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# ANALYSIS OF BRAIN NUCLEI ACCESSIBLE TO GHRELIN PRESENT IN THE CEREBROSPINAL FLUID

**A. CABRAL, G. FERNANDEZ AND M. PERELLO***

*Laboratory of Neurophysiology of the Multidisciplinary Institute of Cell Biology, Argentine Research Council (CONICET) and Scientific Research Commission, La Plata, Province of Buenos Aires (CIC-PBA), Argentina*

**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.** **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: 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; 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; Willesen 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-cerebroventricular (ICV) 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 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.

## EXPERIMENTAL PROCEDURES

### 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

*Corresponding author. Address: Calle 526 S/N entre 10 y 11-PO Box 403, La Plata, Buenos Aires 1900, Argentina. Tel: +54-221-4210112.

E-mail addresses: marioperello@yahoo.com, mperello@imbice.gov.ar (M. Perello).

*Abbreviations:* AHA, anterior hypothalamic area; Amb, nucleus ambiguous; AP, antero-posterior; ARC, arcuate nucleus; CA, Ammon's horn; CSF, cerebrospinal fluid; DMH, dorsomedial nucleus; DMV, dorsal motor nucleus of the vagus; DR, dorsal raphe nucleus; DVC, dorsal vagal complex; EW, Edinger Westphal nucleus; GHSR, growth hormone secretagogue receptor; ICV, intra-cerebro-ventricular; IR, immunoreactive; LoC, locus coeruleus; LSD, lateral septal nucleus, dorsal part; MCPC, magnocellular nucleus of the posterior commissure; ME, median eminence; MPOA, medial preoptic area; PVH, paraventricular nucleus; SCh, suprachiasmatic nucleus; SFO, subfornical organ; SN, substantia nigra; VTA, ventral tegmental area.

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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 protocols

Mice were stereotaxically implanted with a single indwelling guide cannula (4 mm long, 22 gauge, Plastics One, Inc. Roanoke, VA, USA) into the lateral ventricle (ICV). The placement coordinates for the lateral ventricle were: antero-posterior (AP): −0.34 mm; lateral: +1 mm and ventral: −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 four mice. Brains were removed, post-fixed, immersed in 20% sucrose, and cut coronally at 25 μm 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 cromogenic immunostaining. For fluorescent immunohistochemistry, brain sections were treated with blocking solution (3% normal donkey serum and 0.25% TritonX in phosphate buffered saline (PBS)) and incubated with goat anti-fluorescein antibody conjugate to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA, cat# A-11096, 1:100) for 2 days at 4 °C. For cromogenic immunohistochemistry, brain sections were pretreated with 0.5% $H_2O_2$, treated with blocking solution and incubated with goat anti-fluorescein antibody (Molecular Probes, cat# A-11095, 1:1,500) for 2 days at 4 °C. Then, sections were overnight treated with biotinylated donkey anti-goat antibody (Vector Laboratories, Burlingame, CA, USA, cat# BA-1000, 1:1,000) and with Vectastain Elite ABC kit (Vector Laboratories, cat# PK-6200) for 1 h, according to the 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-μm-thick immuno-stained 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 ± SEM and analyzed by a two-way analysis of variance (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

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