Both acyl and des-acyl ghrelin regulate adiposity and glucose metabolism via CNS ghrelin receptors.

Short Title: CNS ghrelin receptor regulation of metabolism

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Abstract

Ghrelin receptors (GHSRs) in the central nervous system (CNS) mediate hyperphagia and adiposity induced by acyl ghrelin (AG). Evidence suggests that des-acyl ghrelin (dAG) has biological activity through GHSR independent mechanisms. We combined *in vitro* and *in vivo* approaches to test possible GHSR-mediated biological activity of dAG. Both AG (100nM) and dAG (100nM) significantly increased IP_3 formation in HEK-293 cells transfected with human GHSR. As expected, intracerebroventricular (icv) infusion of AG in mice increased fat mass (FM), in comparison with the saline-infused controls. Icv-dAG also increased FM at the highest dose tested (5 nmol/day). Chronic icv infusion of AG or dAG increased glucose-stimulated insulin secretion (GSIS). Subcutaneously infused AG regulated FM and GSIS in comparison to saline-infused control mice, whereas dAG failed to regulate these parameters even with doses that were efficacious when delivered icv. Furthermore, icv-dAG failed to regulate FM and induce hyperinsulinemia in GHSR deficient (*Ghsr-/-*) mice. In addition, a hyperinsulinemic-euglycemic clamp suggests that icv-dAG impairs glucose clearance without affecting endogenous glucose production. Taken together, these data demonstrate that dAG is an agonist of GHSR and regulates body adiposity and peripheral glucose metabolism through a CNS GHSR-dependent mechanism.

Key Words

Ghrelin, Glucose Metabolism, Energy Metabolism, Adiposity, Neuroendocrinology

Introduction

Ghrelin, a hormone predominately secreted from the stomach (1), regulates multiple aspects of energy metabolism, including feeding and adiposity (2; 3), and is therefore a potential target for therapeutic strategies to prevent or cure obesity. Ghrelin acts directly on pancreatic islets to modulate glucose-stimulated insulin secretion (GSIS) which has made it a popular target for type 2 diabetes therapies (reviewed in (4)). Ghrelin circulates as an acylated (AG) and des-acylated (dAG) form (1). Although the exact ratio of circulating AG to dAG varies depending on metabolic status (5; 6), the majority of ghrelin circulates in the dAG form. There is currently only one known ghrelin receptor, the growth hormone secretagogue receptor 1a (GHSR). This receptor is expressed in multiple areas of the central nervous system (CNS) where it mediates AG-induced feeding and adiposity. The presence of an acyl side-chain (mainly *n*-octanoic acid) attached to the ghrelin peptide is required for full agonism of GHSR (1). Some *in vitro* evidence suggests that dAG interacts with GHSR, although this occurs at significantly lower levels as compared to AG (1; 7). Therefore, dAG was initially considered an inactive by-product of ghrelin secretion and degradation. Despite this, multiple reports have suggested that dAG acts in peripheral tissues and in the brain to regulate biological actions including the control of feeding (8; 9), body temperature (10), muscle atrophy (11), GSIS (12; 13), and lipid metabolism (14; 15). All of these actions are attributed to GHSR-independent mechanisms.

Many peripherally secreted hormones such as leptin, glucagon or glucagon-like peptide-1 act in the CNS to regulate energy metabolism, and interestingly, a growing body of literature has highlighted that these hormones act in the CNS to regulate glucose homeostasis (16-19). Peripherally derived AG acts centrally to promote a positive energy balance (3) and acts directly

on pancreatic islet cells to regulate insulin secretion (20; 21). However, it is not well established whether AG regulates glucose metabolism through central mechanisms. Rats given chronic central administration of AG have elevated fasting insulin levels (22), which is independent of hyperphagia induced by ghrelin (23) and suggests that AG acts in the CNS to regulate circulating insulin levels.

In addition to the gut, ghrelin is expressed in the brain where both AG and dAG can be detected (24-26), and therefore, understanding the central actions of these peptides is of interest. In contrast to AG, the central action of dAG in regulating energy and glucose metabolism remains poorly characterized. Some reports suggest that dAG action in the brain plays a role in the control of feeding (8; 9). A role for dAG acting in peripheral tissues to regulate insulin secretion has been described (12; 27; 28), although the CNS-control of glucose metabolism by dAG has not been explored. Previous reports demonstrate a lack of dAG-induced activation of GHSR (1), which has led researchers to assume that dAG-mediated effects are solely GHSR independent. However, a potential role for GHSR-mediated dAG action has yet to be directly tested *in vivo*. The aim of this study was to compare CNS-mediated action of AG and dAG on feeding, adiposity, and GSIS in mice. Furthermore, we aimed to determine whether GHSR mediates the biological actions of dAG using a combination of both *in vitro* and *in vivo* techniques.

Materials and Methods

Peptide synthesis

Rat AG and dAG were synthesized using *in situ* neutralization for Boc chemistry, purified by preparative chromatography, and characterized by HPLC and mass spectral analysis, as described previously (3).

Cell culture and transfection

Human embryonic kidney cells (HEK-293) cells were grown in Dulbecco´s modified Eagle´s medium. DMEM was supplemented with 10 % FBS (PAA Laboratories GmbH), 100 U/ml penicillin, 100 µg/ml streptomycin (Biochrom AG) and 2 mM L-glutamine (Invitrogen). For seeding of HEK-293 cells, 48-well plates were coated with Poly-L-Lysine (Biochrome). 83.3 ng plasmid-DNA/well were transfected using 0.9 µl Metafectene^{TM}/well (Biontex).

Measurement of intracellular IP_3 formation

Intracellular inositol triphosphate (IP_3) levels were determined using a luciferase reporter assay (Promega). HEK-293 cells were seeded into 48-well plates ($5x10^4$ cells/well). Equal amounts of receptor-DNA (pcDps) and a reporter construct containing a response element and the firefly luciferase gene under control of the nuclear factor of activated T-cells (NFAT) were co-transfected. Cells were stimulated 2 days after transfection for 6 hours at 37 °C and 5 % CO_2 with AG (10 nM and 100 nM) or with dAG (10 nM and 100 nM) and lysed with 100 µl 1x Passive Lysis Buffer (Promega). IP_3 formation was determined by luciferase activity according to the manufacturer's instructions.

Animals

Male C57BL/6 mice (12 weeks old; Jackson Labs, Bar Harbor, ME) were maintained on a standard chow diet (Teklad, Harlan). After receiving surgery, animals were singly housed and maintained on a 12:12-h light-dark cycle at 22 ℃ with free access to food and water unless noted otherwise. *Ghsr-/-* mice and wild-type (WT) control mice both on a C57BL/6 background were received from Regeneron Pharmaceuticals and bred in our facilities as described previously (29). The average body weight (BW) of the mice used in these studies was 26.8 grams. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

Subcutaneous and intracerebroventricular (icv) infusions in mice

For all surgical procedures, mice were anesthetized using 5 % isoflurane in oxygen in an induction chamber and then maintained on 2.5 % isoflurane delivered by a nose cone. Subcutaneous infusions of AG and dAG were delivered by subcutaneously implanted osmotic-mini pumps (1007D Alzet, Cupertino, CA). For icv infusions, mice were stereotaxically implanted (David Kopf Instruments, Tujunga, CA) with a cannula (brain infusion kit #3, Alzet, Cupertino, CA) placed in the lateral cerebral ventricle as previously described (30). A polyethylene catheter attached the cannula to an osmotic mini-pump (1007D Alzet, Cupertino, CA) that was subcutaneously implanted.

