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Insulin Signals Through the Dorsal Vagal Complex to Regulate Energy Balance

Insulin signaling in the hypothalamus regulates food intake and hepatic glucose production in rodents. Although it is known that insulin also activates insulin receptor in the dorsal vagal complex (DVC) to lower glucose production through an extracellular signal–related kinase 1/2 (Erk1/2)–dependent and phosphatidylinositol 3-kinase (PI3K)–independent pathway, it is unknown whether DVC insulin action regulates food intake. We report here that a single acute infusion of insulin into the DVC decreased food intake in healthy male rats. Chemical and molecular inhibition of Erk1/2 signaling in the DVC negated the acute anorectic effect of insulin in healthy rats, while DVC insulin acute infusion failed to lower food intake in high fat–fed rats. Finally, molecular disruption of Erk1/2 signaling in the DVC of healthy rats per se increased food intake and induced obesity over a period of 2 weeks, whereas a daily repeated acute DVC insulin infusion for 12 days conversely decreased food intake and body weight in healthy rats. In summary, insulin activates Erk1/2 signaling in the DVC to regulate energy balance.

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Hyperphagia and increased body weight are the hallmark features of obesity (1). Obesity incidence has doubled since 1980, and ~1.4 billion adults were overweight and 500 million were obese in 2008. Obesity leads to diabetes, as an elevated BMI is associated with an increased risk for diabetes (2), while abdominal obesity is a strong predictor of diabetes (3). Laboratories have focused on dissecting the regulatory mechanisms of feeding and weight gain that aim to unveil novel antiobesity targets. In this regard, the central nervous system (CNS) has received much attention.

CNS integrates signals generated by the pancreas-, white adipose tissue–, and gastrointestinal-derived hormones to regulate energy homeostasis (1,4–6). Alteration of the CNS sensitivity to these hormones leads to uncontrolled feeding behavior, reduced energy expenditure, and elevated body weight and adiposity (1,4–6). Central insulin administration reduces food intake and body weight in baboons (7), rats (8), mice (9), and humans (10,11). Intracerebroventricular infusion of insulin into the third ventricle (ICV-3) of mice and rats decreases food intake in males (8,9), and intranasal insulin administration in humans also reduces body fat and food intake in males but not females (10,11). ICV-3 injection of small interfering RNA against the insulin receptors reduces hypothalamic insulin receptor expression and induces hyperphagia in male rats (12), and neuronal insulin receptor knockout female (but not male) mice are hyperphagic (13). These data collectively highlight the hypothalamus as an insulin-responsive region that regulates appetite but does not limit insulin action to the hypothalamus. This is because intranasal insulin injection in humans does not limit insulin delivery solely to the hypothalamus (10,11) and insulin

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receptor expression is not only reduced in the hypothalamus of neuronal insulin receptor knockout mice (13).

In fact, insulin receptor knockdown in the catecholaminergic neurons of the ventral tegmental area increases body weight and fat mass in mice (14), whereas direct injection of insulin into the central bed nucleus of the amygdala decreases food intake in healthy but not high fat–fed rodents (15). Insulin responsiveness in the dorsal vagal complex (DVC) and the subsequent control on glucose homeostasis have also been examined (16). First, insulin receptors and the downstream phosphatidylinositol 3-kinase (PI3K) and extracellular signal–related kinase 1/2 (Erk1/2) signaling effectors are all expressed in the DVC. Direct insulin injection into the DVC preferentially activates Erk1/2 instead of PI3K-AKT in a dose-dependent manner. Importantly, insulin triggers a PI3K-independent and Erk1/2-dependent signaling cascade in the DVC to lower glucose production in healthy rodents independent of weight changes, and inability of DVC insulin to control glucose develops after 3 days of high-fat feeding (16).

No study to date has addressed the role of DVC insulin action in the regulation of energy balance (i.e., food intake and body weight gain). In contrast, DVC administration of leptin (17), GLP-1 (18), and leucine (19) lowers food intake, and administration of insulin into the fourth ventricle decreases expression of Hap1-Ahi1 (20). The fact that Hap1-Ahi1 is normally upregulated to stimulate feeding (21) highlights a potential role of DVC insulin action in energy balance regulation. We provide here the first evidence, to our knowledge, that acute insulin infusion into the DVC activates Erk1/2 to lower food intake in healthy but not high fat–fed rats. Direct molecular disruption of DVC Erk1/2 signaling in normal rats induces hyperphagia and obesity over a period of 2 weeks, whereas daily acute repeated DVC insulin infusion for 12 days lowers food intake and body weight in normal rats. These data collectively demonstrate that insulin activates Erk1/2 in the DVC to regulate energy balance.

RESEARCH DESIGN AND METHODS

All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network.

