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The Hypothalamic Glucagon-Like Peptide 1 Receptor Is Sufficient but Not Necessary for the Regulation of Energy Balance and Glucose Homeostasis in Mice

Pharmacological activation of the hypothalamic glucagon-like peptide 1 (GLP-1) receptor (GLP-1R) promotes weight loss and improves glucose tolerance. This demonstrates that the hypothalamic GLP-1R is sufficient but does not show whether it is necessary for the effects of exogenous GLP-1R agonists (GLP-1RA) or endogenous GLP-1 on these parameters. To address this, we crossed mice harboring floxed *Glp1r* alleles to mice expressing Nkx2.1-Cre to knock down *Glp1r* expression throughout the hypothalamus (GLP-1RKD$^{\Delta Nkx2.1cre}$). We also generated mice lacking *Glp1r* expression specifically in two GLP-1RA–responsive hypothalamic feeding nuclei/cell types, the paraventricular nucleus (GLP-1RKD$^{\Delta Sim1cre}$) and proopiomelanocortin neurons (GLP-1RKD$^{\Delta POMCcre}$). Chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice exhibited increased food intake and energy expenditure with no net effect on body weight. When fed a high-fat diet, these mice exhibited normal food intake but elevated energy expenditure, yielding reduced weight gain. None of these phenotypes were observed in GLP-1RKD$^{\Delta Sim1cre}$ and GLP-1RKD$^{\Delta POMCcre}$ mice. The acute anorectic and glucose tolerance effects of peripherally dosed GLP-1RA exendin-4 and liraglutide were preserved in all mouse lines. Chronic liraglutide treatment reduced body weight in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, but this effect was attenuated with high-fat diet feeding. In sum, classic homeostatic control regions are sufficient but not individually necessary for the effects of GLP-1RA on nutrient homeostasis.

Glucagon-like peptide 1 (GLP-1) is a gut-secreted peptide that augments glucose-dependent insulin secretion via a pancreatic GLP-1 receptor (GLP-1R) (1). Long-acting GLP-1R agonists (GLP-1RA) are used for treating type 2 diabetes (2). GLP-1RA also reduce food intake and body weight primarily by targeting the central nervous system (CNS) (3). Intracerebroventricular (ICV) injection of GLP-1RA reduce food intake (4–8) and body weight (9) in rodents. Furthermore, CNS deletion of the *Glp1r* attenuates the anorectic effect of the GLP-1RA liraglutide (3). Hypothalamic GLP-1R signaling has received particular attention as this region regulates energy balance as well as glucose and lipid metabolism (10,11). Peripheral administration of GLP-1RA stimulates hypothalamic neuronal activity (7,12,13). Injection of GLP-1RA to hypothalamic nuclei suppresses feeding in rats (5,14–16), and recent evidence suggests that the arcuate nucleus (ARC) mediates the anorectic effects of liraglutide (17). Furthermore, targeting liraglutide to the ventromedial hypothalamic (VMH) nucleus stimulates brown adipose tissue thermogenesis and adipocyte browning, suggesting that hypothalamic GLP-1R signaling also controls body weight via regulation of energy expenditure (EE) in rodents (15).

The studies described above primarily entailed pharmacological activation of the GLP-1R. Although this demonstrates that the hypothalamic GLP-1R is sufficient to modulate energy balance, it does not address whether it is necessary for the effects of endogenous GLP-1 or clinically utilized GLP-1RA on phenotypes associated with energy balance. ICV delivery of the GLP-1R antagonist exendin (9-39) (Ex9) stimulates food intake in fed rats and blocks the anorectic effects of peripherally

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METABOLISM

administered GLP-1RA, suggesting that the CNS GLP-1R is necessary for the feeding effects of endogenous and exogenous GLP-1RA (7,18). However, this does not identify the specific CNS regions that mediate the satiety effects of GLP-1 or therapeutic GLP-1RA. Furthermore, acute pharmacological approaches do not assess the long-term, day-to-day regulation of energy balance by the CNS GLP-1R. To address this, we generated mice lacking hypothalamic *Glp1r* expression by crossing mice expressing floxed *Glp1r* alleles with Nkx2.1-Cre mice. Nkx2.1-Cre mice exhibit Cre expression throughout the hypothalamus including in the ARC, paraventricular (PVN), VMH, dorsomedial (DMH), and lateral (LH) nuclei (19). As the PVN and ARC have been identified as GLP-1RA–responsive hypothalamic nuclei (14,17), we also generated mice lacking *Glp1r* expression specifically in the PVN and in proopiomelanocortin (POMC) neurons (ARC *Glp1r* expression is primarily in POMC neurons [14]) by crossing floxed *Glp1r* mice with Sim1-Cre and POMC-Cre mice, respectively (20,21). Using these models, we tested the hypothesis that disruption of hypothalamic *Glp1r* expression dysregulates energy balance and that the anorectic effects of peripherally administered GLP-1RA require the hypothalamic GLP-1R. As pharmacological studies suggest a role for the hypothalamic GLP-1R in the maintenance of glucose homeostasis (14), we also tested the hypothesis that disruption of hypothalamic *Glp1r* expression impairs glucose tolerance.

We demonstrate that knockdown of the hypothalamic *Glp1r* has no overall effects on net energy balance, glucose homeostasis, or the response to peripherally administered GLP-1RA. These findings highlight the complexity of CNS GLP-1R–mediated signals modulating feeding behavior and glucose handling and indirectly support a role for extrahypothalamic brain regions in GLP-1R–dependent control of energy homeostasis.

RESEARCH DESIGN AND METHODS

Animals and Housing

We generated mouse lines lacking *Glp1r* expression in the hypothalamus (GLP-1RKD$^{\Delta Nkx2.1cre}$), PVN (GLP-1RKD$^{\Delta Sim1cre}$), and POMC neurons (GLP-1RKD$^{\Delta POMCcre}$) by breeding floxed *Glp1r* mice (GLP-1R$^{f/f}$), generated as described previously (22), with Nk2 homeobox 1 (Nkx2.1)-Cre, single-minded homolog 1 (Sim1)-Cre, and POMC-Cre mice (The Jackson Laboratory, Bar Harbor, ME), respectively (19–21). Experiments were performed in male mice. Chow diet (Harlan Teklad #2016) studies were performed in 3- to 4-month-old animals. Following energy balance assessment, mice were placed on a 60% high-fat diet (HFD) (D12492; Research Diets, New Brunswick, NJ) for 12 weeks, such that HFD-fed studies were performed in 7- to 8-month-old animals. Animals were maintained on a 12:12-h light:dark cycle in facilities at Sanford Burnham Prebys Medical Discovery Institute (SBP) at Lake Nona or the University of Cincinnati (UC). All protocols were approved by the SBP and UC Institutional Animal Care and Use Committees.

Cannula Implantation

Cannulae were implanted as previously described (4). Guide cannulae (Plastics One, Roanoke, VA) targeted the PVN (0.7 mm caudal, 0.3 mm from midline, 5.0 mm ventral), ARC (2.3 mm caudal, 1.1 mm from midline, 5.6 mm ventral, 10° angle), or cortex (0.46 mm caudal, 2.0 mm from midline, 1.75 mm ventral). Verification of proper coordinates was made histologically following injection of 1% Evans-Blue dye. Mice recovered for 7 days before experimentation.

Validation of *Glp1r* Knockdown

Glp1r expression was assessed in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1R$^{f/f}$, and whole-body GLP-1R knockout mice (GLP-1RKO) mice by qRT-PCR. Total RNA was extracted from the hypothalamus using a column-based method (Zymo Research, Irvine, CA) and converted to cDNA (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA). *Glp1r* and ribosomal protein L32 mRNA transcript levels were determined using gene-specific probes in accordance with manufacturer's instructions using a StepOnePlus qPCR instrument (Applied Biosystems). qPCR primers (5′→3′) were as follows: *Glp1r*, F:GATGCTGCCCTCAAGT GGAT, R:ATGAGCAGGAACACCAGTCG and L32, F:ACAT TGCCCTGAATAGTGGT, R:ATCCTCTTGCCCTGATCCTT. qPCR was also performed in whole-brain homogenates excluding the hypothalamus. Gene expression levels were calculated as relative quantification using the $2^{-\Delta\Delta CT}$ method.

