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Glucagon-Like Peptide 1 and Its Analogs Act in the Dorsal Raphe and Modulate Central Serotonin to Reduce Appetite and Body Weight

Diabetes 2017;66:1062–1073 | DOI: 10.2337/db16-0755

Glucagon-like peptide 1 (GLP-1) and serotonin play critical roles in energy balance regulation. Both systems are exploited clinically as antiobesity strategies. Surprisingly, whether they interact in order to regulate energy balance is poorly understood. Here we investigated mechanisms by which GLP-1 and serotonin interact at the level of the central nervous system. Serotonin depletion impaired the ability of exendin-4, a clinically used GLP-1 analog, to reduce body weight in rats, suggesting that serotonin is a critical mediator of the energy balance impact of GLP-1 receptor (GLP-1R) activation. Serotonin turnover and expression of 5-hydroxytryptamine (5-HT) 2A (5-HT_{2A}) and 5-HT_{2C} serotonin receptors in the hypothalamus were altered by GLP-1R activation. We demonstrate that the 5-HT_{2A}, but surprisingly not the 5-HT_{2C}, receptor is critical for weight loss, anorexia, and fat mass reduction induced by central GLP-1R activation. Importantly, central 5-HT_{2A} receptors are also required for peripherally injected liraglutide to reduce feeding and weight. Dorsal raphe (DR) harbors cell bodies of serotonin-producing neurons that supply serotonin to the hypothalamic nuclei. We show that GLP-1R stimulation in DR is sufficient to induce hypophagia and increase the electrical activity of the DR serotonin neurons. Finally, our results disassociate brain metabolic and emotionality pathways impacted by GLP-1R activation. This study identifies serotonin as a new critical neural substrate for GLP-1 impact on energy homeostasis and expands the current map of brain areas impacted by GLP-1R activation.

Glucagon-like peptide 1 (GLP-1), a peptide produced in the brain and in the intestine, is a critical regulator of energy balance; its glucoregulatory and antiobesity properties are currently successfully used in the clinic (1–3). Although the ability of GLP-1 and its stable analogs, exendin-4 (EX4) for example, to reduce food intake is well established, the brain mechanisms governing GLP-1 receptor (GLP-1R)–induced anorexia are still poorly understood.

Serotonin has long been explored for its potential as an antiobesity treatment, with a mixed success history, primarily due to the troublesome side effects of drugs broadly affecting the serotonin system (4). Lorcaserin (Belviq) is currently the only serotonin receptor–activating antiobesity pharmaceutical that is still approved for clinical use; it acts as a 5-hydroxytryptamine (5-HT) 2C (5-HT_{2C}) agonist while, to a lesser extent, also activating 5-HT_{2A} receptors. In line with its anorexic and weight-reducing roles, central injections of serotonin or its precursor reduce food intake (5,6). Conversely, brain serotonin depletion leads to hyperphagia and obesity (7,8), although this effect is not always replicated (9,10). Serotonin signals

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Received 22 June 2016 and accepted 2 January 2017.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-0755/-/DC1.

R.H.A. and J.E.R. contributed equally to the study.

through at least 18 receptors; thus far, the 5-HT_{2C} receptor received most attention for its anorexic and weight loss impact (4,11,12).

Surprisingly little is known about the interaction of the central GLP-1 and serotonin systems. However, existing data suggest that an interaction is possible: *1*) GLP-1 and EX4 have been shown to dose-dependently release serotonin from rat hypothalamic synaptosomes (13); *2*) GLP-1Rs have been identified in the dorsal raphe (DR) nucleus, a nucleus harboring cell bodies of serotonergic neurons supplying serotonin to many forebrain sites, including the hypothalamus (14–16); and *3*) a molecular mechanism for the induction of serotonin by GLP-1R activation is suggested by a recent study (17) showing that, at the level of the colon, EX4 attenuates hyperalgesia by increasing colonic serotonin production. At the level of the colon, the GLP-1-serotonin interaction may be reciprocal since serotonin or serotonin 5-HT_{1B} receptor agonists also increase GLP-1 secretion from the enteroendocrine cells (18,19).

Here we used several methodological approaches to determine whether the two clinically relevant antiobesity systems interact and to identify the neuroanatomical mechanism of this interaction. The behavioral, neuropharmacological, electrophysiological, and neuroanatomical results obtained here support a direct impact of central GLP-1 activation to increase central serotonin neurotransmission, a relationship discovered to be critical for GLP-1–induced weight loss or weight loss maintenance and hypophagia.

RESEARCH DESIGN AND METHODS

Animals

Adult male Sprague-Dawley rats, weighing 200–250 g (Charles River Laboratories, Düsseldorf, Germany) were housed in individual plastic cages under a 12-h dark/light cycle at 20°C and 50% humidity. Adult female and male mGLU-124 Venus yellow fluorescent protein (YFP) transgenic mice (YFP-PPG mice; University of Cambridge, Cambridge, U.K.) (20) were housed in plastic cages. Water and standard chow were available ad libitum. All studies were carried out with ethical permissions from the Animal Welfare Committee of the Göteborg University (permission 195–13), in accordance with legal requirements of the European Community (Decree 86/609/EEC).

Drugs

GLP-1(7-36), EX4 (GLP-1R agonist), Exendin (9-39) (Ex9-39; GLP-1R antagonist), *p*-chlorophenylalanine (PCPA), *R*-96544 (selective 5-HT_{2A} antagonist) (21), SB242084 (5-HT_{2C} antagonist) (22), and angiotensin II were purchased from Tocris Bioscience (Bristol, U.K.). All substances, except for SB242084, liraglutide, and PCPA, were dissolved in artificial cerebrospinal fluid (aCSF), vehicle (VEH) for central injections. Liraglutide (Bachem) was dissolved in 0.9% saline. PCPA was dissolved in 0.9% saline by gentle warming and sonication to a concentration of 100 mg/mL (23,24). SB242084 was dissolved in 16% DMSO. 5-HT_{2C} receptor antagonist SB242084 displays 158-fold and 100-fold selectivity, respectively, over 5-HT_{2A} and 5-HT_{2B} receptors, and it also displays selectivity over a range of other 5-HT, dopamine, and adrenergic receptors. *R*-96544 is a potent, selective 5-HT_{2A} receptor antagonist; *R*-96544 shows 100-fold higher affinity for the human 5-HT_{2A} receptors than 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{5A}, 5-HT_6, and 5-HT_7 receptors and 5-HT transporter, although *R*-96544 has relatively high affinity for 5-HT_{2C} receptors (fourfold less compared with 5-HT_{2A}) (21).

Brain Cannulation

Rats were implanted with a guide cannula (26-gauge cannula; Plastics One, Roanoke, VA) as described previously (25) to allow drug injections into lateral ventricle (LV) or DR. The following injection coordinates (given from midline/bregma/skull) were used: ± 1.6/−0.9/−4.0 mm for LV and 0.0/−7.7/−6.8 mm for DR. The LV placement was verified with the angiotensin II drinking test (26). The microinjection site for the DR guide cannula was verified postmortem by the microinjection of India ink at the same microinjection volume (0.3 μL) used throughout the study.