Indirect calorimetry

For measurements of locomotor activity (LA), energy expenditure (EE) and respiratory exchange ratio (RER) animals were implanted with icv mini-pumps as described above and then placed in an indirect calorimetry system for 7 days (TSE Systems, Gmbh, Bad Homburg, Germany).

Intraperitoneal glucose tolerance test (ipGTT)

Intraperitoneal (ip) glucose tolerance tests were performed by injection of glucose (2 g/kg, 20 % w/v d-glucose, Sigma, in 0.9 % w/v saline) after a 16h fast. Tail vein blood samples for blood glucose (BG) measurements were collected at 0, 10, 30, 60 and 120 min after the injection, and were measured using a handheld glucometer (Freestyle Lite). Tail vein blood samples (60µl per time point) for insulin measurements were collected at 0, 10 and 60 min. Plasma insulin was determined using either a radioimmunoassay (Sensitive Rat Insulin RIA; Millipore) or ELISA (Ultra Sensitive Mouse Insulin ELISA Kit; Crystal Chem) as indicated in the figure legend.

Body composition measurements

Whole-body composition (fat and lean mass) was measured using NMR technology (31)(EchoMRI-100; Echomedical Systems, Houston, TX). Initial body composition for all experiments was taken the day prior to surgery (d (-1)). For dose response icv infusion studies (Figure 2) and indirect calorimetry studies (Figure 3) final body composition was analyzed on d7 following surgery. For studies involving analysis of glucose tolerance (Figure 4, 5 and 6), final body composition was analyzed on d6 immediately after the ipGTT (~20h fasted).

Catheterization for hyperinsulinemic euglycemic clamps

The left common carotid artery and right jugular vein of the mice were catheterized for clamp studies as previously described (32). Immediately upon completion of catheterization, animals were implanted with an icv cannula attached to a subcutaneously placed osmotic mini-pump as described above. Following surgery, animals were given subcutaneous injections of buprenorphin (0.28 mg/kg Buprenex; Reckitt Benckiser Healthcare, Richmond, VA), meloxicam (0.25 mg/100 g body weight Metacam; St. Joseph,MO), and 1 mL warm saline.

Hyperinsulinemic euglycemic clamps (HEC)

On day 5 after surgery, HEC were performed as previously described (32). Mice were conscious during the entire experimental procedure. Following a 5h fast, catheter lines were exteriorized and connected to infusion pumps. A 5-μCi bolus of [3-^{3}H]glucose (Perkin Elmer Life Sciences, Boston, MA) was given, followed by a 0.05 μCi/min infusion for 120 min before insulin infusion. At 100 min a blood sample (50 μl) was taken to determine basal glucose and insulin levels as well as basal glucose turnover. The insulin clamp started at 120 min with a continuous infusion of insulin at a rate of 4 mU/kg/min. During the clamp, the [3-^{3}H]glucose infusion rate was increase to (0.1 μCi/min) to maintain constant specific activity. Dextrose (50 g/100 mL) was infused as necessary to maintain euglycemia (~130 mg/dl) on the basis of feedback from frequent arterial glucose measurements by handheld glucometers (Accu-check Aviva glucometer). Saline-washed erythrocytes previously collected from donor mice were infused throughout the experimental period to prevent a fall of hematocrit. A 12-μCi bolus of 2[^{14}C]deoxyglucose (2[^{14}C]DG) was given at 198 min. Blood samples (20 μl) were taken every 10 min from t = 200–240 min and processed to determine plasma [3-^{3}H]glucose and 2[^{14}C]DG. At the end of the

clamp period, mice were euthanized with an injection of sodium pentobarbital. Tissues were collected and stored at -80 °C for further analysis.

Tracer calculations

Rates of glucose appearance (Ra), endogenous glucose production (EGP), and glucose utilization were calculated using steady state equations as previously described (33). Briefly, EGP was calculated by determining the total Ra (this comprises both glucose production and any exogenous glucose infused to maintain the desired glycemic levels) and subtracting the amount of exogenous glucose infused. Tissue specific glucose uptake was calculated from tissue 2-DG content as previously described (33).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA). Statistical significance was determined either by unpaired student's t-test, one-way ANOVA followed by Tukey's multiple comparison post hoc test or two-way ANOVA followed by Bonferroni's multiple comparison post hoc test. For non-linear regression analysis, data sets were fitted using the least square method following the equation (Y=Bottom + (Top-Bottom)/(1+10^(LogEC50-X))) and compared using the extra sum of squares F-test. All results are given as means ± SEM. Results were considered statistically significant when $p < 0.05$.

Results

AG and dAG activate GHSR in HEK-293 cells

To determine whether dAG activates GHSR *in vitro*, we incubated GHSR transfected HEK-293 cells with dAG. We found that 100 nM dAG challenge resulted in a significant increase IP_3 turnover above the basal constitutive activity of the receptor (Figure 1), which is similar to what other groups have found at this concentration of dAG in stable transfected Chinese hamster ovary cells (34). The IP_3 accumulation induced by AG was approximately 2-fold greater than that induced by dAG (Figure 1).

Food intake and body composition following chronic icv infusion of dAG and AG

Our *in vitro* data suggested that dAG is a weaker agonist of GHSR as compared to AG, and therefore, we hypothesized that infusion of dAG directly into the brain will have similar, but less potent effects as AG on feeding, body weight (BW), and fat mass (FM). To test this, we performed a 7-day chronic central infusion of either AG or dAG at increasing equimolar doses in mice (0, 0.04, 0.2, 1.0 and 5.0 nmol/day). A two-way ANOVA detected a significant effect of the treatment on cumulative 7d food intake ($P<0.01$ AG vs. dAG) and an extra-sum of squares F-test comparing the curve fits for both treatments ($P<0.05$, calculated EC_{50}=0.096 vs. 0.253nmol/ day, for AG and dAG, respectively, Figure 2a). Both treatments increased BW in a dose dependent manner ($P<0.01$ for dose as main effect as calculated by two-way ANOVA), with a similar potency (Figure 2b). Relative to vehicle-infused controls, AG and dAG increased FM to a similar extent with the highest dose tested (5.0 nmol/day). The difference between the dose response curves of AG and dAG was statistically significant ($P<0.05$ by extra-sum of squares F test for the

calculated curve fittings, calculated EC_{50}=0.073 vs. dAG EC_{50}=0.902nmol/ day for AG and dAG, respectively) (Figure 2c). Lean mass (LM) did not differ among the groups (data not shown).

Locomotor activity (LA), energy expenditure (EE) and respiratory exchange ratio (RER) during chronic icv infusion of dAG and AG

Increased FM induced by AG can occur in the absence of hyperphagia which has been demonstrated (3). This occurs, in part, through suppression of LA (35) and decreased fat utilization as a fuel source (3). We did not detect hyperphagia in dAG treated animals, and therefore, we investigated whether dAG increases adiposity through similar alterations in LA and metabolic fuel preference. We gave mice chronic icv infusions of AG or dAG (5 nmol/day) and placed them into an indirect calorimetry system. We did not detect differences in cumulative food intake in either the AG or dAG treated animals (Figure 3a). Both compounds increased BW and FM following 7d of icv infusion (Figure 3b,c). Figure 3d depicts the RER of the mice during the 7d infusion period. When compared to vehicle-treated animals, dAG has a strong tendency to increase RER in the final 4 days of infusion (p=0.054 saline vs. dAG with treatment as the main effect; two-way ANOVA). However, when comparing all groups only AG causes a statistically significant increase in RER (Figure 3e). We did not detect changes in EE in either dAG or AG treated animals over the course of the infusion (Figure 3f) or during the last 4 days of treatment (Figure 3g), which is consistent with other reports that have demonstrated a lack of effect of AG on EE (3). Figure 3h, shows the LA of the animals during the 7d infusion period. AG suppresses LA during the final 4 days of infusion, whereas dAG has no effect on LA during the final 4 days of infusion (Figure 3i).

