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Research report

Central infusion of glucagon-like peptide-1-(7–36) amide (GLP-1) receptor antagonist attenuates lithium chloride-induced c-Fos induction in rat brainstem

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Abstract

Central infusion of glucagon-like peptide-1-(7–36) amide (GLP-1) and intraperitoneal (i.p.) injection of lithium chloride (LiCl) produce similar patterns of c-Fos induction in the rat brain. These similarities led us to assess the hypothesis that neuronal activity caused by i.p. injection of LiCl involves activation of central GLP-1 pathways. We therefore determined if third-ventricular (i3vt) infusion of a GLP-1 receptor antagonist would block LiCl-induced c-Fos expression in the brainstem. Relative to rats pretreated with i3vt infusion of vehicle, pretreatment with the potent GLP-1 receptor antagonist, des His[1] Glu[9] exendin-4 (10.0 µg), significantly attenuated LiCl-induced (76 mg/kg; i.p.) c-Fos expression in several brainstem regions, including the area postrema, the nucleus of the solitary tract, and the lateral parabrachial nucleus. While central infusion of des His[1] Glu[9] exendin-4 also blocked GLP-1-induced (10.0 µg) anorexia and c-Fos expression, the antagonist produced no independent effects on food intake or c-Fos expression. These results suggest that LiCl-induced c-Fos expression in the rat brainstem is mediated, at least in part, by GLP-1 receptor signaling. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Glucagon-like peptide-1 (7–36) amide (GLP-1) is cleaved from proglucagon in mucosal endocrine cells (L-cells) located predominately in the distal intestinal tract [6,8], and in neurons of the brainstem [10]. In the periphery, GLP-1 has a regulatory role in glucose homeostasis by augmenting insulin release [12,15], inhibiting gastric emptying [26], and stimulating the activation of hepatic vagal afferent nerves [16]. While the role of GLP-1 in the CNS has not been as well studied, it has recently been shown that central infusion of exogenous GLP-1 reduces food

intake in rats [22–24]. These findings raise the possibility that GLP-1 is involved with the homeostatic regulation of food intake, the process by which caloric intake and caloric expenditure are matched to keep parameters such as body adiposity within certain limits. In fact, several authors have suggested that central GLP-1 pathways are involved with the regulation of satiety, and have labelled GLP-1 a satiety factor [20,23].

Food intake can also be influenced by nonhomeostatic factors, such as the response to visceral illness. 'Visceral illness' is a term that encompasses the nausea and/or anorexia that are symptomatic of a wide range of clinical conditions spanning ingestion of toxins, infectious diseases, and the effects of certain tumors or cancers [2,3,5,7]. Lithium chloride (LiCl) is an emetic agent that produces aversive side-effects and symptoms associated with visceral illness, such as anorexia, vomiting in emetic species,

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and conditioned taste aversion learning (CTA; [5,18,22]), and is often used experimentally to study these responses. Recent studies have demonstrated that central administration of GLP-1 produces a range of effects similar to those of toxic amounts of LiCl, including the observations that both agents: (1) are capable of supporting CTAs [22,25], (2) reduce locomotor activity [14,23], (3) reduce core body temperature [1,17], (4) decrease gastric emptying [1,26], and (5) produce similar patterns of c-Fos-like-immuno-reactivity (cFLI) in the brain [21,24].

Given the similar effects produced by administration of LiCl and GLP-1, it is possible that the activation of central neuronal pathways following LiCl administration involves GLP-1 receptor signaling. Indeed, using cFLI as an indica-tion of neuronal activation [19], LiCl administration is found to produce robust activation of cells in the nucleus of the solitary tract (NTS) and the area postrema (AP) in the brainstem [21], the brain regions involved with central GLP-1 production [10]. Furthermore, GLP-1 receptors have been identified throughout the NTS and in the paraventric-ular nucleus of the hypothalamus (PVN) in the forebrain [4,9], regions that express strong cFLI activation following either LiCl or GLP-1 administration. Thus, this LiCl-in-duced cFLI may be mediated, in part, by GLP-1 receptor signaling. According to this hypothesis, LiCl activates GLP-1 secreting cells which in turn stimulate the activa-tion of other cells via GLP-1 receptor.