Animal Preparation

Eight-week-old male Sprague-Dawley rats weighing between 270 and 290 g (Charles River Laboratories, Montreal, Quebec, Canada) were housed in individual cages and maintained on a light-dark cycle with access to standard chow and water ad libitum. Rats were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). A 26-gauge bilateral guide cannula made of stainless steel (Plastics One, Roanoke, VA) was stereotactically implanted (David Kopt Instruments, Tujunga, CA) into the DVC, targeting the nucleus of solitary tract (0.0 mm on occipital crest, 0.4 mm lateral to midline, 7.9 mm below skull surface) as previously described (16). The guide cannula was secured in place with mounting screws, cyanoacrylate gel, and dental cement. To create a closed system, the guide cannula was kept free of obstruction by inserting a dummy cannula followed by a dust cap (Plastics One).

Virus Preparation and Injection

Adenovirus expressing the dominant-negative form of MEK1 (MEK1-DN) with a mutated magnesium binding site (D208 mutated to A) to kill the catalytic activity was prepared (16). The purified adenoviruses with plaque forming units of 1.8×10^8 (MEK1-DN) or 1×10^8 (green fluorescent protein [GFP]) were injected 3 μL per site into the DVC of rats (16).

Short-term Feeding Study

Animals whose food intake and body weight returned to baseline after 5 days of DVC surgery underwent feeding studies. Food intake and body weight were measured 48 h (day 5) and 24 h (day 6) prior to the feeding experiments (day 7). On day 7, the rats were fasted at 10:00 A.M. (6 h prior to night cycle) while food intake and body weight were measured. Body weight was monitored at 3:00 P.M. and mitogen-activated protein kinase (MAPK) inhibitor PD98059 (900 μmol/L), PI3K inhibitor LY-294002 (5, 10, 50, 100, and 250 μmol/L) or wortmannin (20 μmol/L), or saline was infused through the DVC catheter at 0.04 μL/min for 5 min (0.2 μL total). At 4:00 P.M. (1 h later; start of the dark cycle), insulin (2, 20, 200, or 2,000 μU/μL) or saline was infused at 0.04 μL/min for 5 min (0.2 μL total) into the DVC and food was returned. Food intake was subsequently measured every 30 min for 4 h. Food intake and body weight were then monitored at 14, 24, and 48 h after DVC infusion.

A separate group of rats was fed a lard oil–enriched high-fat diet (HFD) (TestDiet #571R; Purina Mills, Richmond, IN) for 3 days prior to the day of the experiment, with food intake and body weight monitored daily. The composition of the HFD differs from regular chow with respect to total calorie content (5.14 compared with 3.83 kcal $\cdot$ g^{-1}, respectively), fat content (33% compared with 17%), protein content (22% compared with 31%), and carbohydrate content (45% compared with 52%). Food intake and body weight were monitored 72, 48, and 24 h prior to the experiment.

When the feeding study was performed in the presence of viral injection and insulin acute infusion, the rats were injected with the virus (MEK1-DN or GFP) immediately after the brain surgery. After a 3-day recovery, rats were subsequently pair-fed for an additional 4 days before the feeding and insulin acute infusion study was performed.

Conditioned Taste Aversion Test

Rats underwent DVC surgery, and after 3 days of recovery (day 3), rats underwent the conditioned taste

aversion (CTA) test as previously described (22). Rats were habituated to 1 h daily access to water on day 3. During this hour, two bottles (each containing unflavored water) were placed in each cage. After 7 days, all rats received two bottles containing 0.1% saccharin solution instead of water. Immediately after this 1-h exposure, rats received an injection of saline or insulin (2 mU/μL; 400 μU per site) into the DVC. Independent rats were intraperitoneally injected with either LiCl (22 mg/kg) as positive or saline as negative control. On the next day, rats received 1 h access to two bottles with unflavored water. In the subsequent and final 2 days, a two-bottle choice test was given in which all rats were allowed 1 h access to water and the 0.1% saccharin solution. The position of the two bottles in each cage was switched on the 2nd day of the test.

Long-term Feeding Study

Rats were injected with virus (MEK1-DN or GFP) into the DVC 3 days after DVC surgery. Food intake, body weight, and water consumption were monitored over an 18-day period.

Feeding Study With Repeated Daily Insulin Infusion Into the DVC

Rats were subjected to DVC surgery and had a 1-week recovery before starting daily repeated DVC infusion. Insulin (2 mU/μL) or saline was infused at 4:00 P.M. for 12 days. Body weight and food and water intake were measured daily at the same time as the DVC infusion. Fat pads were removed and measured on day 12.

Statistical Analysis

Analysis was performed with GraphPad Prism by two-way ANOVA to compare across the groups followed by a Bonferroni post hoc test to compare between the groups or one-way ANOVA to compare across the groups followed by Tukey post hoc test to compare between groups. Statistical analysis was accepted as significant with a P value of <0.05. Data are presented as means ± SEM.

