Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus

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Rinaman, Linda. Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R582–R590, 1999.—This study tested the hypothesis that systemic stressors in rats activate glucagon-like peptide-1 (GLP-1)-immunoreactive neurons in the caudal brain stem, including those that project to the paraventricular nucleus of the hypothalamus (PVN). Neural tracer was microinjected into the PVN to retrogradely label brain stem neurons. Seven to ten days later, rats were injected with lithium chloride (LiCl; 50 mg/kg). Additional non-tracer-injected rats were treated with lipopolysaccharide (LPS; 100 µg/kg) or CCK (100 µg/kg) or were allowed to consume a very large meal. Rats were killed 90–120 min after drug treatment or 30 min after the meal. Brains were processed for immunocytochemical localization of c-Fos (a marker of neuronal activation), GLP-1, and, when appropriate, neural tracer. The majority of GLP-1 neurons were labeled after expression of c-Fos after LiCl, LPS, or CCK treatment, including (in LiCl-treated rats) those projecting to the PVN. In contrast, GLP-1 neurons rarely expressed c-Fos after ingestion of a large meal, despite prominent activation of other brain stem neurons. These results suggest that GLP-1 neurons are uniquely activated in situations of interoceptive stress, and may participate in adaptive hypothalamic stress responses.

The paraventricular nucleus of the hypothalamus (PVN) integrates neuroendocrine and autonomic activity to maintain homeostasis during interoceptive stress, such as that posed by toxemia or systemic infection (32). The term “interoceptive stress” is used here to describe physiological responses to systemic treatments that challenge body homeostasis (for discussion, see Ref. 17). Visceral sensory information reaches the caudal brain stem through the vagal and glossopharyngeal nerves and then is relayed to the PVN through direct and indirect central neural pathways. The direct neural pathways are primarily catecholaminergic and arise from the nucleus of the solitary tract (NST), but a relatively small number of noncatecholaminergic neurons located in the caudal NST and reticular formation also project directly to the PVN (31). These neurons coexpress several neuropeptides, including glucagon-like peptide-1 (GLP-1) (11, 15, 21).

GLP-1 is a major splicing product of the preproglucagon peptide precursor in brain (15) that is expressed only by short-axon cells in the glomerular layer of the

METHODS

Experimental procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Animals and Anesthesia

Thirty-eight adult male Sprague-Dawley rats (Zivic Miller, Zelienople, PA), weighing 200–325 g at the time of treatment, were used. Rats were housed singly in stainless steel wire mesh cages in a temperature-controlled room (20–22°C), with lights on from 0700 to 1900. Rats had free access to water and pelleted chow (Purina) except as noted.

Rats were anesthetized with ketamine-xylazine (83 mg ketamine and 17 mg xylazine per ml; 0.05 ml/10 g body wt ip; Fort Dodge Labs) before tracer injection and/or perfusion fixation procedures.

Procedures

PVN neural tracer injection. To retrogradely label brain stem neurons projecting to the PVN, anesthetized rats (n = 10) received a unilateral PVN microinjection of cholera toxin neural tracer (nontoxic beta subunit, CTb; List Biological Laboratories) by means of a previously described stereotaxic procedure (26). CTb neural tracer (50 nl; 0.25% in 0.15 M NaCl) was pressure-injected into the PVN over ~10 min. The syringe was left in place for 15 min after tracer injection and then was withdrawn. Bone wax was applied to the skull opening, and the scalp incision was closed with stainless steel suture clips. After recovery from anesthesia, rats were

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returned to their home cages, where they remained for 7–10 days before experimental treatment.

Experimental treatments. Three experimental models of interoceptive stress were tested to determine whether they activate c-Fos expression in GLP-1 neurons. Rats that 7–10 days earlier had received CTb tracer injections into the PVN were injected intraperitoneally with either lithium chloride (LiCl; Sigma; 0.15 M solution; 50 mg/kg; n = 8) or 0.15 M NaCl (2.0 ml; n = 2). Additional groups of non-tracer-injected rats were treated by intraperitoneal injection of LiCl (as above; n = 4), lipopolysaccharide (LPS; Sigma; 100 µg/kg in 2.0 ml of 0.15 M NaCl; n = 6), CCK octapeptide (Sigma; 100 µg/kg in 2.0 ml of 0.15 M NaCl; n = 9), or 0.15 M NaCl (2.0 ml; n = 4).