To test the hypothesis that GLP-1 pathways are in-volved with LiCl-induced neuronal activation in the brain, we attempted to block LiCl-induced cFLI by first pretreat-ing rats with third ventricular (i3vt) administration of the potent GLP-1 receptor antagonist, des His[1] Glu[9] exendin-4 (dHGex-4). If LiCl-induced neuronal activation involves GLP-1-receptor signaling, then blockade of GLP-1 recep-tor should block (or attenuate) LiCl-induced cFLI. In Experiment 1, we demonstrated the potency of the dHGex-4 by blocking anorexia produced by i3vt infusion of GLP-1 (in vivo), and by blocking GLP-1-induced secretion of insulin from hamster insulinoma cells (HIT; in vitro). In Experiment 2, we tested our primary hypothesis by exam-ining c-Fos expression in rats pretreated with i3vt infusion of dHGex-4, followed by treatment with intraperitoneal (i.p.) injection of LiCl; as a positive control to demonstrate that the dHGex-4 could block GLP-1-induced cFLI, other rats received i3vt infusion of GLP-1 following treatment with the antagonist.

2. Materials and methods

2.1. Animal preparation

Subjects were male Long–Evans rats weighing between 300 and 400 g at the onset of the experiments. They were individually housed in hanging stainless steel cages and maintained on a 12:12 h light:dark cycle. Laboratory rat chow and water were provided ad libitum (except where noted). Under Equithesin (3.3 ml/kg; i.p.) anesthesia, rats were implanted with 21-gauge stainless-steel cannulae (Plastics One, Roanoke, VA) aimed at the third ventricle. With bregma and lambda at the same vertical coordinate, the sagittal venous sinus was carefully displaced laterally with a metal probe, and the cannulae were placed directly on the midline, 2.2 mm posterior to bregma and 7.4 mm ventral to dura, and fixed to the skull with anchor screws and dental acrylic. The cannulae were fitted with remov-able obturators that extended 0.5 mm beyond the tip of the guide cannula. Each rat was given 0.15 ml each of Chloromycetin (100 mg/ml; subcutaneously) and Gentam-icin (40 mg/ml; i.p.) prophylactically. When rats regained their preoperative body weights following surgery (ap-proximately 3 weeks), placement of i3vt cannulae were confirmed by administration of 10 ng angiotensin II in saline through the cannulae while the animals were water replete. Animals that did not drink at least 5 ml of water within 60 min were not used in the studies.

2.2. Experiment 1: effects of exendin on GLP-1-induced HIT cell insulin secretion and anorexia

To assess the effects of exendin on GLP-1 in vitro, HIT cells were first grown to confluence in KRB media over 3 days. Insulin secretion into the culture media was mea-sured after 1 h of incubation with elevated glucose concen-tration and different combinations of peptides: Either 300 mg% glucose alone, 1.0 nM GLP-1 in 300 mg% glucose, 10 nM or 100 nM of exendin-[9–39] added to 1.0 nM GLP-1 in 300 mg% glucose, or 10 nM or 100 nM of des His[1] Glu[9] exendin-4 added to 1.0 nM GLP-1 in 300 mg% glucose. Groups of 4 to 8 separate culture dishes were used for each condition.

To assess the effects of dHGex-4 on GLP-1-induced anorexia, rats were weighed and their food hoppers were removed from the cages 2 h before the beginning of the dark phase (1800 h). Rats were assigned to groups matched for body weight. Approximately 1 h before the beginning of the dark phase, rats were pretreated with an i3vt infu-sion (for all i3vt infusions reported, a 2.5 μl volume was manually infused with a Hamilton syringe over 60 s; see Ref. [24] for details) of either synthetic-CSF (s-CSF) or dHGex-4 (10.0 μg; dissolved in s-CSF). Ten min later, rats were treated with a second i3vt infusion of either s-CSF or GLP-1 (10.0 μg). This study consisted of the following groups that are identified by pretreatment/treat-ment infusions: Group s-CSF/GLP-1 (C/G; $n = 5$), Group dHGex-4/GLP-1 (E/G; $n = 5$), Group s-CSF/s-CSF (C/C; $n = 5$), and Group dHGex-4/s-CSF (E/C; $n = 5$). At the onset of the dark phase, food hoppers were weighed and returned to the rats cages; they were weighed again 4 h later.