Glp1r knockdown in the PVN and ARC of GLP-1RKD$^{\Delta Sim1cre}$ and GLP-1RKD$^{\Delta POMCcre}$ mice was confirmed by RNA in situ hybridization. Whole brains were harvested, and tissue was formalin-fixed (24-h, 10% neutral buffered formalin) and paraffin-embedded (FFPE) and sectioned using a microtome. Five-micrometer coronal sections were stained with RNAscope probes (Advanced Cell Diagnostics, Newark, CA), according to manufacturer's instructions. Sections were treated with test (*Glp1r*) and control (positive, peptidylprolyl isomerase B; negative, dihydrodipicolinate reductase) RNA probes using a single-plex chromogenic-RED RNAscope 2.5 assay kit on a Bond RX automated in situ hybridization slide staining system (Leica Biosystems, Buffalo Grove, IL). The automated protocol included heat retrieval at 88°C for 15 min and protease retrieval at room temperature for 15 min. Stained slides were coverslipped and scanned using an Aperio ScanScope XT instrument (Leica Biosystems).

Body Composition Analysis and Assessment of Energy Balance

Lean, fat, and fluid mass were measured in 5-h fasted mice using a LF90II-TD NMR (Bruker, Billerica, MA). Body weights were recorded weekly over the 12-week HFD feeding period. Energy balance was assessed using a Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH). Animals were acclimated to the CLAMS 24 h prior to the start of experimentation. Forty-eight hour food intake, water intake, locomotor activity, EE, and respiratory exchange ratio (RER) were measured at 15-min intervals as previously described (4).

Food Intake Response to Centrally and Peripherally Administered GLP-1RA

Following cannulation, recovery, and acclimation, 18-h food intake was measured after PVN- or ARC-targeted delivery of 0.0025, 0.005, or 0.025 µg/100 nL GLP-1 or exendin-4 (Ex4) (R&D Systems, Minneapolis, MN) or artificial cerebrospinal fluid (ACSF) (100 nL bilateral) vehicle (Harvard Apparatus, Holliston, MA) in 5-h fasted mice just prior to the onset of the dark cycle at 1800. For peripheral administration, 16-h food intake was measured in mice treated with Ex4 (3 µg/kg BW, i.p.), liraglutide (200 µg/kg BW, s.c.; Novo Nordisk, Copenhagen, Denmark), or saline vehicle. Peripheral doses were chosen based on previously demonstrated anorectic efficacy (3,23).

Glucose Tolerance Tests

Glucose tolerance tests (GTTs) were performed on 5-h fasted mice. In chow-fed mice, 2 g/kg BW glucose was administered orally or intraperitoneally. In HFD-fed animals, 1 g/kg BW glucose was administered orally. GTTs were also performed in animals that received PVN- or ARC-targeted injections of Ex4 (0.025 µg/100 nL, bilateral) concurrent with an intraperitoneal glucose dose at $t = 0$. In additional experiments, GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta Sim1cre}$, and GLP-1R$^{f/f}$ mice were dosed with the GLP-1R antagonist Ex9 (50 µg, i.p.; American Peptide) or liraglutide (400 µg/kg, s.c.) 15 min (Ex9 studies) or 120 min (liraglutide studies) prior to an intraperitoneal GTT. Animals were fasted for 4 h, and glucose was administered at a fixed dose (200 µL 25% dextrose, Ex9 studies) or at 2 g/kg BW for liraglutide studies. Blood glucose measurements were made at the indicated time points. Glucose tolerance was calculated as area under the curve above baseline.

Body Weight Response to Peripheral Administration of Liraglutide

Liraglutide (200 µg/kg BW s.c., BID as described by Secher et al. [17]) or isotonic saline was administered for 14 days in chow- and HFD-fed mice, and body weight was measured daily. Body weight was also measured daily during a 7-day recovery period, over which animals received no injection.

Statistical Analyses

Data are presented as mean ± SEM. Differences between groups were determined by one-way ANOVA followed by Tukey or Newman-Keuls multiple comparison post hoc tests or by two-tailed t tests as appropriate. Statistical significance was set to $P < 0.05$.

RESULTS

Targeted Delivery of GLP-1 or Ex4 to the PVN and ARC Dose Dependently Suppresses Food Intake

Consistent with previous studies in rats (5,14,15), targeting GLP-1 to the PVN (Fig. 1A) but not the ARC (Fig. 1B) significantly reduced food intake in mice. Targeting Ex4 to the PVN (Fig. 1C) or ARC (Fig. 1D) potently suppressed food intake, particularly during the first 12 h after administration. These findings demonstrate that pharmacological activation of the PVN or ARC GLP-1R reduces

food intake in mice, although the magnitude and duration of the response depend on the GLP-1RA administered and region targeted.

Gene Expression of the Hypothalamic *Glp1r* Is Reduced by Cre-Lox Recombination

To test whether the hypothalamic GLP-1R is necessary for chronic maintenance of nutrient homeostasis, we bred floxed *Glp1r* mice with a line expressing Cre recombinase in multiple hypothalamic regions, Nkx2.1-Cre mice (19), to generate hypothalamic *Glp1r* knockout (GLP-1RKD$^{\Delta Nkx2.1cre}$) mice. *Glp1r* mRNA levels were significantly reduced in hypothalami from GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, although not to the same extent as in GLP-1RKO mice (Fig. 2A). *Glp1r* mRNA levels were unaffected in the cortex (nonhypothalamic control) of GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Fig. 2A). As the PVN and ARC have been shown to mediate the anorectic effects of pharmacological GLP-1RA, we also generated mice lacking *Glp1r* expression in the PVN (GLP-1RKD$^{\Delta Sim1cre}$ mice) and POMC neurons (GLP-1RKD$^{\Delta POMCcre}$ mice). Selective deletion in POMC neurons was chosen because the *Glp1r* is expressed in anorexigenic POMC but not in orexigenic NPY/AgRP neurons of the ARC (14). Knockdown of the *Glp1r* in GLP-1RKD$^{\Delta Sim1cre}$ mice was restricted to the PVN (Fig. 2C), and knockdown of the *Glp1r* in GLP-1RKD$^{\Delta POMCcre}$ mice was restricted to the ARC (Fig. 2B).

Disruption of *Glp1r* Expression in Nkx2.1-Expressing Neurons Elevates Food Intake and EE With No Alterations in Body Weight or Composition in Chow-Fed Mice

Total, lean, and fat mass were unaltered in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Fig. 3A), although there was a tendency ($P = 0.07$) for reduced fat mass relative to total body mass as shown by ANCOVA (Supplementary Table 1). Disruption of *Glp1r* expression in chow-fed GLP-1RKD$^{\Delta POMCcre}$ (Fig. 3B) and GLP-1RKD$^{\Delta Sim1cre}$ (Fig. 3C) mice also had no effect on body weight or composition. GLP-1RKD$^{\Delta Nkx2.1cre}$ mice displayed significantly elevated 48-h food (Fig. 3D) and water (Supplementary Fig. 1A) intake. Basal 48-h EE was also elevated in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Fig. 3G). This was not due to an appreciable increase in locomotor activity (Supplementary Fig. 1G), nor was there any change in the RER (Supplementary Fig. 1D). Increased EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice occurred independently from food intake or total body mass (Supplementary Table 1). These results indicate that the hypothalamic *Glp1r* plays a role in the regulation of basal food intake and EE.

