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Analysis of Brain Nuclei Accessible to Ghrelin Present in the Cerebrospinal Fluid

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Abstract

Ghrelin is a stomach-derived peptide hormone that acts in the brain to regulate many important physiological functions. Ghrelin receptor, named the growth hormone secretagogue receptor (GHSR), is present in many brain areas with or without obvious direct access to ghrelin circulating in the bloodstream. Ghrelin is also present in the cerebrospinal fluid (CSF) but the brain targets of CSF ghrelin are unclear. Here, we studied which brain areas are accessible to ghrelin present in the CSF. For this purpose, we centrally injected mice with fluorescein-labeled ghrelin (F-ghrelin) peptide tracer and then systematically mapped the distribution of F-ghrelin signal through the brain. Our results indicated that centrally injected F-ghrelin labels neurons in most of the brain areas where GHSR is present. Also, we detected F-ghrelin uptake in the ependymal cells of both wild type and GHSR-null mice. We conclude that CSF ghrelin is able to reach most of brain areas expressing GHSR. Also, we propose that the accessibility of CSF ghrelin to the brain parenchyma occurs through the ependymal cells in a GHSR-independent manner.

Keywords

GHSR; ependymal cells; hypothalamus; acyl-ghrelin

INTRODUCTION

Ghrelin is an octanoylated peptide hormone, synthesized mainly by endocrine cells located within the gastric oxyntic mucosa (Kojima et al., 1999). Ghrelin acts in the brain to regulate growth hormone secretion, food intake, energy expenditure, glucose homeostasis, anxiety/depression-related behaviors and stress responses (Kojima and Kangawa, 2005). Two subtypes of ghrelin receptors have been described, the growth hormone secretagogue receptor 1a (GHSR-1a) isoform, which is activated by ghrelin, and the splice variant isoform GHSR-1b, which is a functionally inactive truncated form of GHSR (Howard et al., 1996). Analyses of the distribution of GHSR-1a within the rodent brain using many different techniques have shown that GHSR-1a is present in several brain sites (Guan et al., 1997,

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Mitchell et al., 2001, Zigman et al., 2006, Perello et al., 2012). For instance, GHSR-1a mRNA is highly expressed in brain areas that presumably have direct access to circulating ghrelin, such as the hypothalamic arcuate nucleus (ARC) and the dorsal vagal complex (DVC) of the medulla (Guan et al., 1997, Willeesen et al., 1999, Zigman et al., 2006). However, GHSR-1a is also present in some brain areas distantly situated from circumventricular organs and without immediate access to ghrelin circulating in the bloodstream (Guan et al., 1997, Mitchell et al., 2001, Zigman et al., 2006, Perello et al., 2012, Scott et al., 2012). The physiological relevance of GHSR in many of these brain areas is unclear. Since ghrelin is also present in the cerebrospinal fluid (CSF) (Tritos et al., 2003, Grouselle et al., 2008), we decided to study which brain areas have ghrelin targets accessible to CSF ghrelin. For this purpose, we injected mice with fluorescein-labeled ghrelin (F-ghrelin) peptide tracer via an intra-cerebro-ventricular cannula and then systematically mapped the distribution of F-ghrelin signal through the whole brain. Our results indicated that CSF ghrelin is able to access to most of the areas where GHSR-1a is present. Interestingly, we also detected F-ghrelin uptake in the ependymal cells of both wild type and GHSR-null mice, suggesting that ghrelin can interact with these specialized cells in a GHSR-independent manner.

MATERIALS AND METHODS

Animals

Mice were generated in the animal facility of the IMBICE and housed in a 12-h light/dark cycle with regular chow and water available *ad lib*. In this study, we used adult (8-10 weeks old) C57BL6/J wild type and GHSR-null mice. GHSR-null mice were derived from crosses between heterozygous animals back-crossed for more than 10 generations onto a C57BL6/J genetic background (Zigman et al., 2005). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all efforts were made to minimize suffering. All experimentation received approval from the Institutional Animal Care and Use Committees located in Multidisciplinary Institute of Cell Biology (approval ID 10-0112).