Table 1
Insulin secretion in HIT cell cultures

HIT cells in 300 mg% glucose plus	Insulin secretion (mU/ml)
Nothing	10.1 ± 0.36
1.0 nM GLP-1	16.4 ± 0.56[a]
1.0 nM GLP-1 and 10 nM exendin-[9–39]	14.2 ± 0.41[b]
1.0 nM GLP-1 and 100 nM exendin-[9–39]	10.9 ± 0.59[c]
1.0 nM GLP-1 and 10 nM dHGex-4	9.8 ± 0.51[c]
1.0 nM GLP-1 and 100 nM dHGex-4	9.2 ± 0.62[c]

Values are means $\pm$ S.E.M.
[a] $P < 0.05$ relative to Nothing.
[b] $P < 0.05$ relative to Nothing and 1.0 nM GLP-1.
[c] $P < 0.05$ relative 1.0 nM GLP-1 and 1.0 nM GLP-1 and 10 nM exendin-[9–39].

2.3. Experiment 2: effects of dHGex-4 on drug-induced cFLI

To reduce stress-induced c-Fos expression on the test day, rats were regularly handled during recovery from surgery. Rats were pretreated with an i3vt infusion of either dHGex-4 (10.0 μg) or s-CSF, and 10 min later they were treated with either a second i3vt infusion of GLP-1 (10.0 μg) or an i.p. injection of either LiCl (76 mg/kg of a 0.15 M solution; this dose of LiCl produces CTA after a single conditioning episode [21]) or isotonic saline (10 ml/kg; this dose produced volumes equal to LiCl injections). To accommodate perfusion and brain removal procedures (see below), rats were treated at a rate of one every 15 min (order counterbalanced between treatment conditions) between 1000 h and 1600 h on the test day. This study consisted of the following groups that are identified by pretreatment/treatment drug administration: Group s-CSF/GLP-1 (C/G; $n = 4$), Group dHGex-4/GLP-1 (E/G; $n = 4$), Group s-CSF/LiCl (C/L; $n = 5$), group dHGex-4/LiCl (E/L; $n = 5$), and Group s-CSF/saline (C/S; $n = 4$).
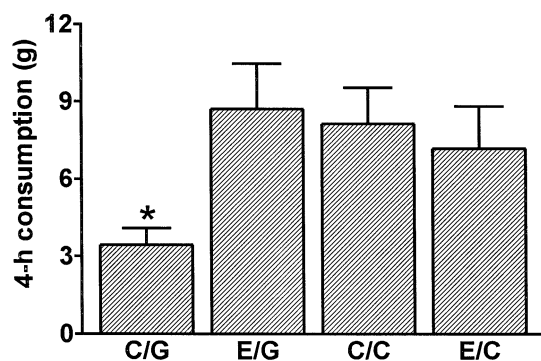
Two hours following treatments, rats were anesthetized with Equithesin (3.3 ml/kg; i.p.) and transcardially perfused with isotonic phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed for approximately 24 h, and then processed for cFLI. Fifty-micrometer slices were cut from the brain with a vibrotome. Slices from the brainstem were made in the horizontal plane to allow visualization of the rostro-caudal extent of the NTS, as well as the AP and the PBN. Tissues were rinsed (3×, PBS), incubated for 20 min in 0.3% H_2O_2 in absolute methanol to quench endogenous peroxidase, rinsed (3×, PBS), and incubated
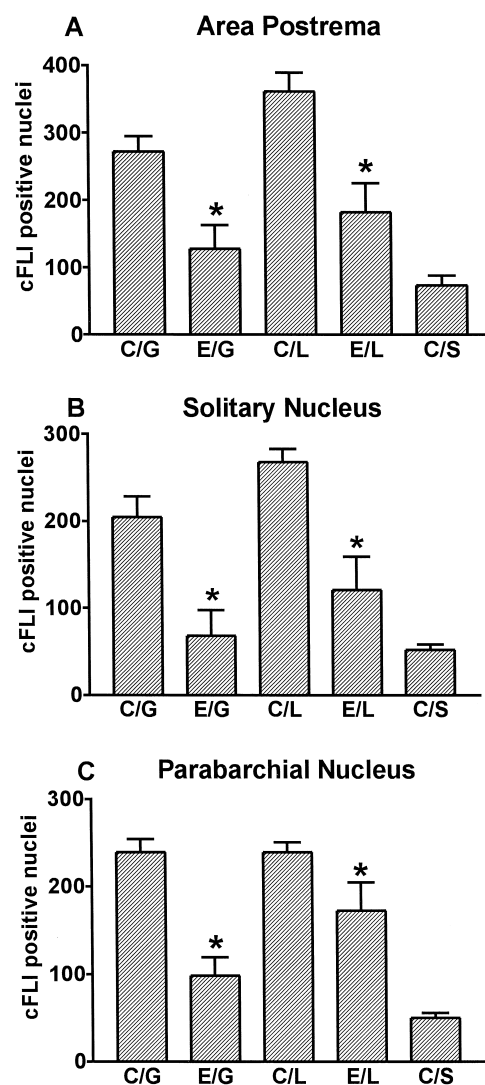

