

Intracellular Signals Mediating the Food Intake-Suppressive Effects of Hindbrain Glucagon-like Peptide-1 Receptor Activation

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SUMMARY

Glucagon-like peptide-1 receptor (GLP-1R) activation within the nucleus tractus solitarius (NTS) suppresses food intake and body weight (BW), but the intracellular signals mediating these effects are unknown. Here, hindbrain (fourth i.c.v.) GLP-1R activation by Exendin-4 (Ex-4) increased PKA and MAPK activity and decreased phosphorylation of AMPK in NTS. PKA and MAPK signaling contribute to food intake and BW suppression by Ex-4, as inhibitors Rp cAMP and U0126 (fourth i.c.v.), respectively, attenuated Ex-4's effects. Hindbrain GLP-1R activation inhibited feeding by reducing meal number, not meal size. This effect was attenuated with stimulation of AMPK activity by AICAR (fourth i.c.v.). The PKA, MAPK, and AMPK signaling responses by Ex-4 were present in immortalized GLP-1R-expressing neurons (GT1-7). In conclusion, hindbrain GLP-1R activation suppresses food intake and BW through coordinated PKA-mediated suppression of AMPK and activation of MAPK. Pharmacotherapies targeting these signaling pathways, which mediate intake-suppressive effects of CNS GLP-1R activation, may prove efficacious in treating obesity.

INTRODUCTION

Glucagon-like peptide-1 (GLP-1), a neuropeptide endogenously produced from "L" cells in the gastrointestinal tract and centrally in neurons of the nucleus tractus solitarius (NTS), plays an important role in glycemic regulation and energy balance (for review, see Hayes et al., 2010a; Holst, 2007). Accumulating clinical and basic science data suggest that pharmacotherapies targeting the GLP-1 system are ameliorative not only for type 2 diabetes Mellitus (T2DM), but for obesity as well (Lovshin and Drucker, 2009). Interestingly, exogenous activation of either peripheral or central GLP-1 receptors (GLP-1Rs) produces a comparable profile of physiological responses that include enhanced

glucose-stimulated insulin secretion (Komatsu et al., 1989), inhibition of gastric emptying (Hayes et al., 2008b; Imeryuz et al., 1997; Wettergren et al., 1993), and inhibition of food intake (Chelikani et al., 2005; Gutzwiller et al., 1999; Hayes et al., 2008b, 2009a; Kinzig et al., 2002; Moran et al., 1996; Turton et al., 1996). Although much is known about the mechanisms by which peripheral GLP-1R activation engages these physiological responses, the relevant central GLP-1R populations and their projections, as well as the intracellular signaling cascades that mediate the food intake inhibitory effects of central GLP-1R-activation, have received considerably less attention.

GLP-1Rs are widely expressed throughout the central nervous system (CNS) in many sites relevant to energy balance regulation, including nuclei in the caudal brainstem (NTS; parabrachial nucleus), hypothalamus (lateral hypothalamus, LH; paraventricular hypothalamus, PVH), and basal forebrain (bed nucleus of stria terminalis; central nucleus of the amygdala) (Goke et al., 1995; Larsen et al., 1997; Merchenthaler et al., 1999). Recent reports highlight the importance of hindbrain GLP-1R-expressing nuclei in the control of food intake and body weight (BW) (Hayes et al., 2008b, 2009a; Kinzig et al., 2002). Specifically, the endogenous activation of GLP-1R within the caudal medial-subnucleus of the NTS (mNTS) was shown to contribute to the normal control of food intake by mediating satiation signaling that arises from mechanical distension of the stomach (Hayes et al., 2009a). The intracellular signaling pathways within GLP-1R-expressing NTS neurons that mediate the food intake and BW-suppressive effects of CNS GLP-1 action have not been characterized.

In pancreatic β cells, GLP-1R activation leads to stimulation of adenylate cyclase, elevated cAMP, and subsequent activation of protein kinase A (PKA) (Gomez et al., 2002; Perfetti and Merkel, 2000). Given that PKA signaling is upstream of many signaling pathways implicated in energy balance regulation, it is likely that the intake-suppressive effects of hindbrain GLP-1R activation involve endemic signaling pathways that alter intracellular Ca^{2+} influx and/or longer-term alterations in gene transcription and protein synthesis to integrate various anorectic signals. Whether the intake-suppressive effects of hindbrain GLP-1R activation are indeed dependent upon an increase in PKA activity within GLP-1R-expressing NTS neurons remains to be tested.

In neurons, elevated cAMP levels increase phosphorylation of the p44/42 mitogen-activated protein kinase (p44/42 MAPK;

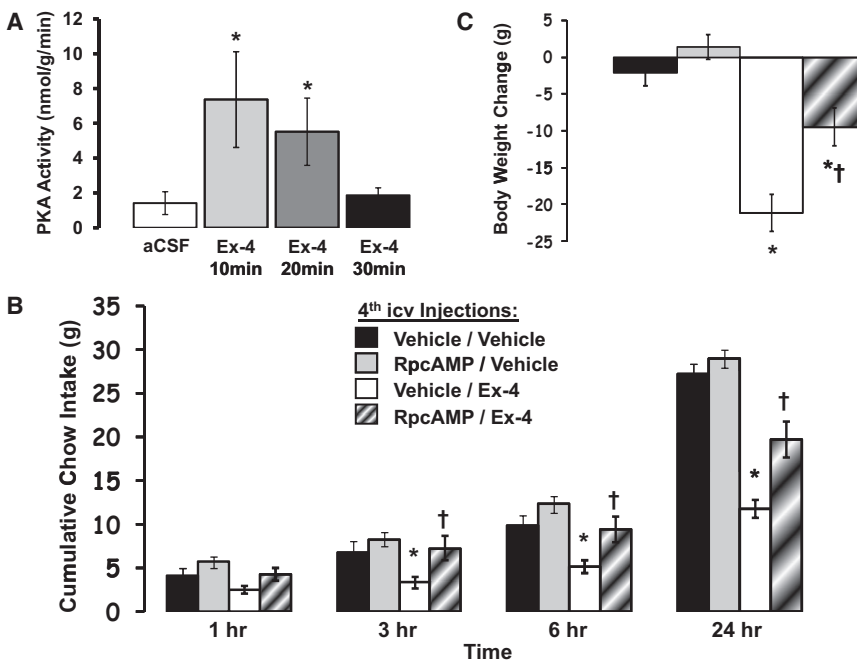


Figure 1. Hindbrain GLP-1R Activation Suppresses Food Intake by Increasing Protein Kinase-A Activity

(A) Increased PKA activity (nmol/g/min) in tissue of the caudal DVC following fourth i.c.v. Ex-4 (0.3 µg) administration. * $p < 0.05$ from aCSF. Cumulative chow intake (B) and 24 hr BW change (C) following fourth i.c.v. delivery of the PKA inhibitor RpcAMP (20 µg) or Ex-4 (0.2 µg) alone or in combination, counterbalanced with vehicle/vehicle (aCSF/aCSF). Data are mean \pm SEM. * $p < 0.05$ from vehicle/vehicle. † $p < 0.05$ from vehicle/Ex-4.

deprivation increases AMPK activity in NTS lysates and that AMPK activity within the NTS is physiologically relevant to the normal control of food intake (Hayes et al., 2009b, 2010b). Together, these data support the hypothesis that reduced AMPK activity in the hindbrain may mediate the suppression of intake that follows GLP-1R activation.

