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Central orexin-A increases colonic motility in conscious rats

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ABSTRACT

Increasing evidence has indicated that brain orexin plays a vital role in the regulation of gastrointestinal physiology such as gastric secretion, gastric motility and pancreatic secretion. However, little is known whether orexin in the brain is involved in the physiology of the lower gastrointestinal tract. The aim of this study was therefore to elucidate whether orexin-A in the brain is involved in the regulation of colonic motility. In this study, we measured fecal pellet output and recorded intraluminal colonic pressure waves in freely moving conscious rats to evaluate the effects of central orexin-A on colonic motor functions. Intracisternal but not intraperitoneal injection of orexin-A dose-dependently (1–10 μg) increased fecal pellet output. Findings obtained from manometric recordings revealed that intracisternal administration of orexin-A at a dose of 10 μg significantly enhanced colonic motor contractions. These results suggest for the first time that orexin-A acts centrally in the brain to enhance fecal pellet output and stimulate colonic motility in conscious rats. The present study would furthermore support our hypothesis that orexin-A in the brain may be an important candidate as a mediator of the cephalic phase gut stimulation including stimulated colonic motility in addition to well known physiological response such as stimulation of gastric acid and pancreatic acid secretion, and gastric motility.

Orexins/hypocretins are novel neuropeptides that are localized in neurons in the lateral hypothalamus [7,27]. On the other hand, orexin-immunoreactive fibers and terminals, and specific orexin receptors are distributed in a wide variety of nuclei in the central nervous system [10]. Based upon these neuroanatomical evidence, orexinergic projection should be involved in a number of biological functions. In fact, orexins may be implicated in a wide variety of physiological functions. These include feeding [27,32], behavioral activity [11], sleep/awake [6], anxiety [29], energy balance [15], neuroendocrinological response [14] and cardiovascular functions [28]. In addition to these functions, we have demonstrated for the first time that orexin-A is involved in central regulation of gastric acid secretion [24,31,33]. In the brainstem, orexin receptors are expressed in the dorsal motor nucleus of the vagus (DMN) in the medulla oblongata [23], and the parasympathetic preganglionic neurons project their axon terminals through the vagus nerve to the digestive system [21]. Intracisternal but not peripheral injection of orexin-A dose-dependently stimulated gastric acid secretion through the vagus nerve in conscious rats [31]. Considering the potent orexigenic action of orexin-A, it may be an important candidate as a mediator of the cephalic phase secretion as proposed by Pavlov [25].

With regard to the roles of brain orexin in gastrointestinal functions other than gastric secretion, a couple of studies demonstrated that orexin-A acts centrally in the brain to stimulate gastric motility. Kobashi et al. [12] have examined the effects of the intracisternal administration of orexin-A on gastric motility in anesthetized rats. Phasic contractions in the distal stomach were facilitated in response to centrally injected orexin-A. Facilitation in the distal stomach was blocked by vagotomy, suggesting that central orexin facilitates distal stomach motility via the vagus nerve. It has been shown that microinjection of orexin-A into the DMN increased intragastric pressure and antral motility in anesthetized rats [13], indicating that orexin-A in the DMN stimulates gastric motor function. In addition to the evidence in anesthetized rats, Bülbül et al. [4] have very recently demonstrated that intracerebroventricular injection of orexin-A at a dose of 10 μg enhanced postprandial gastric motility in conscious rat. Thus, these provided the evidence in conscious rats that orexin-A acts centrally in the brain to increase gastric motility. However, little is known about a role of central orexin-A in colonic motility.

Gastric and colonic motility are regulated by different mechanisms. For instance, centrally administered corticotropin-releasing factor inhibits gastric emptying while simultaneously increasing

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colonic motility, transit and defecation in rats [16,17,19]. The aim of this study was therefore to elucidate whether orexin-A in the brain is involved in the regulation of colonic motility. In this study, we measured fecal pellet output and recorded intraluminal colonic pressure waves in freely moving conscious rats to evaluate the effects of central orexin-A on colonic motor functions.

Male Sprague–Dawley rats weighing about 250 g were housed under controlled light/dark conditions (lights on: 07:00–19:00) with the room temperature regulated to 23–25 °C. Rats were allowed free access to standard rat chow (solid rat chow, Oriental Yeast Co., Tokyo, Japan) and tap water.

Synthetic orexin-A (human/bovine/rat/mouse) was purchased from Peptide Institute Inc., Osaka, Japan and was dissolved in normal saline just before experiments.

Fecal pellet output was measured according to the previous studies [17]. Rats received intracisternal (ic) injection with either orexin-A (1, 3 or 10 μg in 10 μl) or vehicle (saline 10 μl). ic was performed under brief ether anesthesia within a couple of minutes with a 10-μl-Hamilton microsyringe after rats were mounted in a stereotaxic apparatus (David Kopf Instruments, Tijunga, CA, USA) as reported previously [20]. Immediately after ic, rats were put individually in the cage and fecal pellet output was counted for 1 h. To assess the peripheral action of orexin-A on fecal pellet output, orexin-A (10 μg in 0.3 ml) or vehicle (saline 0.3 ml) was administered intraperitoneally under brief ether anesthesia and fecal pellet output was counted for 1 h.