Fig. 2. Mean number ($\pm$ S.E.M.) of nuclei positive for cFLI in the area postrema (A), the nucleus of the solitary tract (B), and the lateral parabrachial nucleus (C) following i3vt infusions. Group C/G was pretreated with infusion of c-CSF followed by infusion of GLP-1; Group E/G was pretreated with infusion of dHGex-4 followed by infusion of GLP-1; Group C/L was pretreated with infusion of s-CSF followed by i.p. injection of LiCl; Group E/L was pretreated with infusion of dHGex-4 followed by injection of LiCl; and Group C/S was pretreated with infusion of s-CSF followed by injection of isotonic saline. ∗ $P < 0.05$ relative to s-CSF pretreated groups.

Fig. 1. Mean ($\pm$ S.E.M.) 4-h food intake following i3vt infusions. Group C/G was pretreated with infusion of s-CSF followed by infusion of GLP-1, Group E/G was pretreated with infusion of dHGex-4 followed by infusion of GLP-1, Group C/C was given two successive infusions of s-CSF, and Group E/C was given pretreatment with dHGex-4 followed by infusion of s-CSF. ∗ $P < 0.05$ relative to all other groups.

1 h in 1% gelatin, 5% normal goat serum in PBS. Slices were then transferred without rinsing to the primary antibody solution, consisting of 0.005 g/ml polyclonal rabbit antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; 1:20 000) which recognizes residues 3–16 of the c-Fos protein. After approximately 48 h of incubation on ice, slices were rinsed (10 ×, PBS) and processed using the ABC method (Vector Laboratories, Burlingame, CA). Slices were transferred to biotinylated goat antirabbit antibody for 1 h, rinsed (6 ×, PBS), transferred to avidin-biotinylated peroxidase for 1 h, rinsed (3 × in PBS, 3 × in PB), and developed with diaminobenzidine substrate intensified with nickel sulfate (6 min). Slices were rinsed (10 ×, PBS), mounted on slides, and cover-slipped with Permount. Camera lucida drawings of c-Fos positive brain structures were prepared by an experimenter naive to group treatments. Care was taken so that each structure was scored in approximately the same plane (one section per subject). Drawings were scored by two blinded raters who recorded the number and location of c-Fos positive nuclei. Scores across raters were averaged for statistical analyses.