RESULTS

Single Acute DVC Insulin Infusion Lowers Food Intake

We monitored food intake and body weight of healthy rats that received a single acute insulin or saline infusion into the DVC (Fig. 1*A*). Food-restricted (6 h) rats were treated with different concentrations of insulin ranging from 2 mU/μL (400 μU per site) to 2 μU/μL (0.4 μU per site) or saline, and food was given back immediately after the DVC administration. All rats that received DVC insulin ate less compared with the saline-infused rats, with a significant difference detected as early as 90 min after infusion (Fig. 1*B*). This difference was lost within 14–24 h after DVC insulin infusion (Supplementary Table 1). A trend toward a drop in body weight at 14 h post–DVC single acute insulin infusion was detected (Supplementary Table 1).

Two groups of rats that received DVC insulin or saline underwent CTA testing (Fig. 1*C*), and the rats did not show a preference for saccharin (Fig. 1*D*). In contrast, rats that received intraperitoneal LiCl injection drank a significantly lower percentage of saccharin as compared with those that received intraperitoneal saline (Fig. 1*D*). Thus, the acute anorectic effect of DVC insulin is not caused by malaise.

DVC Erk1/2 Activation

We decided to carry out all subsequent studies with insulin infused at 2 mU/μL (400 μU per site) into the DVC to mimic the insulin dose used in the hypothalamic feeding studies. Insulin administered at 2 mU/μL into the ICV-3 (e.g., targeting the hypothalamus) was demonstrated as equally effective at lowering feeding in rats (8). Of note, we have previously validated that DVC insulin infused at 2 μU per site activates Erk1/2 but not PI3K-AKT, and such Erk1/2 activation lowers hepatic glucose production (16). However, when the dose of insulin infused into the DVC increases to near 200–400 μU per site, both Erk1/2 and AKT in the DVC are activated (16). These findings open up the possibility that both of these signaling pathways could be involved in the ability of DVC insulin (2 mU/μL) to regulate feeding.

Coinfusion of MAPK inhibitor PD98059 with insulin fully abolished the ability of DVC insulin to lower food intake (Fig. 2*A*), and DVC PD98059 infusion per se did not alter food intake as compared with saline-treated rats (Fig. 2*A*). The current dose of PD98059 administered into the DVC would negate the ability of insulin to activate Erk1/2, as a blockade on insulin-Erk1/2 activation was previously described with a lower dose of PD98059 (16).

We have developed an adenovirus expressing the dominant-negative form of MEK1 (MEK1-DN) (16). MEK1 is a MAPK that phosphorylates and activates Erk1/2. Direct injection of MEK-DN into the DVC negates the ability of insulin to activate Erk1/2 in the DVC (16). We here injected the MEK1-DN into the DVC and evaluated the effect of DVC insulin. DVC MEK1-DN reversed the ability of DVC insulin to lower food intake as compared with the GFP-injected rats (Fig. 2*B*).

Next, DVC insulin was coinfused with PI3K inhibitors LY294002 or wortmannin. Neither DVC LY294002 nor wortmannin infusion negated the ability of insulin to lower food intake (Fig. 2*B*). However, DVC LY294002 or wortmannin alone also lowered food intake to an extent similar to that in association with insulin (Fig. 2*B*). We then performed a dose-response study for DVC LY294002 infusion and discovered that administering LY294002 at as low as 5 μmol/L into the DVC still lowered food intake to an extent similar to that associated with insulin (Fig. 2*C*). A previous study has reported that hypothalamic administration of LY294002 at 10 μmol/L is sufficient to negate insulin's control on glucose production (23).

