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The Role of Central Glucagon-Like Peptide-1 in Mediating the Effects of Visceral Illness: Differential Effects in Rats and Mice

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In rats, central administration of glucagon-like peptide-1 (GLP-1) elicits symptoms of visceral illness like those caused by the toxin lithium chloride (LiCl), including anorexia, conditioned taste aversion (CTA) formation, and neural activation in the hypothalamus and hindbrain including activation of brainstem preproglucagon cells. Most compellingly, pharmacological antagonists of the GLP-1 receptor (GLP-1R) block several effects of LiCl in rat. The major goal of these experiments was to further test the hypothesis that the central nervous system GLP-1 system is critical to the visceral illness actions of LiCl by using mice with a targeted disruption of the only described GLP-1R. First, we observed that, like the rat, LiCl activates preproglucagon neurons in wild-type mice. Second, GLP-1R −/− mice demonstrated normal anorexic and CTA responses to LiCl. To test the possibility that alternate GLP-1Rs mediate aversive effects, we examined the ability of GLP-1 to produce a CTA in GLP1R −/− mice. Although lateral ventricular GLP-1 produced a CTA in wild-type mice, it did not produce a CTA in GLP-1R −/− mice. Furthermore, the same GLP-1R antagonist that can block the aversive effects of LiCl in the rat failed to do so in the mouse. These results support the conclusion that in mouse, unlike in rat, GLP-1R signaling is not required for the visceral illness response to LiCl. Such species differences are an important consideration when comparing results from rat and mouse studies. (*Endocrinology* 146: 458–462, 2005)

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is a posttranslational product of proglucagon that is produced in the intestinal epithelium (1, 2) as well as by a discrete set of neurons in the mammalian hindbrain (3, 4). Rat studies implicate a role for the central GLP-1 system in mediating the responses to visceral illness. First, central GLP-1 administration produces a range of effects similar to those produced by ip administration of LiCl, a commonly used procedure for inducing visceral illness in experimental animals (5–7). These effects include anorexia (8, 9), pica (10), and the formation of conditioned taste aversions (CTA) (6). Second, LiCl and GLP-1 activate a similar pattern of Fos expression in the central nervous system (CNS), including neurons in the hindbrain, hypothalamus, and the central nucleus of the amygdala. Among the populations of neurons activated by LiCl are the GLP-1-producing cells in the nucleus of the solitary tract (11). Finally, central administration of the selective GLP-1R antagonist, des His[1] Glu[9] exendin-4, attenuates end points of both LiCl- and GLP-1-induced visceral illness in rats (10). Furthermore, site-specific administration of GLP-1R antagonists into the central nucleus of the amygdala can attenuate LiCl-induced taste aversions (12). Thus, the pharmacological evidence would suggest that GLP-1R activation is a critical component of the response to visceral illness in the rat. Although such results are consistent with the hypothesis that the CNS GLP-1 system is involved, this evidence comes from experiments in rats using specific GLP-1 receptor (GLP-1R) antagonists and does not provide direct evidence that GLP-1R activation is necessary for the visceral illness response.

Understanding the neural regulation of visceral illness is of great clinical importance because activation of this pathway occurs commonly in clinical medicine and is frequently limiting in many medical conditions. Based on our studies with GLP-1R antagonism and LiCl in rats, we hypothesized that GLP-1 plays a critical role in the coordinated responses to visceral illness. Although these initial results are compelling, there are limitations inherent in studies dependent on the use of pharmacological antagonists. Consequently, alternative approaches are important to validate the role of GLP-1 signaling in mediating the response to LiCl. To this end, we assessed the effects of LiCl in mice with a targeted disruption of the only identified GLP-1R (13). We predicted that mice lacking GLP-1Rs would have attenuated or absent responses to aversive agents such as LiCl. This prediction, however, was incorrect. As a consequence, subsequent experiments sought to understand the critical differences between the pharmacological studies in rats and the genetic studies in mice that support discrepant conclusions about the role GLP-1 signaling plays in visceral illness.