2.4. Data analyses

Data from each study were analyzed using analyses of variance (ANOVA). In Experiment 1, 4-h food intake data
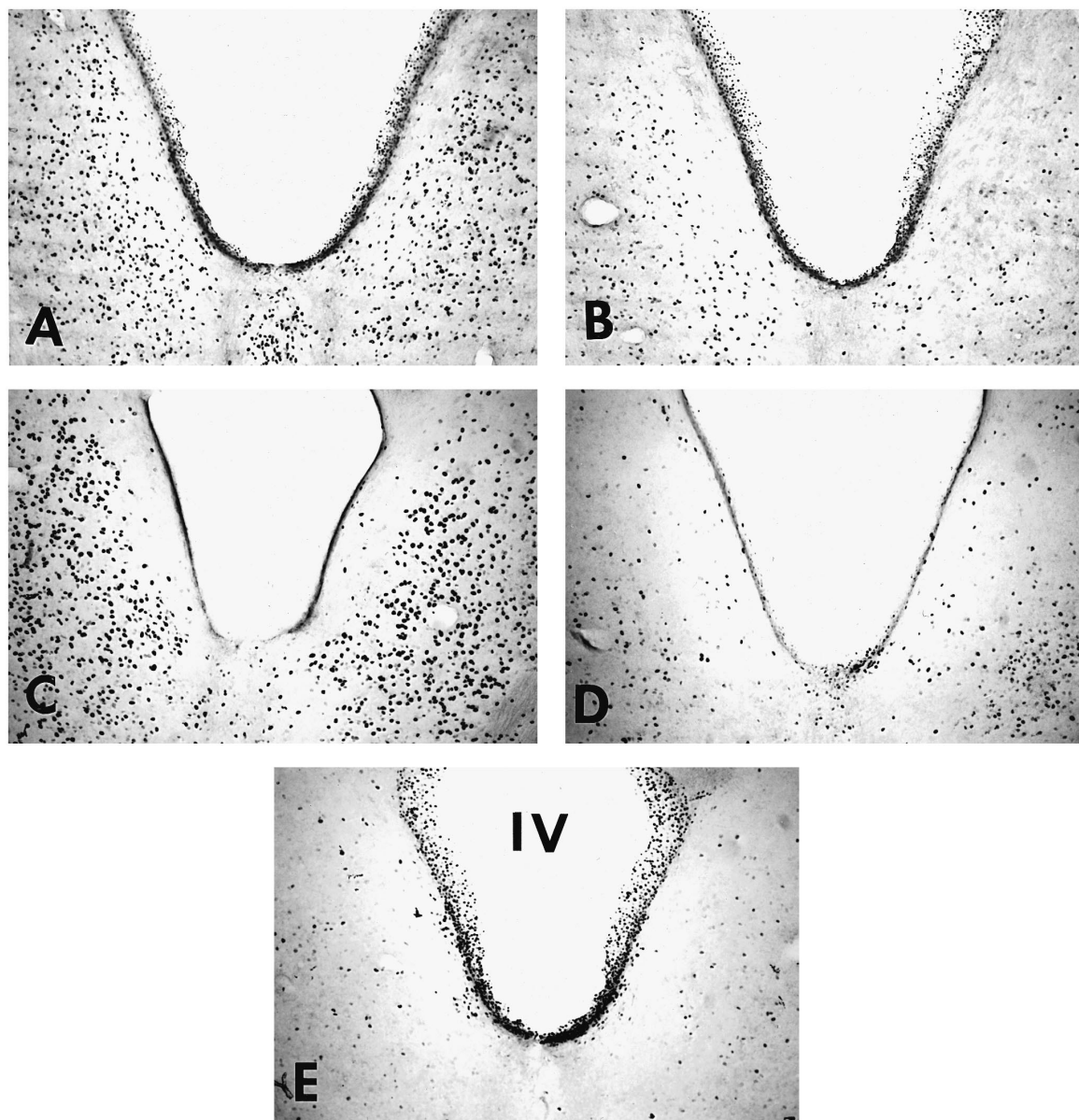

Fig. 3. Representative photomicrographs showing slices cut through the horizontal plane of the nucleus of the solitary tract. Shown are slices from animals (A) pretreated with s-CSF followed by GLP-1 (Group C/G); (B) pretreated with dHGex-4 followed by GLP-1 (Group E/G); (C) pretreated with s-CSF followed by LiCl (Group C/L); (D) pretreated with dHGex-4 followed by LiCl (Group E/L); and (E) pretreated with s-CSF followed by injection of isotonic saline (Group C/S). Also shown is the fourth ventricle (IV).

were analyzed using a two-way, 2×2 (Pretreatment $\times$ Treatment) ANOVA. A one-way ANOVA was run to analyze data from the HIT cell portion of Experiment 1 using insulin secretion (mU/ml) as the dependent measure. Because all groups needed to complete a two-way factorial design were not run in Experiment 2, one-way ANOVAs were run instead to compare groups using cFLI as the dependent measure. Separate ANOVAs were run to analyze cFLI data collected from each brain region. When significant differences were found, post hoc analyses were conducted using t-tests. In all cases, $P < 0.05$ (two-tailed) indicated statistical significance.

