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Distribution of glucagon-like peptide 1-immunopositive neurons in human caudal medulla

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Abstract

In rodents, glucagon-like peptide-1 (GLP-1)-positive neurons within the caudal medulla respond to a broad array of interoceptive signals that suppress food intake and drive the hypothalamic–pituitary–adrenal stress axis. The collective results of experiments utilizing cFos to identify activated neurons in rats and mice indicate that GLP-1 neurons are consistently activated by stimuli that present actual or anticipated threats to bodily homeostasis. The distribution of GLP-1-positive neurons in the human brain is unreported. The present study identified GLP-1-positive neurons and mapped their distribution within the caudal medulla in two adult human subjects (one female, one male). The goal of the study was to obtain structural evidence with which to challenge the general hypothesis that functions ascribed to GLP-1 neurons in rodent species may reflect parallel functions that exist in humans. In both human subjects, GLP-1-immunopositive neurons were located within the dorsal medullary region containing the caudal (visceral) nucleus of the solitary tract and in the nearby medullary reticular formation, similar to the distribution of GLP-1 neurons in rats, mice, and Old World monkeys. Quantitative analysis indicates the presence of approximately 6.5–9.3 K GLP-1-positive neurons bilaterally within the human caudal medulla. It will be important in future studies to map the distribution of GLP-1-positive fibers and terminals within higher regions of the human brain, to improve our understanding of how central GLP-1 signaling pathways might influence stress responsiveness, energy balance, and other physiological and behavioral functions.

Keywords

Preproglucagon; Nucleus of the solitary tract; Medullary reticular formation

Introduction

Within rodent and primate brain, preproglucagon (PPG) gene expression and peptide products have been localized to neurons within the olfactory bulb, the caudal nucleus of the solitary tract, and the caudal medullary reticular formation (Zhang et al. 2009; Merchenthaler et al. 1999; Larsen et al. 1997; Vrang and Grove 2011; Han et al. 1986;

Vrang and Larsen 2010). After posttranslational processing by prohormone convertases PC1/3 and PC2, PPG-expressing neurons generate several distinct protein cleavage products, including glucagon-like peptide-1 (GLP-1), GLP-2, glicentin, intervening peptide-2, and oxyntomodulin (Schafer et al. 1993; Vrang and Larsen 2010; Baggio and Drucker 2007). The potential central signaling functions of the latter three cleavage products are not yet clear. However, the first two products (i.e., GLP-1 and GLP-2) are generated in the same caudal brainstem neurons and transported anterogradely into axon terminals that make asymmetric (i.e., excitatory-type) synaptic contacts with central neural targets in the hypothalamus (Sarkar et al. 2003) and other brain regions. PPG-expressing neurons within the olfactory bulb (Merchenthaler et al. 1999) appear to be interneurons with very short axons (Vrang and Larsen 2010), since GLP-1-positive fibers are absent within brain regions that receive axonal input from the olfactory bulb (i.e., anterior olfactory nucleus, cortical nuclei of amygdala, piriform cortex, olfactory tubercle, lateral entorhinal cortex, and agranular insula; *unpublished observations*). Thus, GLP-1/2-positive fibers and terminals visualized in the thalamus, hypothalamus, limbic forebrain, and other brain regions (Vrang et al. 2007; Vrang and Larsen 2010; Renner et al. 2012; Rinaman 2010; Gu et al. 2013) presumably originate from PPG-expressing, GLP-1-positive neurons within the caudal medulla. Indeed, caudal brainstem GLP-1-positive neurons are retrogradely labeled in rats after tracer deposits into the hypothalamus, limbic forebrain, and other CNS regions that are rich in GLP-1-positive fibers and terminals (Dossat et al. 2011; Vrang et al. 2007; Alhadeff et al. 2012; Rinaman 1999).

