Evidence that central pathways that mediate defecation utilize ghrelin receptors but do not require endogenous ghrelin

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Abstract

In laboratory animals and in human, centrally penetrant ghrelin receptor agonists, given systemically or orally, cause defecation. Animal studies show that the effect is due to activation of ghrelin receptors in the spinal lumbosacral defecation centers. However, it is not known whether there is a physiological role of ghrelin or the ghrelin receptor in the control of defecation. Using immunohistochemistry and immunoassay, we detected and measured ghrelin in the stomach, but were unable to detect ghrelin by either method in the lumbosacral spinal cord, or other regions of the CNS. In rats in which the thoracic spinal cord was transected 5 weeks before, the effects of a ghrelin agonist on colorectal propulsion were significantly enhanced, but defecation caused by water avoidance stress (WAS) was reduced. In knockout rats that expressed no ghrelin and in wild-type rats, WAS-induced defecation was reduced by a ghrelin receptor antagonist, to similar extents. We conclude that the ghrelin receptors of the lumbosacral defecation centers have a physiological role in the control of defecation, but that their role is not dependent on ghrelin. This implies that a transmitter other than ghrelin engages the ghrelin receptor or a ghrelin receptor complex.

Introduction

Activation of the conventional ghrelin receptor (GHSR1a) causes coordinated propulsive activity of the colorectum and increased defecation in rat and mouse (Shimizu et al. 2006; Charoenthongtrakul et al. 2009). Moreover, GHSR1a agonists increase defecation in human (Ejskjaer et al. 2010; Ellis et al. 2015; Acosta et al. 2016). In rat experiments using centrally penetrant GHSR1a agonists with different chemistries have shown the site of action to be the defecation center in the lumbosacral spinal cord (Shimizu et al. 2006; Hirayama et al. 2010; Pustovit et al. 2014; Naitou et al. 2015). The effects of intravenous GHSR1a agonists were mimicked by applying ghrelin intrathecally, but responses could not be evoked by intravenous ghrelin, which does not enter the spinal cord (Hirayama et al. 2010). Defecation in response to centrally penetrant agonists was prevented by cutting the pelvic nerves between the defecation center and the colorectum, but not by cutting the spinal cord rostral to the defecation center (Shimizu et al. 2006; Pustovit et al. 2014; Naitou et al. 2015). Consistent with ghrelin agonists, and intrathecal ghrelin, acting at the lumbosacral defecation center, GHSR1a expression was observed in...
autonomic preganglionic neurons in this spinal cord region by in situ hybridization histochemistry (Ferens et al. 2010a) and in ghrelin receptor reporter mice (Furness et al. 2011).

While these observations imply that GHSR1a is in the pathways of defecation control, ghrelin itself is not found in the mouse spinal cord (Furness et al. 2011). In fact, it is probable that ghrelin is not present even in other parts of the central nervous system (Sakata et al. 2009; Furness et al. 2011; Kern et al. 2014; Cabral et al. 2017).

Despite the apparent absence of ghrelin, the conservation of effect of ghrelin receptor agonists in mouse, rat and human, suggests that GHSR1a receptors in the defecation pathways may have a physiological role. The current experiments were devised to test the proposition that there is a natural stimulant of ghrelin receptors in the defecation center that is released in the spinal cord, but is not ghrelin.

Materials and Methods

Experiments were conducted on 8–12 weeks old Sprague–Dawley rats and Sprague–Dawley rats in which ghrelin were knocked out using CRISPR technology. Procedures were approved by the University of Melbourne Animal Ethics Committee. Rats were supplied with food and water ad libitum prior to the experiments.

Ghrelin knockout rats

Ghrelin was knocked out using Cas9/CRISPR gene editing technology by the Australian Phenomics Network CRISPR service, Monash University. Two microinjection sessions were employed using two different guides. In the first microinjection session, guide 5′-1 GGT was the protospacer adjacent motif (PAM) followed by TGGACTCTTTG 3′ and reverse (5′ TACCTCGACATGGGAC 3′) genotyping primers amplified a product of approximately 550 bp or 400 bp if the modified allele was present. Rats identified as having a modified allele underwent a second PCR to determine homozygosity or heterozygosity. The primers used were 5′CTGCAGTTTTG CTACCTCCTA 3′ and 5′ TGGTGCTTCTGCGATTCTT 3′ generating a band of 689 bp if the rats were heterozygous. For all rats that were used in physiological experiments, the absence of ghrelin was confirmed by immunohistochemistry of gastric sections.