RNA Isolation and mRNA Expression

Hypothalamic gene expression levels were measured after long-term (daily for 10 days) LV injections of EX4 (0.2 μg) or VEH (aCSF). A third group of rats was included to determine whether chronic GLP-1R activation interacts with weight loss–induced changes in serotonin receptors. These rats were pair fed daily to the amount of chow eaten by the EX4-treated rats. The following serotonin receptor genes were examined: *Htr1a*, *Htr2a*, *Htr2c*, and *Htr3a*. These genes were chosen based on their previously shown connection to GLP-1R activation or their well-established role in feeding regulation (see DISCUSSION for details). Brains were rapidly removed 24 h after the last EX4 injection, and the hypothalamus was dissected. Gene expression was determined using TaqMan RT-PCR and primer/probe sets as described previously (26–28) (for reference numbers, see Supplementary Table 1). Gene expression values were calculated based on the $\Delta\Delta C_t$ method (29), with the VEH-injected group set as the calibrator. PPIA (peptidylprolyl isomerase A) was used as a reference gene.

Immunohistochemistry

Mice were anesthetized and perfused transcardially with heparinized saline followed by a buffered fixative solution. The GLP-1 fibers and tryptophan hydroxylase (TPH)–positive neurons were visualized with a confocal microscope (LSM 700; Carl Zeiss AG). Antibodies, manufacturers, and dilutions are listed in Supplementary Table 2.

Whole-Cell Electrophysiology

Coronal slices of rat brain stem, 200 μm thick, containing DR were cut in ice-cold extracellular solution using a vibratome and maintained in an incubation chamber at room temperature. Whole-cell recordings where obtained from visually identified neurons using glass pipettes containing the

following (in mmol/L): 130 K-gluconate, 2 NaCl, 1 MgCl2, 10 HEPES, 0.1 EGTA, 10 Na phosphocreatine, 4 MgATP, 0.5 Na2GTP, and biocytin 0.1%, pH 7.3. Continuous recordings of the membrane potential (V_m) were used to monitor the effect of EX4. The input resistance (R_{input}) was calculated from the response to hyperpolarizing current pulses injected through the recording pipette. EX4 or Ex9-39 were added to the superfusion buffer. Electrophysiological data were analyzed with pClamp10 (Molecular Devices). Pooled data were presented as the mean ± SEM; a repeated-measures ANOVA was used to determine significance because VEH baseline values and EX4 or Ex9-39 and EX4 effects were measured in the same neuron. DR slices were fixed with paraformaldehyde 4% after recording. Fluorescence images of labeled sections were acquired with a confocal microscope (TCS SP5; Leica Microsystems) and reconstructed in three dimensions to assess TPH expression in biocytin-containing cells.

Serotonin Turnover

Brains were dissected 24 h after the last EX4 injection (injections were given daily for 8 days). Brains were rapidly removed, and the hypothalamus was dissected using a brain matrix. Tissue concentrations of serotonin and its metabolite (5-HIAA) were determined via high-performance liquid chromatography (30).

Forced Swim Test

The forced swim test (FST) was originally developed by Porsolt et al. (31) to screen for the antidepressive effect of pharmacotherapeutics. Here we used the modified version of the FST shown to provide a greater reliability for the detection of depressant or antidepressant-like effects for compounds that affect the serotonergic system (32). The test was performed as previously described (26).

Experimental Design

Effect of Serotonin Depletion on Weight Loss Impact of GLP-1R Activation

To assess the impact of serotonin depletion on the weight loss effect of GLP-1R activation, serotonin-depleted rats were injected with EX4, and their body weight was observed for 5 consecutive days of central EX4 injections. Serotonin depletion was performed by three intraperitoneal injections of PCPA, an irreversible inactivator of TPH, an enzyme required for the synthesis of serotonin. Rats received one daily injection of 300 mg/kg for 3 days. This treatment regimen was previously shown to decrease serotonin content to 5–10% of original levels (24,33); in our hands, serotonin turnover in the hypothalamus was still reduced by >40% 1 week after the PCPA treatment (2.53 ± 0.2 vs. 1.57 ± 0.2 serotonin turnover 5-hydroxyindole acetic acid (5-HIAA)/5-HT for VEH-treated and PCPA-treated rats, respectively, $P < 0.01$). Control rats received saline injections. Twenty-four hours after the last PCPA injection, daily EX4 (0.2 μg) or VEH (1 μL aCSF) injections commenced. The dose of EX4 was chosen based on previous studies (25,34,35).

Effect of 5-HT_{2A} or 5-HT_{2C} Receptor Blockade on Feeding and Weight Loss Impact of GLP-1R Activation

Rats were divided into the following four groups, matched for body weight: VEH (1 μL aCSF)/VEH (1 μL aCSF), VEH/EX4 (0.2 μg), *R*-96544 (10 μg)/EX4, and *R*-96544/VEH to test the impact of 5-HT_{2A} blockade. To test the contribution of 5-HT_{2C} receptors, rats were divided into the following four groups, matched for body weight at the start of the experiment: VEH (1 μL 16% DMSO in aCSF)/VEH (1 μL aCSF), VEH/EX4 (0.2 μg), SB242084 (10 μg)/EX4, and SB242084 /VEH. The injection of the antagonist was performed 10 min before the injection of the agonist or the respective VEH. All injections were performed once a day, during mid–light cycle, and intra-LV (total of 8 days of injections). Gonadal white adipose tissue (GWAT) pads were dissected 24 h after the last injection. In an additional group of rats, we tested whether central blockade of the 5-HT_{2A} receptor attenuates the weight loss induced by peripherally injected liraglutide (75 μg/kg i.p.).

Intra-DR GLP-1R Activation

VEH (0.3 μL/aCSF), EX4 (0.05 μg), or GLP-1 (2 μg) were injected into the DR of rats that were mildly food restricted (50% of their normal overnight chow) to promote reliable consumption of chow after the DR injections. The intake of chow was measured at 1 and 24 h; body weight change was measured at 24 h after intra-DR injection. These doses were chosen to be below the LV threshold for effect to avoid a potential confounding effect of potential drug leakage into the cerebral aqueduct located just above the DR.

Statistics

All statistical analysis was performed in GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance was analyzed using ANOVA followed by Holm-Sidak multiple-comparison test or Student *t* test, as appropriate. All data are presented as the mean ± SEM.