The rats were anesthetized with ether, and open-tipped catheter (3-Fr, 1 mm ID; Atom, Tokyo, Japan) for manometric measurement was inserted into the colon at 3 cm from the ileocecal junction (proximal colon). The catheter was held in place by purse-string sutures at the point of exit from colonic wall (2 cm proximal to the recording point), brought out through the abdominal wall musculature, and tunneled subcutaneously to exit at the back of the neck and secured to the skin. The rats were allowed to recover in individual cages for 2–5 days before the experiments.

Colonic motility was measured by manometric methods described in previous studies [1,2]. Conscious animals without fasting were put in wire-bottom and non-restraint polycarbonate cages. Manometric catheter from each animal was threaded through a flexible metal sheath to protect from biting and connected to the infusion swivel (Instech Laboratories, Plymouth Meeting, PA) to allow free movement. It was connected to a pressure transducer (TP-400T; Nihon Koden Kogyo, Tokyo, Japan) and was continuously infused with degassed distilled water at a rate of 1.5 ml/h by a heavy-duty pump (CVF-3100; Nihon Koden). Pressure signals from the transducer were digitized and stored using a PowerLab system (AD Instruments, Colorado Springs, CO). Manometric measurement of pressure wave was started after 1-h of stabilization of animals. First, the basal state of the colonic pressure wave was measured for 1 h. Then, the manometric catheter was disconnected and the rats were removed from polycarbonate cages. Under brief ether anesthesia, rats received orexin-A at a dose of 10 μg/10 μl or saline (10 μl) intracisternally as described above. After that, the rats were put in the cages again and the catheter was re-connected to a pressure transducer. The pressure recording was continued for 2 h after ic.

The motor index (MI) was assessed by area under the manometric trace (AUT). AUT was calculated using a data-acquisition software (LabChart v7, AD instruments, Colorado Springs, CO). The baseline drifting and recording noise due to movement of the animals was very minor. To avoid any baseline drifting, we selected the analysis points with stable baseline. Basal MI was determined by calculating AUT for 1-h period before ic. The %MI was determined by calculating following the formula: (AUT for each 1-h period after orexin-A or vehicle ic)/(basal MI) × 100. Changes in the %MI were compared between orexin-A and vehicle treated group. In this

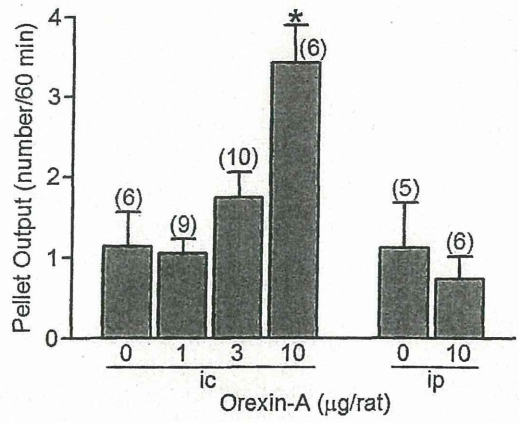

Fig. 1. The effect of intracisternal (ic) or intraperitoneal (ip) orexin-A on fecal pellet output. Fecal pellet output for 1 h was measured in rats injected with either orexin-A or vehicle. Each column represents the mean ± S.E. Number of rats was shown in the parenthesis. *$P < 0.05$ vs vehicle-treated group.

experiment, pressure signals were continuously recorded up to 4 h (1 h for stabilization, 1 h for basal MI and 2 h for determining the changes induced by orexin-A or vehicle ic), but the measurement was temporally stopped in order to perform ic at the middle of the recordings. In relation to ic, we also need the time for recovery from the anesthesia and re-stabilization of baseline of manometric pressure in order to obtain the adequate recordings for the analysis. Therefore, the manometric data during the recovery period from approximately 5 min was excluded from later analysis.

Data were expressed as means ± S.E. Comparison of fecal pellet output was performed by Kruskal–Wallis one-way analysis of variance (ANOVA) followed by non-parametric Bonferroni-type multiple comparison. Comparison of %MI was performed using ANOVA followed by the least significant difference test. Statistica (StatSoft Inc., Tulsa, Okla., USA) was used throughout the study.

The approval of the Research and Development and Animal Care committees at the Asahikawa Medical University was obtained for all studies.

First, we have examined the effect of intracisternal orexin-A on fecal pellet output in freely moving conscious rats. As demonstrated in Fig. 1, ic of orexin-A stimulated pellet output dose-dependently ($H = 12.8$, $P < 0.05$, saline: 1.14 ± 0.42, orexin-A 1 μg: 1.05 ± 0.18, 3 μg: 1.74 ± 0.32, 10 μg: 3.42 ± 0.47, $n = 6$–10). At a dose of 10 μg, it potently increased pellet output ($P < 0.05$ vs saline). By contrast, intraperitoneally administration of orexin-A at the dose of 10 μg failed to increase pellet output (saline: 1.12 ± 0.56, vs orexin-A: 0.73 ± 0.28, $P > 0.05$, $n = 5$–6), suggesting that orexin-A acts centrally in the brain to enhance fecal transit.