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Abbreviations: CNS, Central nervous system; CTA, conditioned taste aversion; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; icv, intracerebroventricular; LiCl, lithium chloride.

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Materials and Methods

Animals

CD-1 mice with a targeted deletion from the second to the fourth transmembrane-spanning regions of the GLP-1R (GLP-1R −/−) and the appropriate control CD-1 mice were generated at The Toronto General Hospital (Toronto, Ontario, Canada) (13) and shipped to the University of Cincinnati (Cincinnati, OH) where experiments 1–4 were done. Experiments 5 and 6 were performed with mice derived from the same breeding colony at Harvard Medical School (Boston, MA) using the appropriate wild-type controls (purchased from Charles River Laboratories, Wilmington, MA). Male mice over 8 wk of age were individually housed under controlled temperature and light (12-h light, 12-h dark cycle) in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. All animal procedures were approved by the Institutional Animal Care and Use Committees either at the University of Cincinnati or Harvard Medical School. There was no difference in the body weights among the groups of wild-type and GLP-1R −/− mice.

Experiment 1: Activation of proglucagon neurons by LiCl in wild-type mice

Ad libitum-fed wild-type mice were given ip injections of 2% body weight by volume of 0.15 M NaCl or 0.15 M LiCl. Two hours later, mice were anesthetized and perfused with 4% paraformaldehyde. Brains were removed, post fixed in 4% paraformaldehyde overnight, and dehydrated in 20% sucrose at 4 C. Brains were blocked in half coronally, and the hindbrains were cut into 25-μm coronal sections using a freezing microtome and refrigerated overnight in 0.1 M PBS with 0.02% sodium azide. After rinsing, the sections were quenched in 0.3% H_2O_2 for 10 min, rinsed again, and blocked in 0.1 M PBS with 0.02% sodium azide, 0.25% Triton X-100, and 3% normal donkey serum for 1 h at room temperature. Immediately after the blocking step, sections were incubated overnight in rabbit anti-Fos (Ab-5; Oncogene Research Products, Boston, MA; 1:50,000 in 0.1 M PBS with 0.02% sodium azide, 0.25% Triton X-100, and 3% normal donkey serum) at room temperature. After rinses, sections were incubated in biotinylated donkey antirabbit secondary (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:1000) for 1 h. Sections were then rinsed, incubated in ABC-elite reagents (Vector Laboratories, Burlingame, CA; 1:500), and rinsed again, and sections were incubated for 10 min in nickel cobalt-enhanced 3,3′ diaminobenzidine chromogen solution. The tissue was immediately processed for preproglucagon immunoreactivity. The sections were rinsed, quenched in 0.5% H_2O_2 for 10 min, rinsed again, and blocked in PBS plus 0.25% Triton X-100 and 1% normal donkey serum. Sections were incubated overnight in rabbit antioxyntomodulin (a peptide product of proglucagon cosecreted with GLP-1; 1:7000) (14) at room temperature. Sections were rinsed three times in 0.1 M Tris-HCl and 0.15 M NaCl (pH 7.5) with 0.05% Tween 20 and blocked in 0.5% Blocking Reagent (TSA Biotin System; PerkinElmer Life Sciences, Inc., Wellesley, MA) for 30 min. Tissue was then incubated in biotinylated donkey antirabbit secondary antibody (1:1000) for 1 h. Sections were rinsed, incubated in ABC-elite reagents (1:250) for 30 min, and washed three times for 5 min each. After washing, tissue was incubated for 10 min in biotinyl tyramide (1:50 in the Amplification Diluent; TSA Biotin System). After washes, tissue was incubated in ABC-elite reagents (1:250) for 30 min. The sections were washed and then treated for 10 min in a 3,3′ diaminobenzidine chromogen solution. After rinsing, sections were mounted on slides and coverslipped. Slides were analyzed by light microscopy. A preproglucagon-positive cell was considered double labeled if the nucleus stained for the blue-black precipitate from the Fos immunodetection.