3. Results

3.1. Experiment 1: effects of exendin on GLP-1-induced HIT cell insulin secretion and anorexia

Average insulin secretion ($\pm$ S.E.M.) assessed from HIT cell cultures in each of the treatment conditions are presented in Table 1. Relative to glucose alone, insulin secretion was significantly increased in HIT cells treated with GLP-1 plus glucose. The addition of 10 nM of exendin-[9–39] slightly attenuated, while the addition of 100 nM of exendin-[9–39] completely blocked, GLP-1-induced augmentation of insulin secretion. Furthermore, both the addition of 10 nM and 100 nM of des His[1] Glu[9] exendin-4 completely blocked GLP-1-induced augmentation of insulin secretion from HIT cells. An ANOVA performed on the data was significant [$F(5,26) = 23.53$], and post hoc tests confirmed the conclusions.

Data illustrating 4-h food intake following i3vt infusions are presented in Fig. 1. Relative to Group C/C, rats pretreated with s-CSF followed by infusion of GLP-1 (Group C/G) had a significant reduction in 4-h food intake, thus providing evidence of GLP-1-induced anorexia. Pretreatment with dHGex-4 attenuated the ability of GLP-1 to reduce food intake, as intake of Group E/G did not differ significantly from that of the control group. Importantly, Group E/G ate significantly more food over 4 h than Group C/G. Finally, because Groups C/C and E/C did not differ reliably, the inference is that dHGex-4 alone did not modulate food intake. ANOVA performed on the data revealed a significant interaction between the pretreatment and treatment effects [$F(1,16) = 4.77$]. Post hoc tests revealed that while the pretreatment condition significantly influenced food intake between groups treated with GLP-1 (i.e., Groups C/G vs. E/G), the pretreatment condition had no influence on rats treated with s-CSF (i.e., Groups C/C vs. E/C).

3.2. Experiment 2: effects of dHGex-4 on drug-induced cFLI

Means nuclei positive for cFLI in the AP, the NTS, and the PBN are depicted in Fig. 2, and representative pho-

tomicrographs through the NTS are presented in Fig. 3. The groups pretreated with i3vt infusion of s-CSF and then treated with either i3vt infusion of GLP-1 (Group C/G) or i.p. injection of LiCl (Group C/L) had significantly more cFLI cells than the control group (C/S); this was the case in the AP (Fig. 2A), the NTS (Fig. 2B), and the PBN (Fig. 2C). However, relative to groups pretreated with s-CSF, those that were given i3vt infusion of dHGex-4 and then either i3vt infusion of GLP-1 (Group E/G) or i.p. injection of LiCl (Group E/L) had significant attenuation of drug-induced cFLI. Again, this was the case in each brain region in which cFLI was assessed. Finally, in both the AP and the NTS, cFLI levels in dHGex-4 pretreated groups did not differ reliably from the c-Fos expression that was observed in the control group (C/S). However, while dHGex-4 attenuated cFLI in the PBN of LiCl treated animals (Group E/L), this group did have greater levels of cFLI in the PBN when compared to Group C/S; Groups E/G and C/S also did not differ reliably. ANOVAs performed on cFLI data collected from the AP [$F(4,17) = 12.20$], the NTS [$F(4,17) = 12.51$], and the PBN [$F(4,17) = 15.72$] each attained significance. Furthermore, post hoc tests confirmed all conclusions discussed above.

4. Discussion

Central infusion of GLP-1 and peripheral administration of LiCl have been observed to produce similar effects, including similar patterns of cFLI in the rat brain [21,24]. The similar actions produced by these drugs led us to assess the hypothesis that the neuronal activation caused by peripheral injection of LiCl is mediated by central GLP-1 receptor signalling. Consistent with our hypothesis, results from Experiment 2 revealed that pretreatment with i3vt infusion of the GLP-1 receptor antagonist, des His[1] Glu[9] exendin-4, attenuated LiCl-induced cFLI in several brainstem regions. Thus, LiCl-induced cFLI in the brainstem is mediated, in part, by GLP-1 receptor signalling. Importantly, while i3vt infusion of the antagonist also blocked cFLI in the brainstem produced by i3vt infusion of GLP-1 (Experiment 2), this antagonist did not produce any independent effects on c-Fos expression or food intake. Furthermore, the results of Experiment 2 replicate the observation that i3vt infusion of GLP-1, and i.p. injection of LiCl, produce robust cFLI in several brainstem regions, including the NTS, the AP, and the PBN [21,24].