Reagents

Octanoylated ghrelin was purchased from Global Peptide (cat# PI-G-03). F-ghrelin (2741 Da) is an 18-amino acid analog of ghrelin conjugated to fluorescein isothiocyanate through the addition of a lysine at its C terminus ([Dpr octanoyl]³, Lys(fluorescein)¹⁹]-ghrelin(1–19)). Fluorescein-ghrelin was synthesized, as previously detailed (McGirr et al., 2011), and provided by Dr. Leonard Luyt from the Department of Chemistry, The University of Western Ontario, Canada. All the reagents were purchased from Biopack (Argentina), except as indicated.

Experimental procedures

Mice were stereotactically implanted with a single indwelling guide cannula (4 mm long, 22 gauge, Plastics One) into the lateral ventricle (intra-cerebroventricular, ICV). The placement coordinates for the lateral ventricle were: AP: −0.34 mm; L: +1 mm and V: −2.3 mm. A 28-gauge obturator was inserted into each cannula. After surgery, animals were individually housed and allowed to recover for at least 5 days. On the morning of the experimental day, animals were ICV injected with 3 µL of vehicle (artificial CSF) alone or containing 16.6 pmol of ghrelin or F-ghrelin. To determine the specificity of the method, an additional set of mice were ICV injected with 3 µL of vehicle containing 16.6 pmol of F-ghrelin plus 166 pg of ghrelin. All ICV injections were made over 2 min through a 30 gauge needle that extend 0.5 mm below the guide cannula and that was connected by polyethylene tubing to a Hamilton syringe. The needle was left in place for 2 min, following the injection, to prevent

back flow of the injected solution. Between injections and sacrifices, mice were exposed to a pre-weighed amount of rodent's food to determine food intake. Then mice were anesthetized 30 min after treatment and perfused with formalin as previously described (Cabral et al., 2012). Each experimental group contained 4 mice. Brains were removed, post-fixed, immersed in 20% sucrose, and cut coronally at 25 μ m into three equal series on a sliding cryostat. Correct placement of the cannula was confirmed by histological observation at the end of the experiment.

Immunohistochemistry

Brain sections were used for visualization in a fluorescent microscope and either fluorescent or chromogenic immunostaining. For fluorescent immunohistochemistry, brain sections were treated with blocking solution (3% normal donkey serum and 0.25% TritonX in PBS) and incubated with goat anti-fluorescein antibody conjugate to Alexa Fluor 488 (Molecular Probes, cat# A-11096, 1:100) for two days at 4 °C. For chromogenic immunohistochemistry, brain sections were pretreated with 0.5 % H₂O₂, treated with blocking solution and incubated with goat anti-fluorescein antibody (Molecular Probes, cat# A-11095, 1:1,500) for two days at 4 °C. Then, sections were overnight treated with biotinylated donkey anti-goat antibody (Vector Laboratories, cat# BA-1000, 1:1,000) and with Vectastain Elite ABC kit (Vector Laboratories, cat# PK-6200) for 1 h, according to manufacturer's protocols. Then, visible signal was developed with 3-3'-diaminobenzidine (DAB)/Nickel solution, giving a black/purple precipitate. Immunostaining of negative control, which did not show any antiserum immunolabeling, included sequential elimination of either the primary or secondary antibody from the staining procedure. Sections were sequentially mounted on glass slides, and cover slipped with mounting media. Fluorescent images were acquired with a Leica TSC SP5 confocal laser scanning microscope. Fluorescein and Alexa 488 were excited at 488 nm and detected in the 496–532 nm range. Bright-field images were acquired with a Nikon Eclipse 50i and a DS-Ri1 Nikon digital camera. An image editing software program, Adobe Photoshop CS2, was used to adjust contrast and brightness of microphotographs.

