bated at 4 °C overnight to allow them to conjugate prior to application to tissue. After application of antibodies, sections were washed 3×10 min in PBS and then incubated with MACH4 HRP polymer (Biocare, Concord, CA, USA) for 30 min at RT. After additional 3×5 min PBS washes, staining was visualized by incubating for 5 min at RT with diaminobenzidine solution (DAB; Dako, Glostrup, Denmark). Sections were washed 3×5 min in distilled water, and then dehydrated prior to applying coverslips using a distyrene plasticizer, xylene mix (DPX) as a mountant.

For immunofluorescence, brain sections were processed following a floating section protocol. In summary, 30 μm thick cryostat sections were collected in PBS in 24-well trays (Costar, Corning Inc., Corning, NY, USA). Sections were incubated in blocking solution, consisting of 10% normal horse serum, 10% Roche Blocking Reagent (Roche Diagnostics, Sydney, Australia), and 0.1% Triton-X100 in PBS for 1 h at RT. Subsequently, they were incubated with primary antibodies, diluted in block solution, overnight at 4 °C. Sections were washed 3×10 min in PBS before incubation with a mixture of secondary antibodies: donkey anti-rabbit IgG conjugated with Alexa 488 (Invitrogen, Melbourne, Australia; 1:1000), and donkey anti-mouse IgG conjugated with Alexa 594 (1:500), or donkey anti-sheep IgG conjugated with Alexa 594 (1:500) in blocking solution, overnight at RT. Sections were washed 3×10 min in PBS and mounted on SuperFrost Plus slides (Menzel GmbH) using a Mowiol (polyvinylalcohol 44–88, Sigma-Aldrich, St Louis, MO)/glycerol mixture (Heimer and Taylor, 1974). Washes were performed under a dissecting microscope to locate the sections in the wells and to carefully aspirate the solutions around them.

Immunofluorescence on spinal cord and stomach cryostat sections (12 μm thickness) was performed after collection of the sections on SuperFrost slides, using the solutions and incubation conditions described above for the floating section protocol.

Reporter Mouse

Ghrelin receptor (GHSR) reporter mice were obtained from the Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis (http://www.mmrrc.org/strains/30942/030942.html). The mice are described by the Gene Expression Nervous System Atlas (GENSAT; http://www.gensat.org). They express EGFP under control of the promoter for GHSR. The EGFP reporter gene is inserted immediately upstream of the coding sequence for the receptor gene.

ELISA

Rats and mice were anesthetized by placing them in a chamber containing isoflurane. Once an animal was anesthetized, 500 μl of blood was drawn from the heart and placed into an EDTA-treated tube (BD Medical, North Ryde, Australia). The carotid arteries were then severed. Aprotinin (500 kIU; Sigma-Aldrich, Sydney, Australia) was added to the collected blood, and samples were centrifuged for 15 min at 3000*g* to separate the plasma. HCl was added to a final concentration of 0.05 N, and samples were stored at −20 °C.

Tissues (stomach, hypothalamus, and lumbo-sacral spinal cord) were dissected on ice in PBS, and collected into radioimmunoprecipitation assay (RIPA) buffer (1 mL per 100 mg tissue) containing protease inhibitor cocktail and 50 μM phenylmethylsulfonyl fluoride (PMSF), all from Sigma-Aldrich. Samples were sonicated on ice to lyse tissue and then centrifuged at 12,000*g* for 10 min to remove tissue debris; the supernatant was collected; HCl was added at a final concentration of 0.05 M; and samples were stored at −20 °C. Thawed tissue samples were centrifuged for a further 30 min at 100,000*g* at 4 °C prior to applying to the ELISA assay.

The rat/mouse ghrelin ELISA kit (Chemicon, Melbourne, Australia) was run according to the manufacturer's instructions, with 20 μL of each plasma or tissue sample per well loaded in duplicate or triplicate. Protein concentrations were determined using the Bio-Rad protein assay DC kit (Bio-Rad Laboratories, Gladesville, Australia), and assay values were normalized against total protein concentration to make results comparable.

RESULTS

Immunohistochemistry

Gastric Endocrine Cells and Nerve Cells in the Rat CNS. All antibodies that were used, RY1601, GO1 and GM2 anti-ghrelin antibodies, and RY1595 anti-des-acyl ghrelin, revealed the ghrelin-containing endocrine cells of the gastric mucosa of the rat and mouse. Using conventional immunofluorescence with antibodies at concentrations that were effective in the stomach, no signal could be found in the CNS of rat or mouse (hypothalamus, medulla oblongata, and spinal cord). We therefore undertook further investigation using higher concentrations and the more sensitive MACH4 detection system.

The antibodies were used in serial dilutions using both the MACH4 HRP-conjugated antibody polymer system and a biotin-streptavidin-HRP system. In the stomach, intensity of staining was concentration-dependent, with ideal concentrations for strong endocrine cells staining and weak background binding for the MACH4 method being 1:10,000 for RY1601, 1:3000 for GO1 and GM2, and 1:10,000 for RY1595. Absorption studies were conducted on stomach sections from rat, in which reactions were performed using diluted antiserum and diluted antiserum equilibrated with peptide (ghrelin or des-acyl ghrelin) at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M (Fig. 1). Immunoreactivity with RY1601 and GO1 was progressively reduced with increasing ghrelin concentration, with slight reduction using 10^{-7} M peptide and abolition of staining with 10^{-5} and 10^{-4} M. The background staining was unchanged by equilibration with ghrelin. Immunoreactivity revealed by GM2 was abolished by 10^{-6} M ghrelin, again with no change in background reaction.