Effects on glucose metabolism following chronic icv infusion of AG and dAG

To investigate the central effects on glucose metabolism, mice received chronic icv infusion of AG or dAG (5 nmol/day). Animals were then exposed to an ipGTT following an overnight (16h) fast. All groups showed similar blood glucose excursions during the ipGTT (Figure 4d). Both AG and dAG significantly increased insulin levels in comparison with vehicle-treated controls, suggesting a higher GSIS (Figure 4e). Animals in all groups experienced a decrease in BW and FM over the course of infusion, which was likely due to the combination of an overnight fast and surgical intervention. However, animals receiving either dAG or AG lost less BW (Figure 4b) and FM (Figure 4c) relative to vehicle-infused controls. Decreases in LM were similar among all groups (data not shown). During the infusion period, AG significantly increased food cumulative 6d food intake, whereas dAG did not alter food intake (Figure 4a).

Effects on energy and glucose metabolism following chronic subcutaneous infusion of AG and dAG

To clarify whether the increases in plasma insulin were due to a centrally mediated action or leakage into peripheral tissues, we chronically infused an equimolar amount of AG and dAG (120 nmol/day) to mice. Animals were exposed to an ipGTT following an overnight (16h) fast. Blood glucose excursions were similar in all groups (Figure 5d). Plasma insulin levels during the ipGTT were elevated in the AG treated group 60 min following glucose injection (Figure 5e). Unlike central administration, subcutaneous infusion of dAG had no effect on GSIS (Figure 5e). Following the infusion period, animals receiving peripheral treatment of AG lost less BW (Figure 5b) and FM (Figure 5c) relative to vehicle-treated controls. Decreases in LM following

the infusion period were also significantly less in animals receiving chronic subcutaneously delivered AG relative to vehicle-treated animals (Saline -7.182 ± 0.5196 g, dAG -5.816 ± 0.3766 g, AG -5.205 ± 0.2920 g; **p< 0.01 Saline vs AG, one-way ANOVA with Tukey's post-hoc). In contrast to icv infusion shown in Figure 4, chronic subcutaneous administration of a larger dose (24 times the amount given centrally) of dAG did not affect BW or FM (Figure 5b,c). Food intake was similar among all groups during the infusion period (Figure 5a).

Energy and glucose metabolism in Ghsr-/- mice administered with chronic icv-dAG

To determine whether the regulation of FM and GSIS by central administration of dAG depends on GHSR activation, we performed chronic central infusion of dAG in WT and age-matched *Ghsr-/-* mice. Administration of an ip glucose bolus following an overnight (16h) fast did not elicit differences in BG excursions regardless of the genotype or the treatment (Figure 6d). Consistent with Figure 4e, dAG increased plasma insulin levels in WT, but not in *Ghsr-/-* mice during the ipGTT (Figure 6e). Cumulative 6d food intake was not altered due to icv-dAG treatment in WT or *Ghsr-/-* animals (Figure 6a). Following the infusion period, dAG-treated WT mice lost less BW and FM relative to vehicle-treated mice; however, dAG did not affect BW or FM in *Ghsr-/-* mice as compared to vehicle-treated *Ghsr-/-* mice (Figure 6b,c). Changes in LM were similar among all groups (data not shown).

HEC clamp in mice treated with chronic icv-dAG

The glucose-stimulated hyperinsulinemia induced by icv-AG or dAG treatment could be a result of the development of insulin resistance in these mice. To detect changes in hepatic and/or

peripheral insulin sensitivity, we performed a HEC in mice that received chronic icv treatment of dAG (5 nmol/day). We used dAG in order to limit the confounding factor of hyperphagia that we commonly see with AG and also because we confirmed that the effects of dAG are mediated through AG's target, GHSR. BG was clamped at similar levels between saline and dAG groups (Figure 7a). The exogenous glucose infusion rate necessary to maintain these steady glucose levels was also similar between groups indicating similar rates of whole-body insulin sensitivity (Figure 7b). Saline and dAG treated animals had a similar basal EGP and insulin similarly suppressed EGP during the clamp period indicating that central dAG treatment did not alter hepatic insulin sensitivity (Figure 7c). However, while glucose clearance was significantly increased during the clamp in saline treated animals (Figure 7d), mice treated with icv-dAG had a minor, but significant impairment in their ability to increase peripheral glucose clearance during the clamp (Figure 7d). Tissue-specific glucose uptake into soleus, inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), and brown adipose tissue (BAT) was similar between groups (Table 1).

Discussion

Cell-based assays have demonstrated that ghrelin requires esterification with an acyl-side-chain on its Ser3 residue to activate GHSR at concentrations within the low nanomolar range (1). This evidence led to the assumption that the biological effects of dAG are independent of GHSR activation. Here, we combined *in vitro* and *in vivo* approaches to demonstrate that dAG can activate GHSR, and activation at the level of the CNS leads to changes in the control of energy balance and glucose metabolism.

Several reports indicate that dAG interacts with GHSR at concentrations within the high nanomolar or micromolar range. While some data indicates that dAG is a weak GHSR agonist (1; 7), other data suggests that dAG is actually a full agonist of GHSR and induces similar maximal receptor stimulation as AG (34). Altogether, our *in vitro* results are consistent with the literature and demonstrate that dAG possess GHSR agonism *in vitro*.

Our experiments consistently show that central infusion of dAG in the brain mimics the effect of AG on adiposity and hyperinsulinemia. Interestingly, the effect of dAG on adiposity is manifested at a higher potency than could be predicted based on functional assays *in vitro.* It should be noted that although central infusion of AG and dAG always led to relative increase in FM in comparison with the control group, we sometimes observe an absolute reduction in FM at the end of the experiment. We attribute that decrease to the impact of our experimental approach, which combines a major surgical procedure and ipGTT following overnight fasting in a relatively short period of time. This should be considered when comparing our results with others obtained in different conditions.

In contrast to the effect on adiposity and insulin secretion, we could not detect any significant effect of dAG on other parameters that are known to be regulated by central AG action including food intake (2), RER (3) or LA (35). It is noteworthy that the effect of AG on adiposity is more consistent across experiments than the effect on food intake. This difference emphasizes that although AG commonly increases food intake, this increase is not necessary for increase in FM induced by AG (3; 30; 35-37).

The reasons underlying the selectivity of dAG towards the control of adiposity over other AG mediated effects as well as the relatively higher than expected potency *in vivo* will require further investigation. The reduced GHSR activation by dAG may explain part of the different outcomes observed between dAG and AG. Alternatively, intrinsic *in vivo* conditions may contribute to a relative increase in GHSR-mediated dAG biological activity, which may determine the selectivity over the control of different physiological effects. A growing area of interest is the ability of GHSR to heterodimerize with other G-protein coupled receptors (GPCRs) (38-41). Formation of these heteromers modifies GHSR basal activity as well as AG action (40). Furthermore, the amount of ghrelin present influences GHSR heterodimer formation as well as coupling to downstream intracellular signaling systems (39). Investigating whether dAG modulates GHSR heterodimerization with other GPCRs could help elucidate the function and molecular mechanisms of endogenous dAG.