It also was of interest in the present study to determine whether GLP-1 neurons are activated to express c-Fos in rats after nonstressful stimulation of visceral afferents. For this purpose, stored brain sections that were generated in an earlier study (25) were used along with new material from additional rats. The stored sections came from rats (n = 4) that were acclimated for 5 days to a feeding schedule with free access to water but access to pelleted chow for only 3 h each day. Each morning, they had 1-h access to an unrestricted amount of liquid diet consisting of 316 g of AIN-76 liquid diet powder (BioServ) blended with 1.1 of 14% dextrose (Fisher Scientific). On the morning of the experiment (day 5 of the acclimation period), they were given 1-h access to an unrestricted amount of liquid diet which had been diluted 50% with water. Rats consumed 30.5 ± 0.2 ml of this diluted liquid diet (25). The new material came from additional rats that were not acclimated to a feeding schedule. These rats initially had free access to pelleted chow and water, but also were given a small amount (1.0 g) of mash made from a 1:1 mixture of smooth peanut butter and powdered chow for 3 days. All rats consumed the entire 1.0 g within 15 min on days 2 and 3. Rats then were deprived of food overnight (their only deprivation experience). After 18 h deprivation, they were given 1-h access to an excess amount (30 g) of mash. Rats (n = 4) consumed 13.8 ± 2.6 g of mash plus an unmeasured amount of water during the 1-h feeding period.

Posttreatment survival times and perfusion fixation. Experimental and control rats were anesthetized and killed by transcardiac perfusion fixation between 1030 and 1130. Posttreatment survival times were based on results from pilot studies and on evidence that nuclear c-Fos protein peaks between 60 and 120 min after peak neural stimulation (9, 30). Based on measured autonomic, endocrine, and behavioral responses, peak neural stimulation occurs within 30 min after systemic administration of LiCl, within 5 min after systemic administration of CCK, and within 60 min after systemic administration of LPS (4, 18, 29, 38, 42). Thus rats that were injected with LiCl, CCK, or 0.15 M NaCl (controls) were perfused 90 min after treatment, whereas LPS-injected rats were perfused 120 min after treatment. Rats used in the feeding protocols were perfused 30 min after the end of the 1-h feeding period, because the rate of voluntary intake of liquid diet or mash had slowed significantly by 30 min into the meal (likely a result of preabsorptive satiety cues) (25).

Perfusates consisted of 100 ml of 0.15 M NaCl followed by 500 ml of 0.1 M sodium phosphate buffer (SPB) containing 4% paraformaldehyde with lysine and sodium metaperiodate (20). Brains were removed from the skull, postfixed at 4°C for 10–18 h, and then cryoprotected for 24–72 h by immersion in aqueous 25% sucrose solution (4°C) before sectioning.

Tissue preparation and immunocytochemistry. Brains were cut coronally at 30 µm on a freezing-stage microtome. Sections were collected from the cervical spinal cord through the anterior commissure in six serially ordered sets, so that each set contained a complete series of brain sections spaced by 180 µm. Tissue sections were stored at −20°C in cryopreservant (43). Before immunocytochemical procedures, sections were removed from cryopreservant, rinsed in SPB, immersed for 10 min in 2% acrolein (Sigma), rinsed in SPB, treated for 30 min in 1% sodium borohydride (Sigma), and rinsed again in SPB.