Immunoreactivity of gastric endocrine cells observed with the anti-des-acyl ghrelin antibody, RY1595, was reduced by des-acyl ghrelin, 10^{-7} and 10^{-6} M, and abolished by 10^{-5} and 10^{-4} M.

Examination of the rat hypothalamus, medulla oblongata, and spinal cord, using the same concentrations of antibodies that were effective in the stomach, revealed very weak or no staining of nerve cells. Staining was revealed by increasing the antibody concentrations to 1:1000 (RY1601), 1:100 (GO1), 1:100 (GM2), and 1:1000 (RY1595). However, even at these concentrations, no immunoreactive nerve fibers were observed. The nerve cell staining using high antibody concentrations was not affected by pre-equilibration of the antibodies with 10^{-4} M peptide (Fig. 2). Stained cell bodies were scattered throughout the hypothalamus, the most prominent being in and around the arcuate nucleus, in the region of the supraoptic nucleus and lateral to the third ventricle. Cells in the medulla were scattered throughout the reticular formation and were obvious in the raphe and in the regions of nucleus tractus solitarius. In the spinal cord, the most prom-

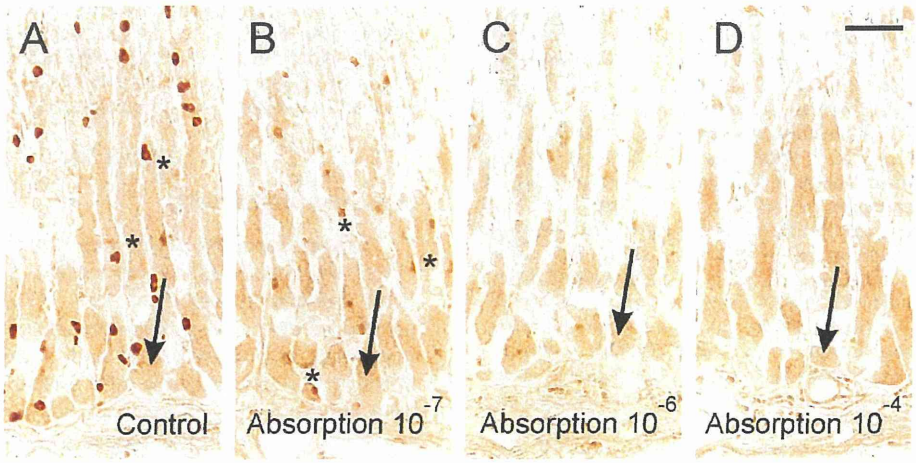

Fig. 1. Location of ghrelin reactive cells in the rat stomach. Gastric endocrine cells (examples adjacent to asterisks) are revealed by anti-ghrelin antibody GO1, 1:3000 (A). After equilibrating the antibodies with ghrelin peptide (10^{-7} M) (B), the endocrine cells were much less intensely stained, and after equilibration with 10^{-6} M peptide, it is very difficult to recognize endocrine cells (C). Equilibration with 10^{-5} or 10^{-4} M (D) abolished all staining of the gastric endocrine cells. Note that the background staining of the gastric glands was unaffected by absorption of antibodies. The arrows indicate the bases of gastric glands. Scale bar: 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

inent staining was of ventral horn cells, although staining was seen in nerve cells of all gray matter regions.

Gastric Endocrine Cells and Nerve Cells in the CNS of the Mouse. In view of the failure to demonstrate specific staining in the rat, we investigated the reactions that could be observed in mice, comparing tissues from six wild-type mice and six ghrelin gene knockout mice. Each of the four antibodies (RY1601, GO1, GM2 anti-ghrelin antibodies, and RY1595 anti-des-acyl ghrelin antibody) revealed endocrine cells in the stomach of wild-type mouse, but not of knockout mice (Fig. 3).

Under the same conditions, a faint staining of nerve cells in the hypothalamus, medulla, and spinal cord was revealed, with a similar distribution to that seen in the rat. This staining appeared no different when sections from wild-type and knockout mice were compared (Fig. 3).

Nerve Fiber Relation with Ghrelin Receptor Expressing Neurons. Peptide-containing nerve fibers are generally revealed very effectively by fluorescence histochemistry in the CNS, and ghrelin-containing nerve terminals might be expected around GHSR-expressing nerve cells. We used double labeling for EGFP of ghrelin receptor reporter mice and ghrelin peptide to investigate this relationship in the hypothalamus and spinal cord. Under conditions in which ghrelin was readily revealed in gastric endocrine cells, no positive nerve terminals could be found in relation to