Using a combination of in vivo and in vitro techniques, we evaluate the cellular signaling pathways that mediate

also known as extracellular signal-regulated kinase [ERK]-1/2) (Dugan et al., 1999; Vossler et al., 1997), presumably through a PKA-dependent pathway (Ma et al., 2007). GLP-1 stimulates p44/42 MAPK phosphorylation in pancreatic β cells through a mitogen-activated protein kinase kinase (MEK)-dependent but Raf/Ras-independent pathway that requires PKA activation, an influx of extracellular Ca^{2+} , and calmodulin-dependent protein kinase II (CaMKII) activation (Gomez et al., 2002). Previous reports highlight a role for p44/42 MAPK signaling within the NTS in food intake control (Sutton et al., 2004, 2005). Yet, direct in vivo and in vitro evaluation of the MAPK pathway mediating the intake suppressive effects of hindbrain GLP-1R activation remains unaddressed.

PKA activation is also known to inhibit calmodulin-dependent protein kinase kinase (CaMKK) (Hurley et al., 2006). CaMKK and LKB1 have been identified as the principal upstream kinases which phosphorylate the fuel-sensing enzyme AMP-activated protein kinase (AMPK) α catalytic subunit at Thr172 (for review, see Xue and Kahn, 2006). Thus, a PKA-induced inhibition of CaMKK activity would lead to decreased AMPK activation (Hurley et al., 2006). AMPK has been implicated in CNS control of energy balance (Minokoshi et al., 2004; Seo et al., 2008; Xue and Kahn, 2006), and its activity is altered by food deprivation (da Silva Xavier et al., 2003; Minokoshi et al., 2004; Xue and Kahn, 2006) and by the effects of leptin, insulin-induced hypoglycemia, or 2-DG cytoglucoopenia (Han et al., 2005; Hayes et al., 2009b, 2010b; Kim and Lee, 2005; Kim et al., 2004b; Minokoshi et al., 2004). In addition, increased hypothalamic AMPK mRNA following food deprivation was attenuated by GLP-1R activation (Seo et al., 2008). Recently, the role of AMPK activity in NTS control of energy balance has also been evaluated (Hayes et al., 2008a). Similar to previous reports evaluating AMPK activity in various hypothalamic nuclei (Minokoshi et al., 2004; Xue and Kahn, 2006), it was found that food

the food intake and BW-suppressive effects of hindbrain GLP-1R activation by the GLP-1R-selective agonist, Exendin-4 (Ex-4). We hypothesize that activation of NTS-GLP-1R-expressing neurons suppresses food intake through coordinated PKA-mediated suppression of AMPK activity and PKA-mediated activation of p44/42 MAPK.

RESULTS

Hindbrain GLP-1R Activation Reduces Food Intake by Increasing PKA Activity

Hindbrain administration of the GLP-1R agonist Ex-4 (0.3 µg; fourth i.c.v.) significantly increased PKA activity in the dorsal vagal complex (DVC; which includes NTS, dorsal motor nucleus of the vagus [DMV], and area postrema [AP]) at 10 and 20 min postinjection compared to aCSF (Figure 1A). By 30 min, the Ex-4-driven elevation in NTS PKA activity had returned to baseline levels.

To test the hypothesis that the intake-suppressive effects of hindbrain GLP-1R activation require PKA activity, intake of standard rodent chow was examined following counterbalanced fourth i.c.v. administration of Ex-4 (0.2 µg) alone or in combination with the widely used PKA inhibitor, RpcAMP (20 µg). Figure 1B shows that cumulative food intake was significantly suppressed at 3, 6, and 24 hr following Ex-4 administration. This intake suppression was dependent on an increase in hindbrain PKA activity, as fourth i.c.v. pretreatment with the PKA inhibitor RpcAMP reversed the intake suppressive effects of fourth i.c.v. Ex-4 at 3 and 6 hr and attenuated the intake suppression at 24 hr. fourth i.c.v. Ex-4 administration also significantly suppressed BW 24 hr after administration compared to vehicle/vehicle (aCSF/aCSF) treatment (Figure 1C). Pretreatment with RpcAMP significantly attenuated the suppression of BW by fourth i.c.v. Ex-4, whereas RpcAMP alone did not affect BW.

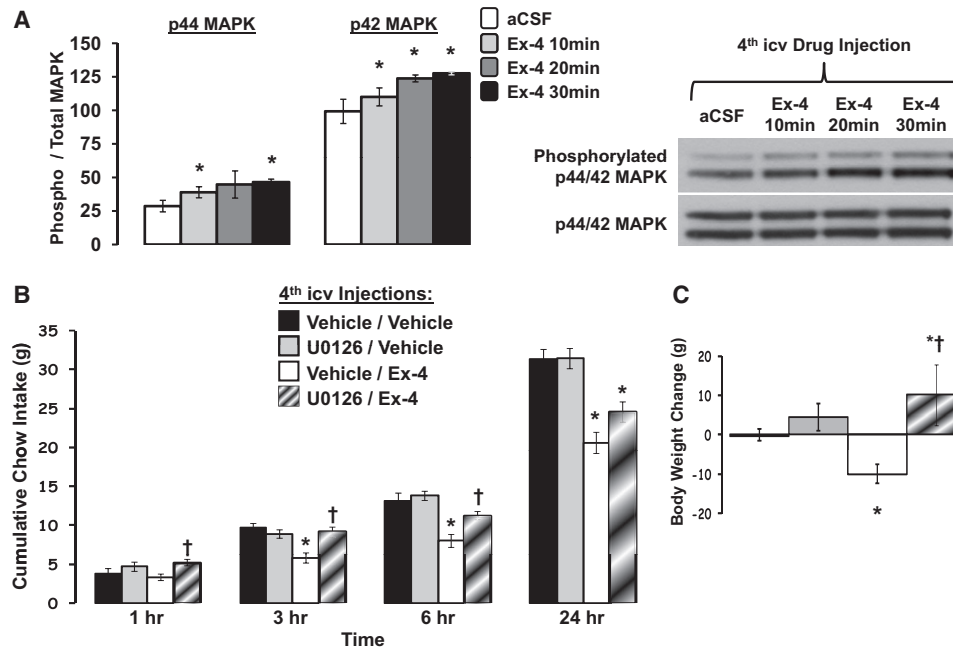


Figure 2. Hindbrain GLP-1R Activation Suppresses Food Intake by Increasing Phosphorylation of Mitogen-Activated Protein Kinase

(A) Increased phosphorylation of p44/42-MAPK in caudal DVC tissue lysates following fourth i.c.v. Ex-4 (0.3 μ g) administration. Representative immunoblots for phosphorylated and total p44/42-MAPK are shown. * $p < 0.05$ from aCSF. Cumulative chow intake (B) and 24 hr BW change (C) following fourth i.c.v. delivery of the MEK inhibitor U0126 (2 μ g) or Ex-4 (0.2 μ g) alone or in combination, counterbalanced with vehicle/vehicle (DMSO/aCSF). Data are mean \pm SEM. * $p < 0.05$ from vehicle/vehicle. † $p < 0.05$ from vehicle/Ex-4.