Next, to clarify whether colonic contractions would be changed by centrally injected orexin-A, we measured intraluminal colonic pressure in freely moving conscious rats. Fig. 2 illustrated the representative recordings of colonic contractions. As demonstrated in Fig. 2A, ic of orexin-A at a dose of 10 μg enhanced the amplitude of colonic pressure waves. The stimulatory effect of colonic contractions was observed immediately after ic of orexin-A, while centrally injected saline did not change (Fig. 2B). As clearly demonstrated in Fig. 3, ic of orexin-A significantly increased MI change of the colon during 0–60 min after the injection ($F = 5.96$, $P < 0.05$, saline: 90.5 ± 5.0, vs orexin-A: 160.8 ± 20.9, $P < 0.05$, $n = 5$–7). The mean value of MI change during 60–120 min in rats treated with central orexin-A was higher but did not reach to the statistical significance ($P > 0.05$, saline: 96.6 ± 6.9, vs orexin-A: 135.1 ± 32.2, $P > 0.05$).

Although increasing evidence has indicated that brain orexin plays a vital role in the regulation of gastrointestinal physiology such as gastric secretion, gastric motility and pancreatic secre-

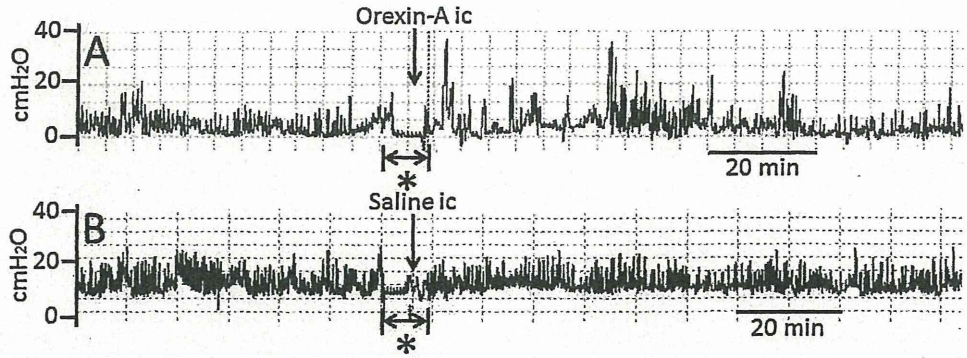

Fig. 2. Representative recordings of the colonic contractions in rat injected intracisternally with either orexin-A (A) or saline (B). *The period which was needed for intracisternal injection and re-stabilization of baseline. The manometric data during the period were omitted from the analysis.

tion, little is known whether orexin in the brain is involved in the physiology of the lower gastrointestinal tract. The current study was performed to clarify whether orexin-A is implicated in the regulation of colonic motor functions. For the aim, we used two methods such as counting pellet output and manometric measurement to assess colonic motility. The major finding in this study is that orexin-A acted centrally to increase fecal pellet output and stimulated colonic contractions in conscious unrestrained rats. The present study therefore suggested for the first time that orexin-A acts centrally in the brain to play a role in the regulation of lower gastrointestinal tract functions.

As demonstrated in the present study, MI was significantly increased to approximately 160% in the first 1-h period after administration of orexin-A at a dose of 10 μg into the cerebrospinal fluid. Bülül et al. [4] demonstrated that the same dose (10 μg) of central orexin-A increased MI to 160% in stomach, and this stimulatory effect was observed immediately after administration and persisted for about 50 min. Thus it is of interest that centrally injected orexin-A at the same dose stimulated both gastric and colonic motor activity at the same degree, suggesting that orexin-A acts in the brain to stimulate not only gastric but also colonic motility under the same condition. As shown by Bülbül et al. [5] central orexin-A-induced change of gastric motility was blocked by atropine and surgical vagotomy in rats, suggesting that vagal cholinergic pathways play a role in the central orexin-A-evoked changes of gastric motility. It is well known that the motor activity of the colon is regulated by the pelvic and vagal cholinergic pathways in rats [3]. All these findings led us to speculate that vagal cholinergic pathways might mediate the stimulated colonic motility by central orexin-A. Further study should be needed to explore the above speculation.

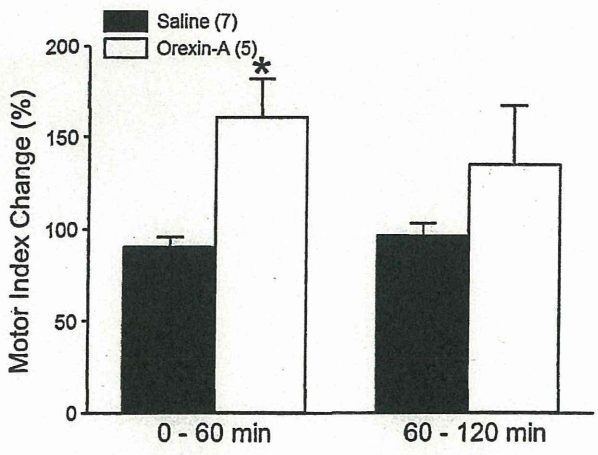

Fig. 3. The effect of intracisternal administration of orexin-A on contractions of proximal colon. Orexin-A significantly increased the motor index as compared to saline treatment at the first 1-h period after administration. However, this effect was no longer observed at next 1-h period. Each column represents the mean ± S.E. Number of rats was shown in the parenthesis. *$P < 0.05$ vs saline-treated group.

