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Central Glucagon-Like Peptide 1 Receptor (Glp1r)-Induced Anorexia Requires Glucose Metabolism-Mediated Suppression of AMPK and is Impaired by Central Fructose.

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Running head: Central Glp1r-mediated anorexia and AMPK

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Abstract

Glucagon-like peptide-1 (Glp1) suppresses food intake via activation of a central (i.e., brain) Glp1 receptor (Glp1r). Central AMP-activated protein kinase (AMPK) is a nutrient-sensitive regulator of food intake that is inhibited by anorectic signals. The anorectic effect elicited by hindbrain Glp1r activation is attenuated by the AMPK stimulator AICAR. This suggests that central Glp1r activation suppresses food intake via inhibition of central AMPK. The present studies examined the mechanism(s) by which central Glp1r activation inhibits AMPK. Supporting previous findings, AICAR attenuated the anorectic effect elicited by intracerebroventricular (ICV) administration of the Glp1r agonist Exendin-4 (Ex4). We demonstrate that Ex4 stimulates glycolysis and suppresses AMPK phosphorylation in a glucose-dependent manner in hypothalamic GT1-7 cells. This suggests that inhibition of AMPK and food intake by Ex4 requires central glucose metabolism. Supporting this, the glycolytic inhibitor 2-deoxyglucose (2-DG) attenuated the anorectic effect of Ex4. However, ICV glucose did not enhance the suppression of food intake by Ex4. AICAR had no effect on Ex4-mediated reduction in locomotor activity. We also tested whether other carbohydrates affect the anorectic response to Ex4. ICV pre-treatment with the sucrose metabolite fructose, an AMPK activator, attenuated the anorectic effect of Ex4. This potentially explains the increased food intake observed in sucrose-fed mice. In summary, we propose a model whereby activation of the central Glp1r reduces food intake via glucose metabolism-dependent inhibition of central AMPK. We also suggest that fructose stimulates food intake by impairing central Glp1r action. This has significant implications given the correlation between sugar consumption and obesity.

Keywords

Glucagon-like peptide 1 (Glp1); AMP-activated kinase (AMPK); Food intake; Glucose; Fructose

Introduction

Health risks associated with obesity, including cardiovascular disease, diabetes and cancer, highlight the importance of understanding mechanisms that control food intake and how they become impaired. Food intake is regulated by neural and hormonal signals that match caloric intake with the chronic and acute energy needs of the organism. Although significant attention has been given to understanding mechanisms associated with adiposity signals such as leptin and insulin, less is known about acute satiety mechanisms regulated by signals such as glucagon-like peptide-1 (Glp1).

Glp1 is secreted from intestinal L-cells in response to nutrient intake and was identified for its ability to stimulate insulin secretion via activation of a pancreatic β-cell Glp1 receptor (Glp1r) (3). Glp1 also regulates processes independently of its pancreatic effects including gastric emptying and cardiac function (57). One of the first extra-pancreatic effects identified for Glp1 is its ability to suppress food intake. Intracerebroventricular (ICV) and targeted hypothalamic or hindbrain administration of Glp1r agonists reduces food intake (12, 20, 55). Conversely, central (i.e., brain) administration of Glp1r antagonists increases food intake (56), demonstrating that endogenous Glp1 is an anorectic factor. Some Glp1-based therapies used for the treatment of type 2 diabetes elicit moderate weight loss (58). The available data (14) suggests that targeting the central Glp1r may be useful as an anti-obesity strategy.

The mechanism(s) by which Glp1 reduces food intake has not been fully elucidated. Prolonged fasting blunts the anorectic effect mediated by the Glp1r agonist Exendin-4 (Ex4) (62). This suggests that nutrient status plays a critical role in reduction of food intake by the Glp1r. Changes in nutrient status modulate the activity of AMP-activated protein kinase (AMPK) in various tissues. Hypothalamic AMPK is a key sensor of nutrient status and plays an integral role in regulating food intake (1, 42). Prolonged fasting stimulates hypothalamic AMPK activity and the subsequent drive to increase food intake (41). Direct pharmacological and genetic activation of hypothalamic AMPK increases food intake (25, 41), whereas its inhibition reduces food intake (23, 41). Moreover, anorectic signals such as leptin (54) and glucose (10, 36, 37) inhibit hypothalamic AMPK activity. ICV Glp1 administration reduces hypothalamic AMPK Thr^{172} phosphorylation, a marker of AMPK activation (6, 49). Ex4 also suppresses

AMPK phosphorylation in the GT1-7 hypothalamic cell line (20). Inhibition of AMPK by Glp1r activation is also observed in non-hypothalamic brain regions. Delivery of Ex4 into the 4th ventricle reduces AMPK phosphorylation in the dorsal vagal complex (DVC) in the hindbrain (20). Furthermore, administration of Ex4 into the 4th ventricle suppresses food intake, and this anorectic effect is attenuated by the AMPK activator AICAR (20). Taken together, these observations suggest that inhibition of AMPK is required for the anorectic effect mediated by central Glp1r activation.

Delineating the signaling pathways mediating the anorectic effect of central Glp1r activation may identify sites where obesogenic diets interfere. High fat diets inhibit the anorectic effects of insulin, leptin and Glp1 (2, 8, 32, 63). Diets rich in sucrose and fructose also promote insulin and leptin resistance (50, 53), but the effects of sucrose or fructose on Glp1 action remain largely unexplored. Increased consumption of sucrose and fructose has been implicated as a culprit in the obesity epidemic, particularly in children (34). Interestingly, ICV administration of the sucrose metabolite fructose stimulates food intake and central (hypothalamic) AMPK activity (28). If inhibition of central AMPK is required for the anorectic effect of Glp1r agonists, then activation of AMPK by fructose could attenuate the satiety effect of Glp1. Assuming that dietary fructose is metabolized in the brain, this could explain reduced satiety associated with consumption of sucrose- and fructose-rich foods and beverages.

The present studies explored the mechanisms by which central Glp1r activation inhibits AMPK and food intake. We employed lateral ventricle administration of AMPK activators and Ex4 to corroborate previous findings that central Glp1r activation suppresses food intake via inhibition of central AMPK (20). Time-course measurements of food and water intake, locomotor activity and energy expenditure were obtained following ICV treatments. The hypothalamic GT1-7 cell line was used to determine a potential mechanism by which Glp1r activation inhibits AMPK. ICV administration of fructose was used to test the hypothesis that this carbohydrate attenuates the anorectic effect mediated by the central Glp1r.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the Sanford-Burnham Medical Research Institute at Lake Nona. Male Glp1r knockout ($Glp1r^{-/-}$) and wild-type ($Glp1r^{+/+}$) mice on a C57Bl/6 background were fed a high starch diet (Research Diets D12328, New Brunswick, NJ). For diet intervention studies, some mice were fed an isocaloric high sucrose diet (Research Diets D12329, New Brunswick, NJ). High starch and high sucrose diets contained 2.44kcal/g of corn starch and sucrose, respectively. Both diets were composed of 10.5, 73.1 and 16.4kcal/g of fat, carbohydrate and protein, respectively. All experiments were performed on 4-5 month-old mice maintained on a standard light-dark cycle (0600–1800h light).

Surgical procedures

ICV cannulae were implanted under isoflurane (2%) anesthesia using a stereotaxic apparatus (David Kopf Instruments, Tujuna, CA). The skull was exposed by an incision and leveled between lambda and bregma. Cannulae (Plastics One, Roanoke, VA) were implanted to target the lateral cerebral ventricle at the following coordinates: 0.3mm caudal, 1.0mm from midline, and 3.2mm ventral. A guide cannula was inserted into the brain by drilling a burr hole through the skull and was fixed into position by two jeweler's screws and cranioplastic cement. Verification of cannula position in the lateral cerebroventricle was made by observing spontaneous flow of cerebrospinal fluid from the tip of the cannula after removal of the obturator. Animals were housed individually after surgery and allowed to recover for 5 days before experimentation. Cannula placement in the lateral cerebroventricle was re-verified by observing increased drinking in response to an ICV administration of angiotensin-II (1μg) on the final day of experimentation.