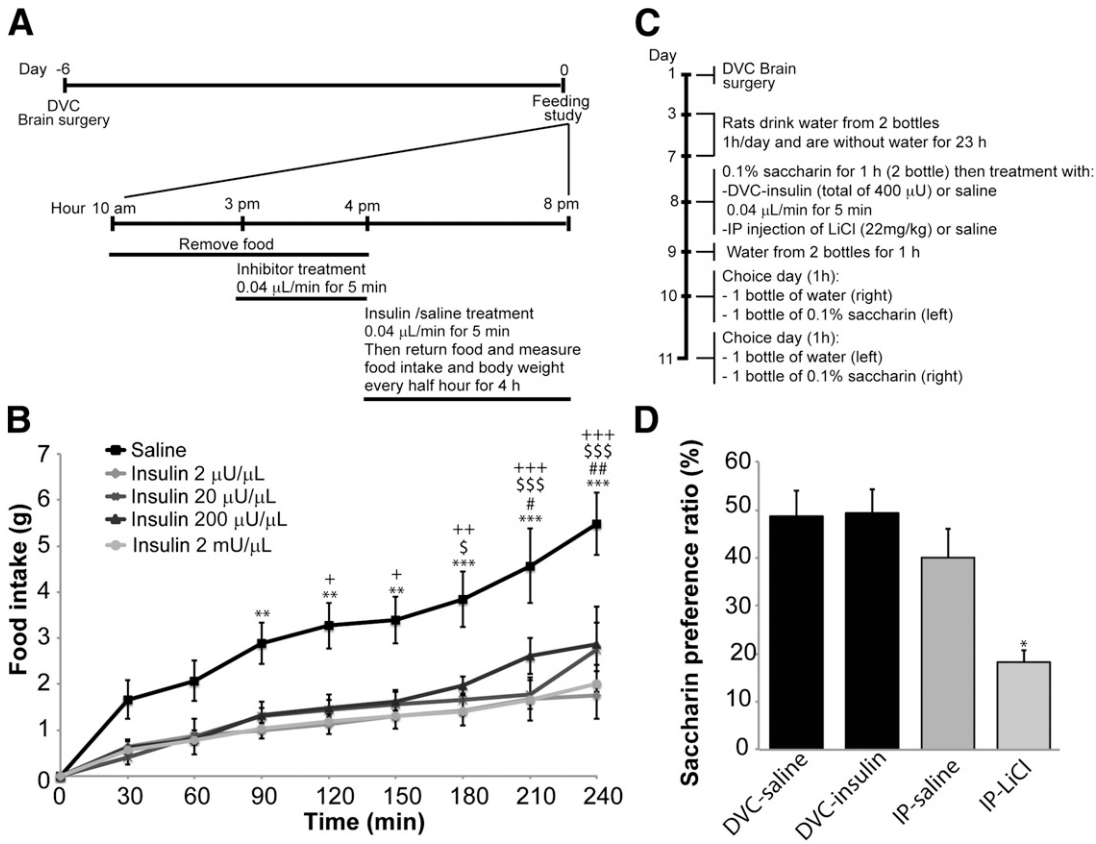

Figure 1—Insulin administration into the DVC inhibits food intake. *A*: Experimental procedure and feeding protocol. *B*: Rats fasted for 6 h were infused with the reported amounts of insulin and saline into the DVC at 0.04 μL/min for 5 min (0.2 μL total). Food was returned and intake was measured every half hour for 4 h. Data are presented as mean ± SEM. $n = 13$ for saline, $n = 11$ for 2 mU/μL insulin, and $n = 5$ for the other insulin concentration. **P* value saline vs. insulin 2 mU/μL; #*P* value saline vs. insulin 200 μU/μL; $*P* value saline vs. insulin 20 μU/μL; +*P* value saline vs. insulin 2 μU/μL. One symbol, $P < 0.05$; two symbols, $P < 0.01$; three symbols, $P < 0.001$. *C*: Experimental procedure and CTA protocol. *D*: Data are presented as % of saccharin over the total liquid ingested. Data are presented as mean + SE. $n = 4$ per group. **P* < 0.05. IP, intraperitoneal.

Thus, insulin signals through an Erk1/2-dependent and PI3K-AKT independent pathway to acutely lower food intake. However, the role of DVC PI3K-AKT signaling in appetite regulation remains ambiguous.

High-Fat Feeding

Rats were fed with a lard oil–enriched HFD for 3 days prior to undergoing the feeding study (Fig. 3*A*). Rats fed with this HFD for 3 days develop hyperphagia and hypothalamic insulin resistance and exhibit increased body weight gain after 1 week (24,25). Thus, this 3-day HFD model was chosen to evaluate whether DVC is a site of insulin resistance that contributes to the early onset of diet-induced obesity. The caloric intake of HFD-fed rats was significantly greater than that of regular chow rats (171.8 ± 4.3 vs. 119.5 ± 3.5 kcal/day, $P < 0.001$). Contrary to the acute DVC insulin response in rats fed with a regular chow diet (Fig. 1*B*), DVC insulin infusion failed to lower food intake in HFD rats (Fig. 3*B*). Together with the fact that 3-day HFD impairs the ability of DVC insulin to activate Erk1/2 (16), these findings indicate that HFD impairs DVC insulin signaling to lower appetite.

To better address whether bidirectional changes of DVC insulin–Erk1/2 signaling alter food intake and body weight, we disrupted DVC Erk1/2 signaling for 18 days or performed daily repeated acute DVC insulin infusion for 12 days in healthy rats.

Molecular Disruption of Erk1/2 Signaling in the DVC

We injected MEK1-DN or GFP viral control directly into the DVC of healthy rats and monitored daily food intake and body weight (Fig. 3*C*). Upon immunohistochemical analysis of GFP-injected rats, we first confirmed that the virus was localized to the DVC (mainly within the nucleus of the solitary tract) (Supplementary Fig. 1). Strikingly, DVC MEK1-DN (vs. GFP) increased cumulative food intake starting at day 6 after viral injection (Fig. 3*D*), and this effect on food intake was sustained until day 18 (Fig. 3*E*). The increase in food intake of DVC MEK1-DN rats was associated with body weight gain that became significant 9 days after viral injection (Fig. 3*F*). The body weight of DVC MEK-DN rats remained elevated up until 18 days (Fig. 3*F*). No difference in the amount of water intake was detected between the groups (data not shown).