In rodent species, GLP-1-positive neurons within the caudal medulla respond to a broad array of interoceptive signals that can suppress food intake and also drive the hypothalamic–pituitary–adrenal (HPA) neuroendocrine stress axis, including hormonal, gastrointestinal, cardiovascular, and inflammatory signals (Gaykema et al. 2009; Rinaman 1999; Vrang et al. 2003; Maniscalco et al. 2013). The collective results of experiments utilizing cFos to identify activated neurons in rats and mice indicate that GLP-1 neurons are consistently activated by stimuli that present actual or anticipated threats to bodily homeostasis (Maniscalco et al. 2013; Rinaman 1999). GLP-1-containing neural circuits have been most extensively described in rodent species, but a very similar central distribution of GLP-2-immunopositive neurons and fibers has been reported in a non-human primate (i.e., *Macaca mulatta*, an Old World monkey) (Vrang and Grove 2011). The presence of GLP-1-receptor mRNA and protein has been documented within the human brain (Alvarez et al. 2005). GLP-1-positive neurons were described within the region of the dorsal motor vagal nucleus in human brain (Drucker and Asa 1988), although specific neural immunolabeling in that report is difficult to discern [see Fig. 4 in (Drucker and Asa 1988)]. Finally, one review article included a photomicrograph of GLP-2-immunopositive fibers in human hypothalamus [see Fig. 3A in (Vrang and Larsen 2010)]. However, the central distribution of GLP-1-immunopositive neurons within the human caudal medulla is unreported. The present study localized GLP-1-immunopositive neurons and mapped their distribution within the human caudal medulla to test the general hypothesis that functions ascribed to central GLP-1 neurons in rodent species may reflect functions that also exist in humans.

Materials and methods

Brain samples were obtained through the tissue bank maintained by the University of Pittsburgh Conte Center for the Neuroscience of Mental Disorders, Western Psychiatric Institute and Clinic Translational Neuroscience Program (Pittsburgh, PA). Both human caudal brainstem specimens ($n = 2$) were originally obtained during autopsies conducted at the Allegheny County Coroner's Office, after consent had been received from the next-of-kin using a mechanism approved by the Institutional Review Board of the University of Pittsburgh. Subject 1 was a 52-year-old female. Subject 2 was a 39-year-old male. Both subjects were Caucasians, and their cause of death was atherosclerotic cardiovascular disease. After postmortem intervals of 15.4 or 22.6 h, brains were removed from the skull, blocked coronally at 2 cm intervals, immersed in ice-cold 4 % paraformaldehyde in phosphate buffer for 48 h, washed in a graded series of sucrose solutions, and stored in an antifreeze solution containing 30 % sucrose.

For the present study, one tissue block through the caudal medulla was selected from each subject. Tissue blocks were removed from antifreeze solution, and transferred through several changes of fresh 20 % sucrose over 3 days at 4 °C. Coronal sections (40- μ m thick) were cut throughout each full block thickness using a Leica freezing-stage sliding microtome. Floating sections were collected in five serial sets and stored in a cryopreservant solution (Watson et al. 1986) at -20 °C. Every fifth section (i.e., sections spaced by 200 μ m) was rinsed in buffer and mounted on Superfrost Plus coated slides (Fisher Scientific, Pittsburgh, PA). After drying overnight, slide-mounted sections were lightly stained for Nissl substance using cresyl violet. Nissl staining was used to provide anatomical reference points that guided subsequent microscopic analysis of GLP-1 immunolabeling, which was performed in an immediately adjacent series of sections.

GLP-1 immunocytochemistry

Primary and secondary antisera were diluted in 0.1 M phosphate buffer containing 0.3 % Triton X-100 and 1 % normal donkey serum. One set of caudal medullary tissue sections from each subject (i.e., sections spaced by 200 μ m) was removed from cryopreservant, rinsed, pre-treated in buffer containing 0.5 % sodium borohydride for 30 min at room temperature, rinsed for 30 min, and then treated in buffer containing 0.3 % H₂O₂ for 15 min at room temperature. After further rinsing, pre-treated sections were incubated for 1 h at room temperature followed by 16 h at 4 °C in a rabbit polyclonal antiserum raised against synthetic GLP-1 (7-37) (Bachem, T-4363; 1:10,000). Sections then were rinsed, incubated for 2 h at room temperature in biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch), rinsed, incubated for 2 h at room temperature in Elite Vectastain ABC reagents, rinsed, and reacted for 10 min in a solution of 0.1 M Tris buffer containing diaminobenzidine (DAB) and 0.03 % H₂O₂ to produce a brown cytoplasmic immunoperoxidase reaction product.

To compare the distribution of GLP-1 neurons with catecholaminergic neurons, an alternate set of tissue sections from each subject was pre-treated and processed for immunoperoxidase localization of GLP-1 as described above, followed by immunofluorescent localization of tyrosine hydroxylase (TH) in the same sections. First, GLP-1 peroxidase labeling was

achieved using a solution of DAB in 0.1 M sodium acetate buffer (pH 6.0) containing 0.025 % nickel sulfate and 0.015 % H₂O₂ to produce a black reaction product. Next, sections were rinsed in buffer and then incubated overnight at room temperature in monoclonal (mouse) anti-TH (1:2,000; Sigma, #T1299), rinsed, incubated overnight at 4 °C in Cy3-conjugated donkey anti-mouse IgG (1:300; Jackson ImmunoResearch), followed by final rinsing.