Intracerebroventricular (ICV) delivery of drugs

Artificial cerebrospinal fluid (ACSF; Harvard Apparatus, Holliston, MA) was used as vehicle and delivered at a volume of 2µl. Exendin-4 (Ex4, Tocris Bioscience, Minneapolis, MN) was administered at a dose of 0.01µg to elicit a 50-70% reduction in 18h food intake. Other compounds were administered as pre-treatments: fructose (Sigma-Aldrich) at 400µg (a dose shown to elicit a short-term increase in food intake (7)); the AMPK activator aminoimidazole carboxamide ribonucleotide (AICAR, Toronto Research Chemicals, Toronto, ON, Canada) at 1 or 10µg; the glycolytic inhibitor 2-deoxyglucose (2-DG, Sigma-Aldrich) at 5mM; and D-glucose (Sigma-Aldrich, St. Louis. MO) at 400µg (a dose shown to elicit a short-term decrease in food intake (7)). Doses of the pre-treatments were selected to produce minimal effects on food intake by themselves. On each study day, mice were fasted for 5h beginning at 1300h. ICV treatments were performed just prior to the onset of the dark cycle at 1800h. In drug combination studies, compounds were administered 15min apart.

Measurement of food intake, water intake, locomotor activity and energy expenditure

Food and water intake, locomotor activity (infrared beam breaks) and energy expenditure were acquired using a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Animals were placed in the CLAMS and acclimated for at least 48h prior to experimentation. Feeding, drinking, locomotor activity and energy expenditure were continuously measured for 18h (1800-1200h) following ICV treatments. Locomotor activity was converted to distance traveled by multiplying the number of beam breaks recorded for ambulatory activity by the distance between the beams. For pica tests, mice were individually housed and acclimated to the presence of Kaolin clay in their home cages for one week. Mice were given ICV treatments of ACSF or Ex4 at the doses and volumes indicated above or an i.p. injection of lithium chloride (0.15 M) prior to the onset of the dark cycle and following a 5h fast. 18h Kaolin clay consumption was measured manually using a precision scale.

Protein immunoblots in isolated hypothalamus

Whole cell extracts from entire hypothalami were obtained by homogenizing tissue in 100µl tissue lysis buffer (1M Tris-HCl, 1M NaCl, 100mM EDTA, 50mM EGTA, 10% NP-40, 25mM sodium pyrophosphate, 100mM sodium orthovanadate, 1M NaF) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Homogenates were centrifuged (15min, 10,000x*g*, 4°C), pellets were discarded, and supernatants were retained for protein determination. Protein content was determined using a Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL). Whole-cell (30µg) extracts were separated on 4–12% Bis-Tris SDS-PAGE gels (Life Technologies, Carlsbad, CA), followed by electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with primary antibodies overnight at 4°C and with secondary antibodies conjugated to alkaline phosphatase at room temperature for 1h. Imaging was performed using a Typhoon FLA9500 imager (GE Healthcare), and densitometry was calculated using ImageJ software (NIH). Antibodies for AMPK, Thr^{172} phosphorylated AMPK and β-actin were from Cell Signaling Technology (Danvers, MA).

Studies in hypothalamic GT1-7 neurons

Hypothalamic GT1-7 neurons, an immortalized mouse hypothalamic cell line expressing the Glp1r (generous gift from P.L. Mellon, (39)), were maintained in DMEM with 4.5g/L glucose, L-glutamine and sodium pyruvate (Mediatech, Manassas, VA), 10% fetal bovine serum (Sigma, St. Louis, MO) and 10ml/L penicillin/streptomycin (Hyclone, Rockford, IL) at 37°C in 5% CO_2. Prior to treatments, cells were plated in 10cm cell culture dishes and allowed to reach 70% confluence. For glucose/Ex4 experiments, cells were washed twice with phosphate buffered saline (PBS) and serum starved in media (Sigma D-5030 supplemented with sodium bicarbonate, L-glutamine and sodium pyruvate) containing 25mM glucose for 3h. For fructose/Ex4 experiments, cells were serum starved in media supplemented with 25mM glucose for 2h and then switched to serum free media containing 25mM fructose (in place of glucose) for 1h. For each experiment, vehicle (PBS) or Ex4 (Abcam Biochemicals, Cambridge, MA) was then added to the plates at the appropriate concentration (1, 10 or 100nM) for

15min. Media was removed and plates were immediately placed on liquid nitrogen. Plates were then placed on ice and cells were scraped into freshly prepared lysis buffer for protein determination and subsequent immunoblot analysis as previously described. For mannitol experiments, 25mM mannitol was used in place of 25mM fructose.

For analysis of glycolysis, GT1-7 cells were seeded in growth media ($1.5 \cdot 10^4$ cells/well) in a V3-PS 96-well plate (Seahorse Bioscience, Billerica, MA). The following day, cells were washed with PBS and incubated in XF Assay media (Seahorse Bioscience, Billerica, MA) supplemented with 1mM pyruvate and 2mM L-glutamine (pH=7.4) for 1h at 37°C in a CO_2-free incubator. Glucose (0 or 16.7mM) and Ex4 (100nM) treatments were prepared in XF assay media containing pyruvate and L-glutamine (pH=7.38–7.42). Extracellular acidification rate (ECAR), an index of lactate production as a result of glycolysis, was measured before and after administration of glucose and Ex4. Measurements were normalized to protein concentration, as determined by Bradford protein assay as described previously.

Statistics analysis

Data are presented as mean ± SEM. Differences between groups were determined by two-tailed *t*-test or by one-way or two-way repeated-measures ANOVA followed by either Dunnet's or Neuman-Keuls Multiple Comparison post hoc test as appropriate. Significance level was defined as $p < 0.05$.

Results

Functional deletion of the Glp1r is associated with elevated basal hypothalamic AMPK signaling and increased food intake.

To determine whether endogenous, basal Glp1r signaling regulates central AMPK activity, AMPK phosphorylation was assessed in hypothalamic extracts from wild-type ($Glp1r^{+/+}$) and Glp1r knockout ($Glp1r^{-/-}$) mice. As shown in Figure 1A, protein levels of phosphorylated AMPK (pAMPK) normalized to total AMPK were significantly higher ($p<0.05$) in hypothalami from 5h-fasted $Glp1r^{-/-}$ mice compared to $Glp1r^{+/+}$ mice. In agreement with previous studies (17), $Glp1r^{-/-}$ mice exhibited significantly increased ($p<0.05$) 24h food intake (Fig. 1B).

ICV pre-treatment with the AMPK activator AICAR attenuates Ex4-mediated suppression of food intake.

Pre-treatment with AICAR significantly attenuated ($p<0.05$) the anorectic effect of Ex4 administered into the lateral ventricle (Fig. 2A). We chose a dose of AICAR that by itself does not stimulate food intake (Fig. 2A), as has been previously observed with higher doses (25). Indeed, a 10-fold higher dose of AICAR did stimulate 18h food intake compared to vehicle (3.77±0.37 vs. 2.61±0.14 g; $p<0.05$, n=7). In agreement with previous reports (38), central Glp1r activation suppressed ($p<0.05$) water intake (Fig. 2B). ICV AICAR alone did not affect water intake. When administered as a pre-treatment, ICV AICAR attenuated ($p<0.05$) the adipsic effect of ICV Ex4 by ~40% (Fig. 2B).

Locomotor activity was measured to rule out the possibility that the observed effects of AICAR pre-treatment are general and not specific to food intake. Supporting previous findings (9), central Glp1r activation reduced locomotor activity (Fig. 2C). Unlike the effects on food and water intake, ICV AICAR pre-treatment did not attenuate the reduction of locomotor activity elicited by ICV Ex4 (Fig. 2C).