In addition to recapitulating central AG action on plasma insulin levels (23), we further expand on the role of both AG and dAG in the central regulation of glucose metabolism. We find that icv infusion of either ghrelin isoform causes hyperinsulinemia in response to a glucose bolus. The glucose-stimulated hyperinsulinemia induced by AG is blunted when AG is infused subcutaneously, whereas the glucose-stimulated hyperinsulinemia induced by dAG is completely ablated when dAG is delivered subcutaneously. Considering the increased efficacy of dAG when administered centrally in comparison to peripherally as well as the absence of effect when administered centrally in *Ghsr-/-* mice, our results demonstrates that CNS-GHSR plays a critical role in mediating the effect of dAG in the control of FM and insulin secretion. Intriguingly, ghrelin is produced in the brain in areas involved in the control of energy balance (24; 26; 42;

43). Whether neurally-derived dAG plays a physiological role in the control of GHSR activity requires additional investigation. The loss of effectiveness of dAG regulating FM in *Ghsr-/-* mice strongly suggests that dAG shares the mechanisms of action whereby AG controls adiposity, including the regulation of the sympathetic nervous system activity (37).

Our results also highlight the role of CNS-GHSR as a positive regulator of insulin secretion. This role is in contrast with the inhibitory action of GHSR in the periphery on GSIS (21; 44). We find a stronger effect on GSIS following icv infusion of AG or dAG as compared to their peripheral administration. The lack of a peripheral action of dAG is consistent with other groups reporting no effect of chronic peripheral dAG treatment on body composition, glucose tolerance or GSIS in chow-fed mice (27). The inhibitory action of peripheral GHSR signaling on insulin release could contribute, at least in part, to counteract the CNS-GHSR mediated hyperinsulinemic action of AG and dAG. Thus, GHSR may play distinct roles in the control of insulin secretion depending on the site of action of AG or dAG. The underpinnings of the control of insulin release by CNS-GHSR activity have not been completely investigated. The HEC suggests that central dAG impairs peripheral glucose clearance, which may contribute to the development of hyperinsulinemia. However, it is likely that other mechanisms, including a direct action in the pancreas, play a role in the hyperinsulinemia induced by the action of both AG and dAG in the brain.

Collectively, our data demonstrate that dAG regulates adiposity and plasma insulin levels through the interaction with GHSR in the CNS and suggest that dAG may be functional endogenous agonist of GHSR. Furthermore, these data emphasize that adiposity and

hyperinsulinemia induced by dAG are independent of changes in food intake. Our data highlight the plurality of the ghrelin system and implicate that further studies are necessary to fully understand each component.

Author Contributions

KMHe was responsible for data collection, study conception and design, data analysis and interpretation, and writing the article; CLP and AM collected, analyzed and interpreted data; NO, KMHa and SS contributed to the design of the experimental approach and collected data; DLS provided essential research tools; RD provided essential research tools and contributed to the discussion of the article; HB contributed to study design, interpretation of the data, and the discussion of the article; PTP and MHT advised study concept and critical revision of the article; DAS contributed to study design, data analysis and interpretation, as well as the discussion and critical revision of the manuscript. DPT advised study conception and design of the experimental approach, and contributed to writing and critical revision of the article. DPT 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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Conflict of Interest

The authors have no conflict of interest to declare.

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Figure Legend

Figure 1: Effect of AG and dAG on IP_3 formation in HEK-293 cells transfected with human GHSR. Functional *in vitro* assays were performed in transiently transfected HEK-293 cells to investigate $G_{q/11}$ mediated IP_3 formation after stimulation with dAG (10 nM and 100 nM) or AG (10 nM and 100 nM) for 6h. Stimulation with 100 nM dAG or 10 and 100 nM AG significantly increased IP_3 formation (*$p<0.05$; ***$p<0.001$). IP_3 formation was determined via a reporter construct containing a response element and the firefly luciferase gene under control of the nuclear factor of activated T-cells (NFAT). All data indicate a fold increase of the empty

vector (pcDps) transfected cells (*MOCK*) basal activity 3961 ± 528 rlu (relative light units) for IP_3, which was set as 1. Data are means ± SEM out of two experiments performed in triplicates for $G_{q/11}$ activation. Statistical significance was determined by Student's unpaired two-tailed t-test.

Figure 2: Effect of chronic icv dAG or AG infusion on food intake, body weight and fat mass. C57/BL6 mice were given icv infusion of saline, dAG or AG for 7 days. AG and dAG were infused using increasing doses (0.04, 0.2, 1.0 and 5.0 nmol/day). There was a significant effect of the treatment on 7d cumulative food intake ($P<0.05$ AG vs dAG; two-way ANOVA). Consistently, a non-linear regression analysis followed by an extra-sum of squares F-test detected a significant difference between the curves fitting AG and dAG effect (a; $P=0.0185$). A two-way ANOVA detected a significant effect of the dose of both AG and dAG on body weight (b; $P<0.05$) and fat mass (c; $P<0.001$). A non-linear regression analysis followed by an extra-sum of squares F-test detected a significant difference ($P=0.0318$) between the potency of AG and dAG on fat mass (c). n= 6-10 animals per group

Figure 3: Effect of chronic icv dAG or AG infusion on respiratory exchange ratio, energy expenditure and locomotor activity. C57/BL6 mice were given icv infusion of saline, dAG or AG (5 nmol/day) for 7d while being housed in an indirect calorimetry system. AG and dAG had no effect on 7d cumulative food intake (a). Both AG and dAG increased body weight (b; *$p<0.05$ vs. saline, one-way ANOVA with Tukey's post hoc test) and fat mass (c; *$p<0.01$, ***$p<0.001$ vs. saline, one-way ANOVA with Tukey's post hoc test). (d) Effect of AG and

dAG on RER. AG significantly increased RER in the final 4 days of infusion (e; *p<0.05, AG vs. saline; two-way ANOVA with Bonferroni post hoc test). Neither AG nor dAG altered EE throughout the 7d infusion period (f,g). (h) Effect of AG and dAG on locomotor activity. Only AG maintained suppression of locomotor activity in the final 4d of infusion (i; *p<0.01, AG vs. saline; two-way ANOVA with Bonferroni post hoc test). n=7-8 animals per group

Figure 4: Effect of chronic icv dAG or AG infusion on energy and glucose metabolism. C57/BL6 mice were given icv infusion of saline, dAG or AG for 6 days (5 nmol/ day). AG significantly increased 6d cumulative food intake (a; *p<0.05 vs. saline, one-way ANOVA with Tukey's post hoc test). Following the infusion period, mice that received icv AG or dAG lost less body weight (b; **p<0.01, ***p<0.001 vs. saline, one-way ANOVA with Tukey's post hoc test) and fat mass relative to saline-treated control animals (c; **p<0.01, ***p<0.001 vs. saline, one-way ANOVA with Tukey's post hoc test). Neither AG nor dAG icv infusion altered ip glucose tolerance (d). Plasma insulin levels were measured by RIA and were elevated in mice treated with AG or dAG (e; *p<0.05 dAG vs. saline, ##p<0.01 AG vs. saline; two-way ANOVA with Bonferroni's post hoc test). n= 8-9 animals per group.

Figure 5: Effect of chronic subcutaneous dAG or AG infusion on energy and glucose metabolism. C57/BL6 mice were subcutaneously implanted with mini-pumps that infused AG or dAG for 6 days (120 nmol/ day). Neither ghrelin compound had a significant effect on 6d cumulative food intake (a). Following the infusion period, mice treated with AG lost less body weight (b) and fat mass relative to saline-treated controls (c), whereas dAG had no effect. Neither AG nor dAG infusion altered ip glucose tolerance (d). Plasma insulin levels were

measured by RIA and were elevated in sc-AG treated mice at 60 minutes following glucose injection (e). (*p<0.05, ***p<0.001 AG vs. saline, one-way ANOVA) (##p<0.01 AG vs. saline; two-way ANOVA with Bonferroni's post-hoc test). n= 8-9 animals per group.