In Experiment 1, we provided the first in vivo demonstration that central infusion of the GLP-1 receptor antagonist, des His[1] Glu[9] exendin-4, blocks the ability of centrally infused GLP-1 to reduce short-term (4-h) food intake. Furthermore, because 10.0 μg of the antagonist completely blocked the ability of 10.0 μg of GLP-1 to produce anorexia, this antagonist is effective when administered in a 1:1 ratio with GLP-1. Thus, des His[1] Glu[9] exendin-4 appears to be a more potent GLP-1 receptor

antagonist than exendin-[9–39], since exendin-[9–39] must be administered in a concentration ten times stronger than GLP-1 in order to block GLP-1-induced anorexia [23]. The results from the HIT cell study confirm this greater potency of des His[1] Glu[9] exendin-4; only 10 nM of this antagonist were needed to completely inhibit insulin release caused by GLP-1 (1.0 nM), while a much larger concentration of exendin-[9–39] (100 nM) was needed to block fully the actions of GLP-1.

While the present evidence suggests that GLP-1-receptor signalling is involved with LiCl-induced neuronal activation in the brainstem, it is unclear what physiological actions associated with LiCl administration may be mediated by these central GLP-1 pathways. Because LiCl is an emetic agent that produces aversive side-effects, such as vomiting and CTA, one possibility is that GLP-1 is a fundamental and key mediator of the aversive symptoms caused by LiCl administration. If so, then the anorexia and other effects caused by GLP-1 could be the result of aversive side-effects. This is consistent with the recent report that every dose of GLP-1 that reduced 4-h food intake was also capable of supporting a conditioned taste aversion when administered via i3vt infusion to rats (in Ref. [25]; but see Ref. [20]). Furthermore, if GLP-1 pathways are involved with LiCl-induced CTA and/or anorexia, then pretreatment with the GLP-1 receptor antagonist should attenuate or even block these actions of LiCl. Although we are presently addressing these issues in our laboratory, preliminary data suggested that the GLP-1 receptor antagonist may weaken LiCl-induced taste aversions.

Importantly, however, it must be recalled that administration of LiCl produces a range of effects that are also associated with physiological systems involved with homeostatic regulation of food intake (e.g., inhibition of gastric emptying and anorexia; [1,5]) and which are not necessarily aversive in nature. Thus, a second possibility is that GLP-1 pathways mediate physiological actions involved with both systems of homeostatic regulation of food intake and with systems underlying physiologically aversive states (e.g., visceral illness).

GLP-1 may play a role in several physiological functions, with distinct anatomical loci within the brain where the peptide is released. Consistent with this, administration of GLP-1 directly into the PVN reduces food intake without producing a CTA [13]. While seemingly at odds with our previous observation that GLP-1 infused into the third ventricle produces CTAs at all doses that reduce food intake [25], peptides that are infused into the third ventricle interact with many brain areas including those near the fourth ventricle [11]. Hence, GLP-1-receptor signalling in the PVN may be critical for reducing food intake, while GLP-1-receptor signalling in the brainstem (e.g., in the NTS) my be critical for producing responses associated with aversive states. Further experiments will be required to sort out these possibilities.

Additional research is needed to verify what actions associated with LiCl administration are mediated by GLP-1 pathways. However, the present results may have important clinical implications. Although not yet conclusively established, GLP-1-receptor signalling may be critical for mediating at least some aspects of the visceral illness associated with LiCl administration. Thus, GLP-1 pathways may also be involved with common human manifestations of visceral illness, including the wasting and debility seen in patients suffering from cancer and AIDS, and their associated chemotherapy treatments. Hence, GLP-1 receptor antagonists may be useful for reversing some or all of these symptoms by increasing appetite and/or reducing the anorexia, nausea, and visceral illness occurring in a broad spectrum of clinical and pathologic conditions.

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