Despite previous reports that Glp1r agonists reduce energy expenditure (4, 29), ICV Ex4 had no significant effect on 18h energy expenditure in the present studies (data not shown).

The anorectic effect elicited by Ex4 at the chosen dose was not attributed to nausea. We measured Kaolin clay consumption (i.e., pica test) as an index of nausea (43) and observed similar 18h Kaolin consumption following ICV ACSF vehicle (0.21±0.05 g) or Ex4 (0.26±0.10 g; n=6). By comparison, administration of lithium chloride (0.15 M, i.p.), a known inducer of nausea in rodents (27) stimulated 18h Kaolin consumption (1.15±0.08 g; $p<0.05$ vs. ICV ACSF).

Ex4 inhibits AMPK and stimulates glycolysis in the GT1-7 hypothalamic cell line.

We next used the Glp1r-expressing GT1-7 hypothalamic cell line (20, 46) to explore the mechanism by which Ex4 inhibits AMPK activity. Ex4 suppressed AMPK activation in these cells in a dose-dependent manner (Fig. 3A), demonstrating the validity of using this cell model. Interestingly, Ex4 treatment did not suppress AMPK activation in GT1-7 cells cultured in the absence of glucose (Fig. 3B). This demonstrates that the ability of Glp1r activation to inhibit AMPK is dependent upon glucose availability. We tested the hypothesis that Ex4 stimulates glucose metabolism in GT1-7 neurons. Using a Seahorse extracellular flux analyzer, we measured glycolytic rates as a surrogate for glucose metabolism. Figure 3C shows that Ex4 increased ($p<0.05$) glycolytic rates beyond the effect of glucose alone.

In vivo inhibition of central glycolysis attenuates the anorectic effect mediated by ICV Ex4.

Based on our observations that Ex4 inhibits AMPK in a glucose-dependent manner and stimulates glycolysis in GT1-7 cells, we tested whether inhibition of glycolysis *in vivo* attenuates the Ex4-mediated suppression of food intake. Central glycolysis was inhibited via ICV administration of the glycolytic inhibitor 2-deoxyglucose (2-DG). The ability of ICV Ex4 to reduce food intake was abolished ($p<0.05$) by pre-treatment with ICV 2-DG (Fig. 4A). ICV 2-DG alone had no significant effect on food intake. Pre-treatment with ICV 2-DG also prevented the adipsic effect of Ex4 by ~50% (Fig. 4B). Neither ICV Ex4 nor ICV 2-DG had a significant effect on 18h energy expenditure (data not shown).

ICV glucose does not enhance the anorectic effect mediated by ICV Ex4.

Our results indicate that central Glp1r activation suppresses food intake via inhibition of AMPK and suggest that this occurs via increased central glucose metabolism. We, therefore, hypothesized that central administration of glucose would enhance the anorectic effect of Ex4. Food intake was measured in mice receiving ICV glucose prior to ICV Ex4 administration. At the chosen dose, ICV glucose alone had no effect on food intake (Fig. 5A). When given as a pre-treatment, ICV glucose did not enhance the anorectic effect mediated by ICV Ex4 (Figure 5A). Pre-treatment with ICV glucose also did not enhance the ability of Ex4 to reduce water intake (Figure 5B). Thus, contrary to our hypothesis, increasing central glucose availability does not enhance the anorectic effect mediated by activation of the central Glp1r.

ICV fructose attenuates Ex4-mediated reduction of food intake and AMPK activity.

Mice fed a high sucrose diet display increased ($p<0.05$) food intake compared to mice fed an isocaloric starch diet (Fig. 6A). We explored whether fructose, a component of sucrose, could affect the anorectic response mediated by Ex4. This is based on the observation that ICV-administered fructose stimulates hypothalamic AMPK activity (28). The anorectic effects of Ex4 were blunted ($p<0.05$) by pre-treatment with ICV fructose (Fig. 6B). At the chosen dose, ICV fructose alone had no significant effect on food intake. ICV fructose also attenuated ($p<0.05$) the reduction in water intake elicited by ICV Ex4 (Fig. 6C). Similar to what was observed in the ICV AICAR and 2DG studies, neither ICV Ex4 nor ICV fructose had a significant effect on 18h energy expenditure (data not shown).

To determine whether fructose can interfere with the inhibition of AMPK by Ex4, GT1-7 cells were cultured in the presence of fructose for 1h prior to treatment with Ex4. A 1h fructose incubation was sufficient to markedly ($p<0.05$) stimulate AMPK (Fig. 7A). Contrasting the ability of Ex4 to reduce AMPK phosphorylation in the presence of glucose (Fig. 3A), Ex4 did not suppress AMPK activation in GT1-7 cells exposed to an equal concentration of fructose (Fig. 7B). Ex4 also did not suppress AMPK activation in the presence of 25 mM mannitol (Figure 7C), demonstrating that inhibition of AMPK by Glp1r activation requires glucose.

295 **Discussion**

296 The ability of Glp1r agonists to suppress food intake has been recognized for over a decade, yet
297 the mechanism by which this occurs remains largely unknown. Hayes and colleagues recently showed
298 that hindbrain Ex4 administration suppresses food intake and inhibits AMPK in the DVC (20).
299 Furthermore, pre-treatment with AICAR blunted the anorectic effect mediated by hindbrain Ex4
300 administration. The present studies support these findings and show that AICAR attenuates the anorectic
301 effect mediated by Ex4 administered into the forebrain lateral ventricle. We extend these findings by
302 showing that the inhibition AMPK and food intake by Ex4 requires glucose metabolism. This is
303 analogous to the dependence on glucose characteristic of pancreatic Glp1 action (59). We further
304 demonstrate that the nature of carbohydrates can influence the central actions of Glp1r agonists. Our
305 results show that the sucrose metabolite fructose impairs the anorectic effect mediated by centrally-
306 administered Ex4. This is likely due to the ability of fructose to prevent the inhibition of hypothalamic
307 AMPK by Ex4. Assuming that dietary fructose is metabolized in the brain, this provides a potential
308 mechanism for the increased food intake associated with sucrose consumption. This has significant
309 clinical relevance given the association between increased sucrose and fructose consumption and the
310 obesity epidemic, particularly in children (34).

311 AMPK is a critical cellular energy sensor that regulates energy homeostasis by sensing changes in
312 nutritional and hormonal signals (64). Modulation of central AMPK activity has direct effects on food
313 intake. Activation of central AMPK by orexigenic factors (e.g., fasting, ghrelin and hypoglycemia)
314 stimulates food intake, whereas its inhibition by anorexigenic factors (e.g., feeding, leptin, insulin and
315 glucose) suppresses food intake (41). We and others have shown that Glp1r agonists inhibit AMPK in the
316 hypothalamus (6, 49) and hindbrain (20). In the present studies, we show that loss of basal Glp1r activity
317 in $Glp1r^{-/-}$ mice is associated with both increased food intake and hypothalamic AMPK activity. The
318 observation that pre-treatment with AICAR in either the 4^{th} ventricle (20) or lateral ventricle, as in the
319 present studies, attenuates the anorectic effect of Ex4 demonstrates that inhibition of AMPK is a general
320 mechanism by which central Glp1r activation suppresses food intake. However, this does not rule out

potential contributions of non-AMPK pathways to the anorectic effect of Glp1r agonists. Hindbrain administration of Ex4 also activates the cAMP-dependent protein kinase (PKA) and p44/42 MAP kinase, which are regulators of feeding-related genes via the CREB transcription factor (20). Future studies in mice with conditional disruption of brain AMPK will be needed to define the potential contribution of non-AMPK pathways to the anorectic effects of Glp1r agonists.