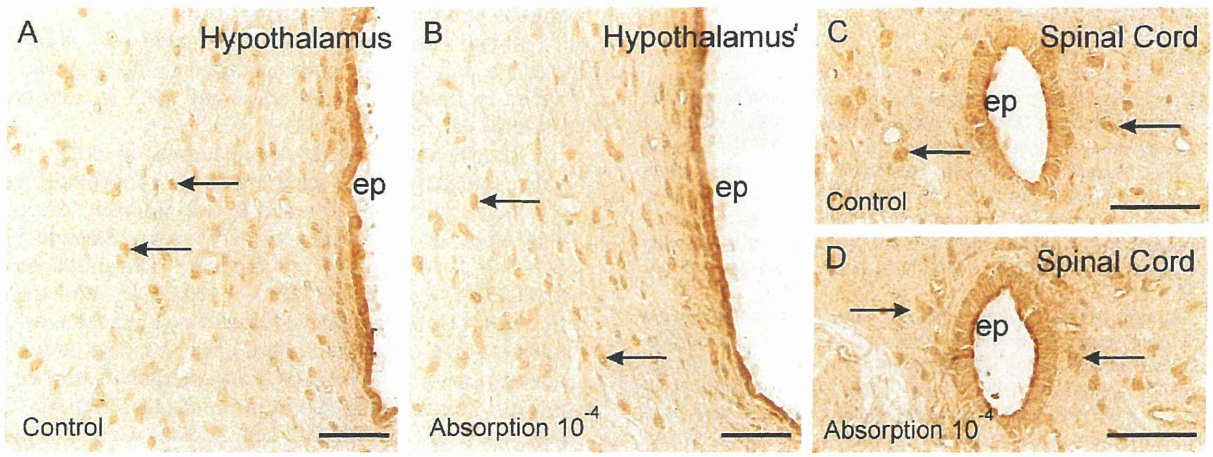

Fig. 2. Staining in the rat hypothalamus and spinal cord using anti-ghrelin GO1 (1:500) with and without equilibrium with ghrelin peptide (10^{-4} M). The appearance of the hypothalamus in the control (A) and using antibody pre-equilibrated with peptide is almost identical. Reaction product is seen in the cell bodies of neurons of the arcuate nucleus (examples at arrows) and in ependymal cells (ep). Likewise, antibody without (C) and with (D) absorption gave similar patterns of staining in the spinal cord. The region around the central canal is shown, and staining can be seen in nerve cells (examples at arrows) and the ependyma (ep.). Scale bars: (A, B), 200 μm; (C, D), 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

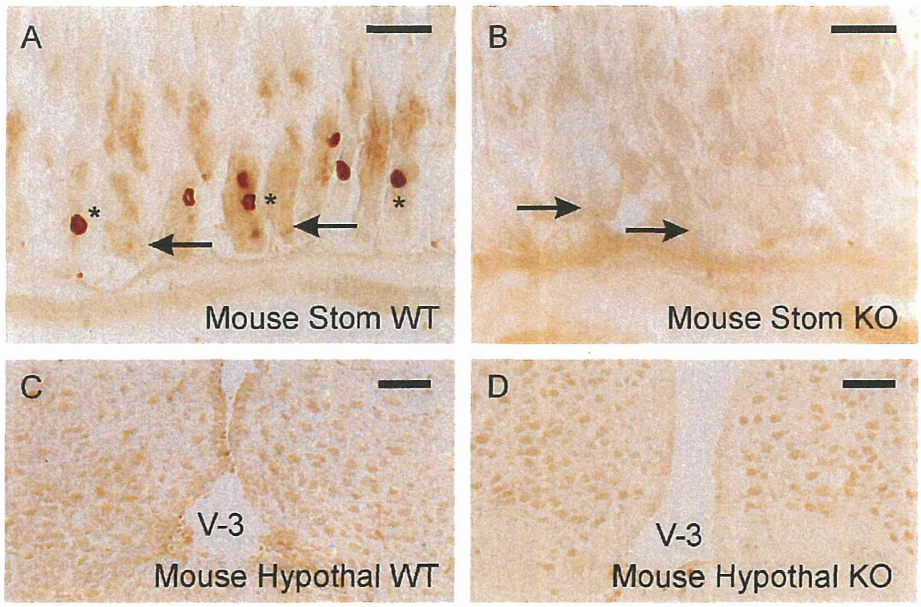

Fig. 3. Immunoreactivity for ghrelin in the stomach and hypothalamus in wild-type and knockout mice, antibody GO1, 1:3000 (stomach) and 1:500 (hypothalamus). In the stomachs of wild-type mice (A), there was strong immunoreactivity in endocrine cells (examples at asterisks), whereas no immunoreactivity could be seen in the knockout animals (B). In the hypothalamus of the wild-type (C) and knockout (D) mice, there was similar staining of nerve cell bodies. V3 = third ventricle. Scale bars: 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

GHSR-expressing neurons, with any of the four antibodies (Fig. 4). As an additional test, we used anti-NPY to verify that terminals could be readily revealed with another anti-peptide antibody. In sections of hypothalamus, medulla oblongata, and spinal cord, anti-NPY antibodies revealed intensely labeled nerve fibers, but no immunoreactivity in nerve terminals was revealed by anti-ghrelin antibodies (Fig. 4D, D′).

ELISA

Assays of extracts from tissues of C57Bl6 wild-type mice revealed high levels of immunoreactive material in the stomach, around 20,000 pg ghrelin per mg protein, which was about 100-fold the level in the extracts of hypothalamus (Fig. 5A). Ghrelin-like activity in extracts from the ghrelin−/− mice was less than 5% of that in the wild-type animals. Plasma ghrelin, which was 300 pg/mL in the wild-type mice, was not detectable in the knockout mice (Fig. 5B). Immunoreactive material in extracts of medulla and spinal cord was in low concentrations compared with stomach, and concentrations in extracts from wild type mice were more than 50% of concentrations in extracts from the ghrelin gene knockout mice (Fig. 5C).