Neuroanatomical Analysis

The mouse brain atlas of Paxinos and Franklin (2001) was used to identify coronal brain sections and to describe the mouse brain nuclei (Paxinos and Franklin, 2001). The location of fluorescein-immunoreactive (IR) signal was determined in 1-in-3 series of 25 μ m thick immunostained sections from the level of the olfactory bulbs down to the cervical spinal cord. Semi-qualitative estimates of fluorescein-IR for the different brain sites were made by considering both signal strength and number of labeled cells as compared to signal observed in negative control samples. Series were used to make projection drawings upon which the IR cells were plotted.

Statistical analyses

The food intake data were expressed as the mean $\pm$ SEM and analyzed by two-ways ANOVA test for comparison of different mean values. Significant differences were considered when $P < 0.05$.

RESULTS

Mice ICV-injected with either ghrelin or F-ghrelin significantly increased food intake (199 ± 19 and 203 ± 30 mg, respectively), as compared to vehicle-treated mice (18 ± 11 mg, Fig. 1A). Of note, the magnitude of F-ghrelin- and ghrelin-induced food intake was not statistically different. Initially, coronal brain slices from the three experimental groups were mounted for examination in the fluorescence microscope. Under this condition, strong cell

body-shaped green fluorescent signal was observed exclusively in F-ghrelin-injected mice and no signal was observed in vehicle- or ghrelin-treated mice. Even though samples were covered with commercial mounting media to prevent photobleaching, endogenous F-ghrelin fluorescence lasted only a brief time and disappeared when brain slices were exposed to light. In order to perform a detailed neuroanatomical characterization of F-ghrelin positive sites, immunostaining against fluorescein was performed. We used either a fluorescent anti-fluorescein antibody or further amplification of the signal achieved by a biotinylated secondary antibody followed by streptavidin-peroxidase, the presence of which was visualized with a chromogenic reaction. Both fluorescent and chromogenic immunostainings for fluorescein showed specificity as no signal was observed when primary or secondary antibodies were omitted or when brain samples from vehicle- or ghrelin-treated mice were used. In addition, F-ghrelin presence in the different brain sites showed specificity since fluorescein-IR signal was significantly reduced by an excess of unlabeled ghrelin (Fig. 1B). Both fluorescent and chromogenic immunostainings against fluorescein showed very similar and consistent patterns of signal. However, chromogenic immunostaining was more sensitive due to the extra amplification steps of the reaction. Thus, chromogenic fluorescein-IR signal in brain samples of mice ICV-injected with F-ghrelin was used for the detailed neuroanatomical analysis of CSF ghrelin accessible sites. The observed fluorescein-IR patterns are outlined in Table 1 and visually summarized in representative schematic diagrams contained in Figure 2. The abbreviations used in the figures and throughout the text are denoted in Table 1.

In general, the pattern of fluorescein-IR in F-ghrelin-injected mice was consistent with previous reports of the distribution of GHSR-1a mRNA (Zigman et al., 2006, Perello et al., 2012). The higher number and strongest intensity of fluorescein-IR cells were observed in the hypothalamus. The ARC was one of the areas with the highest amount of fluorescein-IR cells, which were located across the entire rostral-caudal axis and more concentrated in its medial part (Fig. 3A). The VMH also contained abundant fluorescein-IR signal, which were found throughout the entire nuclei (Fig. 3A). The MPOA contained dense fluorescein-IR signal, which also spanned the rostral-caudal expanse of the nucleus (Fig. 3B). The Pe showed fluorescein-IR cells throughout the whole area surrounding the third ventricle (Fig. 3C). Fluorescein-IR signal within the PVH was moderate and enriched in the parvocellular part of the nucleus (Fig. 3D). The DMH also showed fluorescein-IR signal, with most positive cells located in the caudal part of the nucleus (Fig. 3E). The PMV, AHA and SUM also contained a significant amount of fluorescein-IR cells of moderate intensity (Fig. 3F-H). The Sch contained low presence of fluorescein-IR cells.