The Glp1r is expressed throughout the brain including areas that play a role in the regulation of food intake such as the hypothalamus and hindbrain (15, 40). Since compounds were administered into the lateral ventricle in the present studies, we cannot identify the specific brain region(s) in which inhibition of AMPK by Ex4 results in decreased food intake. We also cannot exclude the possibility that modulation of Glp1r activation and AMPK exert control on food intake via distinct brain regions. Nevertheless, administration of Glp1r agonists into the lateral, 3rd or 4th ventricles, as well as targeted delivery of Glp1r agonists into hypothalamic nuclei or hindbrain regions, all reduce food intake (21, 45, 47, 55, 56) as well as AMPK phosphorylation (6, 20, 49). When administered into the lateral or the 4th ventricles, Glp1r agonists reduce hypothalamic and hindbrain AMPK phosphorylation within 20 min (6, 20). Interestingly, the anorectic effect of hindbrain Glp1r activation is delayed by several hours and maximal at 24h following Ex4 administration (20). In the present studies, the anorectic effect of Ex4 administered into the lateral ventricle was observed within the first hour of dosing, during which food intake was suppressed by ~75% compared to vehicle (data not shown). This is in agreement with previous studies demonstrating a rapid anorectic response to Glp1r agonists delivered to the lateral or 3rd ventricles or directly into the hypothalamus (45, 47, 55, 56). The apparent difference in the onset of anorexia between hindbrain and forebrain Glp1r activation may reflect an effect of experimental design such as different doses of Glp1r agonists used. Alternatively, this could be indicative of differences in the mechanisms by which forebrain and hindbrain Glp1r signaling regulate food intake. However, whether such a discrepancy between forebrain and hindbrain Glp1r signaling exists in mice remains to be determined. Studies using site-specific disruptions of the Glp1r are required to fully elucidate the relative

346 contributions of different brain regions to the regulation of food intake by Glp1r agonists and to identify
347 the signaling pathways downstream of the Glp1r in distinct brain regions.

348 Glp1 acts at multiple sites in both the CNS and periphery to promote insulin secretion, maintain
349 glucose homeostasis and reduce food intake and body weight (60). Glp1 is not only secreted from the
350 gut, but it is also synthesized in the nucleus of the tractus solitarius (NTS) of the brainstem, a brain region
351 that receives vagal inputs from visceral organs and extends axonal projections to other brain regions
352 including the hypothalamus (22, 26, 30, 40). This suggests that Glp1 synthesized in the brainstem may be
353 the principal mediator of the anorectic effects attributed to Glp1. However, there is also evidence of
354 peripheral Glp1 action in the regulation of food intake (61). Thus, the source of Glp1 that regulates
355 feeding behavior as well as the relative contributions of peripheral versus central Glp1r-mediated effects
356 on food intake remain to be clearly identified.

357 AMPK activity is regulated allosterically by changes in the AMP:ATP ratio and covalently via
358 phosphorylation by various AMPK kinases (18). A decrease in the AMP:ATP ratio due to increased
359 metabolism and ATP production causes a conformational change in AMPK that enhances its interaction
360 with inactivating AMPK phosphatases. Glp1r activation increases glucose uptake and ATP production in
361 NSC-34 motor neurons (31). We, therefore, speculated that inhibition of AMPK by Glp1r activation
362 occurred via increased glucose metabolism. This was supported by the observation that in the absence of
363 glucose, Ex4 was unable to suppress AMPK in hypothalamic GT1-7 cells. Furthermore, we show that
364 Ex4 increases glycolysis beyond the effects of glucose alone in GT1-7 cells. We observed a similar effect
365 in another Glp1r-expressing hypothalamic cell line, A2/28 cells (5) (data not shown). As immortalized
366 cell lines, GT1-7 and A2/28 cells display high basal glycolytic rates, making it all the more significant
367 that Ex4 further stimulated glycolysis. This demonstrates a novel mechanism by which Glp1r activation
368 regulates central AMPK activity. It must be noted that inhibition of AMPK by Ex4 in the hypothalamic
369 cell lines was observed in the presence of 25mM glucose, which exceeds the typical glucose
370 concentrations in the brain. Nevertheless, physiological evidence for the proposed glucose-dependent
371 properties of Glp1r-induced anorexia is demonstrated by the fact that inhibition of central glycolysis via

ICV 2-DG administration attenuates the anorectic effect mediated by ICV Ex4 administration. Taken together, our findings propose a model whereby central Glp1r activation enhances glucose metabolism, resulting in decreased AMPK activity and suppression of food intake. This provides a mechanism for feedback inhibition of food intake following a meal.

The proposed model indicates that glucose is not only a stimulus for Glp1 secretion, but it is also a component of the mechanism by which Glp1 suppresses food intake. We, therefore, hypothesized that an increase in central glucose would mimic the fed state and enhance the anorectic effect of Ex4. However, we did not observe such a cooperative effect. We did not measure CNS glucose levels nor did we perform glucose dose response experiments, so it is not clear whether the changes in CNS glucose that occur within a fast-to-fed cycle in mice is sufficient to modulate the response to central Glp1 action. Nevertheless, our studies using 2-DG suggest that glucose metabolism is a necessary component for the anorectic effect of Ex4. Glucose was delivered centrally in the present studies in order to circumvent the effects of other anorectic factors normally secreted in response to oral nutrient intake. However, we cannot exclude the possibility that central Glp1 action is coordinated with pathways activated by hormones such as insulin and leptin or even by the peripheral sensing of glucose.

Components of palatable foods, mainly fat and sugar, can impair anorectic mechanisms and stimulate weight gain. In rats chronically fed sugar solutions, caloric overconsumption and body weight gain occur from an activation of hunger signals and reward components and a depression of satiety signals (33). Moreover, food intake is increased in rats given an oral sucrose preload compared to a starch preload (13). When administered centrally, the sucrose metabolite fructose enhances hypothalamic AMPK activation and subsequently stimulates food intake (7, 24). We expand upon these observations and show that ICV pre-treatment with fructose attenuates the anorectic effect of ICV Ex4. This is likely due to the ability of fructose to prevent the inhibition of hypothalamic AMPK by Ex4, as shown in GT1-7 cells. These findings suggest that sucrose-derived fructose can attenuate the satiety effect mediated by the central Glp1r, resulting in the increased food intake associated with sucrose feeding. This hypothesis assumes that the brain is a site for the metabolism of dietary fructose. The Glut5 fructose transporter is

expressed in the central nervous system, and its expression is increased in response to chronic fructose feeding (52). This is especially significant since fructose itself regulates Glut5 expression, thus, raising the possibility that fructose is taken up and metabolized in the CNS. Furthermore, proteins necessary for the metabolism of fructose including fructokinase and aldolase are expressed in the brain (11). However, it is generally believed that dietary fructose is significantly extracted in the liver (35), and the possibility that sucrose consumption impairs central Glp1 action acutely via central fructose metabolism remains to be tested. We cannot exclude the possibility that central Glp1 resistance results from chronic intake of sucrose. Chronic sucrose consumption results in leptin resistance (19, 51). Given the interaction between central leptin and Glp1 action (16, 46, 48), it is possible that sucrose feeding impairs the anorectic effects of Glp1 indirectly via modulation of leptin action.

Collectively, these findings implicate central AMPK as a critical signaling molecule in the anorectic effect following central Glp1r activation. We provide mechanistic insight by demonstrating a role for glucose metabolism in the regulation of hypothalamic AMPK by Glp1r activation. Glucose dependence is a characteristic of pancreatic Glp1 action, and, in the context of feeding behavior, it provides a safety mechanism ensuring that Glp1 suppresses food intake only in the presence of nutrients. This has significant clinical implications given the increased use of Glp1-based therapies as treatments for type 2 diabetes. Importantly, some Glp1-based therapies are associated with moderate weight loss (58), highlighting the need to understand the potential anti-obesity mechanisms regulated by these compounds. We further demonstrate that fructose can significantly impair the anorectic response to Glp1r agonists. The link between increased consumption of sugary foods and beverages and the obesity epidemic underscores the need to understand the mechanisms by which dietary carbohydrate composition influence satiety and feeding behavior.