Targeted Disruption of *Glp1r* Expression in POMC Neurons and the PVN Elicits Minor Effects on Energy Balance in Chow-Fed Mice

We next determined whether the elevated food intake and EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice was due to loss of *Glp1r* expression in POMC neurons or the PVN. Knockdown of *Glp1r* within POMC neurons conferred no changes in basal food intake (Fig. 3E) or EE (Fig. 3H). Although
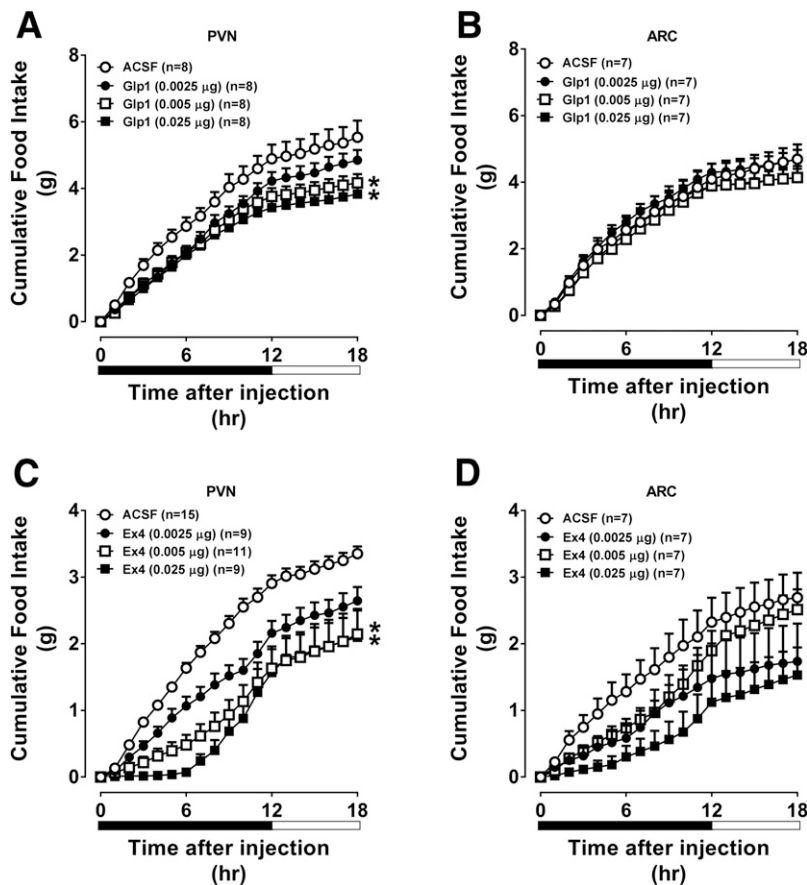
Figure 1—Direct injection of Ex4 into the PVN or ARC of the hypothalamus robustly suppresses food intake, whereas GLP-1 only modestly suppresses food intake. The values are mean ± SEM and represent the time-course of cumulative 18-h food intake in chow-fed C57BL/6J mice receiving GLP-1 or Ex4 (0.0025, 0.005, or 0.025 µg) or ACSF (100 nL bilateral [PVN] or unilateral [ARC]) in the PVN (*A* and *C*) or ARC (*B* and *D*). *n* = 7–15 mice/group. *$P < 0.05$ vs. ACSF.

knockdown of the GLP-1R specifically within the PVN conferred no changes in basal food intake (Fig. 3*F*), there was a significant decrease in EE (Fig. 3*I*). There were no significant effects on water intake, RER, or locomotor activity in GLP-1RKD$^{\Delta POMCcre}$ or GLP-1RKD$^{\Delta Sim1cre}$ mice (Supplementary Fig. 1).

Glp1r Knockdown in Nkx2.1-Expressing Neurons Protects Mice From HFD-Induced Weight Gain and Increased Fat Mass, and This Phenotype Is Associated With Elevated EE

We next determined whether the body composition and energy balance phenotypes observed in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice persist after HFD feeding. GLP-1RKD$^{\Delta Nkx2.1cre}$ mice displayed a significantly reduced HFD-induced weight gain and fat mass (Fig. 4*A* and *D*, respectively). In contrast to chow-fed animals, HFD-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice did not exhibit increased 48-h food intake (Fig. 4*G*). Despite this, 48-h water intake was significantly elevated in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Supplementary Fig. 2*A*). Forty-eight hour EE remained significantly elevated in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Fig. 4*J*), and this was independent of the difference in total body

weight, compared with controls (Supplementary Table 1). Elevated EE was not due to an appreciable difference in locomotor activity (Supplementary Fig. 2*G*). There was no difference in the RER (Supplementary Fig. 2*D*). Thus, loss of *Glp1r* expression in Nkx2.1-expressing neurons increases EE regardless of diet and independently of effects on food intake.

Disruption of Glp1r Expression in POMC Neurons and the PVN Elicits Variable Effects on Energy Balance in HFD-Fed Mice

We assessed whether the protection from HFD-induced obesity and increased EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice was due to loss of *Glp1r* expression in POMC neurons or the PVN. Contrary to GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, GLP-1RKD$^{\Delta POMCcre}$ mice displayed increased HFD-induced weight gain (Fig. 4*B*). However, there were no significant differences in lean or fat mass between genotypes (Fig. 4*E*). No differences in HFD-induced weight gain or body composition were observed in GLP-1RKD$^{\Delta Sim1cre}$ mice (Fig. 4*C* and *F*). Additional parameters of energy balance in these mice were unaltered (Fig. 4*H*, *I*, *K*, and *L* and Supplementary Fig. 2). These findings suggest that the energy balance phenotypes observed in
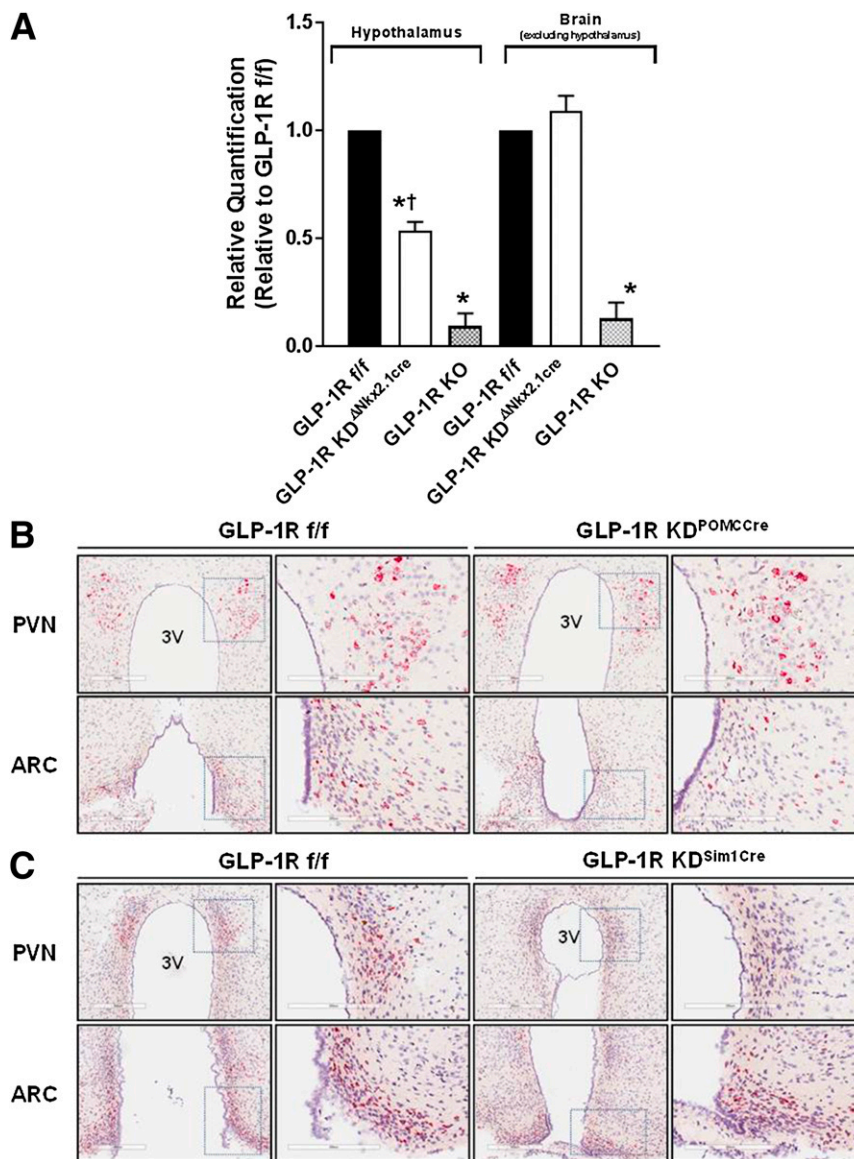
Figure 2—A: Cre-recombinase site-selectively knocks down the *Glp1r* in the hypothalamus. The values are mean $\pm$ SEM and represent gene expression levels of the *Glp1r* in RNA isolates from the hypothalamus or extrahypothalamic brain tissue of GLP-1R[f/f], GLP-1RKD[ΔNkx2.1cre], and GLP-1RKO mice as determined by qRT-PCR. $n = 5$ mice/group. *$P < 0.05$ vs. GLP-1R[f/f]. †$P < 0.05$ vs. GLP-1RKO. Representative histology images showing *Glp1r* RNA expression in PVN and ARC sections of GLP-1RKD[ΔPOMCcre] (*B*) and GLP-1RKD[ΔSim1cre] (*C*) mice, each compared with GLP-1R[f/f] controls. *Glp1r* RNA expression is indicated as a red, chromogenic signal. Images are shown at both 200 μm and 300 μm (magnified area designated by the dashed box). Scale bars are 200 μM and 300 μM for the lower and higher magnification panels, respectively. 3V, third ventricle.