Experiment 2: LiCl-induced anorexia in wild-type mice and GLP-1R −/− mice

Male wild-type and GLP-1R −/− mice (n = 8 per group) had food removed 1 h before lights off. Seventeen hours later, they received 2% of body weight of 0.15 M NaCl, or 0.5, 1.0, or 2.0% of body weight by volume of 0.15 M LiCl. Ten minutes after the injection, food was returned. Intake was measured 30 min later. Over a 10-d interval, each mouse received each of the four treatments in random order with 2 d between successive injections.

Experiment 3: LiCl-induced CTA in wild-type mice and GLP-1R −/− mice

Ad libitum-fed male wild-type and GLP-1R −/− mice (n = 8 per group) were given 1-h access to water at the same time each day until their intakes became consistent. On training d 1, each mouse was given 1-h access to one of two novel flavors (20% sucrose flavored with either grape or cherry Kool-Aid (Kraft, Northfield, IL) with half of each group receiving each flavor) instead of water. Immediately after access to the flavor, each mouse received an injection of either 0.15 M LiCl or 0.15 M NaCl (2% of body weight). On the next day, the mice had 1-h access to water, and on the day after that (d 3), each mouse was given the alternate novel flavor (grape or cherry Kool-Aid) and received the alternate injection (either LiCl or saline). An identical flavor/LiCl-saline sequence was repeated on d 5 and 7. Hence, each mouse had 2 d of exposure to each novel flavor and paired injection so that consumption of one of the flavors was always associated with LiCl and consumption of the other flavor was always associated with NaCl. On the test day (d 9), mice were given access to both flavors simultaneously for 1 h, and the intake of each measured.

Experiment 4: GLP-1-induced CTA in wild-type and GLP-1R −/− mice

Mice were fitted with a 28-gauge stainless steel guide cannula in the lateral ventricle (−1.0 mm lateral to bregma and −3.5 mm dorsal to skull). After a 7-d recovery period, *ad libitum*-fed male wild-type and GLP-1R −/− mice were given 1-h access to water at the same time each day until the intakes stabilized. On the first training day (d 1), each mouse was given 1-h access to a novel flavor (20% sucrose flavored with cherry Kool-Aid) instead of water. Immediately after access to the flavor, each mouse received either an intracerebroventricular (icv) injection of 5 μg GLP-1 (1-μl injection volume; wild-type, n = 6; and GLP-1R −/−, n = 7) or a mock icv injection (wild-type, n = 7; and GLP-1R −/−, n = 8). After a day of 1-h water access, the mice received a second 1-h exposure to the 20% sucrose cherry Kool-Aid solution followed by the matched icv injection. After another water access day, mice received the 20% sucrose cherry Kool-Aid solution, and 1-h intake was measured.

Experiment 5: Effect of GLP-1R blockade on LiCl-induced anorexia in wild-type mice

Male wild-type mice fitted with lateral ventricle cannulas (see details in experiment 4 methods) had food removed 1 h before lights off. After a 17-h fast, mice received an icv injection of either 10 μg des His^1 Glu^9 exendin-4 (1-μl injection volume) or a mock injection (n = 5 per group). Fifteen minutes after the icv treatment, mice received either 2% of body weight of 0.15 M NaCl or 0.15 M LiCl. Fifteen minutes after the ip injection, food was returned, and intake was measured 30 min later. Over a 6-d interval, each mouse received both of the two peripheral treatments in random order with 2 d between successive injections.

Experiment 6: Effect of GLP-1R blockade on GLP-1-induced anorexia in wild-type mice

Male wild-type mice (n = 5) with indwelling lateral ventricle cannulas (see details in experiment 4 methods) had food removed 1 h before lights off. Seventeen hours later, the mice received an icv injection of either 10 μg des His^1 Glu^9 exendin-4 (1-μl injection volume) or a mock injection. Fifteen minutes after the first injection, all mice received 5 μg GLP-1 icv (0.5-μl injection volume). Food was returned 15 min after the GLP-1 injection, and 1-h intake measurements were taken. After a 2-d recovery period, the paradigm was repeated such that each mouse received each condition in a within-subjects design.