RESULTS

Serotonin Depletion Attenuated EX4-Induced Weight Loss Maintenance

Serotonin synthesis is initiated by the hydroxylation of tryptophan, a rate-limiting reaction performed by the enzyme TPH in the brain. PCPA acts as a selective and irreversible inhibitor of TPH. To evaluate whether intact serotonin synthesis is necessary for the energy balance impact of GLP-1R activation, serotonin was depleted by a well-established treatment regimen of 3-day intraperitoneal PCPA injections (24,33). Consistent with a critical downstream role of serotonin in the weight loss effect of GLP-1R stimulation, serotonin depletion resulted in an attenuation of EX4-induced weight loss maintenance (Fig. 1*A*). Two-way repeated-measures ANOVA of body weight data indicated a significant effect of drug treatment ($F_{(3,15)} = 18.69$, $P < 0.0001$) and treatment day ($F_{(4,60)} = 4.655$, $P < 0.005$) but no significant interaction of treatment and time ($F_{(12,60)} = 0.517$, $P = 0.896$).

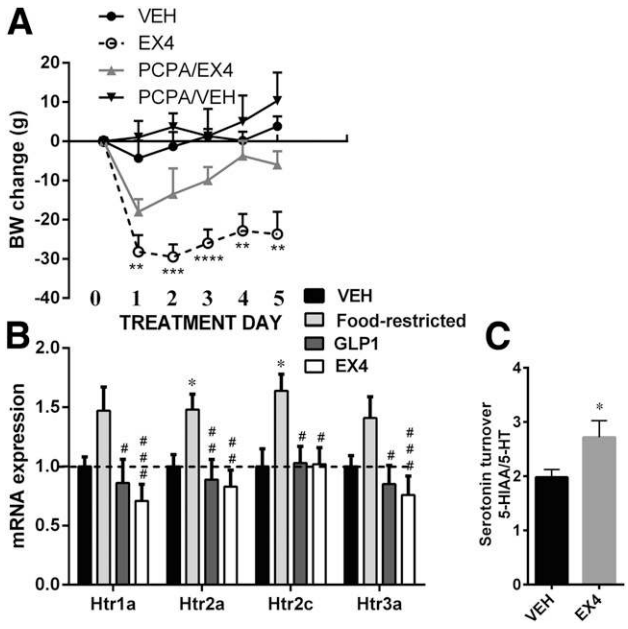

Figure 1—*A*: Serotonin depletion interferes with the weight loss maintenance after central GLP-1R activation. Although daily central EX4 injections in control rats led to a potent reduction of body weight, the weight loss impact of EX4 was markedly attenuated in rats depleted of serotonin by PCPA, suggesting that intact serotonin synthesis is needed for a full expression of EX4-induced weight loss. PCPA/VEH animals did not increase in weight. n = 3–6 per each treatment group. Activation of central GLP-1R ameliorated changes in serotonin receptor genes in the hypothalamus induced by food restriction (*B*) and also increased serotonin turnover in the hypothalamus (*C*). *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 compared with VEH-treated rats. #P < 0.05, ##P < 0.01, ###P < 0.005 compared with pair-fed rats. BW, body weight.

A Holm-Sidak multiple-comparisons test indicated a significant weight loss effect induced by EX4 for all testing days. In contrast, EX4 did not exert any significant effect on body weight in the rat group that has been depleted of serotonin by PCPA compared with VEH-injected rats. A significant difference in weight loss between EX4 and PCPA/EX4 rats was, however, detected 48 h after the first EX4 injection (Holm-Sidak test: day 1, t = 1.7, P = not significant [NS]; day 2, t = 2.7, $P < 0.05$; day 3, t = 2.7, $P < 0.05$; day 4, t = 3.2, $P < 0.01$; day 5, t = 3.0, $P < 0.05$). A significant difference in weight loss was also detected between PCPA/VEH and PCPA/EX4 rats on days 1, 2, and 5 (Holm-Sidak test: day 1, t = 3.0, $P < 0.05$; day 2, t = 2.7, $P < 0.05$; day 3, t = 1.8, P = NS; day 4, t = 1.4, P = NS; day 5, t = 2.5, $P < 0.05$). This indicates that serotonin signaling may not be essential for the weight loss effect of EX4 acutely but is crucial in a subchronic setting. PCPA/VEH animals did not increase in weight; this lack of effect of PCPA alone for male rats is consistent with a previous report (9).

Chronic Central GLP-1R Stimulation Affected Hypothalamic Serotonin Receptor Expression

The hypothalamus is a major target site for the energy balance effect of serotonin. In order to determine whether GLP-1R activation changes hypothalamic serotonin signaling, we measured changes in hypothalamic serotonin receptors and serotonin turnover after central long-term EX4 or GLP-1 treatment. Central injection of EX4 or GLP-1 abolished the increase of hypothalamic 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{3A} induced by the restricted feeding ($F_{(3,41)}$ = 6.85, $P < 0.001$; $F_{(3,399)}$ = 6.35, $P < 0.001$; $F_{(3,41)}$ = 5.14, $P < 0.005$; $F_{(3,41)}$ = 6.34, $P < 0.005$, respectively for each receptor) (Fig. 1*B*). Feeding was restricted to the daily amount voluntarily eaten by EX4-treated rats. Although food restriction elevated the mRNA of all serotonin receptors tested, this change reached significance only for *Htr2a* and *Htr2c* mRNA (Fig. 1*B*). Of note, the food restriction also promoted increased expression of these two receptors when both the control and food-restricted groups did not receive cannulation or injection (Supplementary Fig. 1). Consistent with the idea that GLP-1R activation increases serotonin neurotransmission in order to produce hypophagia, we found that serotonin turnover was increased in the hypothalami of EX4-treated rats (Fig. 1*C*).

5-HT_{2C} Receptor Blockade Does Not Attenuate EX4-Induced Weight Loss or Anorexia

The antagonist used to block 5-HT_{2A} receptors (below), *R*-96544, offers a very high selectivity for all but one serotonin receptor—it only offers fourfold selectivity for the 5-HT_{2C} receptor. Because of the possibility of some contribution of 5-HT_{2C} blockade by *R*-96544 to the results obtained, along with the well-established role of 5-HT_{2C} stimulation in weight regulation, we tested whether a selective 5-HT_{2C} receptor antagonist was sufficient to attenuate the impact of EX4 on body weight, fat deposition, and food intake. Selective blockade of 5-HT_{2C} with SB242084 did not attenuate EX4-induced weight loss (Fig. 2*A*). Surprisingly, blockade of the 5-HT_{2C} receptor slightly and significantly exacerbated the EX4-induced weight loss. Two-way repeated-measures ANOVA of body weight data indicated a significant interaction ($F_{(18,162)}$ = 1.998, $P < 0.05$) and a significant effect of drug treatment ($F_{(3,27)}$ = 34.12, $P < 0.0001$) and treatment day ($F_{(6,162)}$ = 3,099, $P < 0.01$). Although the reduction of food intake by EX4 seemed exacerbated by SB242084, this effect was NS (Fig. 2*B*). Also for food intake a significant interaction ($F_{18,162)}$ = 15.38, $P < 0.0001$) effect of drug treatment ($F_{(3,27)}$ = 49.85, $P < 0.0001$) and treatment day ($F_{(6,162)}$ = 748.7, $P < 0.0001$) were found. The weight of GWAT was clearly reduced by the subchronic central EX4 treatment, but this effect was not altered by coapplication of the 5-HT_{2C} antagonist (Fig. 2*C*) ($F_{(3,27)}$ = 5.329, $P < 0.01$). It is unlikely that the lack of attenuation of the weight loss and anorexia induced by EX4 are due to an insufficient dose of the drug, since previous reports (36,37) have reported even lower doses of the drug applied in the brain (intracerebroventricularly) to be effective in changing food intake in mice or rats. Trends in reduced FST immobility after EX4 treatment were detected (Fig. 2*D*), although these results did not receive