Primary and secondary antisera were diluted in SPB containing 0.3% Triton X-100 and 1% normal donkey serum. Rinses were performed in multiple changes of SPB over 30 min. The c-Fos antisera used in this study (provided by Drs. Philip Larsen and Jens Mikkelsen, Panum Institute, Denmark) was generated in rabbits immunized with amino acids 4–17 of synthetic c-Fos protein. The specificity of this antisera has been demonstrated (27). Tissue sections were incubated for 48 h at 4°C in rabbit anti-c-Fos (1:50,000), rinsed, and then incubated in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch; 1:600) for 1 h at room temperature. After rinsing, sections were treated with Elite Vectastain reagents (Vector) with diaminobenzidine (DAB) intensified with nickel sulfate to produce a blue-black nuclear c-Fos peroxidase reaction product.

One set of c-Fos-labeled hindbrain sections from each rat was subsequently processed for immunoperoxidase localization of GLP-1. Sections were incubated at 4°C for 48 h in rabbit anti-GLP-1 (Peninsula; 1:10,000) and then processed as above with biotinylated secondary antibody and Elite Vectastain reagents. DAB was used alone to produce a brown cytoplasmic GLP-1 peroxidase reaction product. GLP-1 immunostaining was abolished by preabsorption of the antisera with the synthetic peptide immunogen [GLP-1(7–36)amide; Peninsula].

In the 10 rats with hypothalamic injections of CTb neural tracer, a second set of c-Fos- and GLP-1-reacted tissue sections was processed for immunofluorescent localization of CTb. For this purpose, sections were incubated at 4°C for 48 h in goat anti-CTb (List Biological Laboratories; 1:5,000), rinsed, incubated for 2 h at room temperature in BODIPY-conjugated donkey anti-goat IgG (Molecular Probes; 1:200), and rinsed again. The punctate, fibrous nature of the perikaryal GLP-1 immunoperoxidase label allowed concurrent visualization of cytoplasmic DAB peroxidase and BODIPY fluorescent signals within the same cell bodies by means of bright-field and fluorescent illumination, respectively (see RESULTS).

After completion of immunolabeling procedures, tissue sections were rinsed for 1 h in SPB, mounted onto Superfrost Plus microscope slides (Fisher Scientific), dehydrated and defatted in graded ethanols and xylene, and placed under coverslips with Cytoseal 60 (VWR).

Analysis of treatment-induced activation of GLP-1 neurons. One set of tissue sections (180 µm frequency) from each experimental and control animal was used to obtain counts of GLP-1-positive neurons and to determine the proportion that were activated to express c-Fos. Sections containing GLP-1-positive neurons extended from the upper cervical spinal cord through the middle extent of the area postrema; thus 6–8 tissue sections contained GLP-1-positive neurons in each animal. In these sections, GLP-1-immunoreactive profiles that were clearly perikaryal (rather than dendritic, axonal, or ambiguous) were counted as neurons whether or not a nucleus was visible. GLP-1 neurons were considered c-Fos-positive when their nuclei contained visible blue-black immunostaining, regardless of intensity. GLP-1 neurons were considered c-Fos-negative when they displayed either no...
visible nucleus or a nucleus lacking c-Fos immunoreactivity. Single- and double-labeled cells were counted bilaterally; counts were adjusted by means of Abercrombie's correction (1). Data were combined by treatment group and are presented as means ± SE. Differences in cell count values were tested for statistical significance by means of one-way ANOVA with experimental treatment as the independent variable, followed by planned comparisons between treatment groups. Differences were considered statistically significant when P < 0.05.

Analysis of CTb tracer transport and GLP-1 phenotype of hindbrain neurons. The locations of CTb tracer injection sites were determined by microscopic inspection of CTb-immunolabeled forebrain sections, with the use of fluorescent and dark-field illumination to discern white matter tracts and cytoarchitectural subdivisions of the hypothalamus. In animals with accurate tracer injection sites centered in the PVN, the presence of stimulus-activated, retrogradely labeled GLP-1 neurons was evaluated by analysis of hindbrain sections processed for triple localization of c-Fos, CTb, and GLP-1 immunoreactivities. Activated (c-Fos-positive) neurons were identified by the presence of blue-black nuclear immunoperoxidase reaction product, as described above. GLP-1-positive cells were identified by their cytoplasmic content of brown punctate peroxidase labeling. Retrogradely labeled neurons were identified by their cytoplasmic content of green (BODIPY) fluorescence. Retrogradely labeled and/or GLP-1-positive profiles that were clearly perikaryal (rather than dendritic, axonal, or ambiguous) were counted whether or not a nucleus was visible, and the proportion of these neurons that also contained nuclear c-Fos immunostaining was determined.