Results

Experiment 1: Activation of proglucagon neurons by LiCl in wild-type mice

We administered LiCl to wild-type mice to determine whether LiCl activates central GLP-1-producing cells in the

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mouse as it does in the rat. Consistent with previous data obtained in rats (11), we observed LiCl activation of GLP-1 cells in the wild-type mouse (Fig. 1). Using a one-way ANOVA, we found that LiCl treatment of Fos activated significantly more preproglucagon-positive neurons compared with the saline-treated group ($F_{(3,12)} = 40.58$; $P < 0.0001$).

Experiment 2: LiCl-induced anorexia in wild-type mice and GLP-1R −/− mice

To determine whether the GLP-1R is necessary for LiCl-induced anorexia in mice, we measured food intake after NaCl or one of three doses (0.5, 1.0, and 2.0% of body weight) of LiCl in wild-type and GLP-1R −/− mice (Fig. 2). LiCl suppressed 30-min food intake significantly in both groups of mice at the highest dose, and the magnitude of LiCl-induced anorexia was similar in the wild-type and GLP-1R −/− mice. Although there was a tendency for the lower doses to suppress food intake in the GLP-1R −/− mice, these effects were not statistically significant. Using a two-way ANOVA, we found a main LiCl effect ($F_{(3,56)} = 3.299$; $P < 0.05$), no genotype effect ($F_{(1,56)} = 3.801$; $P > 0.05$), and no interaction ($F_{(3,56)} = 1.107$; $P > 0.05$). When the genotype groups were collapsed into saline and 2.0% LiCl groups only, the LiCl group was statistically different using a Tukey's t test ($t_{(30)} = 2.743$; $P < 0.05$ for each group). Thus, LiCl dose-dependently reduced 30-min food intake, with no difference between wild-type and GLP-1R −/− mice in this effect.

Experiment 3: LiCl-induced CTA in wild-type mice and GLP-1R −/− mice

To determine whether the GLP-1R is necessary for the formation of a LiCl-induced CTA, wild-type and GLP-1R −/− mice were given novel flavors paired either with LiCl or saline. Neither genotype demonstrated a clear preference for either flavor of Kool-Aid independent of the injections of saline or LiCl. On the test day (d 11), using a two-way ANOVA, we found a LiCl effect ($F_{(1,28)} = 21.53$; $P < 0.0001$), no genotype effect ($F_{(1,28)} = 0.0632$; $P > 0.05$), and no interaction ($F_{(1,28)} = 0.9660$; $P > 0.05$; Fig. 3). When the genotype groups were collapsed into saline-paired and LiCl-paired groups only, the LiCl-paired group of both genotypes drank significantly more of the NaCl-paired flavor than the LiCl-paired flavor using a Tukey's t test ($t_{(30)} = 5.268$; $P < 0.0001$ for each group). Hence, both genotype groups developed a CTA when administered LiCl, and this aversive action was not different between the wild-type and GLP-1R −/− mice.

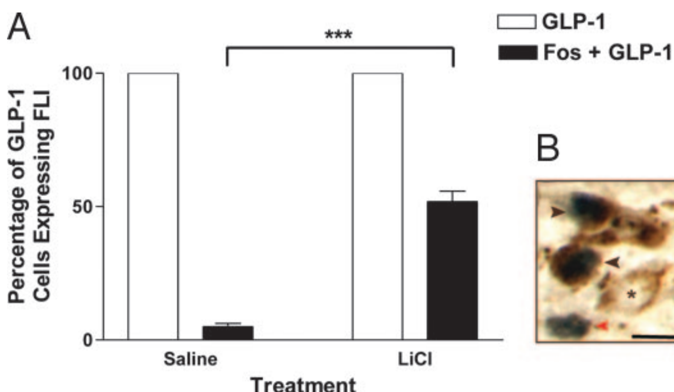

FIG. 1. A, Percentage of GLP-1-producing cells that are Fos activated in response to either saline or LiCl administration in wild-type mice. ***, $P < 0.0001$. B, Photomicrograph of the nucleus of the solitary tract (coronal plane) of a 0.15 M LiCl-treated (2% body weight by volume) wild-type mouse double-labeled for Fos (*purple nuclear staining*) and oxyntomodulin (*brown cytoplasmic staining*). The *black arrows* indicate Fos-activated GLP-1 cells, the *asterisk* points out a quiescent GLP-1 cell, and the *red arrow* shows an activated non-GLP-1 cell. *Scale bar*, 10 μm.