DISCUSSION

In this work, we have used four well-characterized antibodies—three against ghrelin and one against the unacylated form of ghrelin—to investigate whether ghrelin immunoreactivity can be localized in neurons of the rat and mouse CNS. We have concentrated on three regions in which ghrelin might be anticipated to be a neurotransmitter, based on studies that show that functional receptors for ghrelin occur in these regions. These are the hypothalamus, where ghrelin acts to stimulate food intake (Hewson and Dickson, 2000; Nakazato et al., 2001; Andrews et al., 2008); the medulla oblongata, where ghrelin activates pathways controlling sympathetic and parasympathetic nerve pathways (Faulconbridge et al., 2003; Lin et al., 2004; Tsubota et al., 2005); and in the spinal cord, where ghrelin or ghrelin-mimetics act to stimulate defecation, urinary bladder contraction, and vasoconstriction (Shimizu et al., 2006; Ferens et al., 2010a,b). Ghrelin receptors in the hypothalamus and other forebrain regions can be activated by ghrelin after it has crossed the blood–brain barrier (Date et al., 2002; Diano et al., 2006; Chuang et al., 2011), but peripherally administered ghrelin appears to have no access to receptors in the spinal cord (Hirayama et al., 2010). Thus, in this region any endogenous ligand for the ghrelin receptor would be anticipated to originate in the CNS, most likely as a neurotransmitter.

In addition to ghrelin and ghrelin receptor ligands having effects on CNS neurons, it is evident that the ghrelin gene is expressed in the CNS of rat (Kojima et al., 1999), mouse (Wortley et al., 2004), and human (Gahete et al., 2010). Thus it can be predicted that ghrelin may be a transmitter in the CNS. However, we were unable to find any specific immunoreactivity for ghrelin or des-acyl ghrelin in the rat or mouse brain with antibodies that readily revealed ghrelin and des-acyl ghrelin in gastric endocrine cells. In particular, we found no evidence of ghrelin in nerve terminals, where peptide transmitters are present in high amounts and can be readily revealed by immunohistochemistry (Hökfelt et al., 1984; Sasek et al., 1984). As-