Immunolabeled tissue sections were mounted onto Superfrost Plus coated slides (Fisher Scientific), allowed to dry, dehydrated in a graded series of ethanol, defatted in xylene, and coverslipped using Cytoseal 60 (ThermoFisher; Pittsburgh, PA).

The commercially available rabbit polyclonal antiserum used in the present study to localize GLP-1-positive neurons in human tissue has been reported to effectively label GLP-1 neurons in rat brain tissue sections (Maniscalco and Rinaman 2013; Alhadeff et al. 2012). For the present report, the specificity of GLP-1 immunolabeling was tested in two ways: (1) by performing the entire labeling procedure described above after omitting the primary anti-GLP-1 antiserum from the initial incubation solution, and (2) by pre-absorbing the primary antiserum overnight at 4 °C with a tenfold higher concentration of synthetic GLP-1 (7-37) acetate salt (Bachem, H-9560) before tissue incubation. Both procedures eliminated GLP-1 immunolabeling in representative caudal brainstem sections from both human subjects, and also in stored (cryopreserved) perfusion-fixed brainstem sections obtained from an adult male Sprague–Dawley rat.

In tissue samples from both human subjects, the bilateral distribution of all GLP-1-positive neurons was plotted at 20× magnification in consecutive medullary sections spaced by 200 µm. Plots were made using a Nikon light microscope connected to a computerized XY data acquisition system (StereoInvestigator; MBF Bioscience). In addition to mapping the distribution of immunopositive neurons, these plots were used to derive their number, and thereby to estimate the total number of GLP-1-positive neurons present through the rostrocaudal extent of the caudal medulla. Representative immunopositive neurons that displayed a clearly visible nucleus ($n = 14$ neurons per subject) were photographed using a 40× microscope objective to measure neuronal length and width, and thereby to calculate average GLP-1 neuronal size.

Results

In medullary tissue sections from both subjects, GLP-1-positive neurons were located within the dorsal medullary region containing the NST and dorsal motor nucleus of the vagus (DMV). In the same tissue sections (i.e., at the same rostrocaudal levels), GLP-1-positive neurons also were distributed in the medullary reticular formation, extending ventrally and laterally from the DMV (Figs. 1, 2). GLP-1-positive neurons were clearly identified by the presence of dense cytoplasmic immunoperoxidase labeling, which often extended into one or two proximal neurites arising from the labeled cell body (Fig. 3, inset; Fig. 4). GLP-1-positive neurons had an ovoid, slightly elongated shape. In the female subject, GLP-1 neurons averaged $11.5 \mu\text{m} \pm 0.6$ (mean $\pm$ SE) in the short axis (i.e., width), and $22.4 \mu\text{m} \pm 1.2$ in the long axis (i.e., length). In the male subject, GLP-1 neurons averaged $12.9 \mu\text{m} \pm 0.8$ in width, and $25.0 \mu\text{m} \pm 1.4$ in length. These measurements were statistically similar

between subjects (independent samples *t* test: $P = 0.15$ for width measurement comparison, $P = 0.16$ for length measurement comparison). Short GLP-1-positive varicose fibers were occasionally observed within the caudal NST and reticular formation (small arrows in Fig. 3).

The medullary distribution and number of immunopositive neurons were similar between subjects (Figs. 1, 2). GLP-1-positive neurons were present in the most caudal sections derived from the obtained sample blocks, which extended just beyond the pyramidal decussation at the spinomedullary junction; note that the caudal NST extends beyond this level and into the upper cervical cord (Hyde and Miselis 1992). Thus, GLP-1-positive neurons may also be present further caudally in the spinal cord. GLP-1-positive neurons were absent in medullary sections through the rostral gustatory portion of the NST, which continues rostral to the area postrema. GLP-1-positive neurons were distributed in close proximity, but were anatomically separate from TH-positive catecholaminergic neurons within the same tissue sections (Fig. 4), evidence that these neuronal populations are distinct.