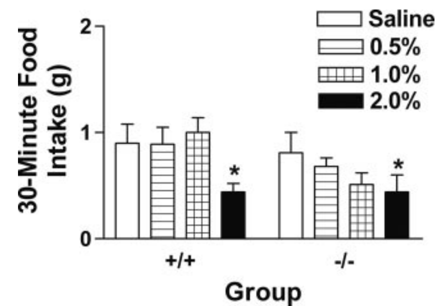

FIG. 2. Thirty-minute food intakes of wild-type (+/+) and GLP-1R −/− (−/−) mice taken after an injection of saline or one of three doses of LiCl. Each mouse received each of the four treatments. *, $P < 0.05$.

Experiment 4: GLP-1-induced CTA in wild-type and GLP-1R −/− mice

The effect of centrally administered GLP-1 to cause anorexia in both rats and mice has been published (10, 13). To determine whether the CNS GLP-1 system also mediates other responses to visceral illness in the mouse as it does in the rat (6), the ability of GLP-1 to elicit a CTA in wild-type mice was evaluated. Pairing central GLP-1 administration to a novel flavor caused wild-type mice to associate the flavor with illness and, therefore, to avoid it in further exposures compared with mice receiving no GLP-1 injections (1-h intake, $t_{(11)} = 6.531$; $P < 0.0001$; Fig. 4). In contrast, there was no effect of central GLP-1 to cause a CTA in GLP-1R −/− mice ($t_{(13)} = 1.520$; $P > 0.05$). This supports the selectivity of GLP-1 on the known GLP-1R in these animals to produce both anorexia and CTA.

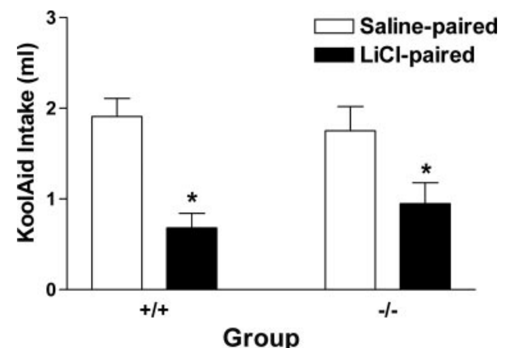

FIG. 3. One-hour Kool-Aid intake of wild-type (+/+) and GLP-1R −/− (−/−) mice after mice are simultaneously exposed to the saline-paired and LiCl-paired Kool-Aid flavors. *, $P < 0.05$.

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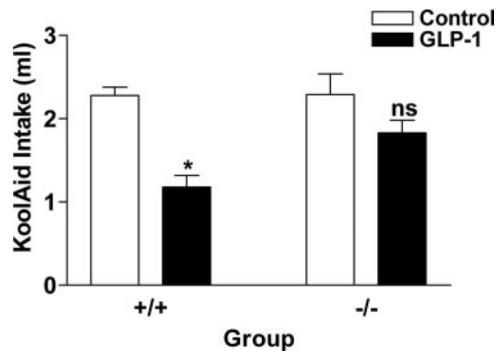

FIG. 4. One-hour Kool-Aid intake of wild-type (+/+) and GLP-1R −/− (−/−) mice that received a previous exposure to the flavor paired with control or icv GLP-1 treatment. *, $P < 0.05$; ns, not significant.