In vivo experiments

Rats were sedated with ketamine hydrochloride (50–60 mg·kg⁻¹, i.m.), following which anesthesia was induced with α-chloralose (60 mg·kg⁻¹, i.v.). The femoral artery was then cannulated for the infusion of anesthetic and blood pressure recording, and the femoral vein was cannulated for delivery of drugs. Blood pressure and heart rate were recorded with a Power Lab recording system using Chart 5 software (both from ADInstruments, Sydney, Australia). Anesthesia was maintained by intraperitoneal infusion of α-chloralose (12–20 mg·kg⁻¹·h⁻¹) plus ketamine (3–5 mg·kg⁻¹·h⁻¹) in phosphate-buffered saline (PBS; 0.15 mol/L NaCl containing 0.01 mol/L sodium phosphate buffer, pH 7.2). The distal colon was cannulated at the colonic flexure, which in the rat is at the junction of the proximal and distal colon, where formed fecal pellets are first observed. A second cannula was inserted into the anus. The colon remained in situ, and the muscle and skin were closed around the proximal cannula. The oral cannula was connected to a Mariotte bottle filled with warm PBS, and the distal cannula to a pressure transducer via a one-way valve. The baseline intraluminal pressure was maintained at 6–10 mmHg by adjusting the heights of the Mariotte bottle and outlet. Expelled fluid was collected in a cylinder distal to the one-way valve, and measured by weighing with a force transducer. Blood pressure measurements were made continuously. At the end of each experiment, the rat was killed with a lethal dose of sodium pentobarbital (300 mg·kg⁻¹ i.v.), while still under anesthesia.

Spinal cord lesion

A total of nine wild-type rats had their spinal cord severed at T4. The rats were lesioned at 8–10 weeks of age and left for 5 weeks to recover after surgery. A total of 10 sham operations were conducted at the same ages. Spinal transections were made using aseptic conditions under inhaled isoflurane anesthesia (3% in oxygen). The muscles connecting to the spines of the vertebrae were
detached and a laminectomy was performed to remove the dorsal aspect of the T4 vertebra. The spinal cord was raised using a curved probe so that complete transection could be observed. The cord was transected with sharp scissors. The laminectomy was closed with three layers of sutures through the adjacent vertebral musculature, the subdermal tissues and the skin. The area was then disinfected with chlorhexidine in 70% alcohol. Sterile saline (1.5 mL/100 g body weight) and analgesic, buprenorphine (0.03 mg kg⁻¹), were administered post-operatively by subcutaneous injection. After recovery from anesthesia, the animals were examined to confirm paralysis of the hind limbs as an index of the completeness of the lesion. Additional injections of sterile saline and analgesic were administered twice daily for the first 3 days postoperatively. For the first 10–14 days after the spinal cord was transected, until the bladders of spinal cord injury (SCI) animals started voiding spontaneously, prophylactic antibiotic (cefazolin 50 mg kg⁻¹) was injected at 12 h intervals, and bladders of SCI rats were manually expressed by external palpation 2–3 times per day.

Water avoidance test

Fecal output in response to water avoidance was measured as previously described (Million et al. 2000; Pustovit et al. 2015). Ghrelin knockout rats and their wild-type littermates were familiarized with handling over a period of 2 weeks. On the experimental day, the rats were injected with the GHSR1a receptor antagonist, YIL781 (Tocris Bioscience, Bristol, UK) were injected at 12 h intervals, and bladders of SCI rats were voided spontaneously, and fecal pellets were manually expressed with water up to 7 cm (1 cm below the top of the platform), located in the middle of a large plastic tub that was filled with water up to 7 cm (1 cm below the top of the platform), for a period of 60 min. The numbers of fecal pellets produced by the rat in the first 10, 15, 30, 45, and 60 min on the platform were counted. Following completion of WAS experiments, the rats were culled and stomachs collected for confirmation of genotype by immunohistochemistry and numbers of residual pellets were counted.