Hindbrain GLP-1R Activation Suppresses Food Intake by Increasing Phosphorylation of MAPK

Immunoblot analysis revealed that fourth i.c.v. Ex-4 (0.3 μ g) administration increased p44/42 MAPK phosphorylation in DVC tissue compared to vehicle (aCSF) (Figure 2A; data presented as phosphorylated/total MAPK). Specifically, hindbrain Ex-4 significantly increased phosphorylation of p44 MAPK at 10 and 30 min postadministration with no change in total p44 levels. Likewise, fourth i.c.v. Ex-4 increased phosphorylation of p42 at 10, 20, and 30 min postadministration. For both p44 and p42 MAPK, the greatest increase in phosphorylation was observed at 30 min following hindbrain GLP-1R activation.

Food intake was significantly suppressed following fourth i.c.v. Ex-4 (0.2 μ g) administration in a separate group of rats at 3, 6, and 24 hr postinjection compared to vehicle/vehicle (DMSO/aCSF) administration (Figure 2B). Blockade of MAPK signaling by fourth i.c.v. administration of the MEK inhibitor, U0126, had no effect on food intake when administered alone. Pretreatment with U0126, however, significantly reversed the inhibition of intake by Ex-4 at 3 and 6 hr. BW was also significantly suppressed 24 hr after fourth i.c.v. Ex-4 administration compared to vehicle/vehicle treatment (Figure 2C). Fourth i.c.v. administration of U0126, while having no effect on 24 hr BW when administered alone, significantly attenuated the suppression of BW by fourth i.c.v. Ex-4.

Hindbrain GLP-1R Activation Reduces Food Intake via Inhibition of AMPK Signaling

Immunoblot analysis revealed that hindbrain GLP-1R activation by fourth i.c.v. Ex-4 (0.3 μ g) significantly reduced phosphoryla-

tion of AMPK α 2 in DVC tissue at 20 and 30 min postadministration, with no change in total AMPK α levels (Figure 3A; data presented as pAMPK α 2/total AMPK α). Inhibition of NTS AMPK activity following hindbrain GLP-1R activation was greatest at 30 min.

In a separate cohort of rats, the AMPK activity promoter, AICAR, was used to investigate whether inhibition of AMPK activity mediates the suppression of food intake by Ex-4 (measured by automated feedometers that record food intake to the nearest \pm 0.1 g/m/24 hr every 1 min). Hindbrain GLP-1R activation by fourth i.c.v. Ex-4 suppressed food intake in the dark cycle by reducing meal number and increasing intermeal interval, not by altering meal size (Figure 4A). A maximum 44% intake suppression by Ex-4 occurred 12 hr postinjection (at the start of light cycle; Figure 3B). Fourth i.c.v. AICAR attenuated Ex-4's intake-suppressive effect by approximately 50% (Figure 3B) and attenuated Ex-4's suppression of meal number and increase in intermeal interval (Figure 4A). The change in 24 hr BW was significantly suppressed by fourth i.c.v. Ex-4 (Figure 3C) compared to BW change following vehicle. AICAR alone did not alter 24 hr BW. Pretreatment with AICAR produced a nonsignificant trend of an attenuation in the suppression of BW by fourth i.c.v. Ex-4 ($p = 0.065$ for BW change comparison between vehicle/Ex-4 and AICAR/Ex-4 treatments), whereas AICAR alone did not alter 24 hr BW.

Hindbrain GLP-1R activation (fourth i.c.v. Ex-4) reduces food intake that results from the suppression in meal number, with no alteration in meal size (see Figures S1A and S1B available online). In a separate group of rats, IP Ex-4 (3.0 μ g/kg) administration suppressed food intake by approximately 47% at 5 hr

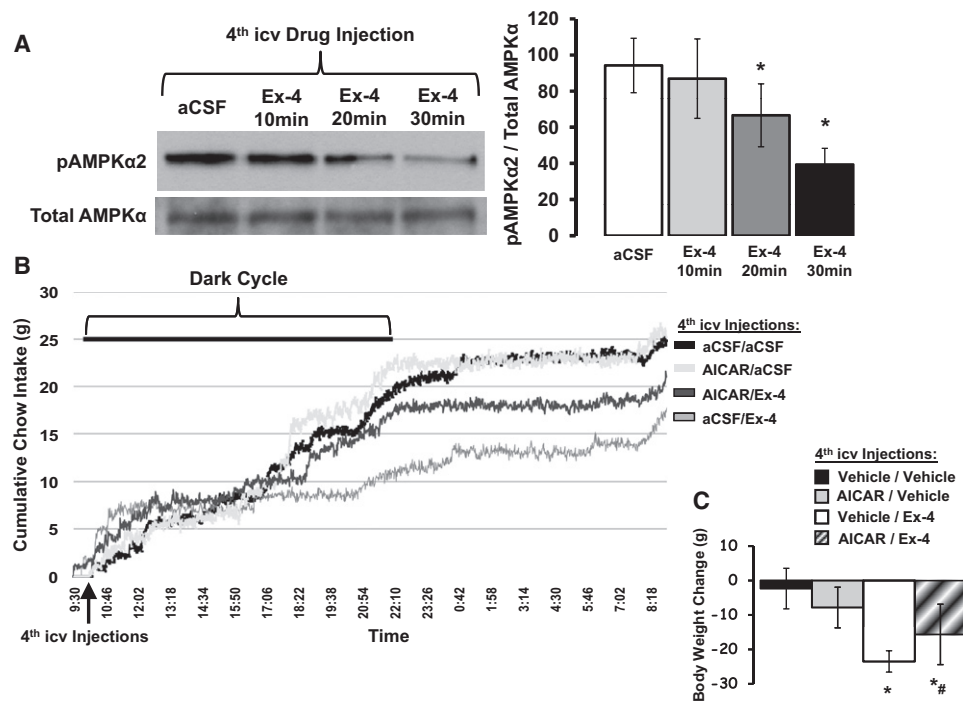


Figure 3. Suppression of Intake by Hindbrain GLP-1R Activation Is Mediated by Reduced AMPK Signaling

(A) Decreased phosphorylation of AMPK α 2 in caudal DVC tissue lysates following fourth i.c.v. Ex-4 (0.3 μ g) administration. Representative immunoblots for pAMPK α 2 and total AMPK α are shown. * $p < 0.05$ from aCSF. Cumulative chow intake (B) and 24 hr BW change (C) following fourth i.c.v. delivery of the AMPK activity promoter AICAR (300 μ g) or Ex-4 (0.1 μ g) alone or in combination, counterbalanced with vehicle/vehicle (aCSF/aCSF). Chow intake recorded via automated feedometers that continuously record food intake to the nearest ± 0.1 g/m²/24 hr. Data are mean \pm SEM. * $p < 0.05$ from vehicle/vehicle. # $p = 0.065$ from vehicle/Ex-4.

post-drug delivery through a reduction in meal size, with no alteration in meal number (Figure S1B). Thus, the time point chosen for meal pattern assessment for fourth i.c.v. and IP Ex-4 administration in Figures S1A and S1B is based on comparable maximum percent suppression of chow intake ($\sim 45\%$ suppression).

The Hindbrain GLP-1R-Mediated Increase in MAPK and Reduction in AMPK Signaling Are PKA Dependent

Immunoblot analysis showed that the increase in MAPK phosphorylation and decrease in pAMPK α 2 by fourth i.c.v. Ex-4 administration is dependent, in part, on PKA activation. Inhibition of hindbrain PKA activity by fourth i.c.v. pretreatment with RpCAMPA (20 μ g) significantly attenuated the Ex-4 (0.3 μ g; fourth i.c.v.)-induced decrease in NTS pAMPK α 2 levels (Figure 4A; data presented as pAMPK α 2/total AMPK α). Likewise, RpCAMPA significantly attenuated the Ex-4-induced increase in phosphorylated p42-MAPK levels, but not p44-MAPK levels in DVC tissue (Figure 4B; data presented as phosphorylated/total MAPK).