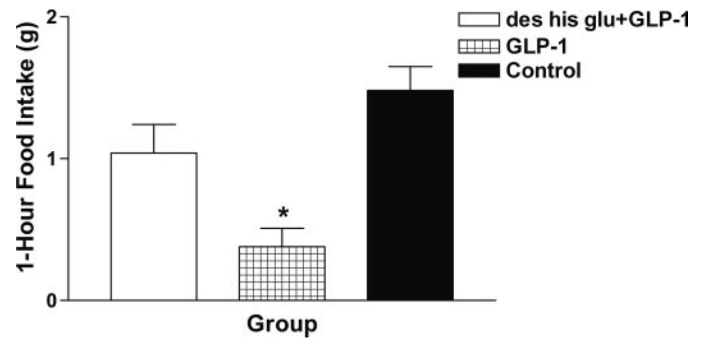

FIG. 6. One-hour food intakes of wild-type mice that received icv administration of GLP-1, GLP-1 after pretreatment with des His^1 Glu^9 exendin-4, or saline. *, $P < 0.05$.

Experiment 5: Effect of GLP-1R blockade on LiCl-induced anorexia in wild-type mice

The GLP-1R antagonist des His^1 Glu^9 exendin-4 was given to wild-type mice to determine whether the central GLP-1 system mediates effects of LiCl in mice as it does in rats. In the two-way ANOVA, there was a significant effect of LiCl to suppress food intake ($F_{(1,16)} = 20.15$; $P > 0.001$; Fig. 5) but no effect of GLP-1R blockade ($F_{(1,16)} = 0.5597$; $P > 0.05$) and, therefore, no interaction ($F_{(1,16)} = 0.3149$; $P > 0.05$). These data indicate that the GLP-1R antagonist, which attenuates LiCl end points in the rat (10), has no effect on LiCl anorexia in the mouse.

Experiment 6: Effect of GLP-1R blockade on GLP-1-induced anorexia in wild-type mice

The GLP-1R antagonist des His^1 Glu^9 exendin-4 was given to wild-type mice to determine whether des His^1 Glu^9 exendin-4 blocks the ability of exogenous GLP-1 to induce anorexia in mice as it does in rats. Using a one-way ANOVA followed by a *post hoc* Tukey's *t* test, we found that mice receiving control treatment followed by 5 μg GLP-1 exhibited significantly reduced 1-h food intake compared with the untreated controls ($t_{(9)} = 4.965$; $P < 0.001$; Fig. 6) and mice receiving pretreatment with 10 μg des His^1 Glu^9 exendin-4 ($t_{(8)} = 2.767$; $P < 0.05$). These results support the conclusion that des His^1 Glu^9 exendin-4 is an effective antagonist of the mouse GLP-1R. There was no difference in food intake between the controls and the mice receiving des His^1 Glu^9 exendin-4 ($P = 0.1256$).

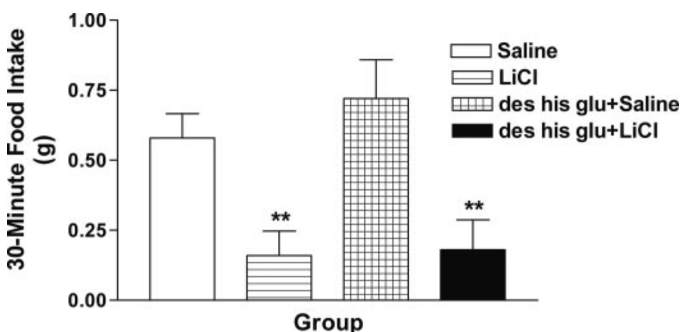

FIG. 5. Thirty-minute food intakes of wild-type mice receiving either a control or des His^1 Glu^9 exendin-4 icv pretreatment followed by injection of saline or 0.15 M LiCl. **, $P < 0.01$.