GLP-1RKD[ΔNkx2.1cre] mice are not due to loss of *Glp1r* expression in the PVN or POMC neurons.

Hypothalamic *Glp1r* Knockdown Mice Exhibit a Normal Anorectic Response to Acute, Peripheral Administration of Ex4

We next tested the hypothesis that the loss of hypothalamic *Glp1r* expression blunts the acute anorectic effect of peripherally dosed GLP-1RA. Suppression of food intake by Ex4 was equivalent in chow-fed GLP-1RKD[ΔNkx2.1cre], GLP-1RKD[ΔPOMCcre], and GLP-1RKD[ΔSim1cre] (Fig. 5A–C), compared with their respective controls. Ex4 also suppressed food

intake similarly in HFD-fed GLP-1RKD[ΔNkx2.1cre], GLP-1RKD[ΔPOMCcre], and GLP-1RKD[ΔSim1cre] (Fig. 5D–F) mice, compared with controls. These findings show that the GLP-1R in Nkx2.1-expressing neurons and classic hypothalamic feeding centers (i.e., PVN and POMC neurons) is not required for the acute anorectic effects of peripherally administered GLP-1RA.

Loss of the *Glp1r* in Nkx2.1-Expressing Neurons Attenuates the Body Weight–Reducing Effect of Chronic Liraglutide Treatment Only in HFD-Fed Mice

Liraglutide does not reduce 24-h food intake in mice with CNS deletion of the GLP-1R (3). We tested the
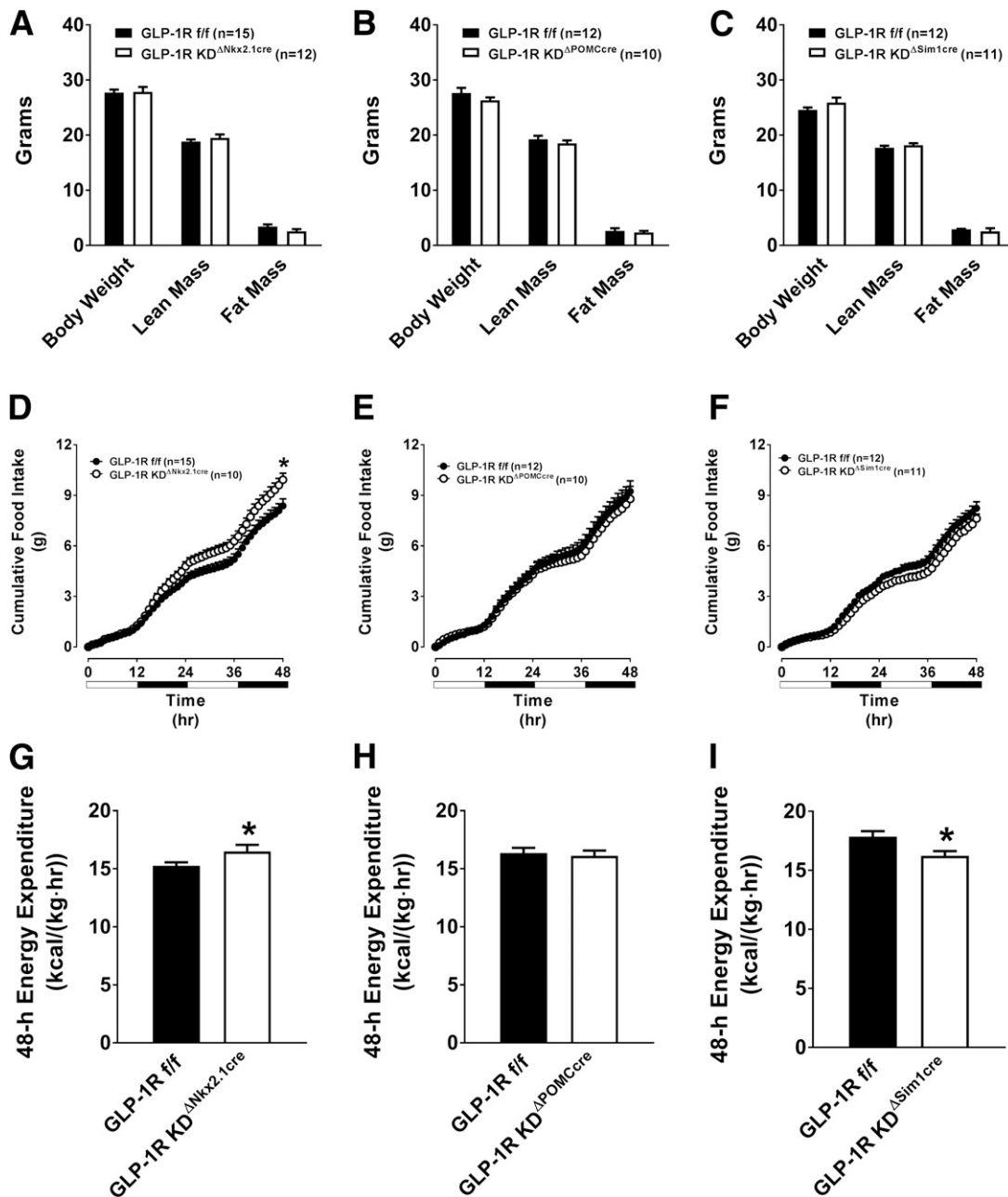
Figure 3—Chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice exhibit elevated food intake and EE, compared with GLP-1R$^{f/f}$ controls. The values are mean ± SEM and represent 5-h–fasted body composition (body weight, lean mass, and fat mass), cumulative 48-h food intake, and 48-h EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ ($n = 12$) (A, D, and G), GLP-1RKD$^{\Delta POMCcre}$ ($n = 10$) (B, E, and H), and GLP-1RKD$^{\Delta Sim1cre}$ ($n = 11$) (C, F, and I) mice, compared with GLP-1R$^{f/f}$ controls ($n = 12$–15). *$P < 0.05$ vs. GLP-1R$^{f/f}$.