Figure 6: Effect of chronic icv dAG infusion on energy and glucose metabolism in *Ghsr -/-* mice. *Ghsr-/-* and age-matched WT mice were given icv infusion of saline or dAG ghrelin for 6 days (5 nmol/ day). Icv-dAG infusion did not alter feeding in WT or *Ghsr-/-* animals (a). Following the infusion period, WT mice treated with icv-dAG lost less body weight (b) and fat mass (c) relative to WT saline-treated controls. Icv-dAG infusion had no effect on body weight (b) or fat mass (c) in *Ghsr-/-* mice. Icv-dAG did not alter ip glucose tolerance in either WT or *Ghsr-/-* animals (d). Plasma insulin levels were measured by ELISA and were elevated in WT mice treated with icv-dAG (e) compared to WT mice treated with icv-saline. Icv-dAG treatment did not alter glucose-stimulated plasma insulin levels in *Ghsr-/-* mice compared to icv saline treated *Ghsr-/-* mice (e). (**p<0.01, ***p<0.001, WT saline vs. WT dAG; two-way ANOVA with Bonferroni's post-hoc test). n= 7-8 animals per group.

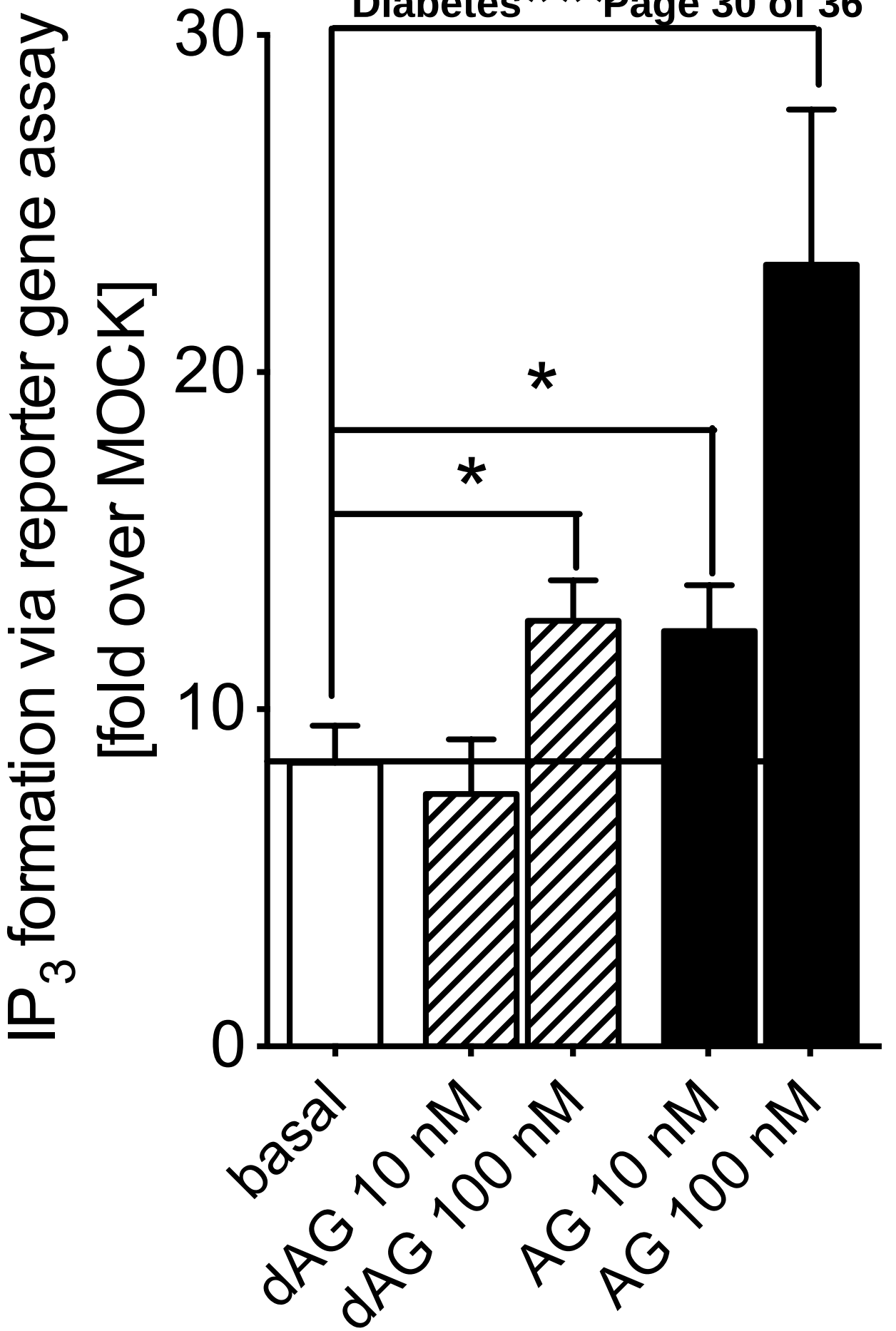
Figure 7: Effect of chronic icv-dAG infusion on peripheral glucose homeostasis during a hyperinsulinemic euglycemic clamp. Mice received chronic icv infusion of dAG (5 nmol/ day) for 5 days prior to a hyperinsulinemic euglycemic clamp. Blood glucose levels were clamped at similar levels in saline and dAG treated mice (a). Exogenous glucose infusion rate was similar in saline and dAG treated mice (b). Basal EGP and suppression of EGP during the hyperinsulinemic euglycemic clamp were similar in saline and dAG treated mice (c). Animals

treated with dAG had impaired insulin-mediated glucose disposal (d; *p<0.05 saline vs. dAG; t-test). n= 9-10 animals per group

Table 1: Effect of chronic icv dAG infusion on tissue specific glucose uptake during a hyperinsulinemic euglycemic clamp.

	Saline Glucose Uptake (mg/kg/min)	dAG Glucose Uptake (mg/kg/min)
BAT	111.70 ± 19.76	112.00 ± 16.88
iWAT	18.13 ± 7.88	15.34 ± 5.18
eWAT	5.49 ± 1.52	3.19 ± 0.72
soleus	11.18 ± 1.11	8.63 ± 3.46

No difference in glucose uptake in BAT, iWAT, eWAT or soleus was detected in icv dAG (5 nmol/ day) treated mice compared to saline treated controls. N =4-7 animals per group