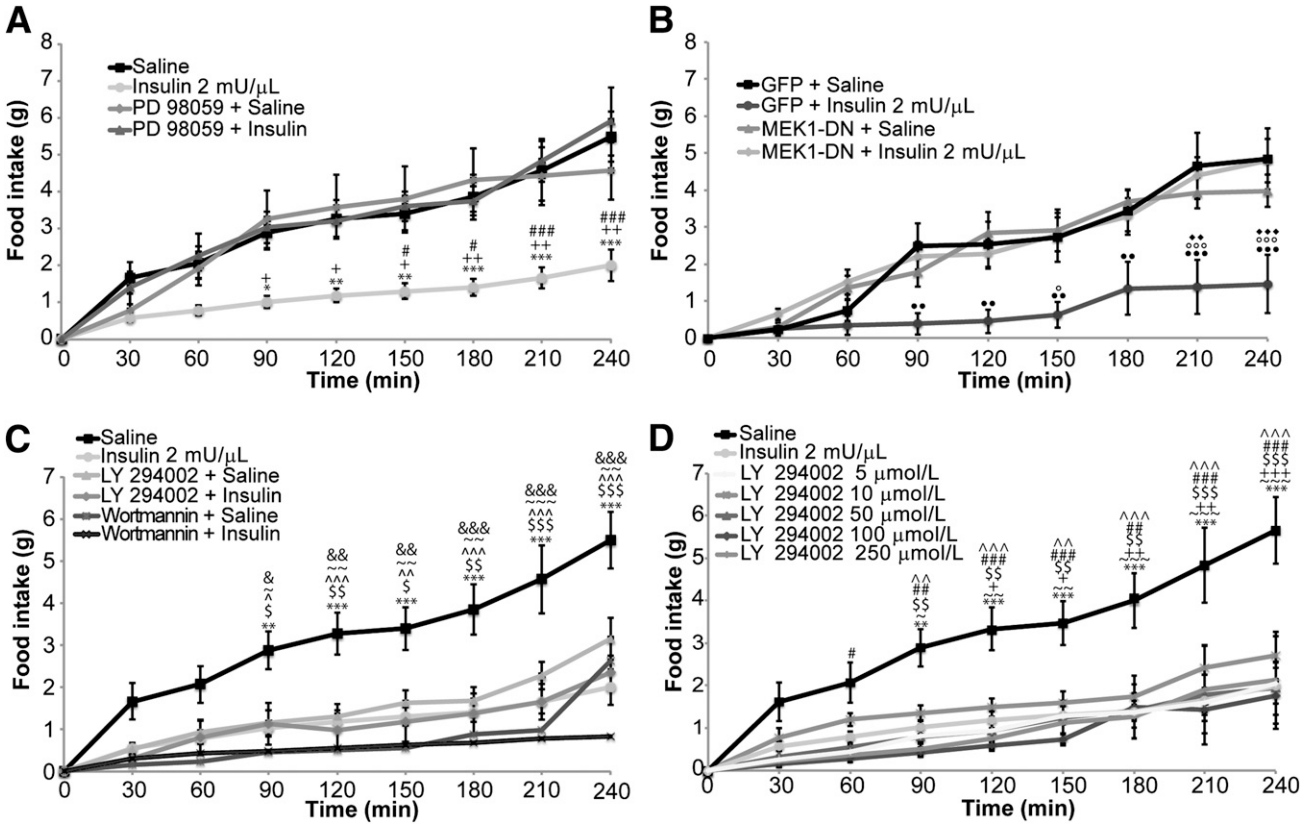

Figure 2—Inhibition of DVC Erk1/2 signaling negates the ability of insulin to lower food intake. *A*: Rats fasted for 6 h were infused with MAPK inhibitor (PD98059, 900 μmol/L) into the DVC at 0.04 μL/min for 5 min (0.2 μL total). An hour later, rats were infused with insulin (2 mU/μL, 400 μU per site) or saline into the DVC at 0.04 μL/min for 5 min. Food was returned and intake was measured every half hour for 4 h. Data are presented as mean ± SEM; n = 13 for saline, n = 11 for insulin 2 mU/μL, n = 6 for insulin or saline with PD98059. **P* value insulin vs. saline; +*P* value insulin vs. PD98059 + saline; #*P* value insulin vs. PD98059 + insulin. *B*: Rats injected with an adenovirus overexpressing MEK1-DN or GFP into the DVC were fasted for 6 h and subsequently infused with insulin (2 mU/μL) or saline into the DVC at 0.04 μL/min for 5 min. Food was returned and intake was measured every half hour for 4 h. Data are presented as mean ± SEM; n = 6 for MEK1-DN with saline or insulin, n = 5 for GFP with saline or insulin. ◆*P* value GFP-insulin vs. GFP-saline; ○*P* value GFP-insulin vs. MEK1-DN-insulin; ●*P* value GFP-insulin vs. MEK1-DN-saline. *C*: Same protocol was used as for panel *A* but PI3K inhibitors (LY294002, 500 μmol/L or wortmannin 20 μmol/L) were infused instead. Data are presented as mean ± SEM; n = 13 for saline, n = 11 for insulin 2 mU/μL, n = 7 for insulin or saline with LY294002, n = 4 for insulin or saline groups with wortmannin. **P* value insulin vs. saline; $*P* value saline vs. LY294002 + saline; ^*P* value saline vs. LY294002 + insulin; ~*P* value saline vs. wortmannin + saline; &*P* value saline vs. wortmannin + insulin. *D*: Rats fasted for 6 h were infused with various doses of LY294002, saline, or insulin. Food was given back and food intake was measured every half hour for 4 h. Data are presented as mean ± SEM. n = 13 for saline; n = 11 for insulin at 2 mU/μL; n = 5 for various doses of LY294002 (5, 10, 50, 100 and 250 μmol/L). **P* value saline vs. insulin 2 mU/μL; ~*P* value saline vs. 5 μmol/L LY294002 + saline; +*P* value saline vs. 10 μmol/L LY294002 + saline; $*P* value saline vs. 50 μmol/L LY294002 + saline; #*P* value saline vs. 100 μmol/L LY294002 + saline; ^*P* value saline vs. 250 μmol/L LY294002 + saline. One symbol, $P < 0.05$; two symbols, $P < 0.01$; three symbols, $P < 0.001$.