Photography and preparation of illustrations. Tissue sections were photographed with Kodak Ektachrome 160T color slide film. A Polaroid SprintScan 35 slide scanner was used to import photographic images from color slides into Adobe Photoshop 5.0 for construction of illustrations. Some images of immunoperoxidase labeling were transformed to grayscale for black-and-white prints. Illustrations were printed on a Fuji Pictrography 3000 printer.

RESULTS

Hindbrain and Hypothalamic GLP-1 Immunoreactivity

The distribution of GLP-1-positive neurons in the present study was consistent with previous reports (11, 15, 21). Taking section thickness into account, the number of GLP-1-positive neurons in acrolein-treated tissue sections in the present study was similar to the number reported previously in colchicine-treated rats (11, 15). The majority of GLP-1-positive neurons were located in or near the medial and lateral subdivisions of the caudal NST and the caudal and lateral dorsal motor nucleus of the vagus (Fig. 1). Additional scattered neurons were observed in an arc-like distribution in the medullary reticular nuclei between the caudal lateral NST and the nucleus ambiguus. A few GLP-1-positive neurons were located near the midline below the hypoglossal nucleus in the region of the paramedian reticular nucleus.

The perikarya of GLP-1-immunopositive neurons were ~15 µm in widest diameter, and displayed a scant cytoplasm that contained punctate, fibrous-like GLP-1 immunoreactivity (Fig. 1B). Immunopositive fibers were sparse and scattered throughout the caudal NST and adjacent reticular formation. In contrast, GLP-1-positive fibers were prominent in the PVN (Fig. 2), as reported previously (11, 15). GLP-1 terminals were especially prevalent in PVN subregions that contain magnocellular OT neurons [i.e., the peripheral borders of the posterior magnocellular subnucleus, lateral division (PVNmpl)], and in regions that contain parvocellular OT and CRH neurons [i.e., medial parvocellular subnucleus, dorsal division (PVNmrd)]. These regions also contained many c-Fos-positive cells in rats after CCK, LPS, or LiCl treatment (Fig. 2B). In contrast, and consistent with previous reports (11, 15), GLP-1-positive fibers were only rarely observed in the center core of the PVNmpl, which contains primarily magnocellular vasopressin (AVP) neurons (Fig. 2).
Treatment-Induced Activation of GLP-1 Neurons

Large proportions of GLP-1-positive neurons were activated to express c-Fos in rats after CCK, LPS, or LiCl treatment (Fig. 3; Table 1). In contrast, GLP-1-immunoreactive neurons rarely expressed c-Fos in control rats, including those with previous injections of CTb neural tracer into the region of the PVN, or in rats that voluntarily consumed a very large meal (Fig. 3C; Table 1). Regarding the latter, similarly robust feeding-induced hindbrain c-Fos expression coupled with rare GLP-1 activation was observed in rats that were acclimated to a feeding schedule before consuming a large final meal of liquid diet and in rats that consumed a large final meal of mash after being food deprived overnight for the first time. Thus quantitative data from these rats were combined into a single “gastric distension” group (Table 1).