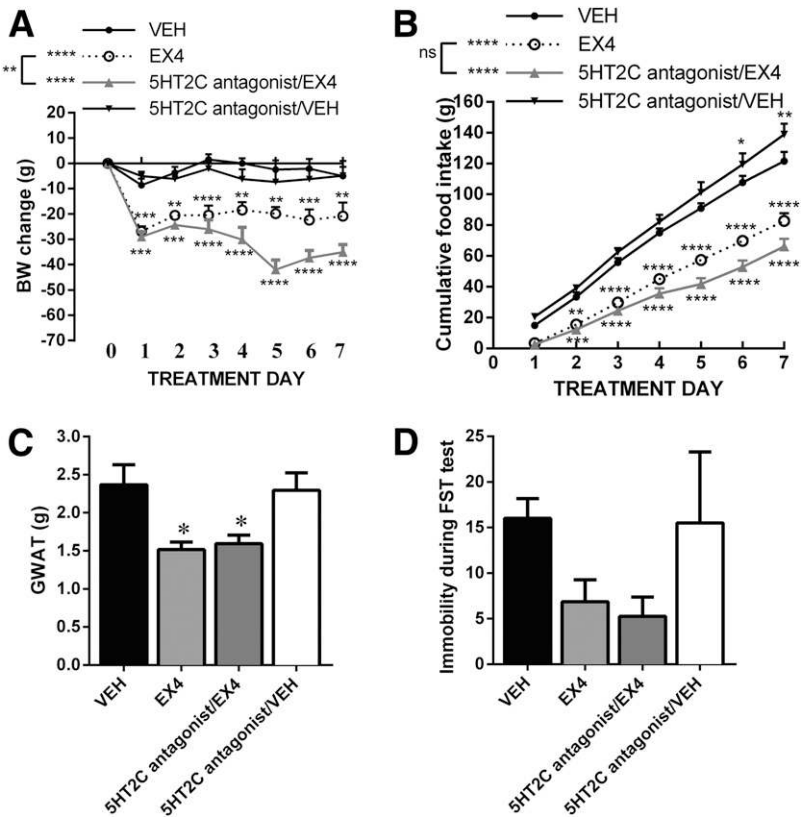

Figure 2—Blockade of central 5-HT_{2C} receptors does not reduce the anorexic or weight loss effect of EX4. *A*: Week-long daily central EX4 treatment produced a profound and sustained weight loss; blockade of 5-HT_{2C} receptor concurrent with the EX4 treatment did not affect the acute EX4-induced weight loss but potentiated the EX4-induced weight loss on the remaining days of the treatment. *B*: In line with the body weight results, EX4 reduced food intake; this reduction was not significantly altered by the 5-HT_{2C} antagonist cotreatment. *C*: Similarly, the reduction in the weight of the gonadal adipose fat pad (GWAT) induced by EX4 was spared by the concurrent blockade of the 5-HT_{2C} receptor. *D*: The impact of EX4 on depression-like behavior was unaltered by the chronic 5-HT_{2C} receptor blockade. n = 7–16 per each treatment group. Data are presented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$. BW, body weight.

statistical significance, irrespective of the antagonist coapplication, due to large variability in the antagonist-administered group.

5-HT_{2A} Receptor Blockade Attenuated EX4-Induced Weight Loss and Anorexia

Because 5-HT_{2A} and 5-HT_{2C} were two hypothalamic receptors that we found to be responsive to food restriction and central chronic EX4 treatment, we hypothesized that their pharmacological blockade should impair the metabolic impact of EX4. In line with this idea, central blockade of 5-HT_{2A} receptor with the selective antagonist *R*-96544 significantly attenuated the weight loss induced by EX4 (Fig. 3*A*). Interestingly, the onset of attenuation was delayed to 48 h after the injections of EX4, suggesting that the 5-HT_{2A} receptor becomes essential to the weight loss effect only after 2 days of treatment, although it seems dispensable for the acutely induced weight loss. Two-way repeated-measures ANOVA of body weight data indicated a significant interaction ($F_{(18,156)}$ = 6.104, $P < 0.0001$), a significant effect of drug treatment ($F_{(3,26)}$ = 13.87, $P < 0.0001$), and a significant effect of treatment day ($F_{(6,156)}$ = 5,893, $P <$ 0.0001). An identical pattern of the effect of treatment impact was detected for food intake (Fig. 3*B*), with a significant interaction ($F_{(18,156)}$ = 25.68, $P < 0.0001$), a significant effect of drug treatment ($F_{(3,26)}$ = 985.6, $P < 0.0001$), and a significant effect of treatment day ($F_{(6,156)}$ = 29.21, $P <$ 0.0001). In line with the food intake and body weight results, the GWAT weight was potently reduced by the subchronic central EX4 treatment, an effect that was abolished by the cotreatment with the 5-HT_{2A} antagonist (Fig. 3*C*). One-way ANOVA indicated a significant effect of the treatment on GWAT weight ($F_{(3,23)}$ = 3.328, $P < 0.05$). Because subchronic (but not acute) central EX4 treatment was recently shown to reduce depression-like behavior (26) in rat FST and serotonin has a well-established role in the regulation of emotionality, we hypothesized that the antidepression action of EX4 may be mediated by the central 5-HT_{2A} receptors. The data obtained did not support this idea and clearly indicate that 5-HT_{2A} blockade did not affect the EX4-induced reduction of immobility in the FST (Fig. 3*D*).