Many extra-hypothalamic areas contained fluorescein-IR signal. Positive cells were found in Ammon's horns of the hippocampus (Fig. 4A), LSD of the septum (Fig. 4B), PN of the thalamus (Fig. 4C) and SFO (Fig. 4D). In the midbrain, fluorescein-IR cells were observed in the VTA (Fig. 4E) and DR (Fig. 4F). Among these regions, the most abundant number of fluorescein-IR cells was found in the DR, and less abundantly in the VTA. In the pons, fluorescein-IR cells were present in the LoC (Fig. 4G). The LDTg and DTg of the brainstem also showed a significant amount of fluorescein-IR cells (Fig. 4H). In the medulla oblongata, fluorescein-IR cells were observed in all three components of the DVC: the AP, the NTS and the DMV (Fig. 4I). Of note, we found little or inconsistent fluorescein-IR cells in the SN, EW, Amb and MCPC, where GHSR-1a mRNA expression has been previously reported (Zigman et al., 2006, Perello et al., 2012, Scott et al., 2012).

As it can be observed in Figures 3 and 4, fluorescein-IR signal also was consistently detected in the ependymal-like cells lining the ventricular system of the brain (Zigman et al., 2006, Perello et al., 2012). Fluorescein-IR cells were observed in the ependymal-like cells of the third ventricle, lateral ventricle, fourth ventricle, aqueduct of Sylvius and central canal. In

the bottom part of the third ventricle, most of ependymal cells lining the floor of the infundibular recess, named tanycytes, were fluorescein-IR (Fig. 5A). Within tanycytes, fluorescein-IR signal was found in the cell body as well as in their cellular processes that extend deep into the median eminence (ME) (Fig. 5A). It has been recently shown that tanycytic-like cells are also present in other circumventricular organs (Langlet et al., 2013b); however, fluorescein-IR signal in cellular processes was exclusively found at the ME level in our experimental conditions. In contrast, distribution of fluorescein-IR signal within the rest of the ependymal layer, including those of the upper part of the third ventricle, showed a patchy profile with positive signal present exclusively in the cell body of some, but not all, ependymal cells (Fig. 5B-D). Fluorescein-IR signal in the ependymal layer was specific as it was significantly reduced by an excess of unlabeled ghrelin (Fig. 6). Interestingly, fluorescein-IR signal was detected exclusively in the ependymal-like cells lining the ventricular system of the brain of GHSR-null mice ICV-injected with F-ghrelin (Fig. 6).

DISCUSSION

The current study provides the first characterization of brain nuclei accessible to ghrelin present in the CSF. For this purpose, we extensively mapped the brain distribution of F-ghrelin in mice ICV injected with this tracer. Our results indicated that CSF ghrelin is able to reach most of the forebrain, midbrain and hindbrain areas where GHSR-1a gene expression has been reported (Zigman et al., 2006, Perello et al., 2012). Interestingly, we also detected F-ghrelin uptake in the ependymal cells of the ventricular system of the brain, where GHSR-1a is presumably not present.

Here we show that F-ghrelin is an excellent fluorescent ghrelin analog to imaging ghrelin binding sites. This peptide tracer is an 18-amino acid analog of ghrelin conjugated to a fluorescent molecule at its C terminus (McGirr et al., 2011). Of note, the receptor-binding region of ghrelin resides within the first five N-terminal amino-acids, which are sufficient for activation of GHSR-1a (Bednarek et al., 2000). Thus, modifications to the C terminus preserve the bioactive side chain of ghrelin. McGirr and colleagues have shown that F-ghrelin behaves similarly to native full-length ghrelin in terms of serum stability, receptor binding and agonist activity (McGirr et al., 2011). Here, we expanded the characterization of this tracer and showed that it increases food intake in the same extent as ghrelin, suggesting that F-ghrelin is fully bioactive *in vivo*. In addition, we showed that F-ghrelin fails to label brain nuclei of GHSR-null mice, confirming that this molecule specifically binds to GHSR. Of note, fluorescence lifetime of F-ghrelin was short in our experimental conditions, which is expected for this compound (Saylor, 1995). Thus, we used anti-fluorescein antibodies in order to stabilize and also increase the sensitivity and intensity of the signal. Overall, this experimental strategy allowed us to fully characterize the brain nuclei accessible CSF ghrelin.