hypothesis that this is due to loss of hypothalamic *Glp1r* expression. Loss of *Glp1r* expression in Nkx2.1-expressing neurons did not affect the acute food intake–suppressive effect of liraglutide (Fig. 6A). We then tested whether loss of hypothalamic *Glp1r* expression attenuates the body weight–reducing effects of a chronic dosing regimen of liraglutide. Secher et al. (17) recently demonstrated that the infusion of the GLP-1R antagonist Ex9 into the ARC partially attenuates the anorectic effects of

a 14-day liraglutide treatment. We show that the body weight–reducing effect of liraglutide was unaffected in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Fig. 6B). However, loss of hypothalamic *Glp1r* expression attenuated the weight-reducing effect of liraglutide in HFD-fed mice (Fig. 6C), suggesting that the hypothalamic GLP-1R is required for peripherally dosed liraglutide to exert its body weight–reducing effect during the metabolic stress of an HFD.
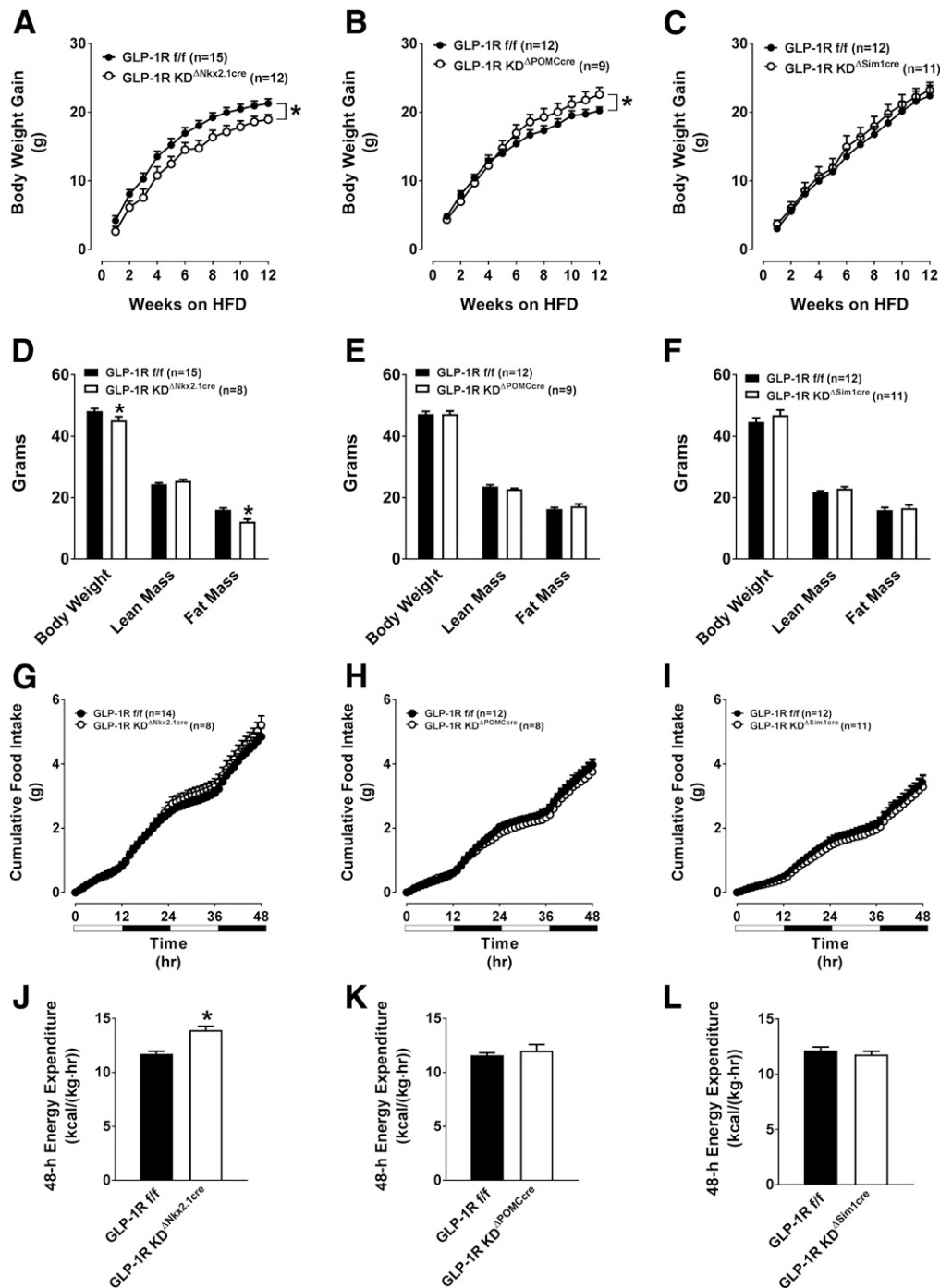
Figure 4—Disruption of the GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$, and GLP-1RKD$^{\Delta Sim1cre}$ mice differentially alters HFD-induced weight gain, body composition, and EE. The values are mean ± SEM and represent HFD-induced body weight gain, body composition (body weight, lean mass, and fat mass), cumulative 48-h food intake, and 48-h EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ (n = 12) (A, D, G, and J), GLP-1RKD$^{\Delta POMCcre}$ (n = 9) (B, E, H, and K), and GLP-1RKD$^{\Delta Sim1cre}$ (n = 11) (C, F, I, and L) mice, compared with GLP-1R$^{f/f}$ controls (n = 12–15). *$P < 0.05$ vs. GLP-1R$^{f/f}$.

Glucose Tolerance Is Improved by Pharmacological Activation of the GLP-1R in the PVN and ARC, but Loss of *Glp1r* Expression in These Brain Regions Does Not Impair Glucose Tolerance

Brain GLP-1R signaling modulates peripheral glucose production and/or utilization (14,24–27). We assessed the effect of targeted delivery of Ex4 to specific hypothalamic nuclei on glucose tolerance. An anorectic dose of Ex4 (0.025 μg) targeted to the PVN (Fig. 7A) or ARC (Fig. 7B), but not the cortex (Fig. 7C), improved glucose tolerance.

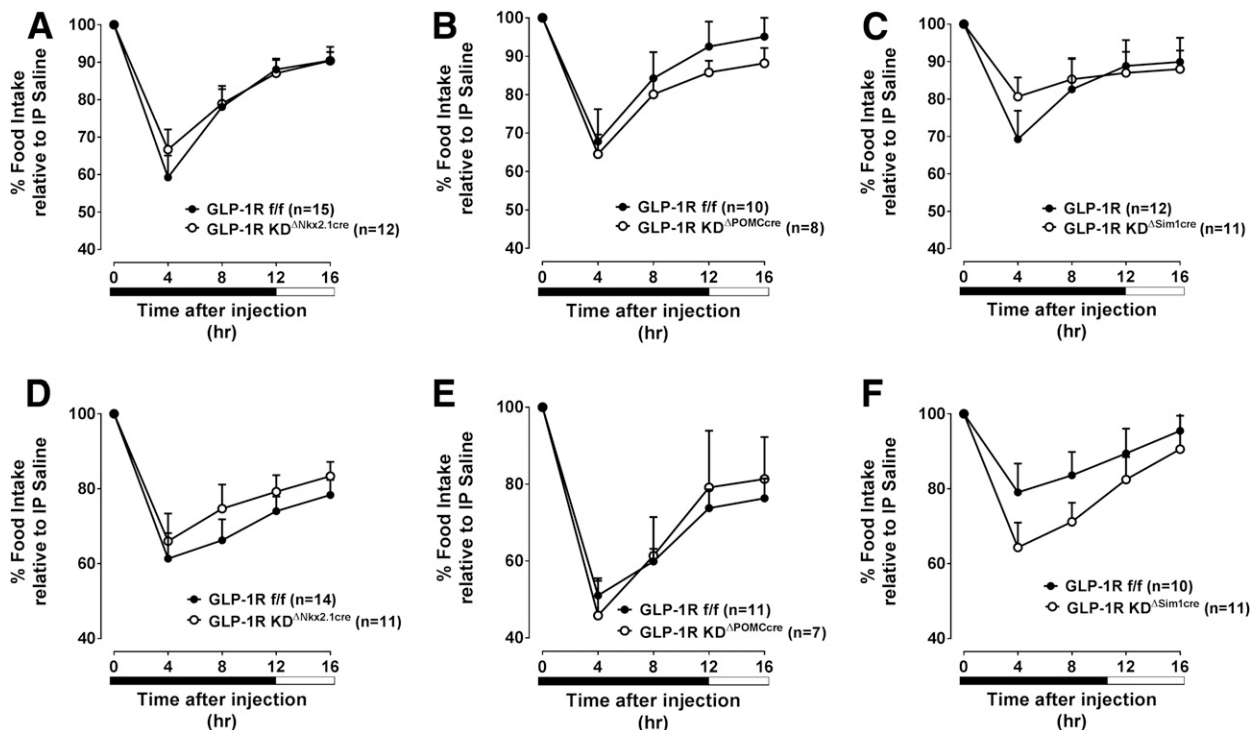We then assessed whether the loss of *Glp1r* expression in the Nkx2.1 neurons, PVN, or POMC neurons impairs