Medial NTS GLP-1Rs Mediate the Intake-Suppressive Effects of Hindbrain Ex-4

Ex-4 was administered intraparenchymally to the mNTS, AP, and DMV to determine the hindbrain-specific GLP-1R-expressing nucleus within the DVC that mediates the intake suppressive effects of fourth i.c.v. Ex-4. First, a subthreshold dose for intake suppression of Ex-4 was determined for fourth i.c.v. delivery, as

fourth i.c.v. Ex-4 (0.05 μ g/1 μ l) significantly suppressed chow intake at 6 and 24 hr postadministration, whereas fourth i.c.v. Ex-4 (0.025 μ g/1 μ l) was without effect (Figure 5A). Unilateral intraparenchymal mNTS administration of Ex-4 at 0.025 μ g/100 nl significantly suppressed chow intake at 3, 6, and 24 hr postadministration; 0.05 μ g/100 nl was also effective (Figure 5B). Conversely, unilateral DMV administration of Ex-4 at 0.05 μ g/100 nl, a dose effective following either fourth i.c.v. or mNTS delivery, was without effect on food intake (Figure 5C). A similar absence of an intake-suppressive effect was observed following intraparenchymal Ex-4 administration to the AP at 0.025 μ g/100 nl and 0.05 μ g/100 nl (Figure 5D).

In addition to postmortem histological verification of intraparenchymal cannula placement for the mNTS, AP, and DMV, functional tests were performed to further confirm placement of mNTS and AP injection sites. The AP has been shown to mediate the intake suppressive effects of the pancreatic hormone amylin and its agonist, salmon calcitonin (sCT) (Mollet et al., 2004; Potes and Lutz, 2010). AP administration of sCT at 0.4 μ g/100 nl, a dose previously established to be just at threshold for intake suppression when administered to the fourth ventricle (Mollet et al., 2004), produced a significant suppression of food intake at all time points measured (Figure 5E), thus providing functional confirmation of our AP site of delivery. To confirm mNTS injection sites, the cytoglucopenia-induced sympathoadrenal-mediated glycemic effect resulting from intraparenchymal injection of 24 μ g of 5-thio-D-glucose in 100 nl of aCSF (Hayes et al.,

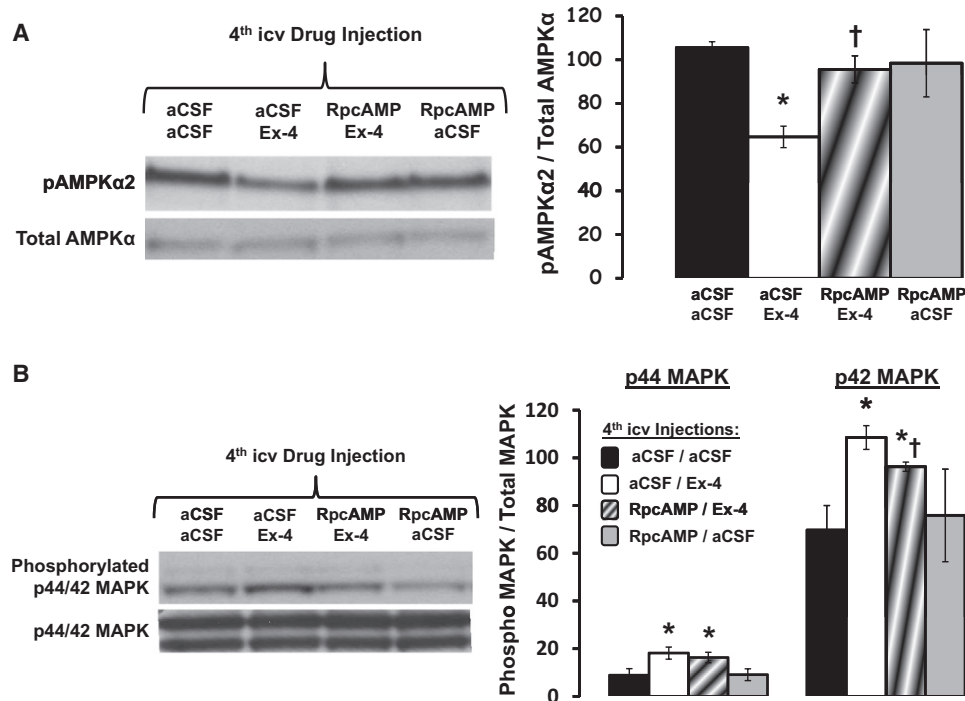


Figure 4. Hindbrain GLP-1R-Mediated Increase in MAPK and Reduction in AMPK Signaling Is PKA Dependent

(A) Fourth i.c.v. Ex-4 (0.3 μg)-driven decrease phosphorylation in AMPKα2 in caudal DVC tissue lysates is PKA dependent, as fourth i.c.v. administration of the PKA inhibitor RpcAMP (20 μg) attenuated the suppression in pAMPKα2 by Ex-4. Representative immunoblots for pAMPKα2 and total AMPKα are shown. *p < 0.05 from aCSF/aCSF. †p < 0.05 from aCSF/Ex-4.

(B) RpcAMP administration attenuated the increased phosphorylation of p42-MAPK by Ex-4 administration, but did not alter the increased phosphorylation of p44-MAPK by Ex-4. Data are mean ± SEM. *p < 0.05 from aCSF/aCSF. †p < 0.05 from aCSF/Ex-4.

2009b, 2010b) was performed. A hyperglycemic response (≥ 100% of basal glycemia) verified cannula placement.

GLP-1R-Expressing GT1-7 Neurons Show Exendin-4-Mediated Increases in cAMP/PKA and MAPK Signaling, and Suppression of AMPK Signaling

Total cAMP levels were evaluated in GLP-1R-expressing GT1-7 neuronal cells (Beak et al., 1998; Yang et al., 2005) following 15 min of Ex-4 treatment (0.0, 0.01, 0.1, 1.0, 10, 100 nmol). Figure 6A shows that GLP-1R activation by Ex-4 dose dependently increased total cAMP levels in GT1-7 neurons, with 0.1, 1.0, and 10.0 nmol of Ex-4 significantly increasing total cAMP levels compared to vehicle (aCSF).

Immunoblot analysis showed a significant dose-dependent decrease in pAMPKα2 levels in GT1-7 neurons following 15 min Ex-4 treatment (0.0, 1.0, 10, and 100 nmol; Figure 6B; data presented as pAMPKα2/total AMPKα). In addition, Ex-4 treatment dose-dependently increased phosphorylated p44/42 MAPK levels in GT1-7 neurons (Figure 6C; data presented as phosphorylated/total MAPK). Ex-4 treatment did not, however, significantly alter either total AMPKα or total p44/42-MAPK levels.