Central blockade of the 5-HT_{2A} receptor also significantly attenuated the weight loss induced by peripherally

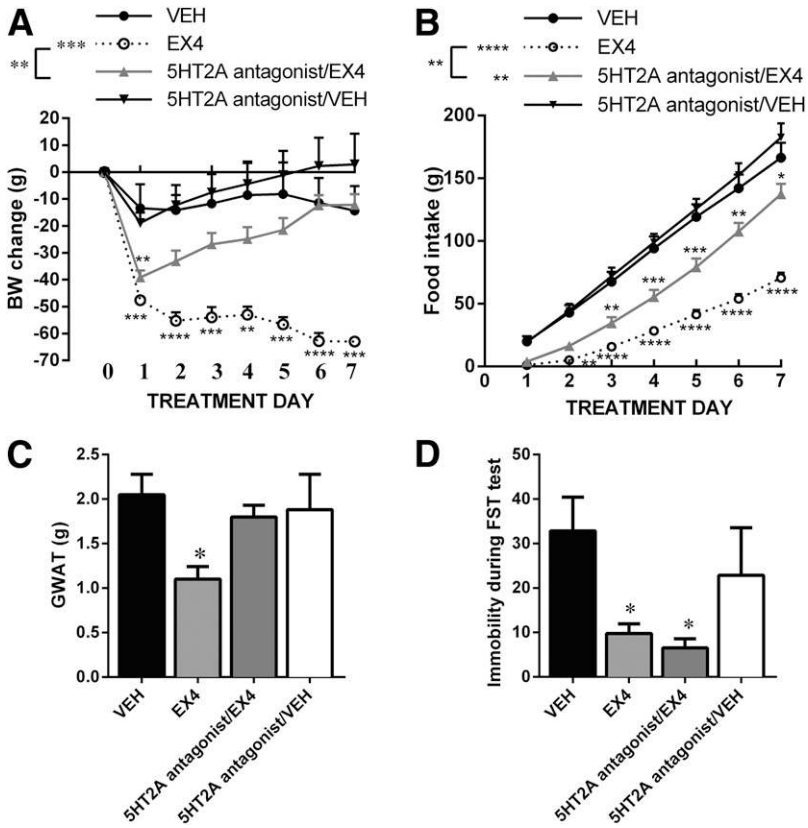

Figure 3—Blockade of the central 5-HT_{2A} receptors attenuates the anorexic and weight loss effect of EX4. *A*: Subchronic, daily central EX4 treatment produced profound and sustained weight loss; blockade of 5-HT_{2A} receptors concurrent with the EX4 treatment did not affect the acute EX4-induced weight loss but abolished EX4-induced weight loss after 1 week. *B*: In line with the body weight results, EX4 produced a profound food intake reduction which was abolished after 5-HT_{2A} antagonist treatment, but only after 3 days of cotreatment. *C*: Similarly, the reduction in the weight of the gonadal adipose fat pad (GWAT) induced by EX4 was completely abolished by the concurrent blockade of the 5-HT_{2A} receptor. *D*: In contrast, the impact of EX4 on depression-like behavior was unaltered by chronic 5-HT_{2A} receptor blockade. n = 6–8 per each treatment group. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 compared with VEH-treated rats. BW, body weight.

injected liraglutide (Fig. 4*A*). The pattern of results was somewhat different from that obtained with EX4, since the intraperitoneally injected liraglutide was much less potent in reducing body weight and, interestingly, the 5-HT_{2A} blockade was already effective at 24 h. Two-way repeated-measures ANOVA of body weight data indicated a significant interaction, a significant effect of drug treatment ($F_{(3,32)}$ = 6.282, $P < 0.005$) and a significant effect of treatment day ($F_{(4,128)}$ = 184.5, $P < 0.0001$). A similar pattern of effect was detected for food intake (Fig. 4*B*): significant interaction, significant effect of drug treatment ($F_{(3,32)}$ = 5.82, $P < 0.005$), and a significant effect of treatment day ($F_{(6,156)}$ = 29.21, $P < 0.0001$) were found. The amount of weight loss differed among the four GLP-1 analog–treated rat groups (Figs. 1*A*, 2*A*, 3*A*, and 4*A*), this is likely because each of these groups received different VEH treatments for the accompanying serotonin signaling–targeting drug, different GLP-1 analogs displaying different potency of effect, or a different route of administration (peripheral vs. intracerebroventricular), resulting in a different effect strength. Importantly, however, the interaction with serotonin discovered here persisted irrespective of the degree of weight loss induced by the GLP-1 analogs. Thus, the interaction is not restricted to cases where GLP-1 analogs result in the largest weight loss.

Acute Intra-DR Administration of GLP-1 or EX4 Is Sufficient to Reduce Food Intake

Acute injections of EX4 (0.2 μg) or GLP-1 (10 μg) into the brain ventricles (here intra-LV) reduced food intake at 1 h (ANOVA; $F_{(2,30)}$ = 9.37, $P < 0.001$) for both EX4 and GLP-1 and at 24 h ($F_{(2,30)}$ = 8.34, $P < 0.005$) for EX4 (Fig. 5*A*). This application route likely delivers the GLP-1R agonists to many brain sites from which a potential anorexic effect can be elicited. Because both serotonin depletion and the 5-HT_{2A} receptor blockade suggested an interaction of GLP-1R activation with serotonin, we hypothesized that GLP-1 may be exerting a part of its anorexigenic and weight loss effects by acting directly on the DR. Rats microinjected with GLP-1 or EX4 into the DR ate significantly less chow both at 1 h (ANOVA; $F_{(2,23)}$ = 4.17, $P < 0.05$) and 24 h ($F_{(2,23)}$ = 5.01, $P < 0.05$) (Fig. 5*B*). The

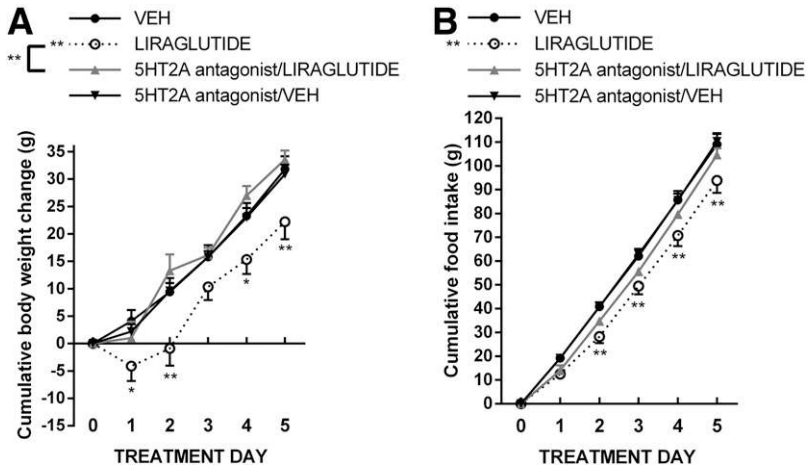

Figure 4—Blockade of the central 5-HT_{2A} receptors attenuates the anorexic and weight loss effect of peripherally injected liraglutide. *A*: Daily peripheral (intraperitoneal) injections of liraglutide (75 μg/kg) promoted weight loss; the blockade of 5-HT_{2A} receptors concurrent with the liraglutide treatment abolished liraglutide-induced weight loss. *B*: In line with the body weight results, liraglutide reduced food intake, an effect that was attenuated by 5-HT_{2A} receptor antagonist treatment. n = 7–10 per each treatment group. Data are presented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with VEH-treated rats.

weight loss of rats injected with either EX4 or GLP-1 did not, however, reach statistical significance ($F_{(2,23)} = 1.39$, $P = 0.26$) (Fig. 5*C*). Intra-DR GLP-2 microinjections did not change food intake or body weight (Supplementary Fig. 2).