The pattern of F-ghrelin labeling was in general agreement with previous studies using *in situ* hybridization immunohistochemistry for visualization of GHSR-1a mRNA in the mouse brain (Zigman et al., 2006). The only nucleus in which we found fluorescein-IR signal that had not previously been recognized as containing GHSR-1a is the LoC (see below). Also, we observed fluorescein-IR in the SFO, which was not listed as a GHSR-expressing brain region by Zigman and colleagues (Zigman et al., 2006, Perello et al., 2012). However, Pulman et al. have shown that SFO neurons express GHSR and dose-dependently respond to ghrelin in rats (Pulman et al., 2006). In contrast, we observed low or no fluorescein-IR signal in some brain regions that had previously been recognized as containing GHSR-1a mRNA (Zigman et al., 2006, Perello et al., 2012, Scott et al., 2012). These brain nuclei include the SCh in the hypothalamus as well as the Amb, EW, MCPC and SN. Thus, these brain regions appear to be inaccessible to CSF ghrelin. Alternatively, GHSR-1a mRNA may

be expressed in low levels in these nuclei and, therefore, only detected by a sensitive method such as the radioactive *in situ* hybridization immunohistochemistry. It is also possible that GHSR-1a mRNA is expressed in these brain areas but the resulting mRNA is not translated to protein, or that GHSR-1a protein is transported to the synaptic terminals of these neurons and not located in their cell body. In fact, a presynaptic mode of action of ghrelin in some brain areas has been suggested (Cowley et al., 2003, Wiedmer et al., 2011). Despite minor inconsistencies, our data provide strong evidence that CSF ghrelin is able to interact *in vivo* with the majority of the areas where GHSR-1a is expressed.

To our knowledge, this is the first *in vivo* analysis of brain nuclei accessible to CSF ghrelin. Previously, incubation of biotin-labeled ghrelin on coronal brain slices has been used to determine ghrelin binding sites. These *in vitro* binding studies have shown that ghrelin binds to several hypothalamic areas (including the ARC, DMH, AHA, MPOA and PVH), the VTA and the CA regions of the hippocampus (Cowley et al., 2003, Abizaid et al., 2006, Diano et al., 2006, Wiedmer et al., 2011). Similar pattern of ghrelin binding sites within the hypothalamus was detected by performing *in vitro* labeling of brain sections with either [¹²⁵I-*his*⁹]-ghrelin followed by quantitative autoradiography or native ghrelin followed by immunostaining against ghrelin (Harrold et al., 2008, Cabral et al., 2012). Here, we have not only confirmed previous studies showing the presence of ghrelin binding sites in these brain areas but also shown that CSF ghrelin is able to access them. Current observations are in agreement with previous reports showing that the marker of cellular activation c-fos can be detected in most of these brain areas after ICV-injections of ghrelin (Nakazato et al., 2001). Of note, a recent study have used *in vivo* multi-photon microscopy together with peripheral administration of fluorescently labeled ghrelin to show that circulating ghrelin is rapidly transported through the fenestrated capillaries of the ME and binds to neurons of the ARC (Schaeffer et al., 2013). Thus, at least some brain ghrelin binding sites seems to be accessible to both bloodstream and CSF ghrelin.