Discussion

These experiments tested the hypothesis that central GLP-1R signaling mediates the effects of peripheral LiCl in mice. The hypothesis was based on previously published work in rats demonstrating that GLP-1-producing neurons are activated in response to noxious stimuli (7, 11) and that central administration of GLP-1 elicits the same characteristic illness behaviors as peripheral LiCl, including anorexia and CTA formation (10), as well as our previous studies demonstrating that pharmacological antagonism of GLP-1R attenuates these physiological and behavioral responses to LiCl in rats (7, 10). Thus, we predicted that mice with targeted disruption of the GLP-1R would have absent or attenuated responses to LiCl.

Consistent with the hypothesis that the GLP-1 system has parallel functions in mediating visceral illness in rats and mice, mice exhibited comparable anorexia and CTA formation after either LiCl or central GLP-1 administration (Figs. 2–4). Additionally, our double-label immunohistochemistry in wild-type mice revealed that over 50% of the GLP-1-positive neurons in the nucleus of the solitary tract were also positive for Fos (Fig. 1). This is comparable to what has been reported in rat (11) and indicates that LiCl increases activity in GLP-1-producing neurons.

Although these experiments support the hypothesis that there is a similar role for GLP-1 signaling to mediate visceral illness in the mouse as has been demonstrated in the rat, the critical experiment is to test the response to LiCl in mice lacking GLP-1Rs. LiCl is equally capable of suppressing food intake or producing a CTA in GLP-1R −/− mice and their wild-type controls (Figs. 2 and 3). Thus, despite the data using pharmacological antagonists in the rat, GLP-1R signaling is not required for at least some of the symptoms of visceral illness produced by LiCl in mice.

The question that we must now face is how to reconcile the disparate conclusions derived from using pharmacological antagonists in rats and genetic disruptions in mice. As the use of genetic manipulations has increased, such discrepancies have become more common. One possibility is that the critical actions for GLP-1 to induce visceral illness are the result of binding to a receptor other than the GLP-1R targeted in

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these mice. This seems unlikely given that no other GLP-1Rs have been identified to date and that GLP-1 is ineffective to produce anorexia (13) and a CTA (Fig. 4) in GLP-1R −/− mice.

Another possibility is that mice with targeted disruption of GLP-1R compensate for the lack of GLP-1R signaling during development and recruit alternative systems to mediate the responses to visceral illness. To test this possibility, we assessed the ability of des His^1 Glu^9 exendin-4 to act as a GLP-1R antagonist. As in the rat, des His^1 Glu^9 exendin-4 blocked the ability of GLP-1 to produce anorexia in wild-type mice (Fig. 6). Knowing that des His^1 Glu^9 exendin-4 could act as a functional antagonist to the GLP-1R in the mouse, we could then test the ability of the GLP-1R antagonist to block the effects of LiCl in the mouse. Central administration of the GLP-1R antagonist was unable to block the effects of LiCl like it had in the rat (Fig. 5). This outcome suggests that GLP-1R signaling plays only a minor role in mediating behavioral responses to LiCl toxicity in mice and/or that there is considerable redundancy in the murine visceral illness pathway.

Taken together, these results indicate that there is a significant species difference between rats and mice in the organization of the response to visceral illness. Interestingly, this species difference is neither the result of differences in the ability of LiCl to increase activity of preproglucagon neurons (Fig. 1) nor the result of GLP-1 to produce symptoms of visceral illness (Fig. 4). Rather, the species difference is the result of differing levels of dependence on GLP-1R signaling to mediate the effects of aversive agents such as LiCl. One lesson from such an outcome is that generalizations between rat and mouse need to be done with great care. Although intermixing experiments in rat and mouse can greatly increase the ability to test specific hypotheses, the possibility for significant species differences cannot be ignored.

Needless to say, the relevant question arising from the current work is whether primates (including humans) are more similar to rats or mice in the central organization of the visceral illness response. Future research will need to address the organization and function of the GLP-1 system in the human CNS. The issue has added importance because several GLP-1-related therapies continue to progress toward the clinical treatment of type 2 diabetes. It is critical that we understand not only the role of the peripheral GLP-1 system to control insulin secretion but also the various roles of GLP-1 signaling pathways within the CNS.

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