The cephalic phase of GI response produces coordinated GI alterations that prime the gut to assist digestion of the impending meal. Since the alterations in response to cephalic stimulation in stomach and pancreas were mimicked by orexin-A [12,18,31], this peptide is thought to be a candidate molecule which triggers the process of cephalic phase stimulation [23]. Moreover, it has been reported that cephalic stimulation also increases colonic motility [26]. The present evidence that orexin-A acts centrally to increase colonic motility may furthermore support our hypothesis that orexin-A in the brain plays a role as a trigger molecule that undergoes cephalic phase gut stimulation.

Functional gastrointestinal disorders (FGIDs) are characterized as chronic or recurrent GI symptoms, which are not explained by structural or biochemical abnormalities. Brain–gut interaction plays an important role in the pathophysiology of FGIDs [8]. The patients with functional dyspepsia (FD), which is one of the FGIDs frequently complain of GI symptoms after meals, and postprandial antral hypomotility or reduced fundic accommodation was reported to be one of the physiological mechanisms of FD [9,30]. Since fundic accommodation and hypermotility in the distal stomach were observed in rats treated with intracerebroventricular orexin-A in rats [12], we suggest in turn decreased orexin signaling may contribute to the pathophysiology of FD as we have shown in recent publications [22,23]. In addition to the role of brain orexin in the physiology of upper GI tract, the present finding that orexin-A acts centrally in the brain to increase colonic motility might support a speculation that orexin signaling would contribute to the pathophysiology of FGIDs which are associated with disturbance of lower GI tract motility.

We have shown for the first time that central orexin-A stimulated colonic motility. This result may further support the hypothesis that orexin-A is a possible molecule mediating the cephalic phase response of digestive system.

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Review

Ghrelin, des-acyl ghrelin and obestatin on the gastrointestinal motility

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ABSTRACT

Ghrelin, des-acyl ghrelin and obestatin are derived from a common prohormone, preproghrelin by post-translational processing, originating from endocrine cells in the stomach. Ghrelin exerts stimulatory effects on the motility of antrum and duodenum in both fed and fasted state of animals. On the other hand, des-acyl ghrelin exerts inhibitory effects on the motility of antrum but not on the motility of duodenum in the fasted state of animals. Obestatin exerts inhibitory effects on the motility of antrum and duodenum in the fed state but not in the fasted state of animals. NPY Y2 and Y4 receptors in the brain may mediate the action of ghrelin, CRF type 2 receptor in the brain may mediate the action of des-acyl ghrelin, whereas CRF type 1 and type 2 receptors in the brain may mediate the action of obestatin.

Contents

1. Introduction

Within several decades a number of brain–gut peptides have been newly discovered and their actions on the gastrointestinal (GI) functions have been widely investigated. The receptor agonists and antagonists have subsequently determined and the site of action on the GI motility has been elucidated. Long-lasting efforts to discover new peptides and their receptor analogs lead to obtain therapeutic strategies on the functional disorders in the GI tracts. After discovery of ghrelin, prokinetic effects of ghrelin on the functional GI disorders have been widely documented [9,12,19,22,28]. In this review we show different effects of ghrelin, des-acyl ghrelin and obestatin on the GI motility, and involvement of different hypothalamic peptides mediating the action of these peptides. This might be helpful to understand the possible prokinetic effects of these peptides.

2. Ghrelin, des-acyl ghrelin and obestatin containing cells in the GI tracts

Ghrelin has been first isolated from rat and human stomach [23], and the localization of ghrelin in the stomach has been studied in various animals by using the specific antibody for ghrelin [11,27]; however, the localization of des-acyl ghrelin in the stomach has been scarcely examined. Our group developed antibodies specific for ghrelin (anti-rat octanoyl ghrelin (1–15)-cys-KLH serum) and for des-acyl ghrelin (anti-rat des-octanoyl ghrelin (1–15)-cis-KLH

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serum) and successfully detected the different localization of ghrelin and des-acyl ghrelin in the rat stomach [24].

Both ghrelin- and des-acyl ghrelin-immunoreative cells were distributed in the oxyntic and antral mucosa of the rat stomach, with higher density in the antral mucosa than oxyntic mucosa. Immunofluorescence double staining showed that ghrelin- and des-acyl ghrelin-positive reactions overlapped in closed-type round cells, whereas des-acyl ghrelin-positive reaction was found in open-type cells in which ghrelin was negative. Ghrelin/des-acyl ghrelin-positive closed-type cells contain obestatin, on the other hand des-acyl ghrelin-positive open-type cells contain somatostatin [24].

The characteristic features of open-type cells that contain des-acyl ghrelin and closed-type cells that contain ghrelin indicate that they may respond differently to intraluminal factors. It is highly possible that open-type cells may react to luminal stimuli more than closed-type cells. In fact, the release of ghrelin was not affected by intragastric pH, whereas the release of des-acyl ghrelin was increased at intragastric pH 2 compared to that at intragastric pH 4 [24]. This result suggests that des-acyl ghrelin-containing cells may sense the intragastric pH via their cytoplasmic processes and release the peptide in accordance with the lower intragastric pH. The fact that the release of des-acyl ghrelin is stimulated by lower intragastric pH seems reasonable because des-acyl ghrelin may act as a satiety signal [2,8] in the fed state of animals.

