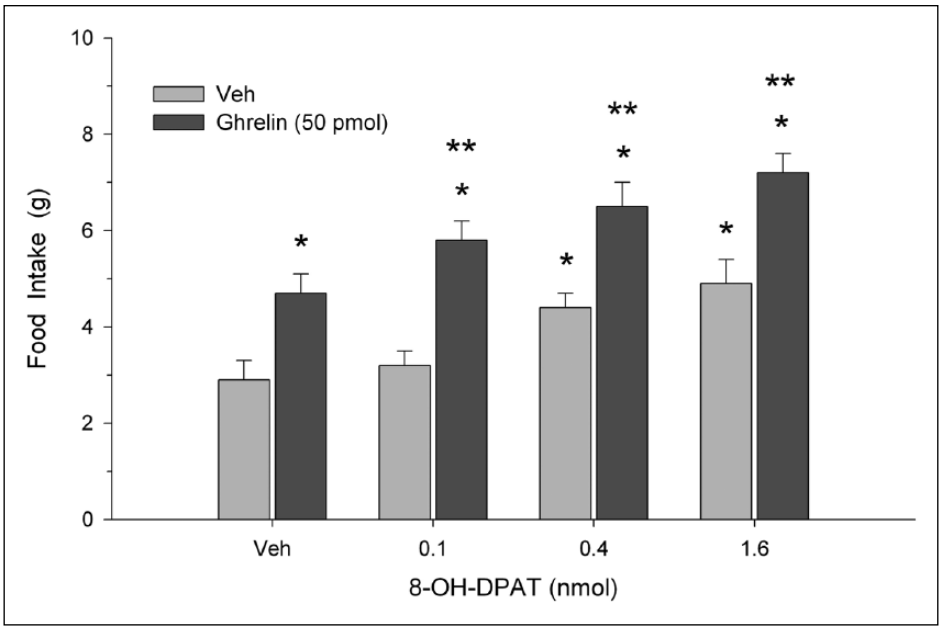

Figure 7. Effect of MRN 8-OH-DPAT pretreatment on the orexigenic action of PVN ghrelin. Values represent mean food intake ± SEM measured over 2 h. *p<0.05 compared with vehicle (Veh). **p<0.05 compared with ghrelin; n=8.

ghrelin levels are increased in fasted rats (Nonogaki et al., 2006). The increase in 5-$HT_{1/2}$ receptor expression is proportional to increases in plasma ghrelin. Further, elevations in plasma ghrelin are inhibited by administration of 5-HT agonists. In the present study, MRN injection of 8-OH-DPAT increased food intake and potentiated the orexigenic effect of PVN ghrelin. Our findings, therefore, are in agreement with the hypothesis that activation of 5-HT_{1A} somatodendritic receptors in the MRN alters serotonergic tone in the hypothalamic PVN, thereby altering the impact of 5-HT signaling in control of feeding as well as the metabolic and anxiogenic action of PVN ghrelin. Indeed, changes in 5-HT_{1A} receptor-mediated transmission are reported to affect the expression of defensive responses associated with anxiogenic behavior, including inhibitory avoidance (Bordukalo-Niksic et al., 2010; Li et al., 2012). This work concurs with numerous studies implicating midbrain and forebrain/limbic 5-HT_{1A} receptors in the neural control of emotion, including anxiety, stress, and depressive-like behaviors (Bonn et al., 2013; Leite-Panissi et al., 2006; Savitz and Drevets, 2013; Vicente et al., 2008; Villela de Andrade Strauss et al., 2013).

Previous reports have indicated that ghrelin administration elicits anxiogenic and anxiolytic behaviors (Alvarez-Crespo et al., 2012; Carlini et al., 2002, 2004; Schuette et al., 2012). While it remains unclear why ghrelin may both increase and decrease anxiety-like behavior in animal models, such bidirectional effects on emotional responding could be attributed, at least in part, to differential effects of dose, route of peptide administration, or GHSR1a distribution and anatomical localization within the brain. In fact, we have recently proposed that ghrelin's effects on anxiety may be influenced by the basal anxiety state, in turn influenced by basal levels of corticosterone (Schuette et al., 2012). Specifically, when the effects of ghrelin on performance in the EPM are examined, the peptide appears to be most effective in eliciting an anxiogenic response under conditions where animals show minimal stress or anxiety prior to ghrelin treatment. In contrast, animals exhibiting higher baseline stress levels might be expected to exhibit an anxiolytic response after ghrelin treatment. Consequently, whether or not ghrelin evokes anxiogenic or anxiolytic-like behaviors could be related to underlying anxiety state, as rats exhibiting reduced basal levels of anxiety would have comparable reduced levels of stress hormones, including corticosterone. Numerous studies have demonstrated a relationship between ghrelin and corticosterone. For example, central administration of the corticotropin releasing hormone (CRH) antagonist alpha-helical CRH9-41 attenuates the anxiogenic action of ghrelin (Asakawa et al., 2001), and we have recently reported that PVN administration of the CRH-related peptide urocortin 1 (UCN1) attenuates the feeding and metabolic effects of PVN ghrelin (Currie et al., 2011). In addition to these opposing interactive effects, central UCN1 alone elicits an increase in anxiety-like behavior (Dono and Currie, 2012; Gehlert et al., 2005; Spiga et al., 2006). Overall, this work suggests that ghrelin interacts with CRH, as it does with 5-HT neurons, in the control of eating, in energy metabolism, and in anxiety/stress-related neural circuits of the hypothalamus. It is also in agreement with recent evidence suggesting that ghrelin reduces anxiety after acute stress by stimulating the hypothalamic–pituitary–adrenal axis via the anterior pituitary (Spencer et al., 2012).

In conclusion, hypothalamic PVN injections of ghrelin increase food intake and elicit anxiogenesis, whereas activation of midbrain 5-HT_{1A} somatodendritic receptors results in both orexigenic and anxiolytic responses. Activation of MRN 5-HT_{1A} receptors potentiates eating stimulated by PVN ghrelin but attenuates ghrelin's effects on anxiety behavior. Accordingly, based on these findings, we propose that ghrelinergic and serotonergic signaling may contribute to an integrated neural axis, extending from midbrain raphe neurons to terminal projections of the

forebrain. This neural integration, in turn, modulates orexigenic and anxiety-like behaviors. Such a hypothesis is consistent with very recent work (Butt et al., 2014) demonstrating that alterations in 5-HT_{1A} receptor expression in mice are associated with changes in the expression of feeding-related neuropeptides and receptor function in the hypothalamus. However, while Funk et al. (2005) have previously reported that MRN 8-OH-DPAT administration induces c-Fos expression in a number of brain regions including the medial prefrontal cortex, the lateral septum, the dorsal bed nucleus of the stria terminalis, and the ventral hippocampus, in their report PVN c-Fos activation was not observed, possibly suggesting that MRN 5-HT neurons impact PVN ghrelin signaling via indirect neuronal pathways.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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