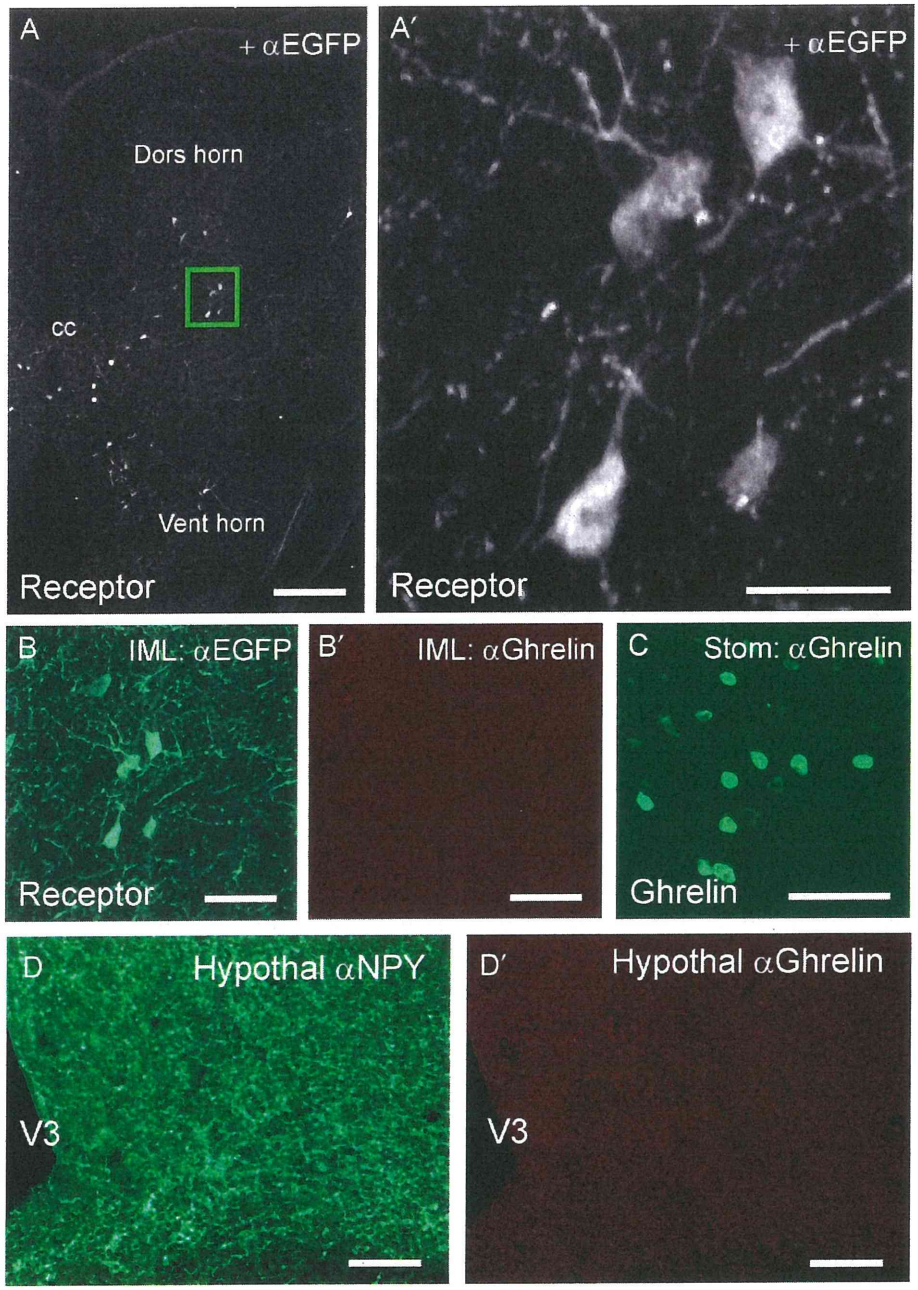

Fig. 4. Ghrelin receptor neurons and the localization of ghrelin peptide in a ghrelin receptor reporter mouse. (A) Locations of neurons that express the ghrelin receptor in a section through the lumbar spinal cord. An area of the intermediolateral cell nucleus (IML), containing ghrelin receptor expressing neurons, is outlined. The neurons have been localized using anti-EGFP. Landmarks labeled are the central canal (cc), the dorsal horn (Dors horn), and the ventral horn (Vent horn). (A′) Higher power view of the area that is marked in A. Receptor expression is seen in nerve cells and the initial parts of their dendrites, and in varicosities within the IML. (B, B′) Ghrelin receptor (B) and ghrelin (B′) immunoreactivity in the same field, using double staining. Note that no ghrelin is revealed in B′. (C) Localization of ghrelin in gastric endocrine cells of the reporter mouse using the identical immunohistochemical method of B, B′. Ghrelin immunoreactivity is strong in the endocrine cells. Results using antibody RY1601 are illustrated; the same observation was made with the other antibodies. (D, D′) Presence of NPY (D) and lack of ghrelin immunoreactivity (D′) in nerve terminals of the rat hypothalamus. The region illustrated is adjacent to the third ventricle (V3), an area that contains neurons with ghrelin receptors. Nerve terminals are revealed with anti-NPY, but none are revealed by anti-ghrelin (antibody GO1, 1:2000). Scale bars: (A), 250 μm; (A′), 25 μm; (B, B′ and C), 250 μm; (D, D′ and C), 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

say for ghrelin using ELISA showed that ghrelin-like material occurs in very low concentrations in extracts of CNS, compared to stomach, despite the fact that the gastric endocrine cells are a small proportion of the cells of the

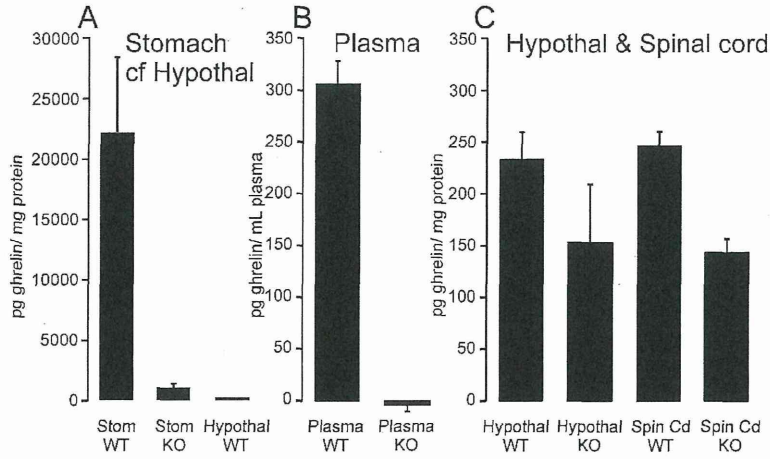

Fig. 5. Concentrations of immunoreactive ghrelin equivalents, determined by ELISA, in tissues and plasma of wild-type and ghrelin−/− mice. (A) Ghrelin-like material occurred in high concentrations in extracts of stomach of wild-type mice, but only in low amounts in the knockouts. Concentrations in the hypothalamus were about one hundredth of gastric concentrations. (B) Ghrelin-like material assayed in normal plasma was eliminated when the ghrelin gene was knocked out. (C) The small amounts of ghrelin-like material in the hypothalamus and spinal cord extracts were not significantly reduced by knock out of the ghrelin gene.

stomach wall. This parallels previous observations in rat (Hosoda et al., 2000) and sheep (Grouselle et al., 2008). In ghrelin knockout mice, the amount of ghrelin-like immunoreactivity in hypothalamic extracts was only partly reduced, suggesting that there may be a ghrelin-like substance in the CNS that is not a product of the ghrelin gene. If such a substance exists, it could have a low affinity for the ELISA assay, and thus it might be present in significant amount. Our result is consistent with the observations of Wortley et al. (2004), who found that ghrelin-like immunoreactivity of CNS neurons was unaffected by ablation of the ghrelin gene. It is possible that other products of the ghrelin gene are present in the wild-type animals. Ghrelin is derived from a longer 117 amino acid translation product, preproghrelin. This is predicted to provide several post-translation products, some of which contain ghrelin or ghrelin fragments (Seim et al., 2011). Moreover, alternative splicing may yield other species of preproghrelin, including partly frame-shifted products. In mouse hypothalamus, a transcript that retains intron 2 is the most abundant product of the ghrelin gene (Kineman et al., 2007). Thus, it is feasible that peptides are produced in the CNS that contain amino acid sequences in common with ghrelin, but that are not authentic ghrelin and that may or may not be acylated. It is also feasible that some anti-ghrelin antibodies react with such related peptides, which may explain some results that have been reported. Variations in published work include differences in places of localization of immunoreactivity and differences in cellular localization, for example different abilities to reveal nerve cells and nerve terminals, as we have summarized in the Introduction. At least some of the ghrelin-mimicking substance in mice is probably not a product of the ghrelin gene because, as elaborated in the Introduction, ghrelin reporter mice do not reveal expression in the CNS, whereas signal was observed in gastric endocrine cells (Wortley et al., 2004; Kageyama et al., 2008; Sakata et al., 2009). Although it might be considered that expression of transgenes is incomplete in the reporter mice, the results with the different reporter constructs all show lack of ghrelin, as do the present immunohistochemical studies. It is possible that authentic 28 amino-acid acylated ghrelin may be produced in some conditions of changed environment, physiological state, or behavior. Relevant to this consideration is the observation that ghrelin mRNA expression fluctuates with a circadian or ultradian rhythm and that the gene contains CLOCK-responsive E-box elements (Turek et al., 2005; Fick et al., 2010). The 24-h variation in ghrelin mRNA is abolished, and absolute levels are substantially reduced in *Clock* mutant mice (Turek et al., 2005). It is feasible that there are also circadian or ultradian influences on the products of ghrelin gene transcripts.