DISCUSSION

Pharmacological agents targeting the peripheral GLP-1 system are used to treat T2DM and may also prove to be efficacious in

obesity treatment (Hayes et al., 2010a; Holst, 2007; Larsen et al., 2003; Lovshin and Drucker, 2009). A variety of evidence indicates that activation of central GLP-1Rs engages a behavioral and physiological profile of responses that is similar to that observed following activation of peripheral GLP-1Rs and therefore may provide another pharmacological strategy to regulate blood glucose levels and reduce excessive adiposity by decreasing energy intake. Of the various brain regions containing GLP-1R-expressing neurons, previous reports establish those in the NTS as a physiologically relevant population of central GLP-1Rs in food intake control (Hayes et al., 2008b, 2009a; Kinzig et al., 2002). The intracellular signaling pathways mediating food intake suppression following NTS GLP-1R activation have not been previously established. Here we show that the suppression of food intake and BW following activation of hindbrain GLP-1Rs involves a coordinated PKA-mediated suppression in AMPK activity and activation of p44/42-MAPK signaling in the NTS.

Our working model (Figure 7, modified from Hayes et al., 2010a) derives from the current data, as well as previous reports examining the intracellular signaling cascades following GLP-1R activation in pancreatic β cells (Gomez et al., 2002; Perfetti and Merkel, 2000), from known signaling pathways downstream of cAMP/PKA activation in neuronal cells (Dugan et al., 1999; Vossler et al., 1997), and from recent reports showing that increased AMPK mRNA in the hypothalamus following food deprivation is attenuated by GLP-1R activity

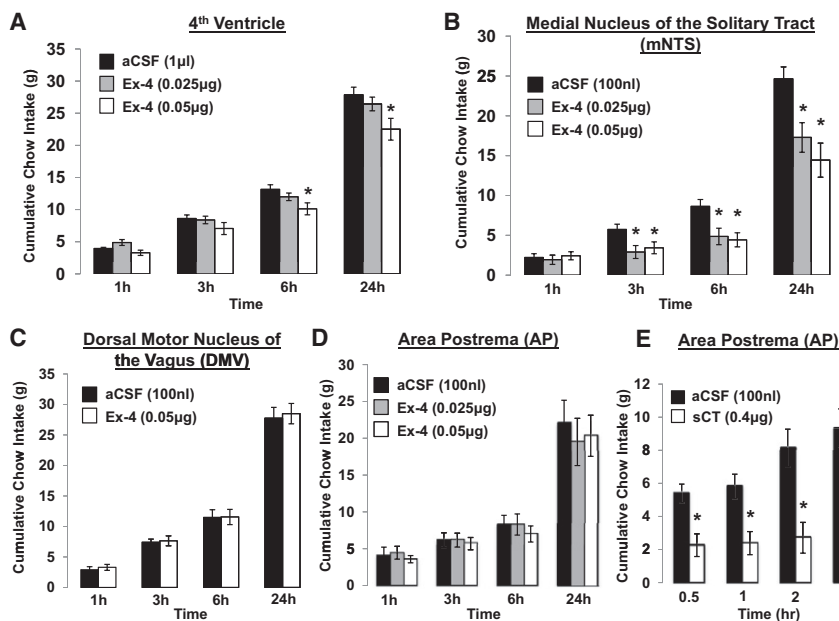


Figure 5. Medial NTS GLP-1R Activation Suppresses Food Intake

(A–D) Cumulative chow intake following (A) hindbrain (fourth i.c.v.) delivery of Ex-4 (0.025 μg and 0.05 μg in 1 μl), (B) unilateral intraparenchymal mNTS delivery of Ex-4 (0.025 μg and 0.05 μg in 100 nl), (C) unilateral intraparenchymal DMV delivery of Ex-4 (0.05 μg in 100 nl), (D) intraparenchymal AP delivery of Ex-4 (0.025 μg and 0.05 μg in 100 nl), counterbalanced with aCSF.

(E) Functional verification of AP injections measured by cumulative 4 hr chow intake following AP administration of the amylin (calcitonin) receptor agonist, salmon calcitonin (sCT) (0.4 μg in 100 nl) counterbalanced with aCSF. Data are mean ± SEM. * = $p < 0.05$ from aCSF.

(Seo et al., 2008). It is likely that the intake-suppressive effects following NTS GLP-1R activation that involve increased PKA and MAPK signaling and decreased AMPK signaling engage additional intracellular signaling cascades, as well as neuronal projections to other brain regions relevant to energy balance control. In addition to the intake-inhibitory responses produced by fourth i.c.v. Ex-4 administration, hindbrain GLP-1R signaling also triggers energy expenditure responses including hypothermia and tachycardia (Hayes et al., 2008b). Interestingly, inhibition of hindbrain AMPK activity also increases heart rate and locomotor activity in rats. Therefore, it is plausible that energy expenditure responses produced by hindbrain GLP-1R activation would involve AMPK signaling (Hayes et al., 2009b). However, it remains to be determined whether energetic responses mediated by hindbrain GLP-1R activation involve the same PKA, MAPK, and AMPK intracellular signaling cascades as described here for food intake.

Previous reports show that the GLP-1R-expressing NTS neurons are the likely mediators of the intake-suppressive effects of hindbrain ventricular Ex-4 administration (Hayes et al., 2008b, 2009a). Direct pharmacological blockade of mNTS GLP-1Rs increases food intake (Hayes et al., 2009a), indicating that endogenous NTS GLP-1R activation contributes to the physiological control of food intake. Vagal afferent signals arising from distension of the stomach appear to engage NTS GLP-1R signaling (Hayes et al., 2009a) following activation of the proglucagon-expressing neurons of the NTS (Vrang et al., 2003), which increases endogenous CNS GLP-1 ligand availability. Data presented in this report show that the food intake-suppressive effects of DVC parenchymal application of a GLP-1R ligand are mediated by mNTS neurons, as a significant suppression of intake was observed following Ex-4 delivery to the mNTS at a fourth i.c.v. subthreshold dose (0.025 μg), effects not observed following administration of Ex-4 at the same dose or even at a ventricular-effective dose (0.05 μg) to other hindbrain

GLP-1R-expressing nuclei (i.e., AP or DMV). Moreover, using a combination of in vivo and in vitro techniques with the GLP-1R ligand Ex-4, we demonstrate that the intake-suppressive effects of NTS-GLP-1R activation occur through a coordinated PKA-mediated suppression of AMPK activity and activation of p44/42 MAPK signaling. These effects may promote Ca^{2+} -dependent depolarization of the GLP-1R-expressing neurons and longer-term cAMP response element-binding protein (CREB)-mediated transcriptional effects. The present findings further suggest that the intake-suppressive effects of gastric distension may also be mediated in part by an increase in PKA, MAPK, and possibly CREB signaling, as well as inhibition of AMPK signaling within NTS neurons. It may also be the case that NTS mediation of the intake inhibitory responses of other anorectic signals (e.g., cholecystokinin, melanocortin, leptin) that engage these intracellular signaling pathways (Hayes et al., 2009b, 2010b; Sutton et al., 2004, 2005) could potentially reduce food intake in a synergistic fashion with either CNS GLP-1R ligands and/or gastric distension. Given that PKA inhibition did not completely reverse the 24 hr intake suppression by fourth i.c.v. Ex-4, it is also reasonable to assume that other non-PKA-dependent signaling pathways may mediate the suppression of intake by hindbrain GLP-1R activation.