Immunohistochemistry

Three Sprague–Dawley wild-type rats were anesthetized with a mixture of ketamine hydrochloride (100 mg kg⁻¹) and xylazine (20 mg kg⁻¹), both from Troy Laboratories (Sydney, Australia), and a perfusion needle was inserted transcardially into the aorta. The right atrium was cut open and the animal was perfused with heparinized PBS followed with 2% formaldehyde plus 0.2% picric acid in 0.1 mol/L sodium phosphate buffer, pH 7.2. After perfusion was completed, the stomach and lumbosacral spinal cord were removed and post-fixed in the same fixative overnight at 4°C. Fixative was washed out with 3 × 10 min washes in DMSO followed by 3 × 10 min washes in PBS and then placed in PBS sucrose azide (PBS containing 30% sucrose as a cryoprotectant and 0.1% sodium azide) and stored at 4°C. Tissue was transferred to a mixture of PBS sucrose and optimum cutting temperature (OCT) compound (Tissue Tek, Elkhart, IN, USA) in a ratio of 1:1 for 24 h before being embedded in 100% OCT and sectioned (12 µm thick) on a cryostat.

Antibodies

Antibodies that were used are listed in Table 1. Antibody RY1601 was raised against synthetic rat acylated ghrelin (1–15)-Cys, coupled to keyhole limpet hemocyanin (KLH) and antibody RY1595 was raised against synthetic rat desacylated ghrelin (1–15)-Cys, similarly coupled to KLH (Mizutani et al. 2009). Both antibodies were raised in rabbits. Previously published results show that immunoreactivity using RY1601 was abolished when the antibody was absorbed against ghrelin, but was not absorbed by desacyl ghrelin; conversely RY1595 was absorbed by desacyl ghrelin, but was not absorbed by ghrelin itself (Mizutani et al. 2009). Lack of

Table 1. Primary antisera, their respective hosts, dilutions, reference codes and sources, used in this study.

| Tissue antigen or label | Host species | Antibody code | Dilution | Source or reference |
|------------------------|--------------|---------------|----------|---------------------|
| Acylated ghrelin       | Rabbit       | RY1601        | 1:10,000 (stomach); 1:1000 (spinal cord) | Mizutani et al. (2009); Furness et al. (2011) |
| Acylated ghrelin       | Rabbit       | G01           | 1:3000 (stomach); 1:500 (spinal cord) | Patterson et al. (2005); Furness et al. (2011) |
| Acylated ghrelin       | Chicken      | Ab15861       | 1:800 (stomach and spinal cord) | Abcam, Cambridge, UK |
| Desacyl ghrelin        | Rabbit       | RY1595        | 1:10,000 (stomach); 1:1000 (spinal cord) | Mizutani et al. (2009); Furness et al. (2011) |
| Neuronal NOS (nNOS)    | Sheep        | V205          | 1:5000 (stomach and spinal cord) | Williamson et al. (1996) |
| Neuropeptide Y         | Sheep        | E2210         | 1:400 (stomach and spinal cord) | Furness et al. (1985) |
Sigma-Aldrich, Sydney, Australia) was added to the tissue containing protease inhibitor cocktail (P8340, USA A21206, Invitrogen, Carlsbad, CA). Tubes, snap frozen in liquid nitrogen and stored at −80°C for 10 min at 4°C to remove cellular debris. The supernatant was collected and further diluted in dH2O (wild-type stomach 1:100, other tissues 1:10) and HCl was added at a final concentration of 0.05 mol/L.

The ghrelin rat/mouse ELISA kit (EZRAGRA-90K; Millipore, Darmstadt, Germany) was run as per manufacturer’s instructions, with 20 µL of diluted tissue sample loaded into wells in duplicate. Prior to acidification, protein concentrations of tissue samples were determined using a Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Gladesville, Australia), and ghrelin assay values were normalized against total protein concentration to make results comparable.

### Data and statistics

Data are expressed as mean ± SEM. Statistical comparisons are made using Student’s t-test or one-way ANOVA with Tukey’s multiple comparisons test, as indicated in the text. Results were considered to differ significantly when P ≤ 0.05.

### Results

#### Immunohistochemical localization of ghrelin

Each of the three anti-acyl ghrelin antibodies and the anti-desacyl ghrelin antibody revealed ghrelin immunoreactive enteroendocrine cells (EEC) in the rat stomach (Fig. 1). By contrast, in sections of the rat spinal cord, no immunoreactivity for ghrelin was detected. To further investigate this observation, concentrations of antibodies were increased over the concentrations that gave strong immunoreactivity in gastric EEC (RY1601, 10-fold; G01, 6-fold; RY1595 10-fold). The higher concentrations resulted in a diffuse background stain in the white and gray matter of the spinal cord, light, even staining of large ventral horn cells, but no staining of nerve terminals. By contrast, antibodies against a neuropeptide known to be in the spinal cord, NPY, revealed bright immunoreactivity in the dorsal horns and in the intermediolateral cell columns. Neuronal NOS immunoreactivity was used to specifically locate the intermediolateral cell columns where ghrelin ligands act. No acyl ghrelin or desacyl ghrelin terminals were observed in these cell groups (Fig. 1). Double labeling for ghrelin and neuronal NOS and for ghrelin and NPY was also conducted in the stomach. Both were revealed, indicating that localizing neuronal NOS or NPY did not interfere with ghrelin immunoreactivity.