Daily Repeated Acute Infusion of Insulin Into the DVC

Conversely, rats received daily repeated acute infusion of insulin (2 mU/μL) or saline into the DVC for 12 days (Fig. 4*A*). DVC insulin substantially reduced cumulative food intake (Fig. 4*B*) and body weight (Fig. 4*C*) and had a strong tendency to lower total fat mass with the most significant reduction detected in visceral fat (Fig. 4*D*).

Together with the loss-of-function experiments targeting Erk1/2 signaling, these gain-of-function experiments targeting DVC insulin signaling demonstrated that insulin-Erk1/2 signaling in the DVC regulates food intake and body weight.

DISCUSSION

We have first demonstrated that insulin action in the DVC lowers food intake in male rats. To date, the regulation of appetite by brain insulin action appears to be sex specific, as hypothalamic insulin administration in rats and intranasal insulin delivery in humans only lowers food intake in healthy males but not females (8,10,11). Similarly, leptin and GLP-1 receptor signaling in the DVC lowers feeding in male rats (17,18). Although we have yet to explore the sex specificity of insulin action in the DVC, we do demonstrate that insulin given at 2 μU per site into the DVC is sufficient to not only lower glucose production in male rats (16) but also lower

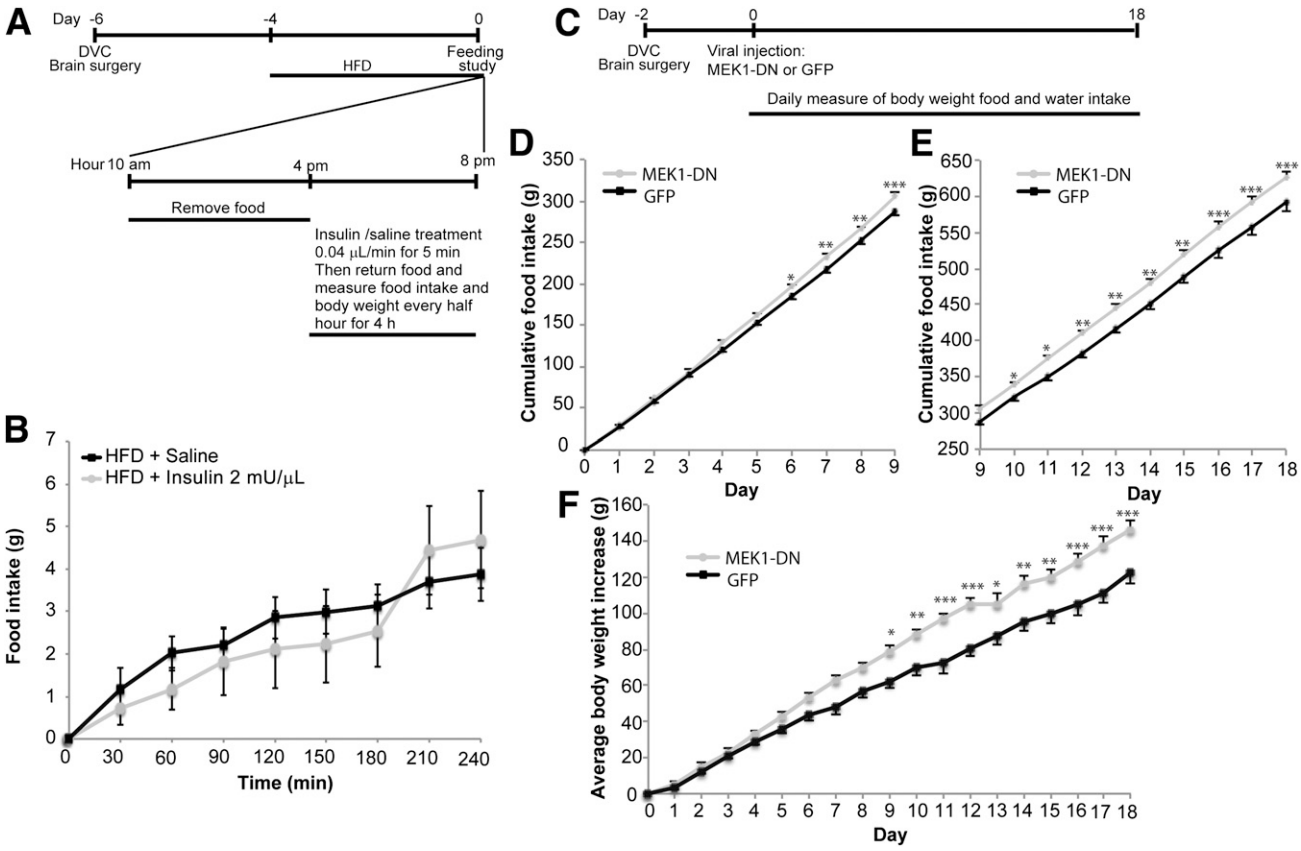

Figure 3—High-fat feeding negates the ability of DVC insulin to lower food intake while molecular disruption of DVC Erk1/2 signaling causes obesity. *A*: Experimental procedure and high-fat feeding protocol. *B*: Rats fasted for 6 h were infused with insulin (2 mU/μL) or saline into the DVC at 0.04 μL/min for 5 min. Food was returned and intake was measured every half hour for 4 h. Data are presented as mean ± SE. $n = 5$ per group. *C*: Experimental procedure and feeding protocol. Rats were injected with MEK1-DN or GFP virus into the DVC 3 days after brain surgery. *D*: Cumulative food intake from days 0 to 9. *E*: Cumulative food intake from days 9 to 18. *F*: Increase in body weight recorded over 18 days. Data are presented as mean ± SEM. $n = 8$ for MEK1-DN, and $n = 9$ for GFP. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