IP3 formation via reporter gene assay
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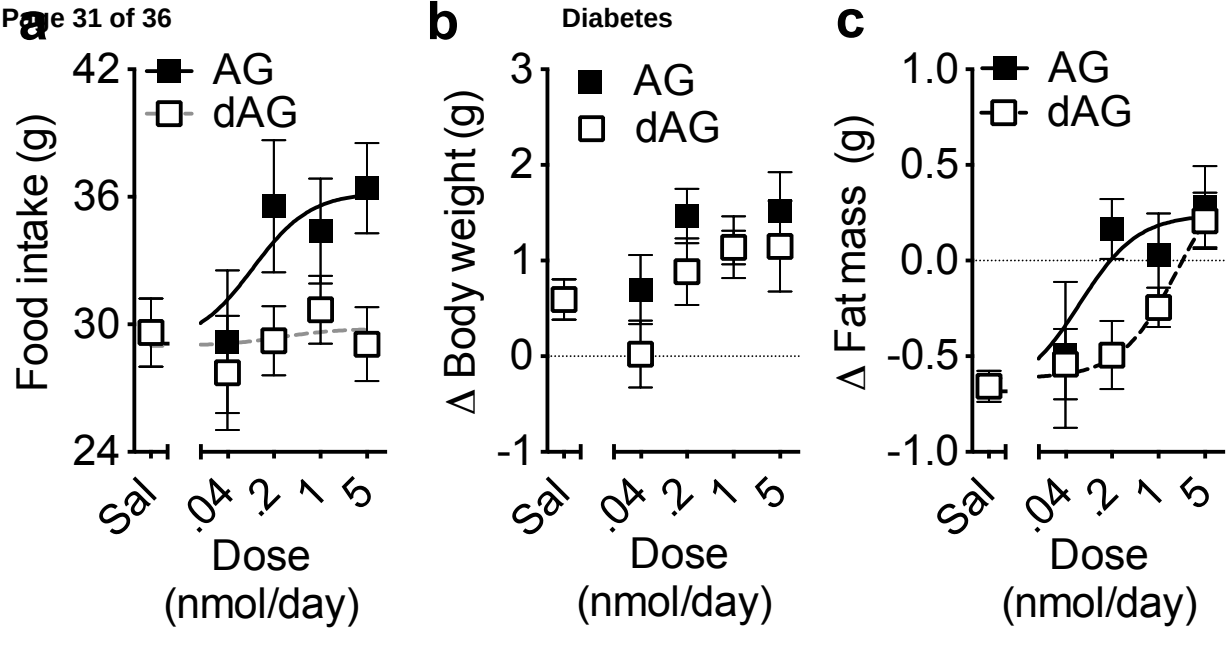
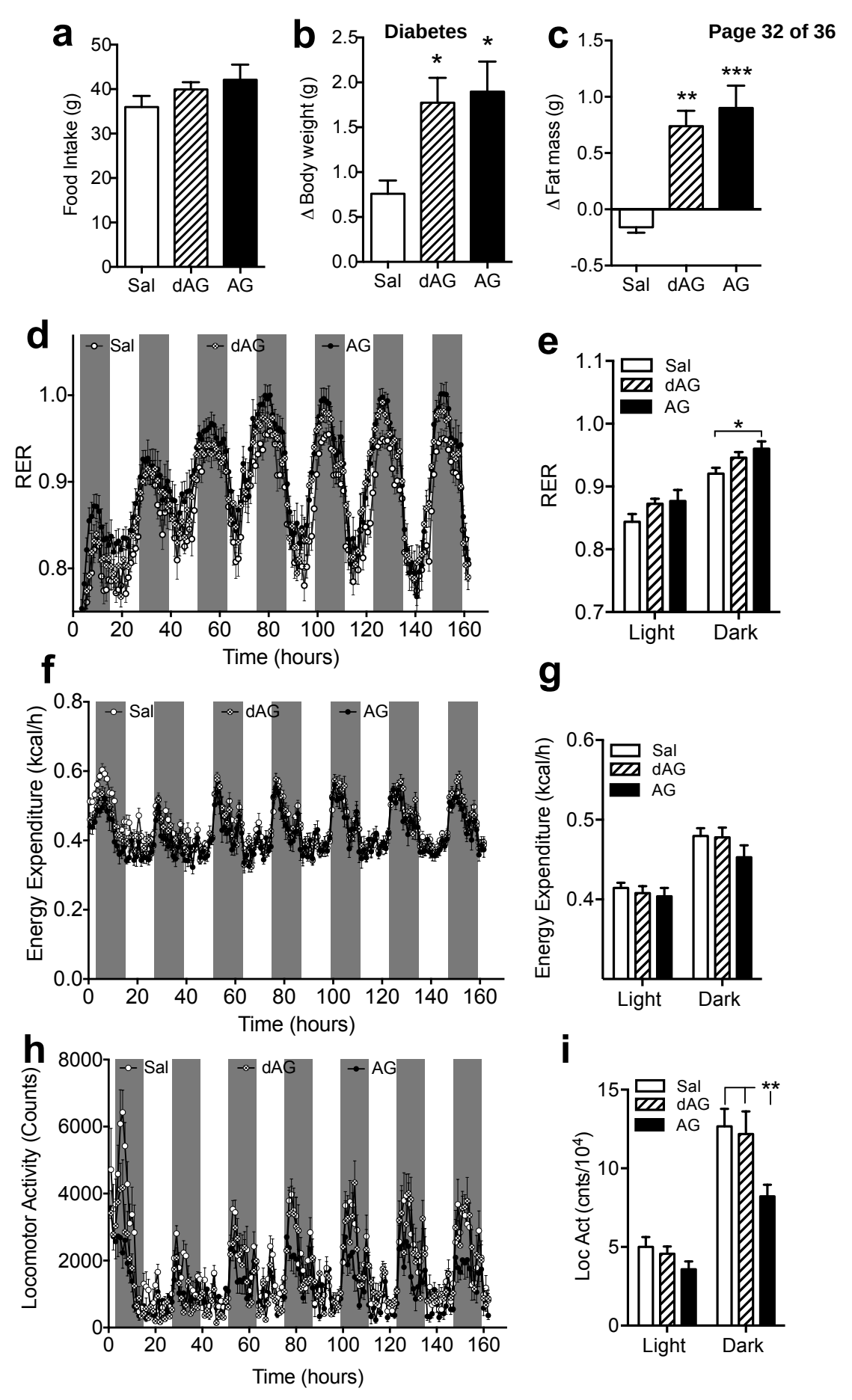