### Table 2. Secondary antisera their respective fluorophores, dilutions and sources, used in this study.

| Antibody          | Fluorophore | Dilution | Source and product code |
|-------------------|-------------|----------|-------------------------|
| Donkey anti-rabbit| Alexa Fluor 488 | 1:1000 | Invitrogen, Carlsbad, CA, USA A21206 |
| Donkey anti-sheep | Alexa Fluor 594 | 1:500 | Invitrogen, Carlsbad, CA, USA A11016 |
| Donkey anti-chicken | Alexa Fluor 488 | 1:500 | Jackson Immuno Research Laboratories Inc., West Grove, PA, USA, 703-545-155 |

Rats were anesthetized by placing them in a chamber containing isoflurane. Once the animal was sedated the carotid artery was severed. Stomach, hypothalamus, lower brain stem, and the lumbarosacral region of the spinal cord were then dissected from the animal, placed in microfuge tubes, snap frozen in liquid nitrogen and stored at −80°C. Five volumes of sterile dH2O (0.5 mL per 100 mg tissue) containing protease inhibitor cocktail (P8340, Sigma-Aldrich, Sydney, Australia) was added to the tissue and tubes were immediately placed in a floating rack in a beaker of boiling water on a hotplate and boiled for 5 min, then sonicated on ice and centrifuged at 11,000 g for 10 min at 4°C to remove cellular debris. The supernatant was collected and further diluted in dH2O (wild-type stomach 1:100, other tissues 1:10) and HCl was added at a final concentration of 0.05 mol/L.

ELISA

Rats were anesthetized by placing them in a chamber containing isoflurane. Once the animal was sedated the carotid artery was severed. Stomach, hypothalamus, lower brain stem, and the lumbarosacral region of the spinal cord were then dissected from the animal, placed in microfuge tubes, snap frozen in liquid nitrogen and stored at −80°C. Five volumes of sterile dH2O (0.5 mL per 100 mg tissue) containing protease inhibitor cocktail (P8340, Sigma-Aldrich, Sydney, Australia) was added to the tissue and tubes were immediately placed in a floating rack in a beaker of boiling water on a hotplate and boiled for 5 min, then sonicated on ice and centrifuged at 11,000 g for 10 min at 4°C to remove cellular debris. The supernatant was collected and further diluted in dH2O (wild-type stomach 1:100, other tissues 1:10) and HCl was added at a final concentration of 0.05 mol/L.

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Ghrelin immunoassay

Ghrelin immunoreactivity was quantified in extracts of stomach, hypothalamus, lower brain stem (medulla) and lumbosacral spinal cord, from both wild-type and ghrelin knockout rats, using ELISA. The knockout of ghrelin was investigated by genotyping and by immunohistochemistry for ghrelin in the stomach and CNS. Genotyping showed gene inactivation and immunohistochemistry showed that there was no detectable ghrelin in either the antrum or the corpus in the ghrelin knockout rats (Fig. 2).

Immunoassay of extracts from the stomach revealed high concentrations of ghrelin, despite the ghrelin-containing enteroendocrine cells being a low proportion of the cell types in the stomach. The signal detected by this ELISA was reduced to background levels in gastric extracts from the ghrelin $--/--$ rats, compared to the wild-type rats (Fig. 3). There were very low levels of binding in the extracts from the hypothalamus, lower brain stem and lumbosacral spinal cord. The small signals in these CNS regions were not different in tissue from ghrelin knockout and wild-type rats (Fig. 3). This indicates that there is background binding in this immunoassay, as there can also be background binding in immunohistochemistry for ghrelin (Furness et al. 2011).