LiCl-Induced Activation of GLP-1 Neurons Projecting to the PVN

Six of the ten tracer-injected rats had injection sites centered in the medial PVN (Fig. 4), as intended. These rats displayed numerous retrogradely labeled neurons in the caudal NST and reticular formation (Fig. 5). One of these rats was a control (injected with 0.15 M NaCl ip) and exhibited very little hindbrain c-Fos expression, including a lack of activation of retrogradely labeled neurons. In contrast, all five of the LiCl-treated rats displayed prominent hindbrain c-Fos expression, including activation of retrogradely labeled neurons (Fig. 3D). In these five rats, 28.3 ± 9.5% of GLP-1-positive neurons were retrogradely labeled (out of 135.3 ± 4.1 GLP-1 neurons counted). The majority (79.3 ± 11.2%) of these retrogradely labeled GLP-1 neurons were activated to express c-Fos.
The other four CTb tracer-injected rats (1 control and 3 LiCl treated) had CTb injection sites centered in the anterior hypothalamic area or in the thalamic nucleus reuniens. In these four rats, few or no neurons in the hindbrain regions of interest were retrogradely labeled. 

Table 1. Treatment-induced activation of GLP-1 neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>No. of GLP-1 Cells</th>
<th>% of GLP-1 Cells Expressing c-Fos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6; 2 with prior neural tracer injection</td>
<td>142 ± 6.5</td>
<td>3 ± 2.8</td>
</tr>
<tr>
<td>LiCl</td>
<td>12; 8 with prior neural tracer injection</td>
<td>131 ± 4.3</td>
<td>68 ± 11.4*</td>
</tr>
<tr>
<td>LPS</td>
<td>6</td>
<td>143 ± 6.8</td>
<td>60 ± 9.2*</td>
</tr>
<tr>
<td>CCK</td>
<td>9</td>
<td>124 ± 6.0</td>
<td>57 ± 9.7*</td>
</tr>
<tr>
<td>Gastric distension</td>
<td>8</td>
<td>134 ± 5.4</td>
<td>4 ± 3.1</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = no. of rats. Glucagon-like peptide-1 (GLP-1)-positive neurons were counted bilaterally in every 6th tissue section (180 µm frequency) from upper cervical spinal cord through area postrema. Counts were adjusted with Abercrombie’s correction (1). *Significantly greater than in control rats (P < 0.001) but not significantly different from one another (P > 0.05 for each comparison). LiCl, lithium chloride; LPS, lipopolysaccharide.

Treatment-Induced Activation of Other Brain Stem Neurons

Although the apparent majority of GLP-1-positive neurons were activated to express c-Fos in rats after...
LiCl, LPS, or CCK treatment (Table 1), this small group of peptidergic neurons comprised only a small subset of the brain stem neurons activated by these treatments. For example, in the five LiCl-treated rats with PVN neural tracer injections, the NST caudal to the area postrema contained $487 \pm 642$ c-Fos-positive cells per section (3 sections counted per rat), and only $0.9 \pm 0.3\%$ of these activated cells were GLP-1-positive. Furthermore, most ($88.6 \pm 7.4\%$) of the activated, retrogradely labeled neurons in the NST and ventrolateral medulla (VLM) were not GLP-1 immunoreactive. The neurochemical phenotype of these activated neurons was not determined in the present study, but their morphology and distribution was consistent with that of catecholaminergic neurons, which provide the major ascending input to the PVN from the NST and VLM (cf. Ref. 32).

**DISCUSSION**

Results from this study provide new evidence that three different types of interoceptive stress are associated with similar activation of GLP-1-positive neurons in the caudal brain stem, including (in the case of LiCl treatment) those that project to the PVN. These results also support and extend a previous report that CCK treatment activates both catecholaminergic and noncatecholaminergic (i.e., peptidergic) caudal brain stem neurons that project to the PVN (26). In contrast, GLP-1 neurons are not activated to express c-Fos after nonstressful interoceptive stimuli associated with the voluntary consumption of a very large meal, and in rats that were food-deprived overnight for the first time before consuming a large final meal of palatable mash. These observations indicate that neither a “practiced” nor a novel food deprivation-refeeding experience activates GLP-1 neurons, despite significant feeding-induced activation of other NST neurons, including catecholaminergic neurons (25).