The plotted distribution of GLP-1-positive neurons in the first (female) subject spanned 26 tissue sections that were spaced 200 μm apart, corresponding to a 5.2-mm rostrocaudal extent within the caudal medulla (three of these sections are depicted in Fig. 1). In the second (male) subject, GLP-1-positive neurons spanned 30 tissue sections, corresponding to a 6.0-mm rostrocaudal extent (three sections are depicted in Fig. 2). A bilateral total of 2,406 GLP-1-positive neurons were plotted in the female subject, and 2,138 GLP-1-positive neurons were plotted in the male subject. As reported above, the average width $\times$ length of GLP-1-positive neurons in the female subject was $11.5 \mu\text{m} \times 22.4 \mu\text{m}$, and was $12.9 \mu\text{m} \times 25.0 \mu\text{m}$ in the male subject. Abercrombie's (1946) formula was applied to calculate the number of GLP-1-positive neurons in both subjects after correcting for neuron size and tissue section thickness:

$$\begin{aligned} \text{Female subject: } N &= 2,406 \times [40 \mu\text{m} / (40 \mu\text{m} \\ &\quad + 11.5 \text{ (or } 22.4) \mu\text{m})] = 1,542 - 1,869; \\ N \times 5 &= 7,710 - 9,345 \text{ GLP-1 neurons} \end{aligned}$$

$$\begin{aligned} \text{Male subject: } N &= 2,138 \times [40 \mu\text{m} / (40 \mu\text{m} \\ &\quad + 12.9 \text{ (or } 25.0) \mu\text{m})] = 1,316 - 1,617; \\ N \times 5 &= 6,580 - 8,085 \text{ GLP-1 neurons} \end{aligned}$$

Note that N is reported as a range in both subjects, because its calculation depends on whether one represents GLP-1 neuronal size as the average neuronal length or width. After multiplying this corrected range of cell count values by 5 (to account for the 1:5 sampling frequency used for immunolabeling and plotting), and considering the values obtained in both subjects, we estimate that there are approximately 6,580–9,345 GLP-1-positive neurons present within the adult human caudal medulla.

Discussion

The present study is the first to map the location of GLP-1-immunopositive neurons within the human brain. The caudal medullary distribution of GLP-1-positive neurons is strikingly similar to that reported previously in rodent species, and also in *M. mulatta*, a non-human primate. Across species, GLP-1 neurons are concentrated within the caudal (visceral) portion of the NST, and also within the medullary reticular formation at the same rostrocaudal levels. In the present study, double immunolabeling demonstrated that GLP-1 neurons are located in close proximity to TH-positive catecholaminergic neurons in human caudal medulla. However, these two neural populations apparently are distinct in human brainstem (Fig. 4), as they are in rats (Larsen et al. 1997; Jelsing et al. 2009) and mice (Llewellyn-Smith et al. 2013).

The human medulla contains a relatively large number of GLP-1 neurons, corresponding to a bilateral average of ~70–90 neurons (across both subjects) per 40- μ m-thick tissue section, and a combined total of approximately 6.5–9.3 K GLP-1 neurons. Although GLP-1 cell count values are relatively similar between subjects, they are not identical. Differences could be due to differences in age, sex, and/or PMI. By comparison, using similar counting and Abercrombie correction methods, we estimate that the caudal medulla of adult male Sprague–Dawley rats contains a bilateral total of approximately 800 GLP-1-positive neurons (unpublished observations), which amounts to only ~10 % of the estimated total number in human medulla. Interestingly, however, the somatic size of individual GLP-1-positive neurons in rats (~10.9 μ m wide $\times$ 19.5 μ m long; *unpublished observations*) is similar to GLP-1 neuronal size in both human subjects analyzed in the present study. A previous study investigating the distribution of central GLP-2 immunolabeling in the Old World monkey, *M. mulatta*, did not calculate the number of labeled neurons (Vrang and Grove 2011). However, labeling plots included in their report suggest a bilateral peak of ~50 neurons per 25- μ m-thick tissue section at the rostrocaudal level of the area postrema, with more caudal levels of the NST and reticular formation containing fewer GLP-2 neurons per section [see Fig. 3 in (Vrang and Grove 2011)]. These estimated “neuron per section” counts of GLP-2 neurons in *M. mulatta* are relatively close to the current counts per section in human medulla if one considers the difference in tissue section thickness (i.e., 25 μ m in the non-human primate report vs. 40 μ m in the present report).