In order to reach its brain targets, CSF ghrelin needs to cross the CSF–brain barrier constituted by the ependymal cells lining the ventricles. Here we report that fluorescein-IR signal is found in ependymal cells, including tanycytes lining the floor of the third ventricle, which show no detectable GHSR-1a mRNA (Zigman et al., 2006, Perello et al., 2012). Additionally, we found F-ghrelin signal in ependymal cells of GHSR-deficient mice. These findings can be interpreted as ependymal cells accumulate and transport CSF ghrelin to the brain in a GHSR-independent manner. The mechanisms transporting CSF molecules through the ependymal layer are currently unclear; however, tracing studies indicate that they are very selective. For instance, ICV-injected wheat-germ lectin is accumulated in ependymal cells, including tanycytes, and not transported to brain parenchyma (Cheunsuang et al., 2006). In contrast, ICV-injections of a FluoroGold equivalent or horseradish peroxidase result in uptake of the tracers in ependymal cells but tanycytes lining the floor of the infundibular recess (Cheunsuang et al., 2006, Rodriguez et al., 2010). Additionally, ICV-injected cholera toxin subunit B or Evans blue are accumulated by ependymal cells and also access to neurons of the raphe nuclei or the ARC, respectively (Mikkelsen et al., 1997, Mullier et al., 2010). Studies using ICV administration of digoxigenin-labeled hormones have shown that leptin is accumulated by ependymal cells and serotonergic neurons in the raphe nuclei, while insulin and insulin-like growth factor-I are accumulated in tanycytes and cells of the ARC and ME (Fernandez-Galaz et al., 1996, Fernandez-Galaz et al., 2002). Thus, CSF ghrelin appears to have a higher accessibility, as compared to other hormones, and quickly access to different brain areas. Interestingly, it has been shown that noradrenergic neurons of the avian LoC accumulate CSF components, which are initially endocytosed and transcytosed by ependymal cells (Feng et al., 2011). This uptake of molecules from the CSF is highly selective as internalizes nerve growth factor and urotensin-1, but not other neurotrophic factors (Feng et al., 2011). Here we report that

fluorescein-IR cells are present in the LoC, which presumably lacks GHSR-1a (Zigman et al., 2006, Perello et al., 2012). Thus, similar trafficking pathways as shown in avian LoC may take place for CSF ghrelin in mice. Future studies will be needed to elucidate the mechanism by which ependymal cells internalize and traffic CSF ghrelin.

CSF ghrelin concentrations are subjected to a dynamic regulation and respond to energy balance status (Tritos et al., 2003, Grouselle et al., 2008). However, the origin of CSF ghrelin is currently unclear. It has been suggested that peripheral ghrelin can enter to the CSF by crossing the blood–brain barrier. In support of this possibility, it has been shown that CSF ghrelin levels correlates with plasma ghrelin levels and that CSF ghrelin increases around 30 min after a peripheral injection of ghrelin in a ewe model (Grouselle et al., 2008). Also, CSF ghrelin concentration shows a pulsatile profile, and around half of CSF ghrelin peaks are preceded by a peak of plasma ghrelin (Grouselle et al., 2008). Experimental studies assessing the permeability of the blood–brain barrier to ghrelin in mice have showed that human ghrelin can be transported from brain-to-blood and from blood to-brain by saturable systems (Banks et al., 2002). Mouse ghrelin, which differs from human ghrelin by two amino acids, crosses the mouse blood-brain barrier predominantly in the brain-to-blood direction (Banks et al., 2002). In order to reach the CSF, peripheral ghrelin could cross the blood–CSF barrier in the choroids plexus and/or to be transported to the ventricles from the brain, after going across the blood–brain barrier at the cerebral endothelium (Banks et al., 2002). A central origin of CSF ghrelin in mice appears to be unlikely since most studies have failed to confirm the presence of ghrelin-producing cells in the mouse brain (Sakata et al., 2009, Furness et al., 2011). However, future studies will be required in order to determine if significant amounts of brain-derived ghrelin can reach the CSF under particular physiological conditions, such as food deprivation, when hypothalamic ghrelin production is presumably increased (Gahete et al., 2010, Kageyama et al., 2010). In this regard, current data allow us to hypothesize that central ghrelin-IR cells detected in some studies could due to ghrelin uptake, rather than CNS-production (Kojima et al., 1999, Lu et al., 2002, Cowley et al., 2003, Mondal et al., 2005). Of note, studies of CSF ghrelin are difficult to address in mice since the available assays for ghrelin require large amounts of fluid. Thus, future studies employing different experimental systems will be required to get insights about the origin of CSF ghrelin.