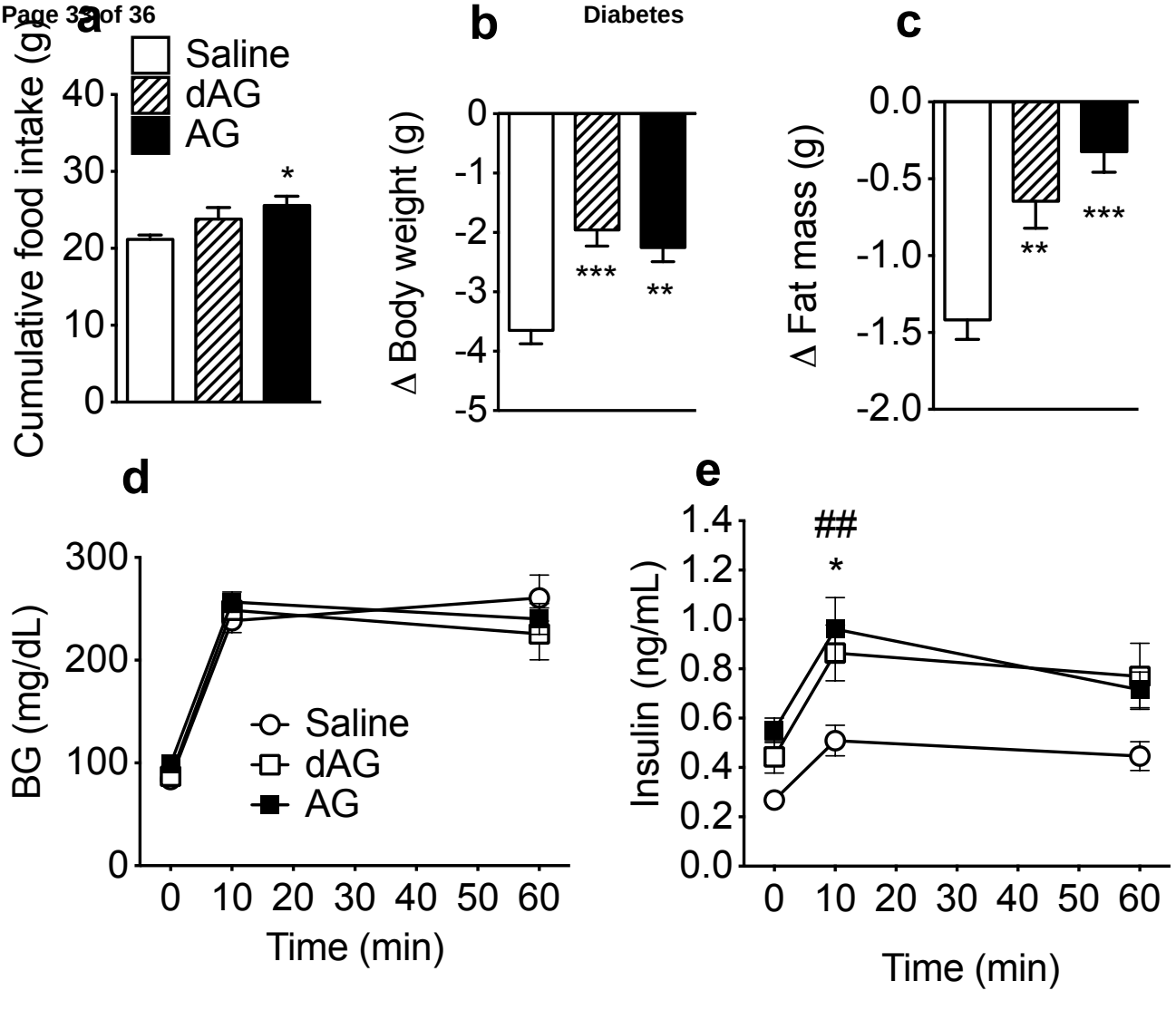
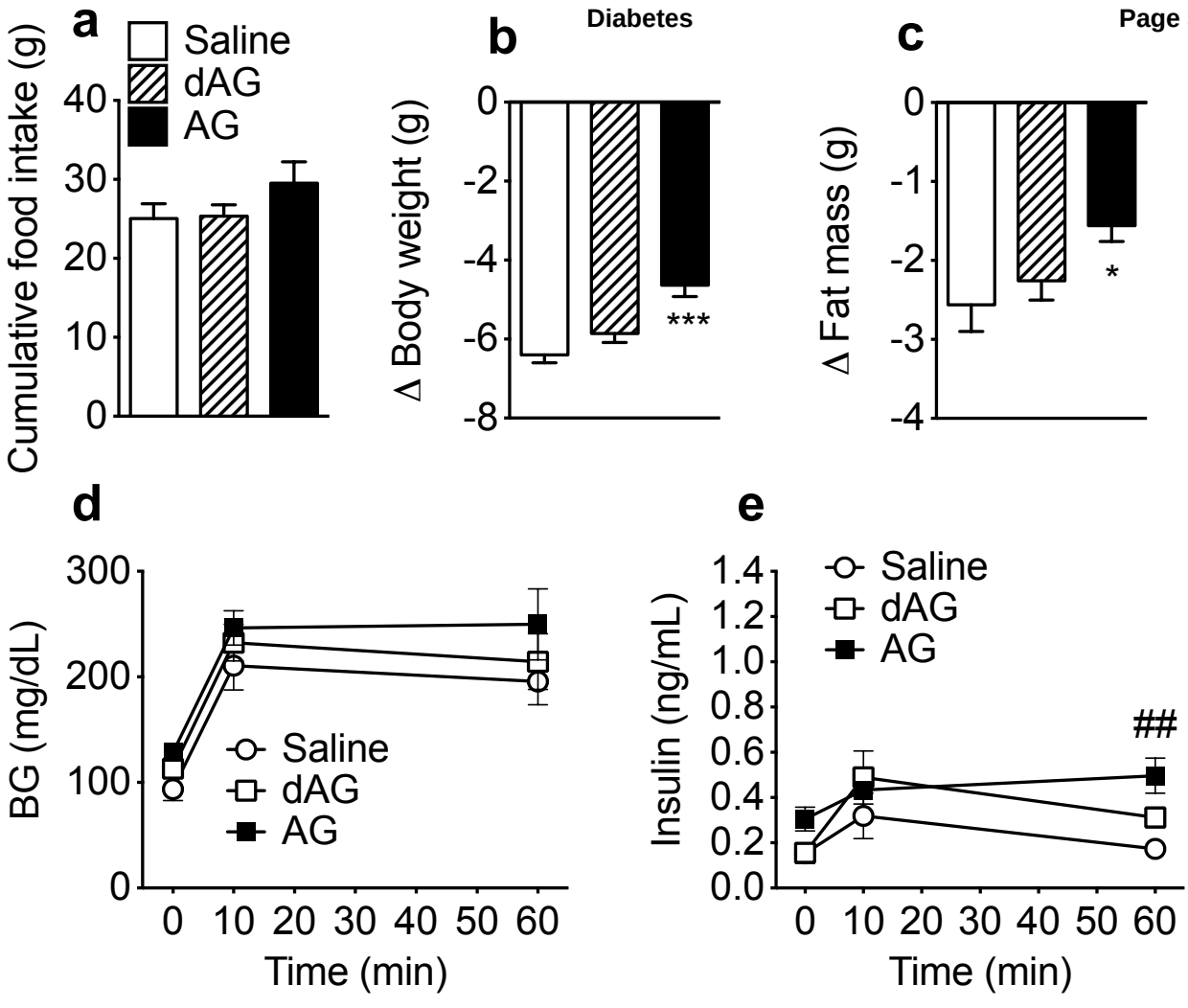
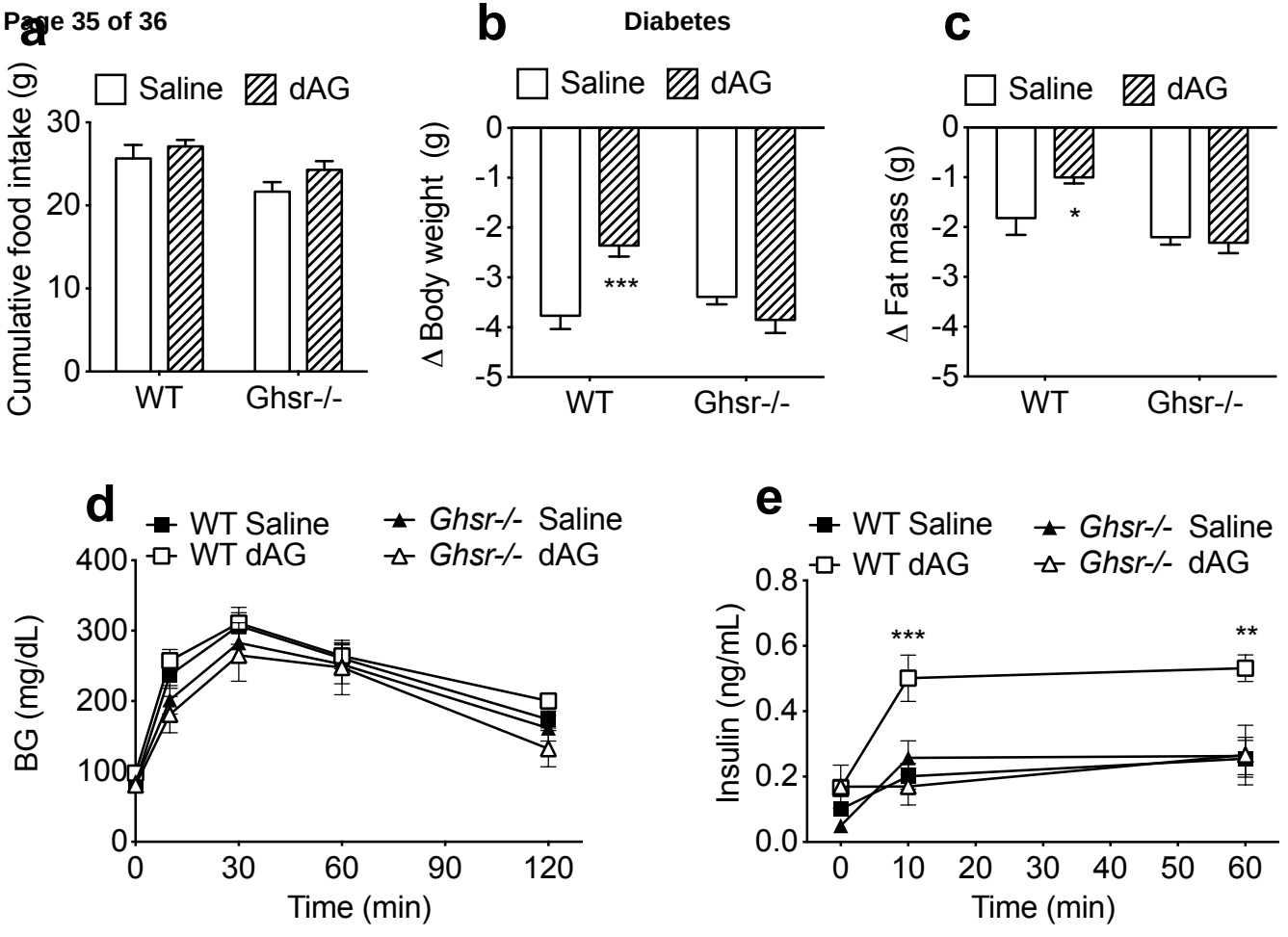
30
20
10
0
*
*
***
basal
dAG 10 nM
dAG 100 nM
AG 10 nM
AG 100 nM