3. Release of ghrelin, des-acyl ghrelin and obestatin, and experimental model for GI motility

Although ghrelin, des-acyl ghrelin and obestatin are colocalized in the same endocrine cells in the stomach, they are released by different stimuli and then exert different effects on the GI motility. The plasma level of ghrelin is elevated before meal and suppressed after meal [30], therefore the release of ghrelin is regulated by blood glucose and insulin levels [17]. However the release of des-acyl ghrelin is stimulated after meal by lowering the intragastric pH [24]. Plasma obestatin levels are suppressed by meal in humans [20], but constant during fasting and refeeding in rats [32].

To investigate the effects of these peptides on the GI motility, previous studies have preferentially used gastric emptying as a parameter to evaluate the upper GI motility. By the development of methods for real time measurement of GI motility in conscious rats or mice [5,13,29], more information could be obtained for the physiological fed and fasted motor activity than gastric emptying. For example, previous studies showed negative effects of obestatin on the gastric emptying [4,18], however detailed analysis for fed and fasted motor activity led positive effects of obestatin on the gastroduodenal motility [3].

4. Regulation of ghrelin on the GI motility and involvement of NPY in the hypothalamus

The effects of ghrelin on the gastric emptying have been well documented, however no previous studies have examined the effects of ghrelin on the fasted motor activities in the GI tracts. Therefore conscious rat model with manometric measurements of fed and fasted motility has been applied to investigate the role of ghrelin [16]. Intracerebroventricular (i.c.v.) and intravenous (i.v.) injection of ghrelin stimulated the % motor index (%MI) in the antrum and induced the fasted motor activity in the duodenum when given in the fed state of animals [16,29]. I.c.v. and i.v. injection of ghrelin increased the frequency of phase III-like contractions in both antrum and duodenum when given in the fasted state of animals [16]. The effects of i.v. injection of ghrelin on gastroduodenal motility were blocked by i.v. injection of GHS-R antagonist but not by i.c.v. injection of GHS-R antagonist [16]. These stimulatory roles of ghrelin on the fasting motility seem to be physiological, because in normal rats GHS-R antagonist abolished the spontaneous phase III-like contractions in the stomach and duodenum [5].

The involvement of NPY neurons in the hypothalamus in feeding stimulatory effect of ghrelin has been investigated [1], however few studies have shown the involvement of hypothalamic peptide in regulating the GI motility. We found that immunoneutralization of NPY in the brain completely blocked the phase III-like contractions in the duodenum of normal rats, and Y2 and Y4 receptor agonists injected i.c.v. induced the phase III-like contractions in the duodenum when given in the fed state of animals, suggesting that fasted motility in the duodenum is under regulation of hypothalamic NPY neurons [13]. NPY neurons in the brain are tightly related to the role of ghrelin on the GI motility. In fact, immunoneutralization of NPY in the brain blocked the stimulatory effects of ghrelin on the gastroduodenal motility [16], suggesting that NPY neurons in the brain mediate the action of ghrelin on the gastroduodenal motility. Ghrelin may stimulate gastroduodenal motility by activating the GHS-R on vagal afferent nerve terminals and affect NPY neurons in the hypothalamus, Y2 and/or Y4 receptors in the brain may mediate the action of ghrelin (Table 1).

5. Regulation of des-acyl ghrelin on the GI motility and brain CRF receptors

Des-acyl ghrelin has been shown to suppress food intake and gastric emptying [2]. The effects of des-acyl ghrelin on fed and fasted motor activities in the stomach and duodenum were examined. I.c.v. and i.v. injections of des-acyl ghrelin disrupted fasted motility in the antrum but not in the duodenum [8]. The frequencies of fasted motility in the antrum were decreased to 58.9% and 54.5% by des-acyl ghrelin injected i.c.v. and i.v., respectively [8]. However i.c.v. and i.v. injections of des-acyl ghrelin did not alter fed motor activity in both the antrum and duodenum [8]. These data indicate that the effects of exogenous des-acyl ghrelin affects fasted motility in the antrum but not in the duodenum.

Different hypothalamic peptides are involved in the action of des-acyl ghrelin from ghrelin. Capsaicin treatment did not alter the disruptive effect of i.v. injection of des-acyl ghrelin on fasted motility in the antrum [8]. Difference in the involvement of vagal afferent pathways in the action of ghrelin and des-acy ghrelin was confirmed by c-*Fos* expression in the NTS. I.p. injection of ghrelin significantly increased the density of c-*Fos*-positive cells in the NTS, while i.p. injection of des-acyl ghrelin induced no change in the density of c-*Fos*-positive cells in the NTS compared with vehicle-injected controls [8]. Therefore des-acyl ghrelin may cross the blood–brain barrier (BBB) and act directly on the brain receptor and disrupt the fasted motility in the antrum. The centrally administered CRF type 2 receptor antagonist, but not the CRF type 1 receptor antagonist, blocked the effects of centrally and peripherally administered des-acyl ghrelin on gastric motility [8]. Between two CRF receptor subtypes, CRF type 1 receptor is highly involved in anxiety-related behavior and CRF type 2 receptor is involved in regulating food intake and peripheral functions such as gastric acid secretion or gastric emptying. CRF is a relatively selective ligand for CRF type 1 receptor, whereas urocortin 2 is a ligand more selective for CRF type 2 receptor [6,10]. The density of c-*Fos*-positive cells in the PVN was significantly increased by i.p. injection of des-acyl ghrelin compared to vehicle-injected controls [8]. These data suggest that peripherally administered des-acyl ghrelin may activate neurons in the PVN by crossing the BBB, and exert inhibitory effects on the antral motility via CRF type 2 receptor in the brain (Table 1).