Regardless of its origin, the observation that CSF ghrelin can access to most ghrelin responsive brain nuclei may have important physiological implications. In particular, it could offer a role for brain nuclei expressing GHSR-1a without immediate access to circulating ghrelin. In terms of appetite, ghrelin not only increases food intake itself but also regulates hedonic aspects of eating (Perello et al., 2010, Dickson et al., 2011, Perello and Zigman, 2012). Thus, it can be hypothesized that brain areas responsive to plasma and/or CSF ghrelin may have different but complementary roles. Peripheral administration of ghrelin stimulates feeding almost instantly, suggesting that this effect is mainly mediated by brain areas accessible to circulating hormone, such as the ARC and the DVC (Cummings, 2006). In addition, plasma ghrelin may be able to act on VTA neurons, which strongly drives motivation for food (Abizaid et al., 2006, Chuang et al., 2011). However, some aspects of hedonic eating behaviors would also involve actions of ghrelin on hippocampal or meso-limbic regions that are not easily accessible to peripheral ghrelin but would be reachable by CSF ghrelin. Similarly, ghrelin effects on more complex functions such as stress responses, learning, sleep duration, memory consolidation, anxiety and depression symptoms may be also modulated by hormone present in the CSF (Carlini et al., 2002, Diano et al., 2006, Lutter et al., 2008, Cabral et al., 2012). Recent evidences indicate that the permeability of the blood-CSF barrier in the hypothalamus undergoes to dynamic changes in response to the metabolic status (Langlet et al., 2013a). Thus, if the accessibility of CSF

ghrelin to brain nuclei can be modulated by different physiological conditions is an interesting possibility that deserves to be specifically investigated.

In summary, we conclude that CSF ghrelin is able to reach most of GHSR-expressing brain areas, and that the accessibility of CSF ghrelin to the brain parenchyma presumably occurs through the ependymal cells in GHSR-independent manner. Future studies will be needed to elucidate the molecular mechanisms mediating the transcellular trafficking of ghrelin through ependymal layer as well as the physiological consequences of the high accessibility of CSF ghrelin to the brain.

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- Cerebrospinal fluid ghrelin reaches most brain areas expressing ghrelin receptors.
- Ependymal cells are able to uptake ghrelin present in the cerebrospinal fluid.
- Ghrelin uptake in ependymal cells occurs in a ghrelin receptor-independent manner.

Figure 1. F-ghrelin increases food intake and specifically binds to brain areas in mice
 Panel A shows 30 min food intake in mice ICV injected with vehicle alone or containing 16.6 pmol of ghrelin or F-ghrelin. Data represent the mean $\pm$ SEM. **, $p < 0.01$. Panel B shows confocal representative photomicrographs of fluorescein-IR signal in ARC coronal sections of mice that were ICV injected with 16.6 pmol of F-ghrelin alone (left) or plus 166 pmol of ghrelin (right). Insert shows high magnification of the area marked in the low magnification image. Arrows point fluorescein-IR cells. Scale bars, 100 μ m (low magnification), 10 μ m (high magnification).

Figure 2. Profile of fluorescein-IR cells in mice ICV-injected with F-ghrelin

A series of line drawings illustrating the fluorescein-IR sites in mice ICV-injected with F-ghrelin. Sections are arranged in a rostral-to-caudal manner. Panels A to L illustrate the following bregma levels: 1.10, 0.26, -0.34, -0.58, -1.82, -2.30, -2.80, -3.52, -4.60, -5.20, -5.80, -7.48 mm, respectively. Each dot represents a positive cell.

Figure 3. Fluorescein-IR cells are found in several hypothalamic nuclei of F-ghrelin-injected mice

Panels show a series of representative microphotographs of coronal sections of different hypothalamic regions after chromogenic immunohistochemistry for fluorescein (black/purple signal). Panels A to G show the ARC and VMH (A, bregma -1.82 mm), MPOA (B, bregma 0.26 mm), Pe (C, bregma -0.34 mm), PVH (D, bregma -0.58 mm), DMH (E, bregma -1.82 mm), PMV (F, bregma -2.30 mm), AHA (G, bregma -0.58 mm) and SUM (H, bregma -2.80 mm) regions. Scale bar: $100\ \mu\text{m}$.