While caudal brainstem processing (in the absence of fore-brain communication) is sufficient to mediate the food intake-suppressive effects following fourth i.c.v. Ex-4 administration (Hayes et al., 2008b), the NTS is not the only CNS GLP-1R-expressing nucleus relevant to energy balance control (e.g., PVH; central nucleus of the amygdala, CeA; dorsal medial hypothalamus, DMH). Indeed, parenchymal application of GLP-1(7-36) in the PVH suppresses food intake in rats (McMahon and Wellman, 1997, 1998). Whether the intracellular signaling cascades for PVH or other GLP-1R-expressing neurons are identical to those reported here for the DVC GLP-1R remains to be determined. It may be possible that in GLP-1R-expressing nuclei outside the NTS, different intracellular signaling pathways may mediate different behavioral/physiological responses produced by GLP-1R activation. Such a case has been made previously for the angiotensin receptor (Daniels et al., 2005). Nonetheless,

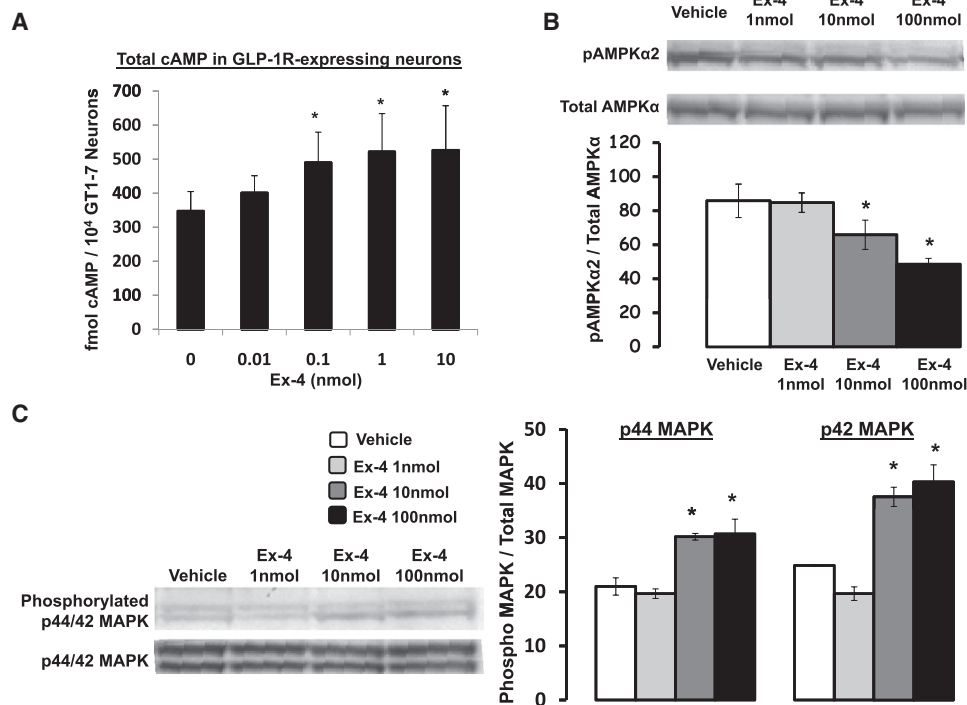


Figure 6. GLP-1R-Expressing GT1-7 Neurons Show Exendin-4-Mediated Increases in cAMP/PKA and MAPK Signaling, and Suppression of AMPK Signaling

(A) Total cAMP levels in 3 hr serum-starved GLP-1R-expressing GT1-7 neuronal cells following 15 min treatment with Ex-4 (0.0, 0.01, 0.1, 1.0, 10 nmol). * $p < 0.05$ from vehicle (0.0 nmol Ex-4). (B) Ex-4 treatment (0.0, 1.0, 10, and 100 nmol; 15 min) dose-dependently decreased pAMPK α 2 and (C) increased phosphorylated p44/42 MAPK in the GLP-1R-expressing GT1-7 neurons. Data are mean \pm SEM. * $p < 0.05$ from vehicle (0.0 nmol Ex-4).

it is reasonable to assume that the signaling cascades for GLP-1R-expressing neurons are similar throughout the brain, as the ex vivo signaling responses reported here for the DVC tissue lysates were also found in vitro in immortalized hypothalamic GLP-1R-expressing GT1-7 cells. It is virtually impossible to rule out the possibility that intracellular signaling inhibitors/activators (e.g., U0126/AICAR) attenuate the behavioral intake suppression of fourth i.c.v. Ex-4 by acting on both GLP-1R- and non-GLP-1R-expressing neurons.

Hindbrain Ex-4 administration rapidly increases NTS PKA activity, with a peak in activity occurring 10 min postadministration and a return to baseline (vehicle) levels seen at 30 min postadministration. Yet behavioral analyses show that the suppression of food intake by fourth i.c.v. Ex-4 is observed by the third hour postadministration, an effect dependent upon an increase in PKA and MAPK activity, as well as a decrease in AMPK activity. This distinction in the time course effects for the intracellular signaling cascades from tissue lysates and those for the food intake response have been reported previously for other anorectic ligands and intracellular signals acting within the brain (Morton et al., 2009; Niswender et al., 2001; Sutton et al., 2005). These observations can be interpreted to suggest that although PKA, MAPK, and AMPK signaling are required to mediate the suppression of intake by NTS GLP-1R activation, other downstream signaling cascades and recruitment of neurons of other CNS nuclei are also required to mediate the intake-suppressive action of hind-

brain Ex-4. These additional signaling cascades and neuronal projections arising from NTS GLP-1R-expressing neurons remain to be identified.

That suppression in meal number accounts for the reduced food intake resulting from hindbrain Ex-4 supports the notion that the central GLP-1 system is different, but complementary to, the peripheral GLP-1 system. This conclusion is based in part on data presented here, as well as on previous observations (Ruttimann et al., 2009; Scott and Moran, 2007) that show that the short-term intake-inhibitory effects of peripheral GLP-1R activation result from a reduction in meal size, with minimal effect on meal number. It may be possible that peripheral administration of Ex-4 or other DPP-IV-resistant GLP-1R ligands with longer half-lives than GLP-1(7-36) (e.g., liraglutide) may reduce food intake across a longer period of time by simultaneous activation of both peripheral and central GLP-1 systems.

In conclusion, current findings demonstrate that the food intake- and BW-suppressive effects of hindbrain GLP-1R activation are dependent upon an increase in PKA activity and subsequent simultaneous phosphorylation of p44/42-MAPK and inhibition of AMPK activity in NTS neurons. Ex-4 activation of the same signaling cascades in GT1-7 neuronal cells and in NTS lysates supports the view that these pathways occur in GLP-1R-expressing neurons. Together with our previous reports (Hayes et al., 2008b, 2009a), current data demonstrate that NTS GLP-1R-expressing neurons play a physiological role in

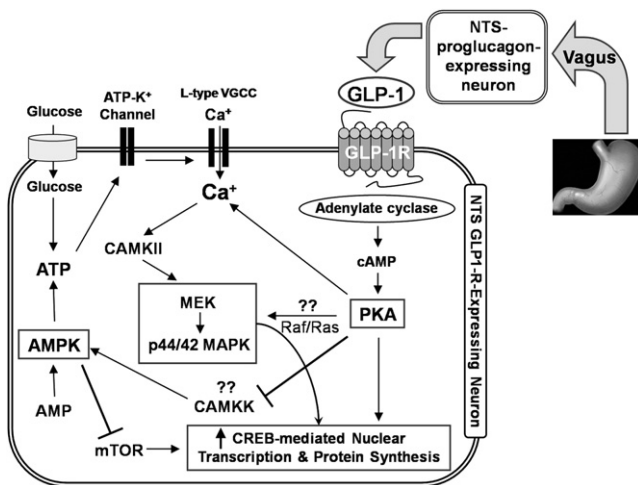


Figure 7. Intracellular Signaling Pathways in NTS GLP-1R-Expressing Neurons Mediating Food Intake Suppression by Hindbrain GLP-1R Ligands