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Disclosures

The authors have no disclosures.

Author Contributions

Conception, design and performance of experiments, data analysis and interpretation of results (MAB, JA and JEA); drafting of manuscript (MAB); editing and revising manuscript (MAB, JA, DJD and JEA).

441 **Figure Legends**

442

443 **Figure 1. Glp1r$^{-/-}$ mice display increased AMPK activation in the hypothalamus and elevated 24h**
444 **food intake compared to Glp1r$^{+/+}$ littermates. (A)** Representative protein immunoblots and group data
445 for phosphorylated AMPK (pAMPK), total AMPK (AMPK) and β-actin in 5h-fasted Glp1r$^{+/+}$ vs. Glp1r$^{-/-}$
446 mice. The values are mean ± SEM and represent quantification of the ratio of pAMPK:AMPK (each
447 normalized individually to β-actin) for group data (n=7/group). *p<0.05 Glp1r$^{-/-}$ vs. Glp1r$^{+/+}$. **(B)** The
448 values are mean ± SEM and represent cumulative 24h food intake in Glp1r$^{+/+}$ vs. Glp1r$^{-/-}$ (n=4/genotype)
449 mice fed a high starch diet. *p<0.05 Glp1r$^{-/-}$ vs. Glp1r$^{+/+}$.

450

451 **Figure 2. ICV pre-treatment with the AMPK activator AICAR attenuates Ex4-mediated**
452 **suppression of food intake.** The values are mean ± SEM and represent cumulative **(A)** food intake, **(B)**
453 water intake and **(C)** locomotor activity in Glp1r$^{+/+}$ mice treated with ICV ACSF, Ex4, AICAR or AICAR
454 + Ex4 (n=8/treatment). *p<0.05 vs. ACSF. †p<0.05 vs. AICAR. ‡ p<0.05 vs. Ex4.

455

456 **Figure 3. Ex4 inhibits AMPK and stimulates glycolysis in the GT1-7 hypothalamic cell line. (A)**
457 Representative protein immunoblots and group data (n=4/group) for phosphorylated AMPK (pAMPK),
458 total AMPK (AMPK) and β-actin in GT1-7 cells cultured in 25mM glucose and treated with either PBS
459 vehicle or Ex4 at 1, 10 or 100nM. The values are mean ± SEM and represent quantification of the ratio of
460 pAMPK:AMPK (normalized to β-actin). *p<0.05 vs. Vehicle. **(B)** Representative protein immunoblots
461 and group data (n=4/group) for phosphorylated AMPK (pAMPK), total AMPK (AMPK) and β-actin in
462 GT1-7 cells cultured in the absence of glucose and treated with either PBS vehicle or Ex4 at 1, 10 or
463 100nM. The values are mean ± SEM and represent quantification of the ratio of pAMPK:AMPK
464 (normalized to β-actin). **(C)** Extracellular acidification rate (ECAR) measured over 28min in GT1-7
465 cells exposed to either 0 or 16.7mM glucose and treated with either PBS vehicle or Ex4 (100 nM) (n=3).
466 Data are expressed as fold change in ECAR in vehicle– vs. Ex4-treated cells relative to the time point at

which vehicle or Ex4 was injected (t=0min). The values are mean ± SEM. *p<0.05 vs. 0mM glucose. Inset: Changes in ECAR following treatment with vehicle or Ex4 in the presence of either 0mM or 16.7mM glucose measured over 28min in the same cells for which data is presented in Figure 3C.

Figure 4. *In vivo* inhibition of central glycolysis attenuates the anorectic effect mediated by ICV Ex4. The values are mean ± SEM and represent cumulative **(A)** food intake and **(B)** water intake in $Glp1r^{+/+}$ mice treated with ICV ACSF, Ex4, 2-DG or 2-DG + Ex4 (n=7-15/treatment). *p<0.05 vs. ACSF. †p<0.05 vs. 2-DG. ‡ p<0.05 vs. Ex4.

Figure 5. ICV glucose does not enhance the anorectic effect mediated by ICV Ex4. The values are mean ± SEM and represent cumulative **(A)** food intake and **(B)** water intake in $Glp1r^{+/+}$ mice treated with ICV ACSF, Ex4, Glucose or Glucose + Ex4 (n=10/treatment). *p<0.05 vs. ACSF. †p<0.05 vs. Glucose. ‡ p<0.05 vs. Ex4.

Figure 6. Sucrose diet stimulates food intake and ICV fructose attenuates the Ex4-mediated reductions in food intake and AMPK activity. (A) 24h food intake in $Glp1r^{+/+}$ mice fed isocaloric sucrose- vs. starch-based diets (n=6/diet). Cumulative **(B)** food intake and **(C)** water intake in $Glp1r^{+/+}$ mice treated with ICV ACSF, Ex4, Fructose or Fructose + Ex4 (n=8-12/treatment). The values are mean ± SEM . *p<0.05 vs. ACSF. †p<0.05 vs. Fructose. ‡ p<0.05 vs. Ex4.

Figure 7. Fructose stimulates AMPK and prevents inhibition of AMPK by Ex4 in GT1-7 cells. (**A**) Representative protein immunoblots and group data (n=4/group) for phosphorylated AMPK (pAMPK), total AMPK (AMPK) and β-actin in GT1-7 cells cultured in fructose for up to 60 min. *p<0.05 vs. t=0 min. **(B)** Representative protein immunoblots and group data (n=4/group) for phosphorylated AMPK (pAMPK), total AMPK (AMPK) and β-actin in GT1-7 cells cultured in 25mM fructose for 60 min and treated with either PBS vehicle or Ex4 at 1, 10 or 100nM. The values are mean ± SEM and represent

493 quantification of the ratio of pAMPK:AMPK (normalized to β-actin). **(C)** Representative protein
494 immunoblots and group data (n=3/group) for phosphorylated AMPK (pAMPK), total AMPK (AMPK)
495 and β-actin in GT1-7 cells cultured in 25mM mannitol for 60 min and treated with either PBS vehicle or
496 Ex4 at 1, 10 or 100nM. The values are mean ± SEM and represent quantification of the ratio of
497 pAMPK:AMPK (normalized to β-actin).
498