GLP-1R Activation Increased the Electrical Activity of DR 5-HT Neurons

To investigate the effect of EX4 on excitability of the DR 5-HT neurons, we carried out whole-cell electrophysiology. Neurons recovered after electrical recordings were identified as serotoninergic by their TPH expression (Fig. 5*F*). EX4 (1 μmol/L) applied to the superfusion buffer induced a rapid and reversible depolarization of the V_m, which, in some cells, was sufficient to trigger action potential firing (Fig. 5*G*). The mean depolarization was 5.7 ± 1.0 mV ($P < 0.002$) (Fig. 5*H*). There was no change in R_{input} (Fig. 5*I*). The effect of EX4 was then tested after preincubation with Ex9-39 (1 μmol/L) to verify the specificity of its effect on 5-HT neurons. In this condition, EX4 did not change V_m (mean depolarization −0.7 ± 0.4 mV, $P < 0.12$) (Fig. 5*H*), confirming that the EX4 effect is mediated by the GLP-1R. These results are in agreement with an excitatory effect of GLP-1R activation on DR 5-HT neurons.

Fibers of GLP-1–Producing Neurons Innervate the DR

To determine whether GLP-1–producing neurons innervate the DR, thereby providing a route via which the endogenous peptide could reach DR serotonin neurons, mice engineered to express YFP in GLP-1–producing preproglucagon (PPG) neurons were used (38) along with immunohistochemical detection of TPH to identify serotonin neurons. YFP-immunoreactive innervation was assessed in coronal brain slices taken throughout the rostrocaudal extent of the DR in YFP-PPG mice (38). YFP-immunoreactive fibers were found throughout the DR (Fig. 6); however, the dorsal and dorsolateral divisions of the DR contained the highest density of the YFP-immunoreactive axons. In this area, many TPH-positive neurons were found to receive innervation from GLP-1–producing neurons (Fig. 6 and Supplementary Fig. 3 [video file]). Z-line three-dimensional reconstruction of a confocal image stack focused on a single TPH-positive YFP-innervated neuron was used to confirm the YFP innervation and to differentiate fibers terminating on the TPH-positive neuron from passing fibers. Only very sparse PPG neuron innervation was detected at the level of the ventral DR (Supplementary Fig. 4).

DISCUSSION

The results presented here demonstrate that intact brain serotonin signaling is critical for the maintenance of weight loss induced by GLP-1R activation. The time course and specific serotonin receptor contribution discovered here are unexpected but are consistent with recently emerging literature (39). Moreover, congruent results from electrophysiological, immunohistochemical, and neuropharmacological studies place the DR on the brain map of sites directly impacted by GLP-1 to regulate energy homeostasis.

Considering the well-established role of the 5-HT_{2C} receptor in energy balance along with several studies indicating an opposing effect of 5-HT_{2A} and 5-HT_{2C} receptors in behavioral inhibition (40,41), the critical role of the 5-HT_{2A} receptor in GLP-1–induced weight loss is unexpected. However 5-HT_{2A} and 5-HT_{2C} receptors may have similar molecular structures (42,43). Furthermore, a careful investigation of the literature does suggest a perhaps overlooked role of the 2A receptor in energy balance regulation. Hypermethylation of the 5-HT_{2A} receptor is associated with elevated baseline insulin levels and reduced sensitivity to the weight loss effect of dietary