food intake. This is in contrast to previous findings in which a much higher insulin dose in the hypothalamus was required to regulate feeding than glucose homeostasis (8,23).

Also in contrast, insulin signals via a PI3K-independent and Erk1/2 signaling–dependent pathway in the DVC to lower glucose production (16). At an insulin dose that activates both Erk1/2 and PI3K-AKT in the DVC (16), we report here that it is still Erk1/2 signaling in the DVC that is necessary for insulin to lower feeding. Importantly, bidirectional changes of insulin-Erk1/2 signaling in the DVC for ~2 weeks are sufficient to alter food intake and body weight, strengthening the role of DVC insulin–Erk1/2 signaling. Together with a recent report that indicates DVC Erk1/2 signaling is necessary for leucine to regulate feeding (19), these findings collectively highlight the integrative and sufficient role of DVC Erk1/2 signaling in the regulation of energy balance.

The neuronal population that mediates DVC insulin to regulate feeding remains unknown. Given that both insulin and leptin signal through the PI3K in the hypothalamus to regulate feeding (1) and that leptin action in the DVC lowers feeding (17), future studies are warranted to begin addressing a potential overlap between insulin and leptin receptor coexpressing neurons in the DVC. Although the bilateral cannula was inserted into the DVC, targeting the nucleus of the solitary tract, other regions, such as the dorsal motor nucleus and the area postrema, within the DVC could be possible targets of insulin action. Also, the role of PI3K signaling in the DVC remains unclear. The chemical inhibition of DVC PI3K not only failed to negate the anorectic effect of insulin but also mimicked the effect of insulin: to lower food intake. This finding suggests that basal PI3K activity in the DVC promotes feeding to maintain energy balance. Although this working hypothesis remains to be tested, it is noteworthy that enhanced PI3K activation in the ventromedial hypothalamic SF-1 neurons likewise promotes hyperphagia and obesity in mice (26). Of note, the role of IGF-1 receptor in the DVC in mediating the anorectic