Figure 5—Disruption of the GLP-1R in Nkx2.1 neurons, POMC neurons, or the PVN does not blunt the food intake–suppressive effect of peripherally dosed Ex4. The values are mean ± SEM and represent 16-h food intake following treatment with Ex4 (3 μg, i.p.) in chow- or HFD-fed (top and bottom panels, respectively) GLP-1RKD$^{\Delta Nkx2.1cre}$ (n = 11–12) (A and D), GLP-1RKD$^{\Delta POMCcre}$ (n = 7–8) (B and E), and GLP-1RKD$^{\Delta Sim1cre}$ (n = 11) (C and F) mice, compared with GLP-1R$^{f/f}$ controls (n = 10–15). Data are shown at 4-h intervals and expressed as a percentage of the food intake response observed following treatment with vehicle.

glucose handling. Oral (Fig. 7D–F) and intraperitoneal (Fig. 7G–I) glucose tolerance was identical in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$, and GLP-1RKD$^{\Delta Sim1cre}$ mice, compared with respective controls. Similarly, oral glucose tolerance was unaffected by the loss of *Glp1r* expression in these brain regions in HFD-fed mice (Fig. 7J–L). Pretreatment with the GLP-1R antagonist Ex9 impaired glucose tolerance similarly in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice (Fig. 8A). Thus, although pharmacological activation of the hypothalamic GLP-1R improves glucose tolerance, GLP-1R in this brain region is not necessary for glucose handling.

Liraglutide Improves Glucose Tolerance Independently of the Hypothalamic GLP-1R
Neuronal-wide deletion of the GLP-1R does not affect the ability of liraglutide to improve glucose tolerance (3). However, global CNS deletion of GLP-1R could mask the contributions of specific brain regions to the regulation of glucose tolerance. Pretreatment with liraglutide improves intraperitoneal glucose tolerance similarly in both GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice (Fig. 8B), suggesting that the hypothalamic GLP-1R is not required for the glucoregulatory effect of peripherally administered liraglutide.

DISCUSSION
GLP-1RA suppress food intake and reduce body weight via food intake–regulatory brain regions including the hypothalamus and brain stem (17,28). We and others have shown that GLP-1RA targeted to the hindbrain (29) and hypothalamic nuclei, including the PVN (30), DMH (5), and ARC, reduce food intake. However, these acute pharmacological interventions do not reflect the physiological, long-term regulation of feeding by brain GLP-1R. The present studies assessed the impact of selectively knocking down hypothalamic *Glp1r* expression on overall energy balance. *Glp1r* expression was disrupted in Nkx2.1-expressing neurons, PVN, or POMC neurons to address whether these regions are necessary for the anorectic and glucoregulatory effects of GLP-1RA. We report that loss of *Glp1r* expression in these three regions does not affect energy balance or responsiveness to peripheral administration of GLP-1RA. This is surprising as targeted administration of GLP-1RA to these regions potently reduces food intake and improves glucose tolerance. These findings demonstrate that classic GLP-1RA–responsive hypothalamic neurons are not necessary for the anorectic and glucoregulatory effects of GLP-1RA.

The GLP-1R is expressed in extrahypothalamic food intake–regulatory regions, including the central amygdala, hindbrain, and area postrema (31). Several studies have
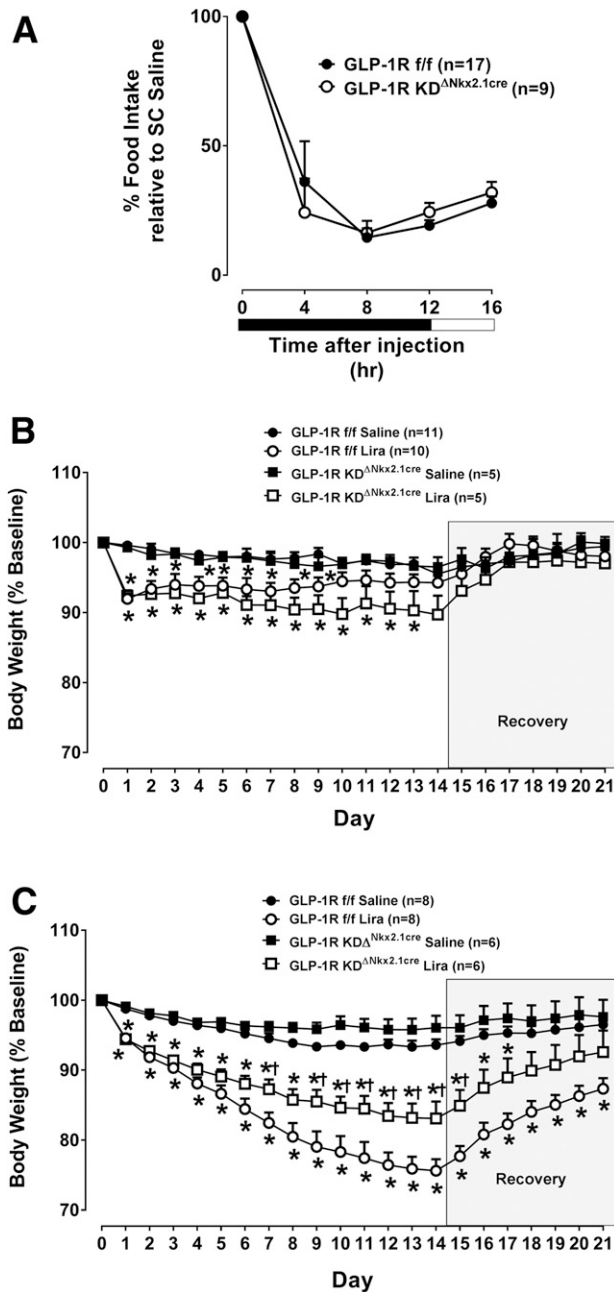
Figure 6—Disruption of the GLP-1R in Nkx2.1 neurons does not blunt the food intake–suppressive effect of peripherally dosed liraglutide but does impact the compound's body weight–lowering effect in HFD-fed mice. *A*: The values are mean ± SEM and represent 16-h food intake following treatment with liraglutide (Lira) (200 μg, s.c.) in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ ($n = 9$) mice, compared with GLP-1R$^{f/f}$ controls ($n = 17$). Data are shown at 4-h intervals and expressed as a percentage of the food intake response observed following treatment with vehicle. *B* and *C*: The values are mean ± SEM and represent daily body weight over the course of 21 days in chow-fed (*B*) or HFD-fed (*C*) GLP-1RKD$^{\Delta Nkx2.1cre}$ ($n = 5$–6), compared with GLP-1R$^{f/f}$ controls ($n = 8$–11) treated with liraglutide. Data are expressed as a percentage of baseline (i.e., prior to liraglutide treatment) body weight. On days 0–13, morning body weight was measured, and animals received a twice-daily injection of liraglutide (200 μg/kg BW, s.c.) or vehicle. On recovery days 14–21, only morning body weight was measured. *$P < 0.05$ vs. saline. †$P < 0.05$ vs. GLP-1R$^{f/f}$.