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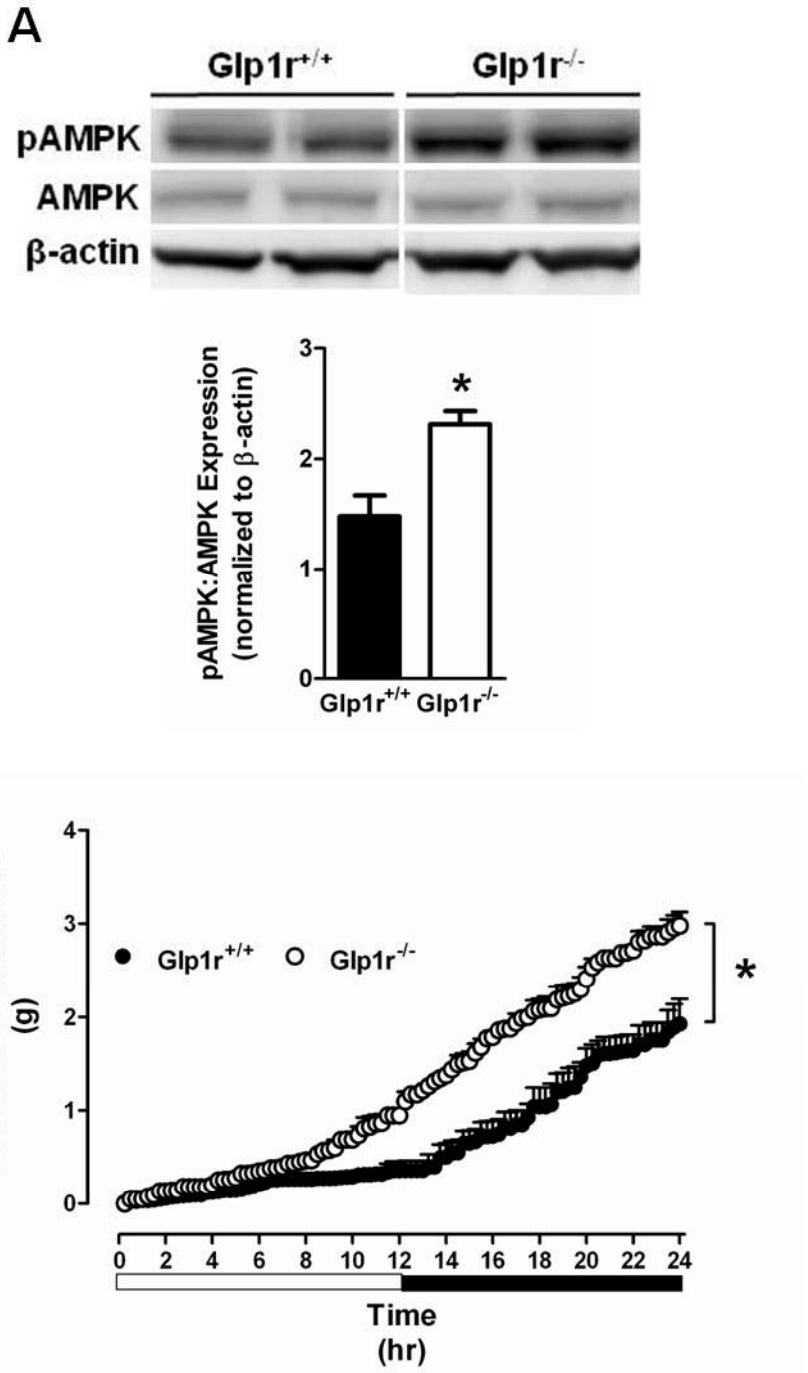
Figure 1



*p<0.05 vs. Glp1r$^{+/+}$

Figure 2

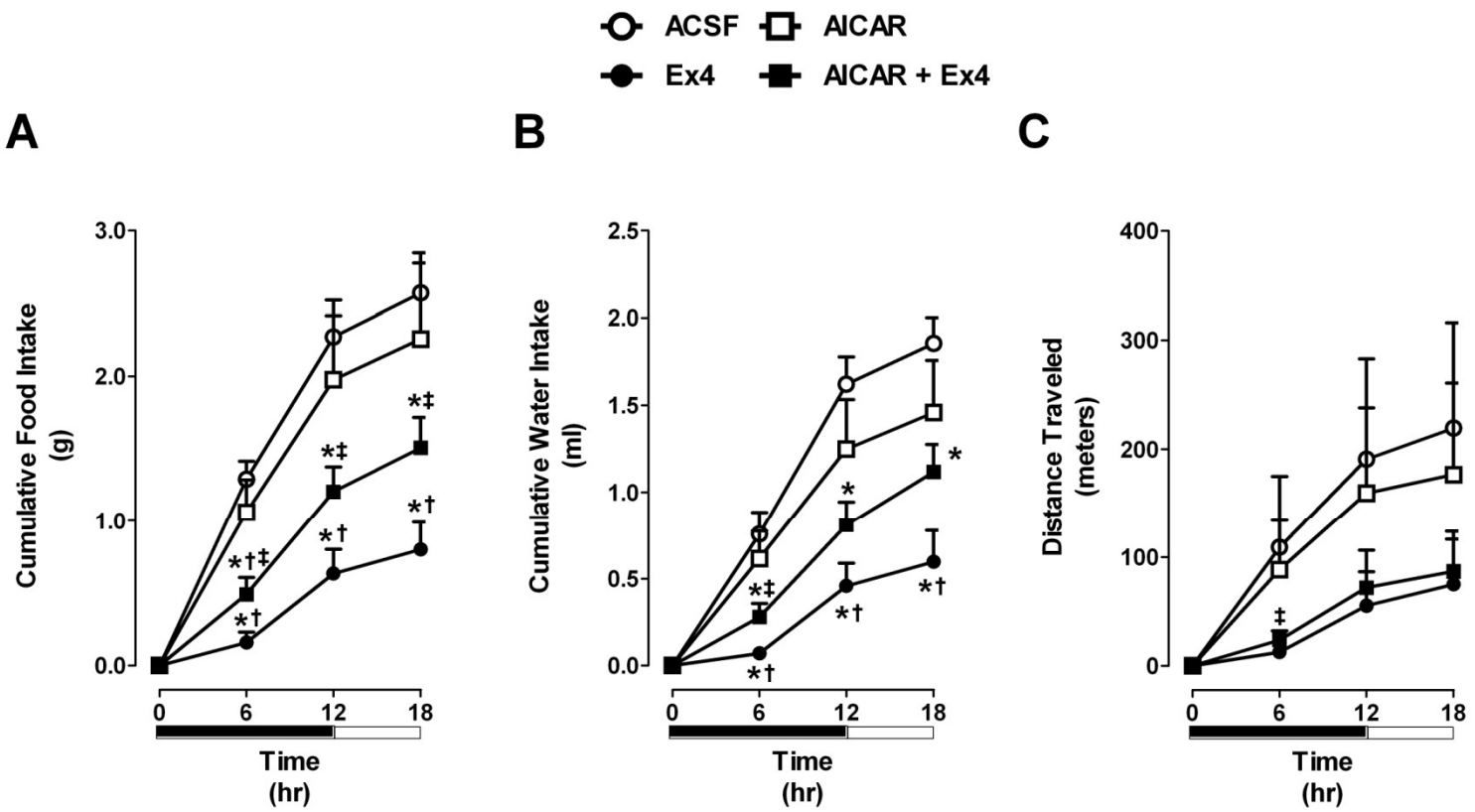
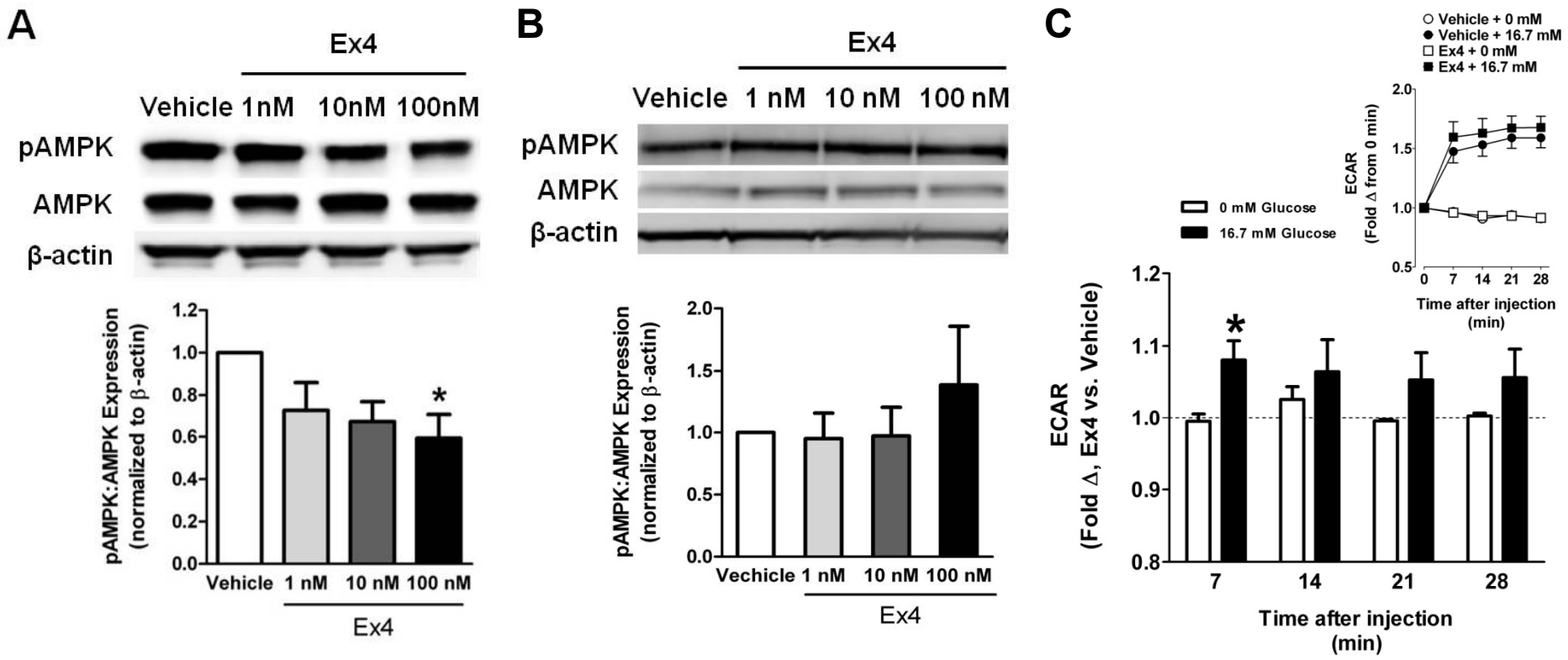