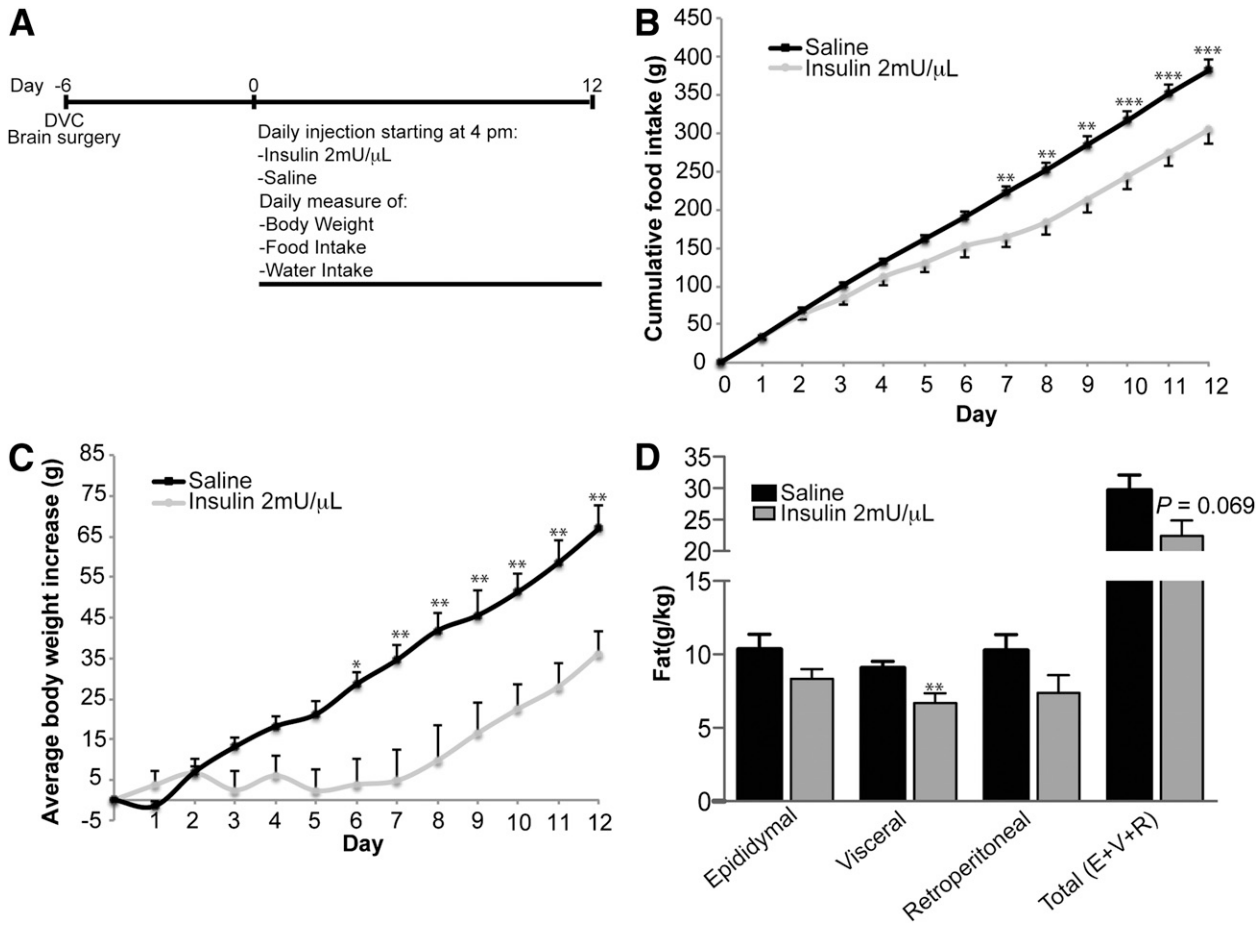

Figure 4—Daily repeated acute infusion of insulin into the DVC lowers food intake, body weight, and fat deposition. *A*: Experimental procedure and feeding protocol. Rats were injected with insulin (2 mU/μL) or saline every day at 4:00 P.M. *B*: Cumulative food intake recorded over a 12-day period. *C*: Relative daily increase in body weight over a 12-day period. *D*: Relative fat mass at end of 12-day period. Data are presented as mean ± SEM. $n = 6$ for insulin-treated rats, and $n = 5$ for saline-treated rats. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

effect of insulin remains to be explored, as insulin could interact with IGF-1 receptor (27).

HFD impairs the ability of DVC insulin infusion to lower food intake. This finding is of importance, as it unveils an extrahypothalamic site of insulin resistance to control feeding. Given that HFD disrupts the ability of insulin to activate Erk1/2 in the DVC (16) and that disruption of DVC Erk1/2 in healthy rats induces hyperphagia and obesity, future studies aimed at characterizing the signaling events involved in insulin-mediated Erk1/2 activation could prove useful to restore DVC insulin action to lower body weight gain in obesity. The mechanisms responsible for HFD-induced DVC insulin resistance may mirror the events of hypothalamic insulin resistance, with an induction of inflammation and ER stress being implicated in the development of insulin resistance (6). Lastly, it is possible that DVC insulin resistance increases food intake via alterations in hedonic food reward signaling, as intact brain insulin signaling is demonstrated to decrease food reward in nonobese humans and rodents (28,29). Future studies are needed to address these working hypotheses.

In summary, the current set of data unveils the DVC as a novel site of insulin action capable of lowering food intake and body weight and suggests that DVC insulin resistance can contribute to weight gain in obesity.

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

Author Contributions. B.M.F. conducted and designed the experiments, performed data analyses, and wrote the manuscript. A.B., M.A.A., F.A.D., and J.T.Y.Y. assisted with experiments. T.K.T.L. supervised the project, designed the experiments, and edited the manuscript. T.K.T.L. 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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