also shown that targeting GLP-1RA to reward regions, including the ventral tegmental area (32), nucleus accumbens (33), and lateral parabrachial nucleus (LPBN) (34), suppresses food intake. Thus, CNS GLP-1R signaling could reduce food intake and body weight by decreasing the hedonic value of food (35). Anorectic effects of GLP-1RA may also be secondary to homeostatic stress (36,37). GLP-1 signaling in the PVN and hindbrain regulates the behavioral, autonomic, and neuroendocrine responses to stress by activating both the "fight or flight" response acutely and the hypothalamic-pituitary-adrenal axis chronically (36–40). Moreover, the central GLP-1R system is activated in response to stressful stimuli, which can themselves reduce food intake and elevate blood glucose (37,41). The GLP-1R is also expressed in peripheral vagal afferent neurons (42), yet the extent to which these neurons relay gut-derived GLP-1R signals to the brain to mediate satiating and glucoregulatory responses remains controversial. Kanoski et al. (18) demonstrated that the anorectic effects of peripherally dosed Ex4 and liraglutide involve the activation of GLP-1R both on vagal afferents and in the CNS, whereas Secher et al. (17) report that subdiaphragmatic vagal afferent deafferentation does not impact the food intake– and body weight–lowering effects of peripherally dosed liraglutide.

GLP-1RKD$^{\Delta Nkx2.1cre}$ mice display elevated 48-h food intake and EE. The net effect likely explains the absence of a body weight effect in these mice. Knockdown of the GLP-1R in the PVN or POMC neurons did not recapitulate the increased food intake and EE phenotypes observed in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, suggesting that alternative *Glp1r*-expressing hypothalamic regions, such as the VMH, DMH, or LH (31), may be involved in regulating these processes. We then hypothesized that the loss of hypothalamic *Glp1r* would exacerbate the metabolic derangements of HFD feeding. On the contrary, GLP-1RKD$^{\Delta Nkx2.1cre}$ mice were protected from HFD-induced weight and fat mass gain. This was not due to reduced food intake. Instead, the elevated EE observed in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice persisted with HFD feeding, demonstrating that loss of hypothalamic *Glp1r* expression increases EE independently of food intake. This is surprising because ICV or hypothalamic administration of GLP-1RA increases EE (15,43). Loss of hypothalamic *Glp1r* expression may increase sensitivity to GLP-1 in other brain regions, thus elevating EE. However, whole-body *Glp1r* KO mice also exhibit increased EE (44). Paradoxically, loss of POMC *Glp1r* expression increased HFD-induced weight gain, suggesting distinct roles for GLP-1R signaling in different hypothalamic regions. Elevated HFD-induced weight gain in GLP-1RKD$^{\Delta POMCcre}$ mice was not associated with significant changes in body composition, food intake, or EE. One potential explanation is that weight gain was measured in ad libitum–fed conditions, whereas body composition was measured in 5-h fasted mice. Thus, POMC GLP-1R may regulate the handling of nutrient stores during fasting. Unlike
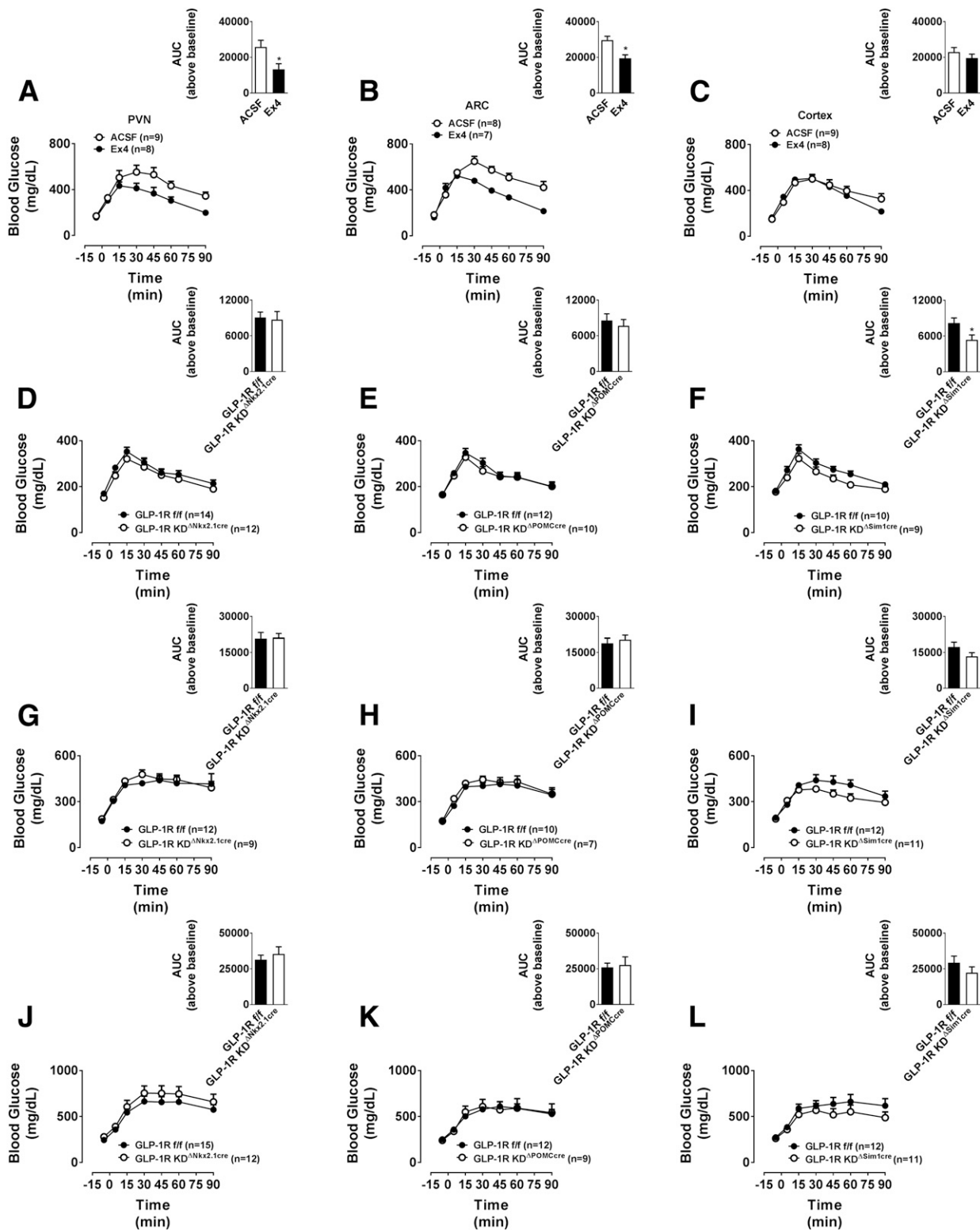
Figure 7—Direct injection of Ex4 into the PVN or ARC of the hypothalamus robustly improves glucose tolerance, whereas disruption of the hypothalamic GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$, and GLP-1RKD$^{\Delta Sim1cre}$ mice does not affect glucose tolerance. The values are mean ± SEM and represent glucose excursion in chow-fed C57BL/6J mice following a gavage of glucose (2 g/kg BW, i.p.) and treatment with Ex4 (0.025 μg) or ACSF (100 nL) in the PVN (n = 8–9) (A), ARC (n = 7–8) (B), or cortex (n = 8–9) (C) at t = 0 min. Inset: Area under the curve (AUC) above baseline for each group. *P < 0.05 vs. ACSF. The values are mean ± SEM and represent glucose excursion in chow- or HFD-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n = 9–12) (D, G, and J), GLP-1RKD$^{\Delta POMCcre}$ (n = 7–10) (E, H, and K), and GLP-1RKD$^{\Delta Sim1cre}$ (n = 9–11) (F, I, and L) mice, compared with GLP-1R$^{f/f}$ controls (n = 10–15). D, E, and F: chow diet, oral GTT; G, H, and I: chow diet, intraperitoneal GTT; J, K, and L: HFD, oral GTT. Inset: AUC above baseline for each group. *P < 0.05 vs. ACSF.
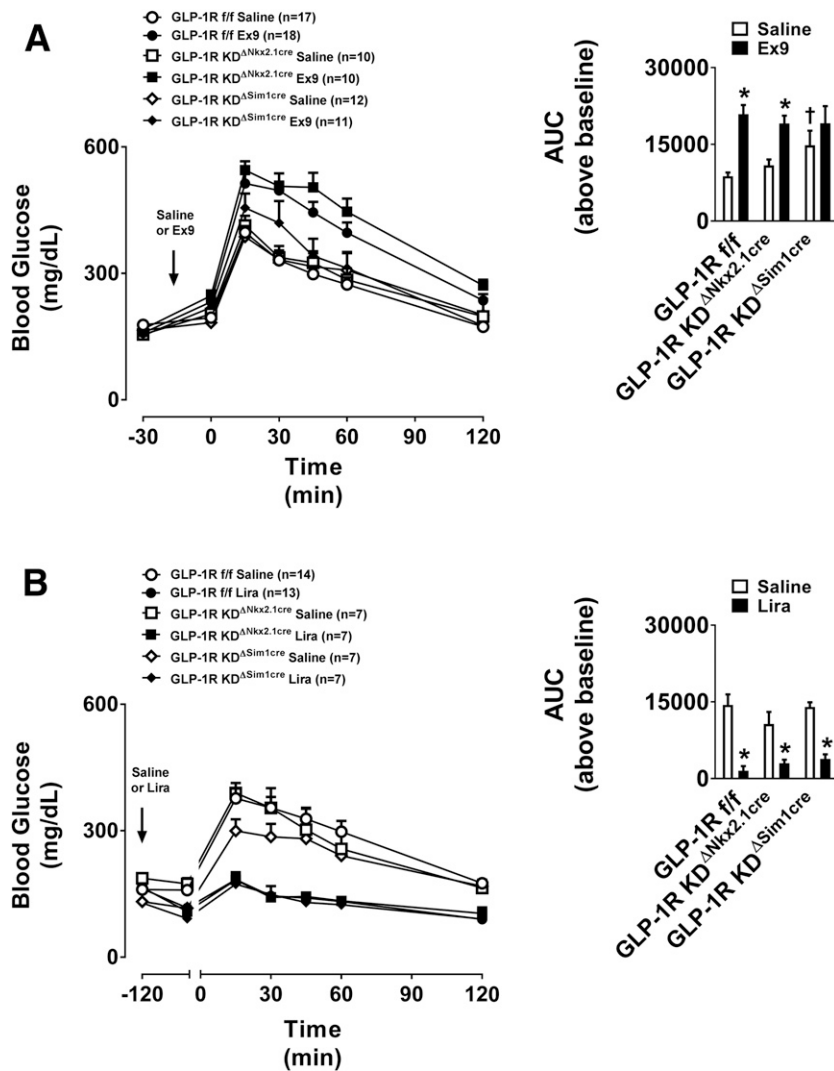
Figure 8—Disruption of the GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice does not impact glucose tolerance following pretreatment with Ex9 or liraglutide (Lira). The values are mean ± SEM and represent glucose excursion in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ ($n = 7$–10) and GLP-1RKD$^{\Delta Sim1cre}$ ($n = 7$–12) pretreated with Ex9 (50 μg, i.p.) vs. vehicle (*A*) or liraglutide (400 μg/kg BW, s.c.) vs. vehicle (*B*) 15 min (for Ex9 studies) or 120 min (for liraglutide studies) prior to an intraperitoneal glucose challenge. Inset: Area under the curve (AUC) above baseline for each group. *$P < 0.05$ vs. saline. †$P < 0.05$ vs. GLP-1R$^{f/f}$.