Figure 4. Fluorescein-IR cells are found in several extra-hypothalamic nuclei of F-ghrelin-injected mice

Panels show a series of representative microphotographs of coronal sections of different extrahypothalamic regions after chromogenic immunohistochemistry for fluorescein (black/purple signal). Panels A to G show the Ammon's horns of the hippocampus (A, bregma -1.82 mm), LSD of the septum (B, bregma 1.10 mm), PN of the thalamus (C, bregma -0.58 mm), SFO (D, bregma -0.58 mm), VTA (E, bregma -3.52 mm), DR (F, bregma -4.60 mm) and LoC (G, bregma -5.80 mm). Panel H shows the LDTg and DTg (bregma -5.20 mm), and panel I shows the AP, NTS and DMV (bregma -7.48 mm). Scale bar: $100\ \mu\text{m}$.

Figure 5. Fluorescein-IR signal is observed in the ependymal cells of F-ghrelin-injected mice Panels show a series of representative microphotographs of coronal sections of different regions of the ventricular system of the brain after immunohistochemistry for fluorescein (black/purple signal). Panels A to D show the ependymal cells lining the bottom (A) or upper (B) part of the third ventricle, the aqueduct of Sylvius (C) and the central canal (D). Arrowheads point fluorescein-IR cellular processes and arrows point fluorescein-IR cell bodies. Scale bar: 20 μ m.

Figure 6. Fluorescein-IR signal in the ependymal layer of F-ghrelin-injected mice is specific and independent of GHSR expression

Left (A and D) and center (B and E) panels show representative photomicrographs of fluorescein-IR signal in ependymal layer of wild-type mice ICV treated with F-ghrelin alone or with a combination of F-ghrelin and ghrelin, respectively. Right (C and F) panels show representative photomicrographs of fluorescein-IR signal in ependymal layer of GHSR-null mice ICV treated with F-ghrelin. Upper (A-C) and bottom (D-F) horizontal panels show the upper and bottom part of the third ventricle, respectively. Scale bars, 100 μ m.

TABLE 1

Relative density of fluorescein-IR in the central nervous system of F-ghrelin injected mice¹

Hippocampus and septum	
Ammon's horn, CA1	+
Ammon's horn, CA2	+
Ammon's horn, CA3	+
Lateral septal nucleus -LS	+
Thalamus	
Paraventricular thalamic nucleus -PV	++
Hypothalamus	
Anterior hypothalamic area -LHA	+
Arcuate hypothalamic nucleus -ARC	+++
Dorsomedial nucleus -DMH	+
Paraventricular nucleus -PVH	++
Periventricular hypothalamic nucleus -Pe	++
Premamillary nucleus, ventral -PMV	++
Supramammillary nucleus -SuM	+
Suprachiasmatic nucleus -SCH	+/-
Ventromedial nucleus -VMH	+++
Ventromedial preoptic nucleus -VMPO	+++
Subfornical organ -SFO	++
Midbrain, pons, and medulla oblongata	
Area postrema -AP	++
Dorsal motor nucleus of the vagus -DMNV	+
Dorsal raphe nucleus -DR	++
Dorsal tegmental nucleus -DTg	++
Edinger Westphal nucleus -EW	+/-
Laterodorsal tegmental nucleus -LDTg	++
Locus coeruleus -LoC	++
Magnocellular nucleus of the posterior commissure -MCPC	+/-
Nucleus ambiguus -Amb	+/-
Nucleus of the solitary tract -NTS	++
Substantia nigra -SN	+/-
Ventral tegmental area -VTA	+

¹ Qualitative estimates of fluorescein-IR were made by considering both signal strength and the number of labeled cells: +++, high density; ++, moderate density; +, low density; +/-, inconsistent visualization.