ACSF
AICAR
Ex4
AICAR + Ex4
A
B
C
Cumulative Food Intake (g)
Cumulative Water Intake (ml)
Distance Traveled (meters)
Time (hr)
0
6
12
18
*p<0.05 vs. ACSF
†p<0.05 vs. AICAR
‡p<0.05 vs. Ex4

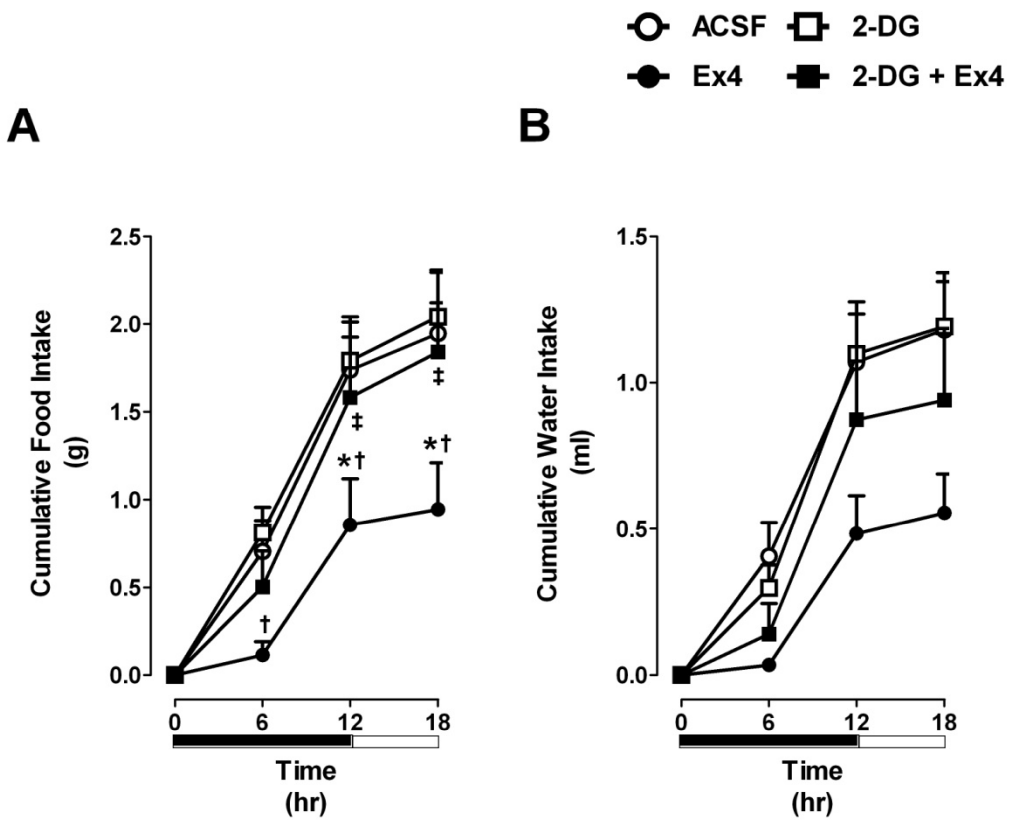
Figure 3



*p<0.05 vs. Vehicle

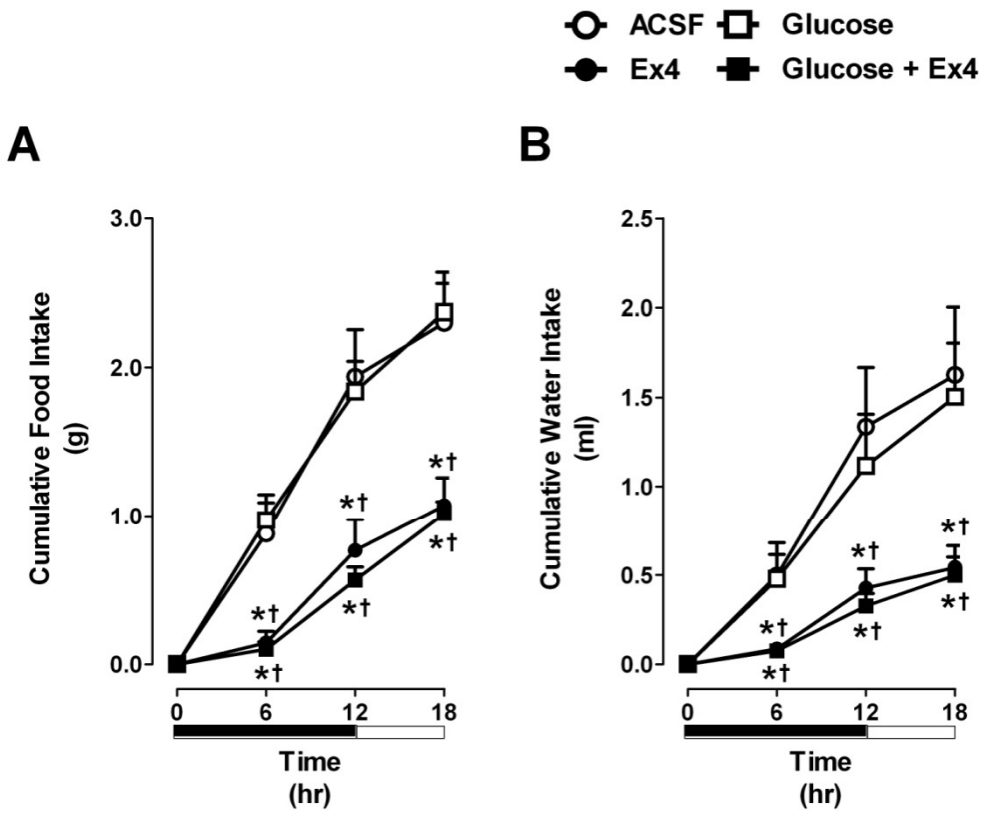
*p<0.05 vs. 0 mM Glucose

Figure 4



*p<0.05 vs. ACSF
†p<0.05 vs. 2-DG
‡p<0.05 vs. Ex4

Figure 5