While this study and others suggest that ghrelin is not expressed in significant amounts in neurons in the CNS of rats or mice, the peptide may be present in other species. In particular, an immunohistochemical study of human brain revealed strong labeling of nerve fibers in hypothalamic nuclei, while no reaction was detected in neuronal cell bodies (Menyhért et al., 2006). The antibodies used in that study were raised against the unacylated form of ghrelin and reveal a 13 kDa proghrelin in Western blots (Cowley et al., 2003).

Although agonists of ghrelin receptors have effects within the forebrain, brain stem, and spinal cord, it is only in the forebrain that current data provides indications of roles of endogenous ghrelin on central neurons in addition to its established role in the stimulation of appetite. Peripherally administered ghrelin accesses a number of forebrain regions, including the hippocampus (Diano et al., 2006). Ghrelin enhances learning and memory and increases numbers of synapses per dendritic area in the hippocampus (Diano et al., 2006). Other central effects of increased circulating ghrelin, in physiologically relevant amounts, are anxiolytic and antidepressant actions (Lutter et al., 2008),

and increased expression of food-reward behavior (Perello et al., 2010; Chuang et al., 2011). Chuang et al. (2011) found that hedonic eating behavior induced by peripherally administered ghrelin was lost if ghrelin receptor expression was deleted and was restored when receptor expression was rescued selectively in catecholamine neurons. These data indicate that peripheral ghrelin accesses catecholamine neurons of the ventral tegmental area.

In the spinal cord, ghrelin receptor agonists or direct intrathecal administration of ghrelin activates autonomic preganglionic neurons that raise blood pressure, and increase colo-rectal motility and contractility of the bladder, but peripherally administered ghrelin is not effective (Shimizu et al., 2006; Ferens et al., 2010a,b; Hirayama et al., 2010). In the present work, we have shown that ghrelin-containing nerve endings do not impinge on the autonomic preganglionic neurons. The presence of functional ghrelin receptors on neurons, not innervated by ghrelin-containing synapses, that are in places that are not accessible to circulating or peripherally administered ghrelin suggests the presence of a natural, but unknown, receptor ligand in nerve terminals. In addition, the ghrelin receptor is constitutively active (Holst and Schwartz, 2004), and it is possible that modification of this constitutive activity, perhaps through an endogenous inverse agonist, has a controlling role for autonomic pathways. Thus, there is a need to look for effects of antagonists or inverse agonists on specific functions in order to determine roles for ghrelin receptors in regions of the CNS such as the spinal cord.

CONCLUSIONS

We conclude that there is little or no authentic ghrelin in the CNS of rats and mice housed under laboratory conditions. Ghrelin-like immunoreactivity that has been reported in these species is likely to be due to detection of precursors or substances that have sufficient similarity to ghrelin to cross-react with the antibodies. Neurons that express the ghrelin receptor are not innervated by nerve terminals that contain detectable ghrelin. Thus, the natural endogenous ligand for these receptors is unlikely to be ghrelin.

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Regulation of Gastroduodenal Motility: Acyl Ghrelin, Des-Acyl Ghrelin and Obestatin and Hypothalamic Peptides

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Key Words
Gastroduodenal motility, regulation · Acyl ghrelin · Des-acyl ghrelin · Obestatin · Hypothalamic peptides

Abstract
Real-time measurements for gut motility in conscious rats or mice combined with intracerebroventricular or intravenous injection of peptide agonists or antagonists allow us to understand the regulatory mechanism of gastrointestinal motility. Neuropeptide Y (NPY) in the arcuate nucleus in the hypothalamus stimulates the fasted motility in the duodenum, while urocortin in the paraventricular nucleus inhibits fed and fasted motility in the antrum and duodenum. Acyl ghrelin exerts stimulatory effects on the motility of the antrum and duodenum in both the fed and fasted state of animals. NPY Y2 and Y4 receptors in the brain may mediate the action of acyl ghrelin, and vagal afferent pathways might be involved in this mechanism. Des-acyl ghrelin exerts inhibitory effects on the motility of the antrum but not on the motility of the duodenum in the fasted state of animals. CRF type 2 receptor in the brain may mediate the action of des-acyl ghrelin, and vagal afferent pathways might not be involved in this mechanism. Obestatin exerts inhibitory effects on the motility of the antrum and duodenum in the fed state but not in the fasted state of animals. CRF type 1 and type 2 receptors in the brain may mediate the action of obestatin, and vagal afferent pathways might be partially involved in this mechanism.