Table 1
Comparison of the effects of ghrelin, des-acyl ghrelin and obestatin on the gastroduodenal motility.

	Ghrelin		Des-acyl ghrelin		Obestatin	
	Fasted motility	Fed motility	Fasted motility	Fed motility	Fasted motility	Fed motility
Stomach	↑	↑	↓	–	–	↓
Duodenum	↑	↑	–	–	–	↓
Hypothalamic neuron	NPY		Urocortin 2		CRF, urocortin2	
Brain receptor	Y2, Y4		CRF type 2		CRF type 1, type 2	
Vagal afferent pathway	+		–		+	

6. Regulation of obestatin on the GI motility and brain CRF receptors

Obestatin was initially reported to be the endogenous ligand for GPR39 [32], however recent studies have found no specific binding of obestatin to various types of GPR39-expressing cells [7,21,31]. In the first report obestatin has been shown to suppress food intake and gastric emptying, however a series of following studies denied the effects of obestatin on feeding behavior as well as GI motility [4,18,25]. The detailed study on the fed and fasted motor activity of the GI tracts led positive effects of obestatin. The motor activity in the antrum and duodenum was inhibited when obestatin was given i.v. to conscious rats in the fed state but not when it was given in the fasted state [3]. I.v. injection of obestatin decreased the %MI of fed motility in the antrum and prolonged the time before the return of fasted motility in the duodenum [3]. Such inhibitory actions were the opposite of those obtained with ghrelin [16]. The results showed that the inhibitory action of obestatin appeared 30–90 min after i.v. injection [3], which is consistent with the timing of the effects of i.v. injection of ghrelin (~30 min) on gastroduodenal motility [16].

The brain mechanism in the action of obestatin is different from des-acyl ghrelin or ghrelin. I.v. injection of obestatin induced a significant increase in the number of c-*Fos*-positive cells in the PVN compared to saline-injected controls [3]. Immunofluorescence overlap staining showed that the PVN neurons activated by i.v. injection of obestatin contain CRF or urocortin 2 [3]. The involvement of CRF type 1 and type 2 receptors in the action of obestatin on the gastroduodenal motility was examined [3]. Results showed that the inhibitory action of i.v. injection of obestatin on the motor activities in the antrum and duodenum were blocked by i.c.v. injection of CRF type 1 and type 2 receptor antagonists, suggesting that both types of CRF receptors in the brain may mediate the action of peripherally injected obestatin on gastroduodenal motility [3]. The results showed that vagal afferent nerve blockade by capsaicin reverses the inhibitory effects of obestatin on duodenal motility but does not alter the inhibitory effects of obestatin on antral motility [3]. These results suggest that vagal afferent pathways might be involved partially, but not entirely, in the action of obestatin. Involvement of vagal afferent pathways was confirmed by the finding that the number of c-*Fos*-positive neurons in the NTS was increased by i.v. injection of obestatin [3]. In addition to vagal afferent pathways, it is possible that circulating obestatin acts on brain targets directly by crossing the BBB, because a previous study has shown that there is a rapid influx of i.v.-injected ^{125}I-labeled obestatin from the blood to the brain [26]. Therefore the lack of effects of obestatin on antral motility during capsaicin treatment might be explained by direct action of peripherally injected obestatin on brain targets by crossing the BBB, similar to what has been observed for des-acyl ghrelin. We further examined whether obestatin can antagonize the stimulatory effects of ghrelin on gastroduodenal motility [3]. Obestatin failed to antagonize the ability of ghrelin either to stimulate the %MI in the antrum or to accelerate the initiation of fasted motility in the duodenum when administrated in the fed state [3]. These results were consistent with previous studies in which obestatin failed to antagonize the ability of ghrelin to stimulate gastric emptying or to shorten the MMC cycle time [4]. In the brain, CRF- and urocortin 2-containing neurons might be activated by i.v. injection of obestatin, and at the level, CRF type 1 and type 2 receptors might be involved in the inhibitory action of obestatin on antral and duodenal motility (Table 1). Vagal afferent pathways might be involved partially, but not entirely, in these actions of obestatin (Table 1).

7. Conclusions

Ghrelin, des-acyl ghrelin and obestatin are included in the endocrine cells in the stomach and regulate the upper GI motility by activating hypothalamic peptides [14,15]. Since hypothalamic peptides are strongly affected by stress or anxiety, such brain–gut interaction seems to be important to understand the pathogenesis of functional disorder in the GI tracts.