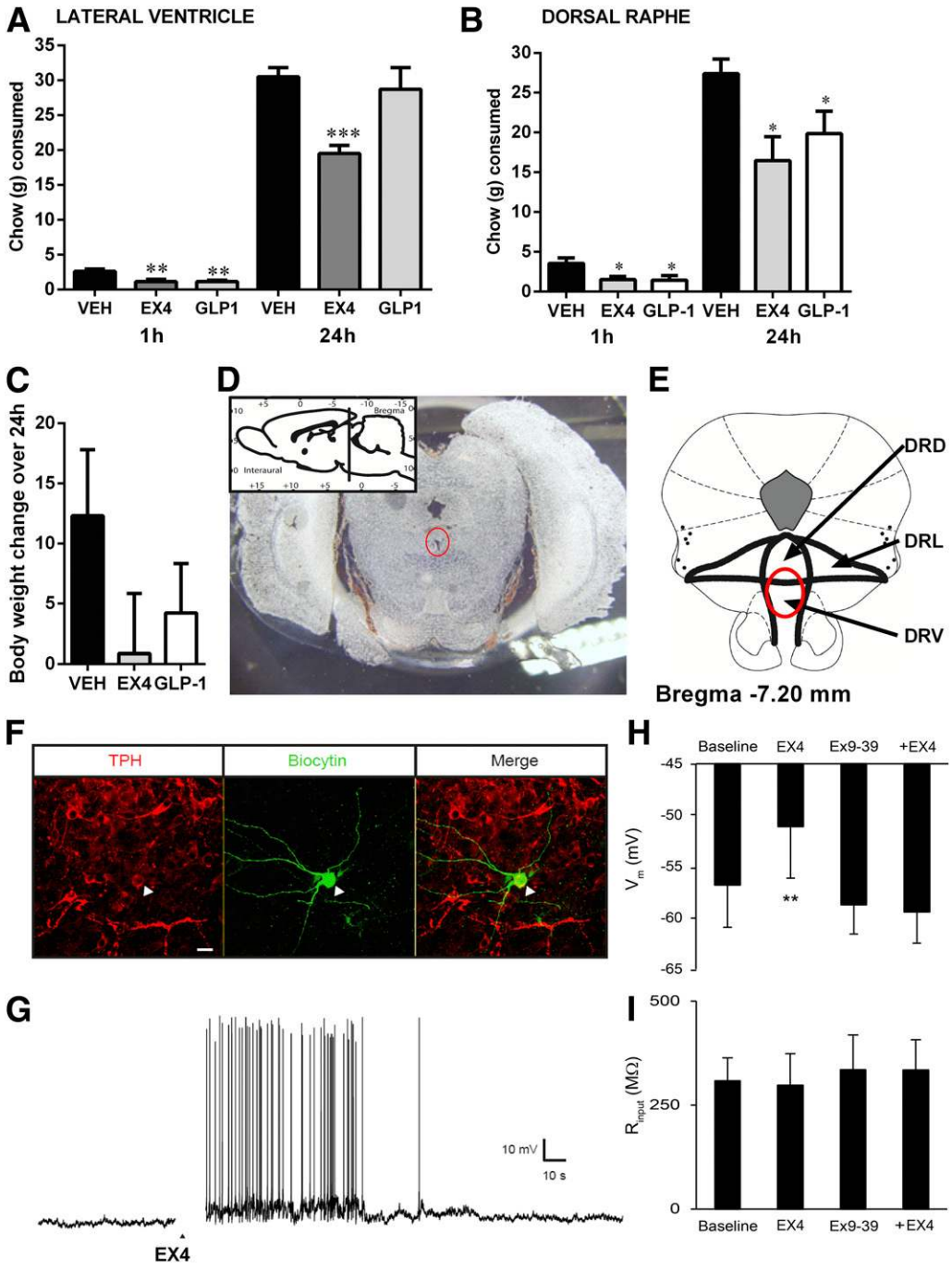

Figure 5—DR GLP-1R activation is sufficient to reduce food intake and body weight and to increase electrical activity of DR 5-HT neurons. *A*: GLP-1 or EX4 potently reduced the amount of chow eaten at 1 h when applied to the LV, but only EX4 remains effective at reducing intake at 24 h. n = 9–14 per each treatment group. *B*: When applied directly to the DR, both EX4 and GLP-1 reduce food intake at 1 and 24 h. *C*: The weight loss at 24 h did not reach significance for either of the two compounds. n = 7–10 per each treatment group. Photomicrograph of a 40-μm coronal section of rat brain illustrating the injection site (*D*) and schematic representation of the DR according to the rat brain atlas (*E*). The red circle illustrates the detected diffusion of ink. *F*: Immunohistochemical identification of TPH expression (red) in a biocytin-filled (green) DR 5-HT neuron (arrowhead). Scale bar: 20 μm. *G*: Voltage trace obtained from whole-cell recordings from DR 5-HT neurons showing the response of a DR 5-HT neuron to EX4 (1 μmol/L, arrowhead). Bar graphs summarizing EX4 effect of V_m (*H*) and R_{input} (*I*) on DR 5-HT neurons in baseline condition or after preincubation with Ex9-39 1 μmol/L (n = 6). DRD, dorsal subdivision of DR; DRL, lateral subdivision of DR; and DRV, ventral subdivision of the DR. Data are presented as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

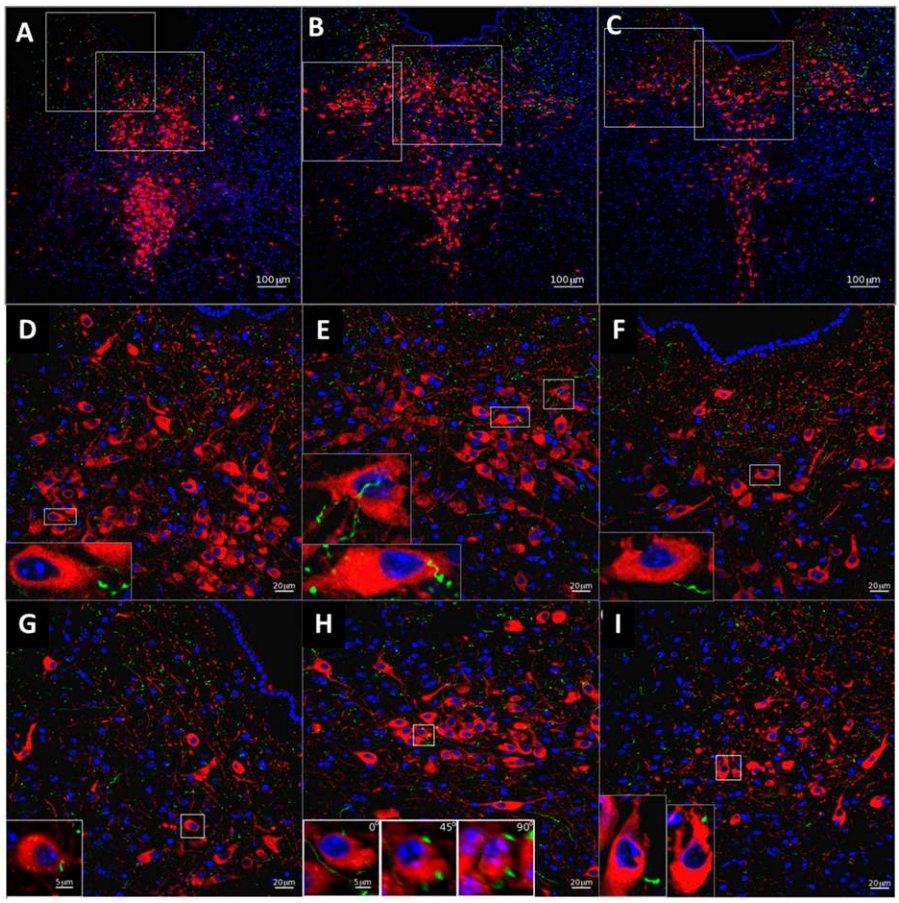

Figure 6—Immunohistochemical labeling for YFP in YFP-PPG neurons (green) and 5-HT in serotonin neurons (red) in coronal sections through the DR nucleus of YFP-PPG mice. *A–C*: Low-magnification micrographs showing the rostrocaudal extent of the DR examined. *D–I*: Groups of red 5-HT-immmunoreactive cell bodies are shown at higher magnification. Higher magnification of the dorsal subregion of the DR is displayed in *D* and *E*, and of the lateral subregion in *G–I*. For each of these subregions, examples of individual TPH-positive neurons receiving YFP-labeled innervation from GLP-1–producing neurons are shown in the bottom left corner of each panel. Innervation of chosen individual neurons was confirmed by creating a three-dimensional reconstruction of *z*-stack images (Supplementary Fig. 3 [video file]). High levels of immunoreactive YFP fibers are found in both the dorsolateral and dorsal section of the DR but not in the ventral section (Supplementary Fig. 4).

interventions in human patients (44). 5-HT_{2A} activation in the hypothalamus attenuates hunger induced by food deprivation or neuropeptide Y administration (45). The stimulation of 5-HT_{2A}, but not 5-HT_{2C} or 5-HT_1 receptors, in the paraventricular nucleus reduced neuropeptide Y–induced hyperphagia, possibly through the activation of corticotropin-releasing factor release (45). Finally, a recent study (39) suggests that the 2A receptor, like the 5-HT_{2C} receptor, is expressed on hypothalamic proopiomelanocortin neurons. In this study, pharmacological activation of the brain 5-HT_{2A} receptors resulted in reduced food intake. Thus, the current results are consistent with several published reports that show an anorexic potential of 5-HT_{2A} receptors at the level of the brain. However, one study (46) reported that peripheral injections of a 5-HT_{2A} receptor antagonist decrease body weight in mice. Thus, it seems that the anorexic effect may be driven by the central 5-HT_{2A} receptors, whereas manipulations that include peripheral receptors may have other effects, especially because the 5-HT_{2A} receptors are expressed on vagal afferents and other tissues, for example in platelets. Also, 5-HT_{2A} receptor KO mice do not show altered body weight or feeding when maintained on laboratory chow, suggesting that some degree of compensation may be present in the KO mice (47). Data showing that pharmacological, but not genetic, blockade of 5-HT_{2C} receptors attenuates lipopolysaccharide anorexia suggest that the manipulation of serotonin receptors is prone to compensatory reorganization (48).