Nkx2.1 neuron- and POMC-specific *Glp1r* knockdown models, GLP-1RKD$^{\Delta Sim1cre}$ mice displayed no effects on body composition or energy balance parameters. These observations highlight the complexity of brain GLP-1R actions on the maintenance of energy balance and suggest that different hypothalamic nuclei mediate distinct effects of GLP-1, consistent with previous findings demonstrating nuclei-specific effects of GLP-1R signaling in visceral illness and hypothalamic-pituitary-adrenal axis activation (16).

Peripheral administration of GLP-1RA reduces food intake and body weight, likely due, in part, to their ability to cross the blood-brain barrier (17,45). Indeed, disruption of pan-neuronal *Glp1r* expression inhibits the anorectic effects of peripherally administered liraglutide (3). We hypothesized that this is due to the loss of hypothalamic *Glp1r* expression. Surprisingly, the acute anorectic effects of peripherally dosed Ex4 and liraglutide were preserved in our hypothalamic GLP-1RKO models. We then tested whether the hypothalamic GLP-1R mediates the long-term weight loss effects of GLP-1RA. Targeted delivery of the GLP-1R antagonist Ex9 to the ARC attenuates the weight loss effect of a 14-day liraglutide dosing regimen (17). In the present studies, chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice displayed similar weight loss as controls following a 14-day liraglutide treatment but were refractory to liraglutide-induced weight loss when fed an HFD. Importantly, in both the present studies and those by Secher et al. (17), pharmacological or genetic blockade of the ARC or POMC GLP-1R did not prevent the initial weight loss induced by liraglutide. These findings implicate other hypothalamic regions and/or extrahypothalamic regions as mediators of the anorexigenic effects of GLP-1RA.

Modulation of the brain GLP-1R affects glucose production and utilization under hyperinsulinemic conditions (24,46). We show that glucose tolerance is identical in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta Sim1cre}$, and GLP-1RKD$^{\Delta POMCcre}$ mice, compared with controls. Moreover, Ex9 impaired glucose tolerance in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice, suggesting that these GLP-1R populations do not mediate Ex9-induced impairments in glucose homeostasis. This is distinct from observations in β-cell GLP-1RKO mice in which Ex9 does not impair glucose tolerance (47). Liraglutide improved glucose tolerance in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice to similar degrees as their respective controls. These observations were surprising considering that targeted delivery of Ex4 to the PVN and ARC improved glucose tolerance. Nevertheless, these results support observations by Sisley et al. (3) that loss of CNS GLP-1R expression does not affect glucose tolerance or the glucoregulatory effects of liraglutide.

Although the Cre lines in these studies primarily target hypothalamic neurons, there are interpretative caveats, including incomplete Cre expression and off-target effects. We achieved only a ~50% reduction in *Glp1r* expression in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice. One potential explanation is that Nkx2.1 is expressed in GABAergic but not glutamatergic neurons (48), raising the possibility that GLP-1R may remain expressed in hypothalamic glutamatergic neurons. Sim1 is not only expressed in the PVN, but it is also enriched in the cerebellum, midbrain, and hippocampus (21). Although enriched in the ARC, POMC is also expressed in the hippocampus and hindbrain (20). Moreover, there are non-POMC and non-NPY/AgRP ARC neurons that are GLP-1R positive (14).

These studies demonstrate that GLP-1R expression within two hypothalamic regions typically assigned a prominent role in regulating energy balance, the ARC and PVN, is sufficient but not necessary for the effects of GLP-1RA on energy balance and highlights the importance of combining targeted pharmacological and genetic approaches to unravel the complex mechanisms by which GLP-1RA maintain nutrient homeostasis. The identification of overlapping CNS sites of action and signaling pathways for GLP-1R–mediated regulation of homeostatic and hedonic aspects of feeding behavior may inform the development of more effective therapies for type 2 diabetes and obesity.

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