Gastric vagal afferent signaling increases endogenous NTS-derived GLP-1, which activates endemic NTS GLP-1R-expressing neurons (Hayes et al., 2009a) to engage cAMP-dependent increase in PKA activity. Increased PKA activity concomitantly increases phosphorylation of p44/42-MAPK and decreases phosphorylation of AMPK. The combined increase in PKA and p44/42-MAPK activity together with decreased AMPK activity is hypothesized to increase CREB-mediated nuclear transcription and protein synthesis. mTOR, mammalian target of rapamycin; CAMKK, calmodulin-dependent protein kinase kinase; CAMKII, calcium/calmodulin-dependent protein kinase II; VGCC, voltage gated calcium channel; MEK, mitogen-activated protein kinase kinase.

control of food intake and energy balance regulation. These data provide clues to understanding how NTS neurons mediate anorectic actions of other stimuli involved in meal-to-meal food intake control. Future pharmacotherapies targeting not only NTS GLP-1R but the CNS GLP-1 system as a whole may prove to be efficacious in ameliorating chronic hyperphagia associated with obesity.

EXPERIMENTAL PROCEDURES

Subjects and Materials

Individually housed, adult male Sprague Dawley rats (300–350 g; Charles River), maintained at 23°C with 12 hr light/dark cycle (10:00 hr lights off), had ad libitum access to chow (Purina Rodent Chow 5001) and water except as noted. All procedures conformed to the institutional standards of the animal care and use committee (University of Pennsylvania).

The following compounds were utilized to examine hindbrain GLP-1R-mediated control of food intake: the DPP-IV-resistant GLP-1R agonist, Ex-4 (American Peptide Co.) (Hayes et al., 2008b); adenosine-3',5'-cyclic monophosphorothioate Rp-isomer (RpcAMP; Sigma), a widely used inhibitor of cAMP-dependent activation of PKA (Dostmann et al., 1990; Kelly et al., 2007; Sherif et al., 2003); the AMP-mimicking promoter of AMPK activity, 5-aminoimidazole-4-carboxamide-1-β-D-ribose (AICAR; Fisher Scientific) (Hayes et al., 2009b; Kim et al., 2004a); and the selective MEK inhibitor U0126 (Tocris Bioscience), which blocks p44/42 MAPK phosphorylation (Daniels et al., 2003; Sutton et al., 2005; Sutton et al., 2004). All drugs were administered in unrestrained animals in a counterbalanced fashion, with all drug combinations for a given experiment being administered on any given experimental day. For behavioral testing, each experimental trial was separated by a minimum of 48 hr.

Fourth Intracerebroventricular/mNTS/AP/DMV Cannulation Surgery

Under ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) anesthesia and analgesia (Metacam 2 mg/kg), guide cannulas (Plastics One; 26-gauge) were implanted at the following coordinates: fourth i.c.v., 2.0 mm above fourth ventricle, 2.5 mm anterior to occipital suture, 4.5 mm ventral to dura, and on midline; bilateral caudal mNTS, 2.0 mm above mNTS, 1.0 mm posterior to occipital crest, ±0.7 mm lateral to midline, 6.7 mm ventral from skull surface; AP, 2.0 mm above AP, 1.0 mm posterior to occipital crest, on midline, 6.4 mm ventral from skull surface; bilateral DMV, 2.0 mm above DMV, 1.0 mm posterior to occipital crest, ±0.7 mm lateral to midline, 6.9 mm ventral from skull surface (Hayes and Covasa, 2006; Hayes et al., 2009b, 2010b; Paxinos and Watson, 1998). Intended anatomical positions of fourth i.c.v./mNTS injection sites were evaluated 1 week postsurgery as described (Hayes et al., 2009b, 2010b). Behavioral testing began ~12 days following surgery. All DVC intraparenchymal injection sites were anatomically confirmed via postmortem verification of the position of 100 nl pontamine sky blue injections. Only animals passing both functional and histological verifications were included in final statistical analyses.

Signaling Analysis

Ex Vivo Tissue Collection

Tissue of the caudal DVC of ad libitum chow-fed rats, were prepared as described (Hayes et al., 2009b; Minokoshi et al., 2004). Briefly, at dark cycle onset, rats received a fourth i.c.v. injection of Ex-4 (0.3 μg) or aCSF vehicle and were sacrificed 10, 20, or 30 min later by decapitation. Brains were rapidly removed; tissue lysates were collected from the DVC (Hayes et al., 2009b) and samples flash frozen in isopentane and stored at –80°C. To collect caudal DVC tissue, brains were positioned with dorsal surface up, and the cerebellum was removed. The calamus scriptorius (most caudal portion of the fourth ventricle) provides a landmark for isolating caudal dorsomedial DVC tissue. Coronal sections were made 1 mm anterior and 2 mm posterior to the calamus scriptorius. Bilateral sagittal cuts were made ~2 mm lateral to the midline. A horizontal cut was then made 0.5 mm ventral to the central canal (Hayes et al., 2009b; Huo et al., 2006).

To determine whether the decrease in pAMPKα2 levels and increase in phosphorylated p44/42-MAPK levels by hindbrain GLP-1R activation are dependent on PKA activation, a separate group of ad libitum-fed rats (n = 3/treatment) received a fourth i.c.v. injection of aCSF vehicle (1 μl) counterbalanced with RpcAMP (20 μg) at dark cycle onset. Twenty minutes later, rats received a fourth i.c.v. injection of Ex-4 (0.3 μg) counterbalanced with aCSF. Rats were sacrificed 20 min following the second set of injections, and brains were rapidly removed and tissue of the caudal DVC was collected.

In Vitro GLP-1R-Expressing Immortalized GT1-7 Neuronal Cells

GT1-7 hypothalamic neuroblastoma cells (generous gift from Dr. Sangwon Kim, University of Pennsylvania) that highly express the GLP-1R (Beak et al., 1998; Yang et al., 2005) were maintained at 37°C in 5% CO₂ and were cultured in 1× DMEM with 4.5 mg/ml glucose (GIBCO/Invitrogen), 10% (v/v) fetal bovine serum (Thermo Fisher Scientific Inc.), and 2% penicillin (10,000 I.U./ml)-streptomycin (10,000 ug/ml) solution (Mediatech, Inc.). Three hour serum-starved GT1-7 neurons were treated with Ex-4 (0.0, 1.0, 10, 100 nmol) for 15 min. At the end of the incubation, medium was removed, cells were washed in ice-cold 0.1M PBS, and lysates were prepared by scraping cells into 500 μl of 1% NP-40 lysis buffer containing fresh protease and phosphatase inhibitors and repetitively passing the lysates through a 26-gauge needle followed by 10 min centrifugation (12,000 rpm) at 4°C to pellet out insoluble material.