The 5-HT_{2C} receptor is the most extensively studied serotonin receptor in relation to food intake regulation (4,11,12). It is widely expressed in the central nervous system, and it is also a target of a clinically approved antiobesity drug. Therefore, it was plausible that the blockade of this receptor should interfere with the weight loss and anorexic response to EX4. Our results, however, did not support this hypothesis. The pharmacological blockade of the 5-HT_{2C} receptor did not alter EX4-induced reduction in food intake or fat mass. Furthermore, the

body weight loss produced by long-term EX4 treatment seemed to be potentiated rather than attenuated by the 5-HT_{2C} receptor blockade. This may seem to contrast with one previous report (49) showing that 30-min food intake reduction, induced by peripheral GLP-1 injection in mice, is attenuated in 5-HT_{2C} KO mice. However, the same report states that the attenuation of anorexia was no longer significant at subsequent 1- and 2-h measurements. Another report (50) shows that 5-HT_{2C} KO mice display an attenuated anorexic response to a low dose of GLP-1, but not of liraglutide, at 2 h. Thus, we may speculate that if 5-HT_{2C} has a contribution to anorexia resulting from GLP-1R stimulation, it may only be relevant in a very acute setting ($\leq$2 h). On the other hand, an acute, 1- or 2-h, anorexic response to liraglutide was not attenuated in mice treated with PCPA (51). These findings together with the current results suggest that a chronic, but not an acute, anorexic effect of liraglutide may require serotonin. Furthermore, in line with our results, the activation of neurons indicated by c-Fos induction at 90 min after peripheral GLP-1 injection in mice is actually potentiated in two hypothalamic nuclei: paraventricular and arcuate nuclei (49). It is also important to note that 5-HT_{2C} and 5-HT_{2A} are only 2 of at least 18 serotonin receptors; thus, future studies should evaluate the contribution of the remaining receptors to energy balance regulation, especially because the mRNA of the two other receptors measured in this study also seems to be affected by GLP-1R activation.

Furthermore, although the blockade of 5-HT_{2A} or 5-HT_{2C} receptors did not significantly alter feeding and weight loss responses acutely ($<$24 h), this may only reflect the redundancy of the systems activated by GLP-1R signaling acutely. The redundancy hypothesis is also favored by data demonstrating *1*) that EX4 or GLP-1 activates DR serotonin neurons, *2*) that intra-DR injections of these agonists effectively reduce intake already at 1 h, and *3*) that they also release serotonin from hypothalamic synaptosomes in a matter of minutes (13).

DR is a major serotonin source for the hypothalamus (15,16). Indeed, our data suggest that the hypothalamus could be one of the downstream targets of GLP-1R–activated serotonergic neurons. These results are consistent with the critical role of the hypothalamus in energy balance regulation. That long-term treatment with EX4 increased serotonin turnover in the hypothalamus suggests that increased activity of central serotonin does not display tolerance to the daily GLP-1R agonist treatment. Moreover, the increased expression of 5-HT_{2A} and 5-HT_{2C} receptors in the hypothalamus induced by food restriction was ameliorated by either central EX4 or GLP-1 treatment. These results suggest that GLP-1R activation counteracts the reduced serotonergic activity associated with negative energy balance (52).

Given the importance of serotonin in emotional homeostasis and the clinical application of serotonin-acting pharmaceuticals in the treatment of depression, it was plausible that the antidepression impact of GLP-1 (26,53,54) uses central serotonin signaling. Surprisingly, blockade of either 5-HT_{2A} or 5-HT_{2C} receptors did not influence the potent antidepression impact of chronic GLP-1 activation. Therefore, whereas serotonin signaling through the 5-HT_{2A} receptor proved to be critical for the energy balance effect of EX4, the impact of the drug on emotionality was spared by the 5-HT_{2A} receptor blockade, suggesting that the interaction is critical for metabolic effects of GLP-1 but is not necessary for emotionality regulation. Thus, these findings disassociate the metabolic and emotionality pathways impacted by GLP-1R activation. These results are also consistent with the lack of a depression-like phenotype in the 5-HT_{2A} receptor KO mouse (47).

With serotonin and GLP-1 recently becoming the two most relevant brain systems for the development of antiobesity therapies, it is increasingly important to understand the neural circuitry underlying their metabolically beneficial effects. Surprisingly, little is known about how these two systems may interact. Current data indicate that GLP-1 avails of serotonin signaling in order to achieve its metabolic impact. Unexpectedly, the most widely researched serotonin receptor, 5-$HT_{2C,}$ was not necessary for this interaction; instead, a receptor much less explored with respect to energy balance, 5-$HT_{2A,}$ was shown to be critical for the hypophagic and weight loss impact of a clinically approved GLP-1 analog, EX4. Our data also suggest that the effect of GLP-1 on serotonin is direct since GLP-1R activation increased serotonin neuron activity at the level of the DR, a key brain nucleus supplying serotonin to many forebrain areas, and caudal brain stem GLP-1–producing neurons innervate the DR. Our results also disassociate the brain metabolic and emotionality pathways impacted by GLP-1R activation. Finally, few studies look at the long-term impact of GLP-1 analogs on the brain; this is somewhat surprising since it is the long-term impact that is likely the most relevant for the antiobesity impact of these substances. Our data indicate that the critical neural substrates activated acutely by GLP-1 analogs may differ from those activated with longer-term repeated drug administration. In summary, the present results contribute to our understanding of neural substrates regulating food intake, body fat, and body weight, which is knowledge of considerable clinical potential. Our data are preclinical and thus provide an interesting avenue to explore in humans or larger animal models where the data may translate more readily to humans, but currently can only be considered preliminary in nature.

Acknowledgments. The authors thank Fredrik Anesten (Institute of Neuroscience and Physiology, University of Gothenburg) for lending his expertise in immunohistochemistry.

Funding. This research was funded by the Swedish Research Council (2014-2945 and 2013-7107), Novo Nordisk Foundation Excellence project grant, Ragnar Söderberg Foundation Fellowship, Harald Jeanssons Stiftelse and Greta Jeanssons Stiftelse, the Knut and Alice Wallenberg Foundation, and Magnus Bergvalls Stiftelse.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. R.H.A. and L.L.-F. performed the brain surgery, injections, feeding, and body weight measurements. J.E.R. performed the FST scoring, brain surgery, injections, feeding, and body weight measurements. K.E. performed DR immunohistochemistry. E.B. and I.W.A. performed fat dissections. C.H., H.N., and F.B. performed the high-performance liquid chromatography. F.M.G. and F.R. generated the YFP mice. C.M.L. performed all electrophysiology and associated immunohistochemistry. K.P.S. conceived and designed all experiments, supervised experimentation, analyzed data, coordinated the project, and wrote the manuscript. K.P.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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