To the extent that it has been examined, GLP-1 neurons within the rat caudal medulla express the immediate-early gene product cFos (a marker of neural activation) in every experimental situation in which food intake is acutely inhibited and the HPA stress axis is activated (Gaykema et al. 2009; Rinaman 1999; Vrang et al. 2003; Maniscalco et al. 2013). As if to emphasize the close relationship between GLP-1 neural recruitment and endocrine stress responses, systemically administered amylin reduces meal size but does not activate GLP-1 neurons, and amylin does not activate the HPA axis, evidence that amylin induces hypophagia without recruiting GLP-1 neurons and in the absence of a stress response (Potes and Lutz 2010). Acute stressors that activate GLP-1 neurons, inhibit food intake, and activate the HPA axis also inhibit gastric emptying, likely via direct or indirect GLP-1-mediated effects on vagal and perhaps spinal autonomic outflow (Llewellyn-Smith et al. 2011, 2013; Hayes et al. 2009; Grill and Hayes 2012; Maniscalco et al. 2013). The available

evidence supports the view that hindbrain GLP-1 neurons represent an important point of integration in the central control of energy intake and metabolism in response to homeostatic challenge.

The neuroanatomy of GLP-1 axonal projections to other brain regions in rodent species has been reviewed (Maniscalco et al. 2013; Trapp and Richards 2013; Llewellyn-Smith et al. 2011, 2013; Vrang and Larsen 2010). The anatomical location of GLP-1 neurons within and near the caudal visceral NST ideally positions them to receive a diverse compilation of interoceptive signals, as well as inputs from the hypothalamus and limbic forebrain that target the caudal NST (Maniscalco et al. 2013). In rats, the densest GLP-1 terminal fields are located within the thalamic paraventricular nucleus and the hypothalamic dorsomedial, paraventricular, and supraoptic nuclei, with less dense inputs to the thoracic spinal cord, hypothalamic arcuate nuclei, central nucleus of the amygdala, anterior ventral bed nucleus of the stria terminalis, nucleus accumbens, ventral tegmental area, and parabrachial nucleus. A generally similar brainstem and forebrain distribution of GLP-2-positive fibers was reported in *M. mulatta* (Vrang and Grove 2011), although the bed nucleus of the stria terminalis and hypothalamic arcuate nucleus appear to contain a much higher density of GLP-2 input in non-human primates compared to rats. GLP-2-positive fibers have been visualized within human hypothalamus (Vrang and Larsen 2010), but the density and distribution of fibers within the diencephalon or any other human brain region is unreported. In future work, it will be important to complete this mapping in human brain to improve our understanding of how GLP-1 signaling pathways from the caudal medulla might influence forebrain-mediated physiological and behavioral functions, including stress responsiveness and the control of body energy balance.

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Fig. 1.

The distribution of GLP-1-positive neurons is plotted in three caudal medullary sections obtained from a 53-year-old woman. Adjacent sections stained for Nissl substance are merged with the plotted images to provide anatomical reference points. *AP* area postrema, *cc* central canal, *DMV* dorsal motor nucleus of the vagus, *Gr* gracile nucleus, *Hyp* hypoglossal motor nucleus, *IO* inferior olive, *ml* medial lemniscus, *mRF* medullary reticular formation, *NST* nucleus of the solitary tract, *Pyr* pyramidal tract, *4th* fourth ventricle

Fig. 2.

The distribution of GLP-1-positive neurons is plotted in three caudal medullary sections obtained from a 39-year-old man. Adjacent sections stained for Nissl substance are merged with the plotted images to provide anatomical reference points. *AP* area postrema, *cc* central canal, *DMV* dorsal motor nucleus of the vagus, *Gr* gracile nucleus, *Hyp* hypoglossal motor nucleus, *IO* inferior olive, *ml* medial lemniscus, *mRF* medullary reticular formation, *NST* nucleus of the solitary tract, *Pyr* pyramidal tract, *4th* fourth ventricle

Fig. 3. GLP-1-immunoperoxidase labeling within the caudal dorsomedial medulla in a 53-year-old woman. The left side of the large panel is medial. The *three large arrows* point out GLP-1-positive neurons that are depicted at higher magnification in the inset at upper right. The *small arrows* point out a few GLP-1-positive fibers, which may be either dendrites or axons. *AP* area postrema, *NST* nucleus of the solitary tract, *tr* tractus solitarius

Fig. 4.

GLP-1 immunoperoxidase labeling (**a** black neurons, indicated by *arrows*) and TH immunofluorescent labeling [**b** white neurons (*arrows*) and fibers] are shown from the same single tissue section through the caudal medulla in a 39-year-old man. **c** Depicts the entire section photographed in brightfield at a low magnification. The *boxed region* in **c** indicates the dorsal medullary region that is shown at higher magnification in **a** (brightfield) and **b** (fluorescence). *Asterisks* in **a** and **b** mark the same blood vessels for orientation. *Scale bar* in **a** = 100 μ m, applies also to **b**. *Scale bar* in **c** = 2.0 mm