Biochemistry

Caudal DVC tissue and GT1-7 neuronal cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes for immunoblot analysis (Hayes et al., 2009b, 2010b). pAMPKα-Thr 172 rabbit monoclonal antibody (pAMPKα2; Cell Signaling; catalog number 2535S) was used to evaluate AMPK activity and was normalized to total AMPKα (reactive against α1 and α2 subunits; Cell Signaling). Phospho-p44/42 MAPK polyclonal antibody (Thr202/Tyr204; Cell Signaling; catalog number 9101S) was used to assess MAPK signaling and was normalized to total p44/42 MAPK (Cell Signaling; catalog number 9102). Blots were quantified using NIH Image software.

Relative PKA activity of tissue and cell lysates was determined by ELISA (Stressgen Assay Designs; catalog number EKS-390A).

Determination of cAMP Levels

Measurement of total cAMP was conducted using the Biotrak cAMP enzyme immunoassay system (GE Healthcare/Amersham Biosciences, USA). Three hour serum-starved GT1-7 neurons, cultured in a 96-well culture plate (6×10^4 cells/well), were treated with Ex-4 (0.0, 0.01, 0.1, 1.0, 10 nmol) for 15 min. cAMP levels were calculated using a standard curve of fmol of cAMP/ 10^4 GT1-7 neurons.

Feeding Behavior

Hindbrain PKA Activity

One hour prior to the dark cycle, ad libitum-fed rats ($n = 7$) received a fourth i.c.v. injection of aCSF vehicle (1 μ l) counterbalanced with RpcAMP (20 μ g) (dose selection, Kelly et al., 2007) 45 min prior to a fourth i.c.v. injection of Ex-4 (0.2 μ g) (dose subthreshold for intake suppression by IP administration from pilot work [data not shown] and [Mack et al., 2006]) counterbalanced with aCSF. Five minutes following the second drug administration, immediately prior to dark onset, rats were presented with preweighed maintenance rat chow, and intakes were recorded to the nearest 0.1 g at 1, 3, 6, and 24 hr post-food presentation.

Hindbrain MAPK Signaling

One hour prior to the dark cycle, a separate group of ad libitum fed rats ($n = 8$) received a fourth i.c.v. injection of vehicle (dimethylsulfoxide [DMSO]; 1 μ l) counterbalanced with U0126 (2 μ g dissolved in DMSO) 45 min prior to a fourth i.c.v. injection of Ex-4 (0.2 μ g) counterbalanced with aCSF (U0126 dose and injection time course chosen from pilot dose response study [data not shown; and Sutton et al., 2005]). Cumulative chow intake was recorded at 1, 3, 6, and 24 hr.

Hindbrain AMPK Signaling

In a separate group of ad libitum-fed rats ($n = 6$), the role of hindbrain AMPK signaling in mediating the intake suppressive effects of fourth i.c.v. Ex-4 was evaluated using automated feedometers designed in our lab. Briefly, feedometers consisted of a standard Nalgene rodent bin (18 \times 9 \times 8 inches for length, width, and depth, respectively) with permanent, externally mounted food hoppers. Rats had ad libitum access to pelleted chow through a 3.5 \times 2.5 inch square hole in the chamber. The externally mounted food hoppers were housed in and rested on a custom-built reinforced acrylic cup (to catch spillage) attached to a leveled load cell (Omega Engineering, Inc; LCAE-1KG). The weight of the chow + spillage was recorded to the nearest 0.1 g/min and relayed to computer via process meter (Omega Engineering, Inc; DP25B). Data were processed by computer software (National Instruments; LabVIEW; Penn 8 Channel Feed Monitor 1.0.0) to determine food ingestion each 1 min, and a total food intake curve was cumulated for 24 hr. Two hours prior to the dark cycle, automated food intake readings began. One hour later, rats received fourth i.c.v. injection of aCSF (3 μ l) counterbalanced with AICAR (300 μ g dissolved in aCSF), followed 45 min later by a second fourth i.c.v. injection (1 μ l) of Ex-4 (0.1 μ g) counterbalanced with aCSF (Hayes et al., 2009b). A meal was defined as a bout of eating at least 0.25 mg of food followed by 10 min of no eating. These meal parameters accounted for >95% of the 24 hr cumulative food intake.

Meal Pattern Analysis of the Intake-Suppressive Effects Following Peripheral GLP-1R Activation

In a separate group of ad libitum chow-fed rats ($n = 7$), the meal pattern effects mediating the intake suppression following peripheral (IP) Ex-4 (3.0 μ g/kg) administration were evaluated to allow for comparisons with the aforementioned meal pattern effects following fourth i.c.v. Ex-4. As detailed above, data were processed to determine the continuous cumulative intake for 24 hr, as well as analyzed for the meal pattern effects of the drugs injected. Two hours prior to the dark cycle, automated food intake readings began. Fifteen minutes prior to the onset of the dark cycle, rats received an IP injection of Ex-4 counterbalanced with 0.9% saline vehicle (1 ml/kg). Dose and time course selection of drug delivery was chosen from Hayes et al. (2009b) and pilot studies to yield a 40%–50% maximum suppression in food intake.

Intracerebral mNTS/DMV/AP Ex-4 Administration

In separate groups of rats fed chow ad libitum, individual nuclei within the DVC were targeted to determine which site(s) contributed to the mediation of the intake-suppressive effects of hindbrain (fourth i.c.v.) Ex-4 delivery. First, to

establish a fourth i.c.v. dose of Ex-4 that is without effect on intake, we administered Ex-4 fourth i.c.v. at 0.025 μ g and 0.05 μ g counterbalanced with 1 μ l aCSF, 5 min prior to dark onset. Rat ($n = 7$) intake of chow was recorded at 1, 3, 6, and 24 hr.

Having established that the 0.025 μ g dose of Ex-4 is subthreshold for intake suppression when administered to the fourth ventricle, the relevant GLP-1R-expressing nucleus within the DVC mediating the intake suppressive effects of fourth i.c.v. Ex-4 was determined by intracerebral administration of Ex-4 at a 100 nl volume. A separate group of rats ($n = 12$), implanted with bilateral intracerebral mNTS cannula (at the level of the AP), received counterbalanced unilateral intracerebral mNTS injections of Ex-4 (0.025 μ g and 0.05 μ g in 100 nl) or aCSF 5 min prior to dark onset. Likewise, rats ($n = 5$) implanted with DMV cannula received counterbalanced injections of Ex-4 (0.05 μ g in 100 nl) or aCSF 5 min prior to dark onset. Cumulative chow intake was recorded at 1, 3, 6, and 24 hr.

A separate set of rats ($n = 7$) received counterbalanced intracerebral injections of Ex-4 (0.025 μ g and 0.05 μ g in 100 nl) or aCSF into the AP 5 min prior to dark onset. Again, chow intake was recorded at 1, 3, 6, and 24 hr. To functionally verify the AP site of injections, at the completion of the AP-Ex-4 experiment, the same rats received counterbalanced AP administration of the amylin agonist, sCT at 0.4 μ g/100 nl or aCSF 5 min prior to dark onset. Food intake was recorded at 0.5, 1, 2, and 4 hr post-food presentation. Dose and time course of food intake recording for the AP sCT experiment was conducted as reported (Mollet et al., 2004).

Data and Statistical Analyses

Data for each respective study were analyzed separately and expressed as mean \pm SEM. For all experiments, comparisons between treatment means were analyzed by one- or two-way ANOVAs and, if appropriate, post hoc, pairwise comparisons were made using Tukey's honestly significant difference test with $p < 0.05$ considered statistically significant. Analyses were made using PC-SAS (version 8.02, SAS Institute) mixed procedure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article at doi:10.1016/j.cmet.2011.02.001.

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