Systemic administration of LiCl, LPS, or CCK in rats offers three reliable and well-explored models of interoceptive stress. LiCl apparently exerts its effects by acting at chemoreceptors in the area postrema (5, 28). LPS (a fragment of gram-negative bacterial cell walls) stimulates macrophages and monocytes to release proinflammatory cytokines (44). Exogenous CCK stimulates subdiaphragmatic vagal mechanoreceptors and chemoreceptors with synaptic inputs to the NST (23). Each of these agents, at the doses used in the present study, generates stereotypical behavioral and physiological responses that mimic those observed in natural situations of toxemia, bacterial infection, and/or systemic malaise (4, 18, 41, 42). Such responses include anorexia, inhibition of gastric motility, and pituitary secretion of ACTH and either AVP (in human and nonhuman primates) or OT (in rats). The species-specific effects of these agents on posterior pituitary secretion are consistent with evidence that AVP secretion is indicative of stress and nausea in primates, whereas OT secretion is indicative of stress and nausea in rats (18, 38, 40).

![Fig. 5. Retrogradely labeled neurons (X) and GLP-1-positive neurons (O) are plotted in single 30-µm tissue section through caudal NST in rat with CTb neural tracer injection centered in PVN (see Fig. 4). Overlaid symbols (X + O) are double-labeled neurons (i.e., GLP-1 neurons with axonal projections to region of PVN). Only neurons ipsilateral to PVN tracer injection site are plotted. Atlas figure modified from Ref. 33. Cu, cuneate nucleus; Gr, gracile nucleus; IO, inferior olive; LRN, lateral reticular nucleus (m, magnocellular; p, parvocellular); MDRN, medullary reticular nucleus (d, dorsal; v, ventral); NA, nucleus ambiguus; med, medial NST; lat, lateral NST; PMR, paramedian reticular nucleus; pyr, pyramidal tract; ROb, raphe obscurus; RPa, raphe pallidus; SpV, spinal trigeminal nucleus; tr, solitary tract.](http://ajpregu.physiology.org/)
Stressor-Induced Activation of Central GLP-1 Pathways

In summary, the present study provides new evidence that interoceptive stressors activate c-Fos expression in GLP-1-positive neurons in the caudal brain stem, including (in the case of LiCl treatment) GLP-1 neurons that project to the PVN. These findings suggest that central GLP-1 neural pathways are involved in relaying specific types of interoceptive signals to the hypothalamus to thereby initiate or modulate appropriate endocrine, autonomic, and behavioral responses to systemic homeostatic challenges. Alternatively, or in addition, GLP-1 neurons may be activated as a consequence of treatment-related effects on the PVN or other brain regions that project to the caudal brain stem. Further studies will be necessary to address these important questions and to determine the physiological role(s) of central GLP-1 pathways.

Perspectives

The widespread axonal projections of GLP-1 neurons (11, 15, 21) suggest that these neurons might send interoceptive information to the hypothalamus and other forebrain regions through multiple direct and indirect pathways. A potentially important indirect pathway to the PVN might involve a relayed projection through catecholaminergic neurons in the VLM. These neurons receive asymmetric (presumably excitatory) synaptic inputs from noncatecholaminergic neurons in the caudal medial NST (13), which may include inputs from GLP-1 neurons. The presence of GLP-1-positive axon terminals and receptor mRNA in VLM regions that contain catecholaminergic neurons (11, 15, 21) is consistent with this hypothesis. Catecholaminergic VLM neurons, including those that project to the PVN, are activated to express c-Fos in rats after treatments that cause interoceptive stress (7, 26); further, the ascending catecholamine pathways are critical for hypothalamic endocrine responses to these treatments (7, 14, 22, 32). However, VLM catecholamine neurons are not activated to express c-Fos in rats after voluntary consumption of a large meal (25). The lack of feeding-induced activation of catecholaminergic VLM neurons might be due to a lack of appropriate stimulatory input from GLP-1 neurons in the caudal NST. Additional studies will be necessary to examine potential interactions between brain stem and hypothalamic GLP-1 and catecholamine pathways in orchestrating adaptive hypothalamic responses to interoceptive stimuli.

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