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INVESTIGATION OF THE PRESENCE OF GHRELIN IN THE CENTRAL NERVOUS SYSTEM OF THE RAT AND MOUSE

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Abstract—Ghrelin and ghrelin receptor agonist have effects on central neurons in many locations, including the hypothalamus, caudal brain stem, and spinal cord. However, descriptions of the distributions of ghrelin-like immunoreactivity in the CNS in published work are inconsistent. We have used three well-characterized anti-ghrelin antibodies, an antibody to the unacylated form of ghrelin, and a ghrelin peptide assay in rats, mice, ghrelin knockout mice, and ghrelin receptor reporter mice to re-evaluate ghrelin presence in the rodent CNS. The stomach served as a positive control. All antibodies were effective in revealing gastric endocrine cells. However, no specific staining could be found in the brain or spinal cord. Concentrations of antibody 10 to 30 times those effective in the stomach bound to nerve cells in rat and mouse brain, but this binding was not reduced by absorbing concentrations of ghrelin peptide, or by use of ghrelin gene knockout mice. Concentrations of ghrelin-like peptide, detected by enzyme-linked immunosorbent assay in extracts of hypothalamus, were 1% of gastric concentrations. Ghrelin receptor-expressing neurons had no adjacent ghrelin immunoreactive terminals. It is concluded that there are insignificant amounts of authentic ghrelin in neurons in the mouse or rat CNS and that ghrelin receptor-expressing neurons do not receive synaptic inputs from ghrelin-immunoreactive nerve terminals in these species.

Key words: ghrelin, neuropeptides, peptide immunohistochemistry, neurotransmitters.

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Abbreviations: BSA, bovine serum albumen; DAB, diaminobenzidine; DPX, distyrene plasticizer xylene mix; EDTA, ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; GENSAT, Gene Expression Nervous System Atlas; GFP, green fluorescent protein; GHSR, growth hormone secretagogue receptor (ghrelin receptor); HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; MMRRC, Mutant Mouse Regional Resource Center; NPY, neuropeptide Y; OCT, optimum cutting temperature (compound); PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioimmunoprecipitation assay; RT, room temperature; RT-PCR, reverse transcriptase polymerase chain reaction.

The acylated peptide, ghrelin, was discovered in the stomach in 1999 (Kojima et al., 1999). In this discovery paper, it was reported that no Northern blot signal for the ghrelin precursor could be detected in whole rat brain extracts, but ghrelin mRNA could be detected by reverse transcriptase polymerase chain reaction (RT-PCR), and an anti-ghrelin antibody appeared to stain neurons in the hypothalamus (Kojima et al., 1999). Subsequent studies also identified nerve cells that bound anti-ghrelin antibodies, but surprisingly most investigators found that the immunoreactivity was confined to neuronal cell bodies (Kojima et al., 1999; Mondal et al., 2005; Sato et al., 2005; Canpolat et al., 2006). This is unusual for a peptide that might be a neurotransmitter in the CNS because peptide messengers (neurotransmitters or neuromodulators) are generally found in high concentrations in nerve terminals. In fact, peptide messengers are often difficult to locate in neuronal somata, requiring manipulations, such as colchicine treatment, to reveal the bodies of the cells that are the sources of immunoreactive terminals (Ljungdahl et al., 1978; Hökfelt et al., 1984; Boyer et al., 1994; Ekblad et al., 1996). In three reports, ghrelin immunoreactivity has been identified in axons in the hypothalamus (Lu et al., 2002; Cowley et al., 2003; Menyhért et al., 2006). However, these papers use antibodies against the nonacylated peptide, which may not locate the mature ghrelin peptide. Other reports also cast doubt on immunolocalization studies. In one study, ablation of the ghrelin gene, by replacement with the lacZ reporter gene, led to loss of ghrelin-like immunoreactivity in the stomach and small intestine, but staining of neurons in the hypothalamus using the same anti-ghrelin antibody was unaffected (Wortley et al., 2004). Furthermore, this lacZ reporter system failed to reveal ghrelin-expressing cells in the hypothalamus, although they were present in the stomach (Wortley et al., 2004). Direct comparisons of concentrations of ghrelin peptide revealed a 5000–20,000 greater concentration of ghrelin in the stomach compared to brain (Hosoda et al., 2000; Grouselle et al., 2008). Another study also failed to find positive neurons in the CNS of two lines of ghrelin reporter mice, although endocrine cells in the stomachs of these mice were readily revealed (Sakata et al., 2009). Because of their failure to reveal ghrelin-expressing neurons in the CNS, these authors used two different commercially avail-

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able antibodies to investigate possible ghrelin peptide localization. Neither antibody revealed specific immunoreactivity in the CNS, as previously reported (Wortley et al., 2004). Ghrelin reporter mice were also investigated by Kageyama et al. (2008). These authors found the reporter signal, using anti-green fluorescent protein (GFP) immunofluorescence, in gastric endocrine cells that were also immunoreactive for ghrelin. Under the same experimental conditions, no GFP immunoreactivity could be found in the hypothalamus. A faint fluorescence was detected in the hypothalamus with wide-band spectral confocal laser microscopy, but the specificity of this signal was not reported. It is also notable that the distributions of immunoreactive neurons described by different authors vary. These neurons have been placed in the arcuate nucleus in rat and mouse (Kojima et al., 1999; Lu et al., 2002; Mondal et al., 2005) or in cells adjacent to the third ventricle between the dorsal medial, ventral medial, paraventricular and arcuate nuclei in mice (Cowley et al., 2003), and in paraventricular and supraoptic nuclei in rats (Zhang et al., 2008).