Experimental Design for Studying Gastrointestinal Motility and Brain-Gut Axis

Although the regulation of hypothalamic peptides on feeding behavior or energy expenditure has been well established, the regulation on the gastrointestinal (GI) motility has not been fully understood. The detailed studies on the brain-gut axis are somewhat hampered by the methodological difficulties in directly measuring the GI motility in conscious animals with intracerebroventricular (ICV) injection of peptide agonists or antagonists.

We developed a freely moving conscious animal model to measure the GI motility in rats [1] and mice [2], combined with intravenous (IV) or ICV injection. This model permits the real-time measurements of GI motility in

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animals in the physiological fed and fasted states under stimulation of the brain or peripheral administration of peptide agonists or antagonists. In the fasted state, the cyclic change of pressure waves were detected in both antrum and duodenum, including the quiescence period during which relatively low amplitude contractions occur (phase I-like contractions), followed by a grouping of strong contractions (phase III-like contractions). After food intake, such a fasted motor pattern was disrupted and replaced by a fed motor pattern, which consisted of irregular contractions of high frequency. By using this method the effects of brain-gut peptides on the GI motility can be examined.

Neuropeptide Y and Urocortin in the Hypothalamus Regulate the Gastroduodenal Motility

Neuropeptide Y (NPY) is a potent feeding-stimulatory peptide that expresses in the arcuate nucleus of the hypothalamus and projects predominantly to the paraventricular nucleus. Because few previous studies have examined the effects of centrally administered NPY-related peptide on GI motility, we investigated the role of NPY in the control of GI motility using a variety of NPY analogs [1]. ICV injection of Y2 and Y4 receptor agonists induced the phase III-like contractions in the duodenum when given in the fed state of rats; however, Y1 and Y5 receptor agonists had no effects on the motility despite their potent feeding-stimulatory effects [1]. More interestingly, immunoneutralization of NPY by ICV injection of NPY antibody completely blocked the phase III-like contractions in the duodenum [1]. This finding suggests that the fasted motor activity in the upper GI tracts is regulated by brain NPY neurons but not regulated by peripheral mechanism.

CRF and endogenous CRF receptor ligand urocortin are feeding-inhibitory peptides localized at the paraventricular nucleus in the hypothalamus. Urocortin binds both CRF type 1 and 2 receptors but shows a higher affinity for CRF type 2 receptor than type 1 receptor. CRF type 2 receptors are related to the stress-induced alterations of GI functions. ICV or IV injection of urocortin disrupted fasted motor activity in both antrum and duodenum, which were replaced by fed-like motor patterns [3]. When urocortin was given ICV in the fed state, the % motor index (%MI) was decreased in the antrum and increased in the duodenum [3]. Increase the %MI in the duodenum was non-propagated and therefore urocortin suppressed the transit of intestinal contents [3].

Colocalization of Acyl Ghrelin, Des-Acyl Ghrelin and Obestatin in Endocrine Cells in the Stomach

Acyl ghrelin was first isolated from rat and human stomach [4], and the localization of acyl ghrelin in the stomach was studied in various animals by using the specific antibody for acyl ghrelin [4, 5]; however, the localization of des-acyl ghrelin in the stomach has scarcely been examined. Our group developed antibodies specific for acyl ghrelin (anti-rat octanoyl ghrelin (1–15)-cys-KLH serum) and for des-acyl ghrelin (anti-rat des-octanoyl ghrelin (1–15)-cis-KLH serum) and successfully detected the different localization of acyl ghrelin and des-acyl ghrelin in the rat stomach [6].

Both acyl ghrelin- and des-acyl ghrelin-immunoreactive 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 acyl 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 acyl ghrelin was negative (fig. 1a). Acyl ghrelin/des-acyl ghrelin-positive closed-type cells contain obestatin (fig. 1b), on the other hand, des-acyl ghrelin-positive open-type cells contain somatostatin [6].

It is possible that open-type cells may react to luminal stimuli more than closed-type cells. Therefore, we investigated the effects of different intragastric pH levels on the release of acyl ghrelin and that of des-acyl ghrelin from the ex vivo perfused rat stomach [6]. The results showed that the release of acyl 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 [6]. Therefore, the release of des-acyl ghrelin is stimulated after meals by lowering the intragastric pH. The release of acyl ghrelin, on the other hand, is stimulated before meals and the release is regulated by plasma levels of glucose and insulin [7].

Regulation of Acyl Ghrelin on the Gastroduodenal Motility

We examined the effects of acyl ghrelin on the gastroduodenal motility and involvement of hypothalamic peptides mediating this action. Acyl ghrelin stimulated the %MI in the antrum and induced the fasted motor activity in the duodenum when given in the fed state of animals [8]. Acyl ghrelin increased the frequency of phase