*p<0.05 vs. ACSF
†p<0.05 vs. Glucose
‡p<0.05 vs. Ex4

Figure 6

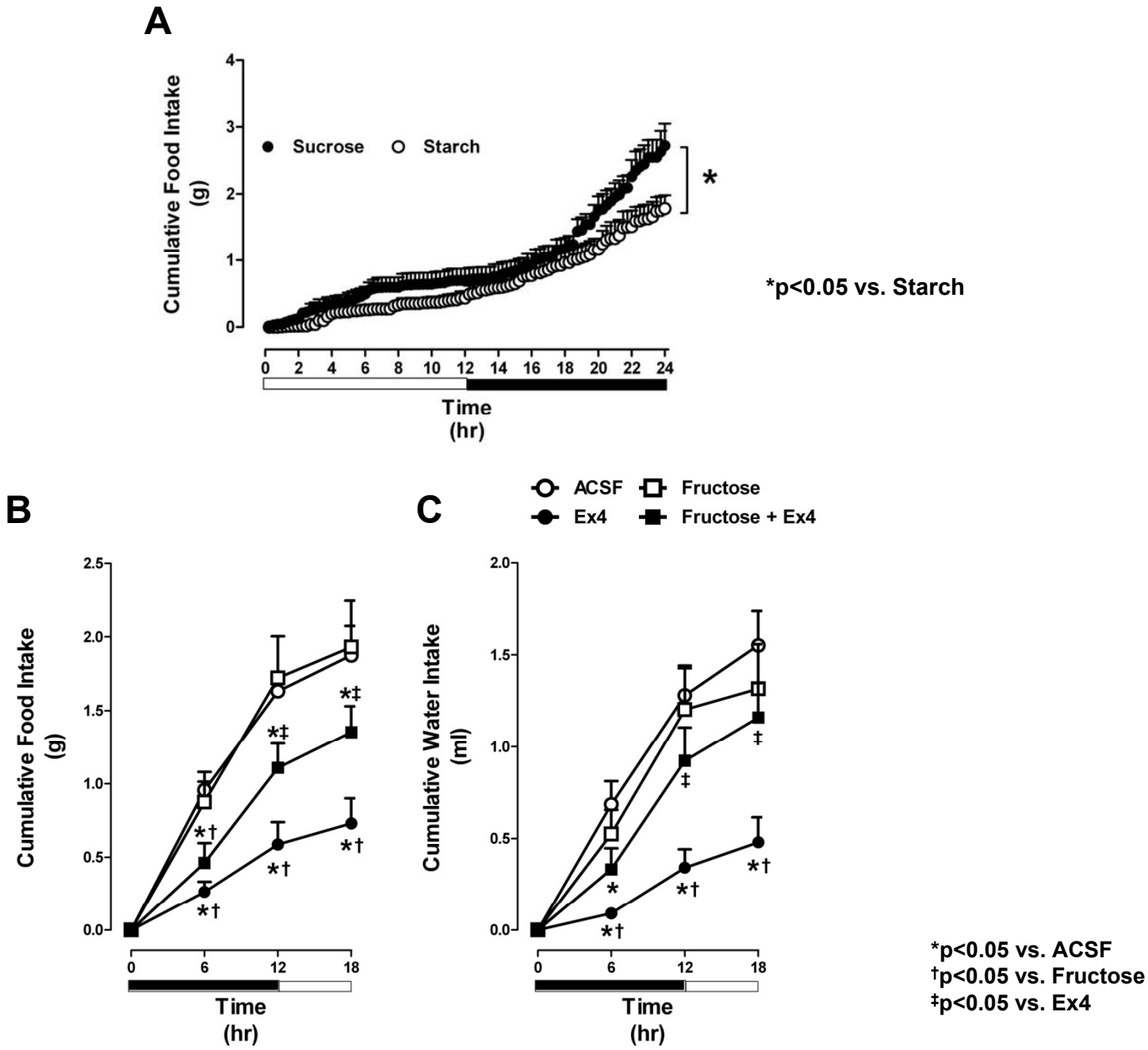
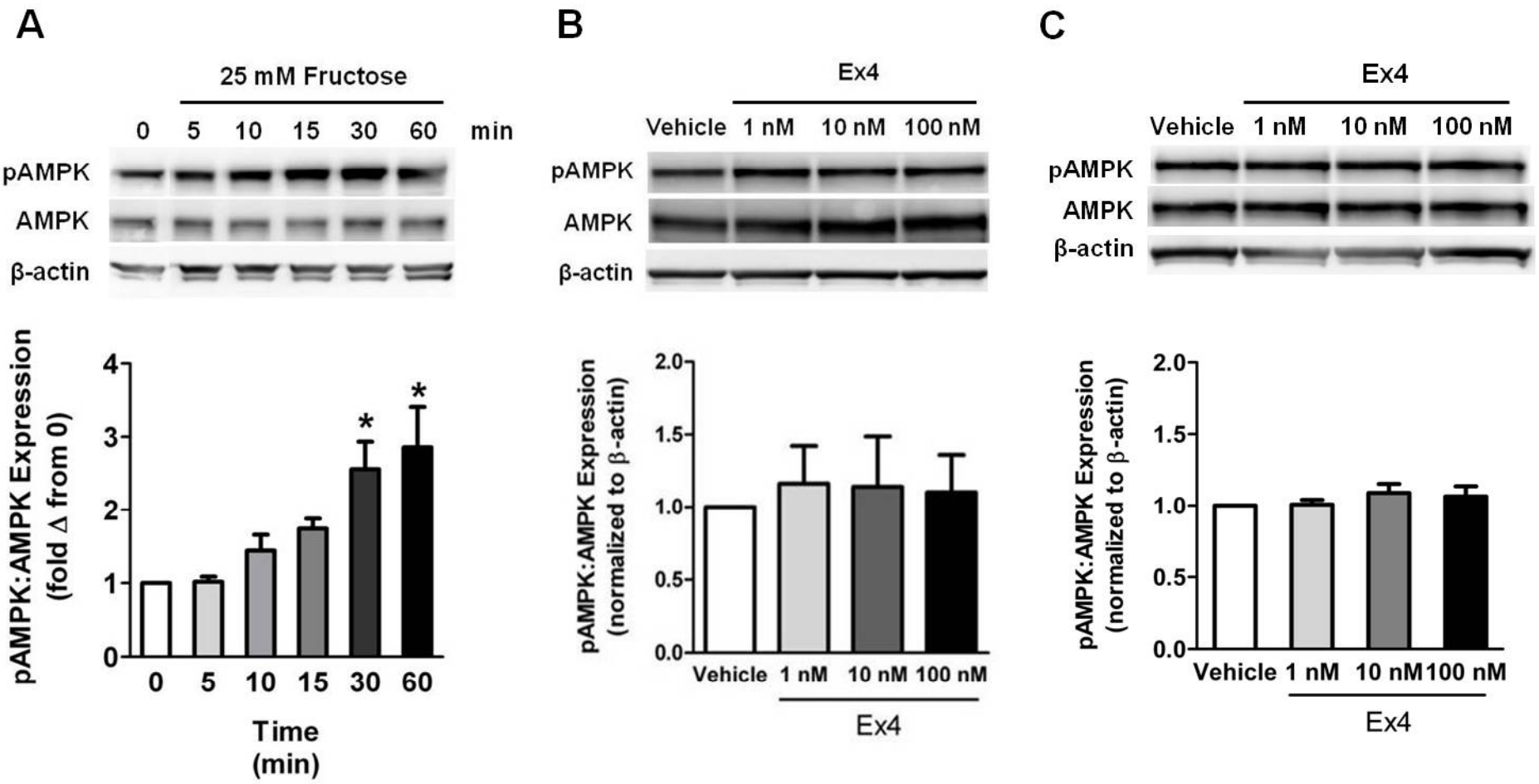

A
Cumulative Food Intake (g)
Sucrose
Starch
*
Time (hr)
*p<0.05 vs. Starch
B
C
ACSF
Fructose
Ex4
Fructose + Ex4
Cumulative Food Intake (g)
Cumulative Water Intake (ml)
Time (hr)
*p<0.05 vs. ACSF
†p<0.05 vs. Fructose
‡p<0.05 vs. Ex4

Figure 7



*p<0.05 vs. 0 min