Effects of spinal cord transection

Fecal release in response to water avoidance stress

Rats that were placed on a platform surrounded by water defecated soon after. The numbers of pellets produced in the first 15 min were fewer in rats that had their spinal cords severed at the 4th thoracic vertebrum, 5 weeks before being placed on the platform, compared with sham operated rats (Fig. 4). Moreover, the numbers of residual pellets in the colorectum 60 min after the water avoidance test were greater in spinal lesioned rats. This indicates that spinal cord transection interrupts the pathways from the cortex that are activated by water avoidance to evoke defecation.
Changes in colorectal sensitivity to a ghrelin receptor agonist

Colorectal contractile activity was measured under anesthesia in rats whose spinal cords had been severed 5 weeks previously or had sham spinal cord surgery. The rats were injected intravenously with the GHSR1a agonist, HM01 (1.0 mg·kg<sup>-1</sup>). This elicited propulsive contractile waves in the colorectum that expelled fluid contents (Fig. 5). The responses in rats with spinal cord transection were about twice those of rats with sham surgery (Fig. 5).
Effect of a ghrelin receptor antagonist in ghrelin-deficient rats

The ghrelin receptor antagonist, YIL781 (3 mg⋅kg\(^{-1}\), intraperitoneal) caused a similar reduction in fecal output in the wild-type and ghrelin-deficient rats (Fig. 6). The reduction in fecal output in the first 10 min after placing rats on the platform surrounded by water was greater than 50%, the reduction in the first 15 min, about 50% and in the hour after being placed on the platform, about 25%. We interpret this diminished effect over time to be metabolism and clearance of YIL781 after a single injection.

Discussion

A problem that has concerned physiologists who use drugs as tools is whether the response that is observed is “pharmacological” or whether it indicates an underlying physiological relevance of the target of the drug? In the case of the effects of centrally penetrant ghrelin receptor agonists that stimulate defecation, the compounds used have been synthetic, nonnatural compounds. Thus, in the current study, we utilized a natural stimulus of defecation, water avoidance stress, and investigated whether it activated ghrelin receptors. This is a practical test in rats, but not in mice. We had previously investigated in detail whether ghrelin is present in the CNS of mice, and found that it was not present (Furness et al. 2011). However, in digestive physiology, there are considerable differences between species, including even that a peptide that has physiological importance in human, motilin, is completely absent in rat and mouse (Furness et al. 2015). Thus, we have repeated the detailed studies on the ghrelin presence using the rat, and found that it is also absent from the CNS of this species. Because it is difficult to absolutely prove a negative (the lack of ghrelin in the spinal cord of wild-type rats) we knocked out the ghrelin gene in the rat and compared effects of a ghrelin receptor antagonist in ghrelin-deficient and wild-type rats. The work shows, as discussed in more detail below, that the effects of synthetic agonists of the ghrelin receptor do reflect a physiological role of the ghrelin receptor in control of colorectal function.

In this study, we found that no ghrelin could be detected by immunohistochemistry in nerve terminals of the lumbosacral spinal cord in the rat, where physiological studies have shown that ghrelin receptor agonists act to stimulate propulsive contractions of the colorectum and increase fecal output (Shimizu et al. 2006; Shafton et al. 2009; Hirayama et al. 2010; Pustovit et al. 2014; Naitou et al. 2015). We used three antibodies to the acylated form of ghrelin, the form with physiological activity at the lumbosacral spinal cord (Hirayama et al. 2010) and an antibody to the unacylated form (desacyl ghrelin). All four antibodies revealed immunoreactive enteroendocrine cells in the stomach, the body’s major source of ghrelin, but none revealed immunoreactivity in the spinal cord, even when the concentrations of antibodies used for localization were increased up to 10-fold. The immunoreactivity of gastric EEC was abolished when the ghrelin gene was disrupted, indicating that the antibodies specifically recognize ghrelin. Neuropeptides are found in high concentrations in nerve terminals and are generally very easy to locate using suitable antibodies (Ljungdahl et al. 1978; Hökfelt et al. 1984; Boyer et al. 1994; Ekblad et al. 1996). To test the preservation of neuropeptides in nerve terminals, we used antibodies to neuropeptide Y (NPY). Strong immunoreactivity for NPY was observed in the...
sections of spinal cord. Thus, four antibodies that are proven to recognize the acylated and unacylated forms of ghrelin in the rat did not reveal immunoreactivity in the spinal cord. We used anti-neuronal NOS to specifically investigate the autonomic nuclei (IML) where the ghrelin receptor expressing neurons that are deduced to be the targets of ghrelin receptor agonist are located (Ferens et al. 2010a,b). No ghrelin immunoreactivity was in the IML. We used a ghrelin-specific ELISA to seek ghrelin in extracts of the lumbosacral spinal cord. There was a low level of binding in the assay that was not different between extracts of wild-type and ghrelin knockout spinal cord, hypothalamus or lower brain stem. We conclude that ghrelin is absent from the lumbosacral spinal cord. These data, although focused on the caudal spinal cord, are consistent with previous studies that indicate that ghrelin is absent from the CNS (Sakata et al. 2009; Furness et al. 2011; Kern et al. 2014; Cabral et al. 2017).