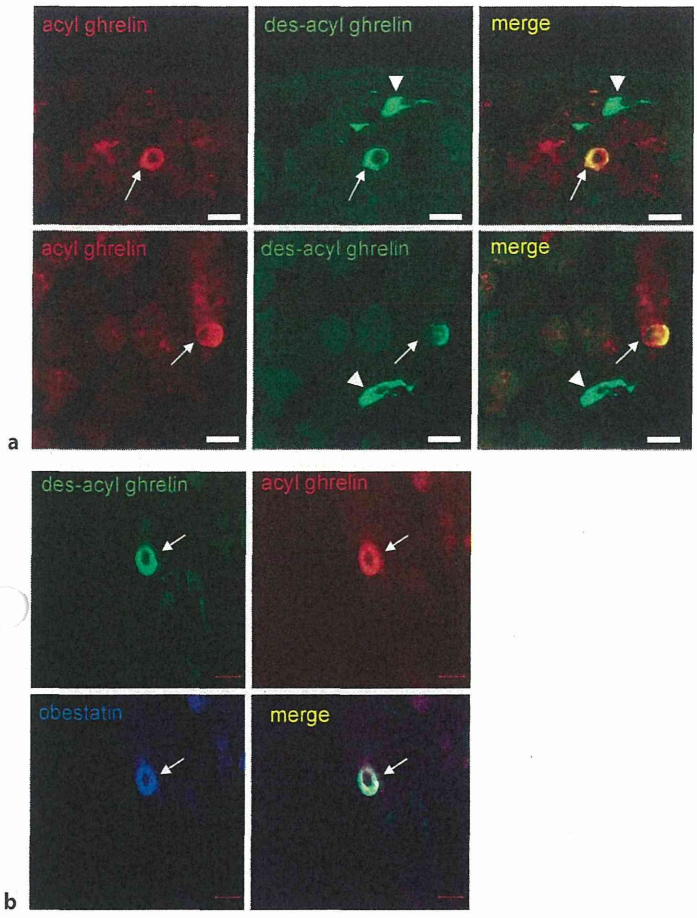

Fig. 1. Localization of acyl ghrelin, des-acyl ghrelin and obestatin in the rat stomach. **a** Immunofluorescence double staining for acyl ghrelin- (red) and des-acyl ghrelin-positive (green) reaction in the antral mucosa of rat stomach. Acyl ghrelin-positive reaction and des-acyl ghrelin-positive reaction are colocalized in closed-type cells (arrows), whereas des-acyl ghrelin-positive reaction is localized in open-type cells (arrowheads). **b** Immunofluorescence triple staining for des-acyl ghrelin (green), acyl ghrelin (red) and obestatin (blue) in the antral mucosa of rat stomach. Three peptides are colocalized in the closed-type cells (arrows). Bars = 10 μm [from 6].

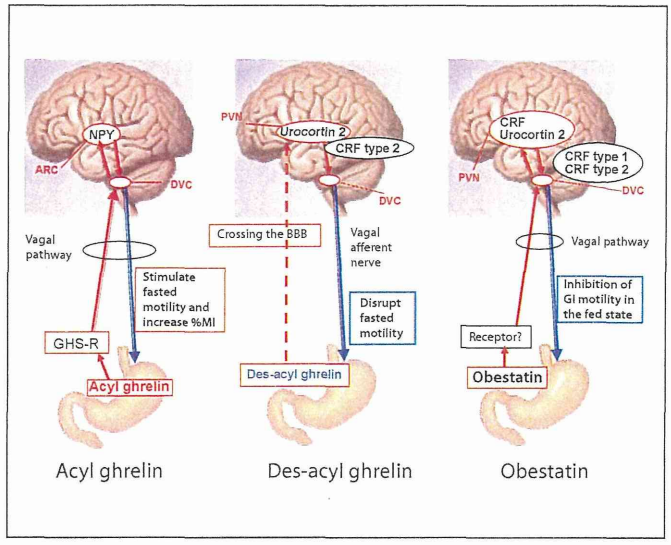

Fig. 2. Different pathways of acyl ghrelin, des-acyl ghrelin and obestatin. Acyl ghrelin released from the endocrine cells in the stomach may act on the ghrelin receptor on vagal afferent nerve terminals, and NPY neurons in the arcuate nucleus may mediate the action of acyl ghrelin to stimulate gastroduodenal motility. Des-acyl ghrelin released from endocrine cells in the stomach may activate urocortin-2 neurons in the paraventricular nucleus by crossing the BBB, and exert inhibitory effects on the antral motility via CRF type 2 receptor in the brain. Obestatin may act on the obestatin receptor on vagal afferent nerve terminals, and CRF and urocortin-2 neurons in the paraventricular nucleus may mediate the action of obestatin to inhibit the gastroduodenal motility via CRF type 1 and type 2 receptors in the brain.

III-like contractions in both antrum and duodenum when given in the fasted state of animals [8]. The effects of IV injection of acyl ghrelin on gastroduodenal motility were blocked by IV injection of GHS-R antagonist [8]. Immunoneutralization of NPY in the brain blocked the stimulatory effects of acyl ghrelin on the gastroduodenal motility [8]. These results indicate that acyl ghrelin released from the stomach may act on the ghrelin receptor on vagal afferent nerve terminals and NPY neurons in the brain may mediate the action of acyl ghrelin on the gastroduodenal motility. Taken together, in normal animals, acyl 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 acyl ghrelin (fig. 2; table 1).

Regulation of Des-Acyl Ghrelin on the Gastroduodenal Motility

Des-acyl ghrelin disrupted fasted motility in the antrum but not in the duodenum; however, des-acyl ghrelin did not alter fed motor activity in both the antrum and duodenum [9]. Capsaicin treatment did not alter the disruptive effect of IV injection of des-acyl ghrelin on fasted motility in the antrum [9]. These results suggest that peripherally administered des-acyl ghrelin may cross the blood-brain barrier (BBB) and act directly on