Although evidence for the occurrence of ghrelin peptide in the CNS is inconsistent, there is good agreement that ghrelin mRNA is expressed in the CNS of several mammalian species (Kojima et al., 1999; Wortley et al., 2004; Turek et al., 2005; Gahete et al., 2010). The question of whether a product of this gene, other than ghrelin, is formed is raised in the Discussion.

Despite the uncertainty concerning ghrelin production by central neurons, both localization and functional studies indicate that the ghrelin receptor occurs in neurons in the brain and spinal cord. Receptor localization has been shown by *in situ* hybridization in rats, mice, and lemurs (Guan et al., 1997; Mitchell et al., 2001; Zigman et al., 2006; Andrews et al., 2008, 2009; Ferens et al., 2010a,b). Moreover, ghrelin and other specific agonists for ghrelin receptors have direct actions on neurons in the brain and spinal cord (Nakazato et al., 2001; Cowley et al., 2003; Lin et al., 2004; Shimizu et al., 2006; Fry and Ferguson, 2009; Ferens et al., 2010a).

The current study was undertaken to investigate, using well-characterized antibodies, whether ghrelin occurs at immunohistochemically detectable concentrations in the CNS and whether it can be revealed in nerve terminals that impinge on neurons that are known to express functional ghrelin receptors. We did this in the knowledge that peptide neurotransmitters occur at high concentrations in nerve terminals and with the assumption that lack of peptide immunoreactivity would be evidence of a lack of a transmitter role of the peptide.

EXPERIMENTAL PROCEDURES

Experiments were conducted on male and female Sprague–Dawley rats in the weight range 200–400 g, C57Bl6 mice of either sex (17–25 g) both supplied by the biomedical sciences animal facility at the University of Melbourne, ghrelin knockout mice on a C57Bl6 background (Andrews et al., 2009) from Monash University, and ghrelin receptor reporter mice that are described below. All procedures were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Antibodies

The following anti-ghrelin antibodies were used. 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 des-acylated 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 des-acyl ghrelin; conversely, RY1595 was absorbed by des-acyl ghrelin, but was not absorbed by ghrelin itself (Mizutani et al., 2009). Lack of cross-reactivity was confirmed by peptide-specific enzyme-linked immunosorbent assay (ELISA) (Mizutani et al., 2009). Antibody GO-1 was raised in a rabbit against synthetic human acylated ghrelin coupled through glutaraldehyde to bovine serum albumen (BSA; Patterson et al., 2005). This antibody has been shown to cross-react 100% with human and rat ghrelin, but not with des-acyl ghrelin of either species, or any other known gastropancreatic peptide or hormone (Patterson et al., 2005). Radioimmunoassay of fractions separated by fast protein liquid chromatography of plasma samples confirmed that antibody GO-1 specifically detects acylated ghrelin (Patterson et al., 2005). Antibody GM-2 was raised in a rabbit against synthetic human acylated ghrelin coupled through carbodiimide to BSA. Anti-neuropeptide Y (NPY; E2210) was raised in a sheep against rat NPY1-36 coupled to BSA. Immunoreactivity revealed by this antibody was eliminated by preincubation with NPY (Furness et al., 1985). Anti-enhanced green fluorescent protein (EGFP), raised against the full-length GFP fusion protein (Rockland Immunochemicals, Gilbertsville, PA, USA; 0.25 μg/mL), was used to enhance the localization of EGFP.

Immunohistochemisty

To prepare tissue for immunohistochemistry, rats and mice were anaesthetized 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 saline followed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. After perfusion was completed, the stomach, hypothalamus, medulla oblongata, and lumbo-sacral spinal cord were removed and post-fixed in the same fixative for 4 h at 4 ^{o}C. Fixative was washed out with 6×10 min washes in phosphate buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and then placed in PBS-sucrose (PBS containing 30% sucrose as a cryoprotectant) and stored at 4 °C overnight. The following day, 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 a further 24 h before being embedded in 100% OCT.

For localization using a horseradish peroxidase (HRP) detection system, sections of 12 μm thickness (or in some cases 30 μm for hypothalamus) were cut and collected on SuperFrost Plus slides (Menzel GmbH, Braunschweig, Germany). Slides were allowed to air dry for 1 h before being incubated in 0.7% hydrogen peroxide for 15 min to remove endogenous peroxidase activity. After washing in PBS, samples were blocked in a mixture of 10% fetal bovine serum, 2.5% skim milk, 1% BSA, and 0.05% sodium azide in PBS for 30 min at room temperature (RT). Additional 3×10 min washes were carried out in PBS, and then primary antibodies were applied overnight in humid chambers at 4 °C. For peptide absorptions, the appropriate peptide and primary antibodies were mixed together at the desired concentration and incu-