We next investigated whether the ghrelin receptors in the lumbosacral spinal cord might nevertheless be involved in physiological responses. We used water avoidance stress (WAS) that acts within the brain to initiate defecation (Million et al. 2000). It has been presumed that WAS communicates to the colorectum via descending spinal cord pathways. To test this, we applied WAS to rats that had their spinal cords severed 5 weeks before. WAS caused about half the number of fecal pellets to be released in 60 min from the spinal cord severed compared with sham rats. Moreover, the spinal cord transected rats had more than twice the number of pellets that remained in the colon at 60 min after WAS. This indicates that there was a deficit in the integrity of the defecation pathways that are activated by WAS when the spinal cord was severed at the lower thoracic level. However, HM01, a centrally penetrant ghrelin agonist that activates the defecation center (Naitou et al. 2015), was effective after the spine was severed. In fact, the responsiveness to HM01 was approximately doubled. One interpretation of this change is that there was a denervation sensitivity, that is, the ghrelin receptors on neurons in the lumbosacral spinal cord were lacking their normal synaptic input after the cord was severed. Denervation hypersensitivity is a well described phenomenon that occurs in both autonomic and somatic pathways when synaptic...
inputs are removed for days or weeks (Kuffler et al. 1971; Ekström and Wahlestedt 1982; Fujiwara et al. 1984). This implies that the ghrelin receptors are physiologically involved in the control of defecation. In a further experiment we investigated the sensitivity of the defecation response to the ghrelin receptor antagonist, YIL781. This compound, formerly named LXRG934, is a centrally penetrant antagonist of the ghrelin receptor, GHSR1a, with nanomolar potency (Esler et al. 2007; Sim et al. 2014). YIL781 caused significant, and similar, reductions in the fecal output in response to WAS in both wild-type rats and in rats lacking ghrelin. The data indicate that defecation has dependency on the ghrelin receptor, GHSR1a, but is not dependent on ghrelin.

Our observation that WAS caused expulsion of some pellets after spinal cord transection deserves comment. After the transection, the number expelled was reduced, on average, from 9 to just under 4 pellets in 60 min (Fig 4A), despite many pellets remaining in the colorectum (Fig 4B). Only about one pellet would be expected to be spontaneously released in this period. An action of stress hormones provides a possible explanation of this spinal cord independent defecation. Peripheral injection of the stress hormone, corticotrophin releasing factor (CRF), mimics stress-induced increases in colonic transit and nonselective CRF antagonists inhibit stress-induced stimulation of colon motility (Taché et al. 2004). An effect through long vagal pathways is also possible, as vagal nerves reach the colon in the rat (Berthoud et al. 1991). However, we are not aware of any physiological data that supports a role of vagal pathways in the colorectal response to stress.

As discussed above, the data indicate that GHSR1a is involved in the spinal pathways of defecation control. It is unknown whether GHSR1a is also involved the pathways that are activated by WAS after spinal transection. If GHSR1a is not involved in these other pathways, the results suggest that ghrelin receptor antagonism blocks most of the effect that is mediated through the spinal pathways and the defecation center.

Previous investigation has similarly identified a functional role of GHSR1a that is independent of ghrelin, in this case, the anorexic effect of dopamine was found to depend on GHSR1a (Kern et al. 2012, 2014). These authors found that GHSR1a forms a heteromer with the dopamine D2 receptor (DRD2). They identified GHSR1a:DRD2 heteromers in a subset of hypothalamic neurons. In wild-type mice and ghrelin-deficient mice, a DRD2 agonist blocked the anorexogenic effect of the DRD2 agonist. These observations resemble those of our investigation. It is thus pertinent that dopamine causes defecation by an action at D2 receptors in the lumbosacral defecation center of the spinal cord of the rat (Naitou et al. 2016). An alternative explanation is that there is a neurotransmitter other than ghrelin that is in descending pathways that acts on GHSR1a.

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**Conflict of Interest**

None declared.

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