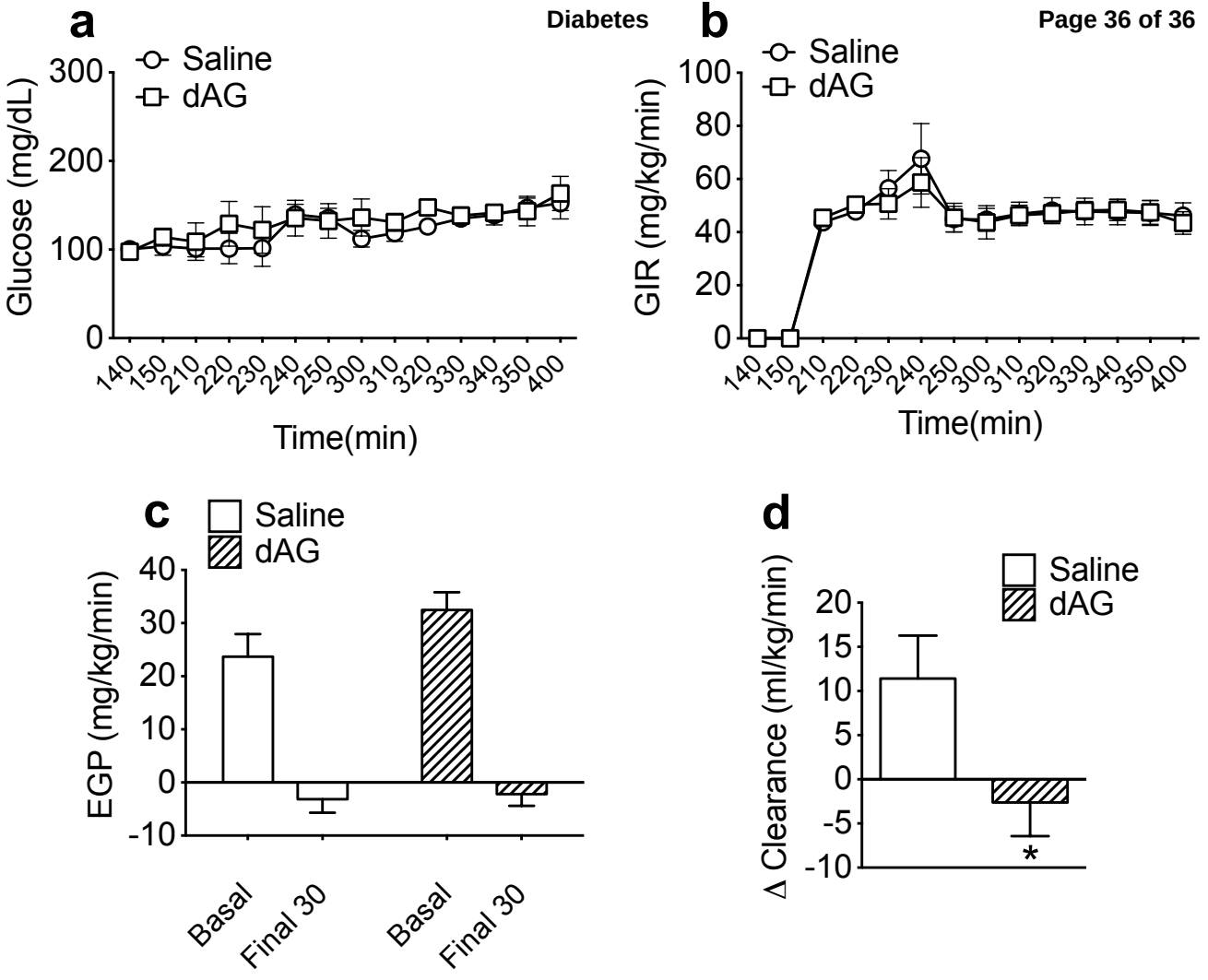

a
Food intake (g)
42
36
30
24
AG
dAG
Sal
.04
.2
1
5
Dose
(nmol/day)
b
Diabetes
Δ Body weight (g)
3
2
1
0
-1
AG
dAG
Sal
.04
.2
1
5
Dose
(nmol/day)
c
Δ Fat mass (g)
1.0
0.5
0.0
-0.5
-1.0
AG
dAG
Sal
.04
.2
1
5
Dose
(nmol/day)


a
Food Intake (g)
Sal
dAG
AG
b
Diabetes
Δ Body weight (g)
c
Δ Fat mass (g)
d
RER
Time (hours)
e
Light
Dark
f
Energy Expenditure (kcal/h)
g
h
Locomotor Activity (Counts)
i
Loc Act (cnts/10⁴)


a
Saline
dAG
AG
Cumulative food intake (g)
40
30
20
10
0
*
b
Diabetes
Δ Body weight (g)
0
-1
-2
-3
-4
-5
***
**
c
Δ Fat mass (g)
0.0
-0.5
-1.0
-1.5
-2.0
**
***
d
BG (mg/dL)
300
200
100
0
Saline
dAG
AG
0 10 20 30 40 50 60
Time (min)
e
Insulin (ng/mL)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
*
0 10 20 30 40 50 60
Time (min)


a
Saline
dAG
AG
Cumulative food intake (g)
40
30
20
10
0
b
Δ Body weight (g)
0
-2
-4
-6
-8
***
c
Δ Fat mass (g)
0
-1
-2
-3
-4
*
d
BG (mg/dL)
300
200
100
0
Saline
dAG
AG
0 10 20 30 40 50 60
Time (min)
e
Insulin (ng/mL)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Saline
dAG
AG
##
0 10 20 30 40 50 60
Time (min)


a
Cumulative food intake (g)
Saline
dAG
30
20
10
0
WT
Ghsr-/-
b
Diabetes
Saline
dAG
Δ Body weight (g)
0
-1
-2
-3
-4
-5
***
WT
Ghsr-/-
c
Saline
dAG
Δ Fat mass (g)
0
-1
-2
-3
-4
-5
*
WT
Ghsr-/-
d
WT Saline
WT dAG
Ghsr-/- Saline
Ghsr-/- dAG
BG (mg/dL)
400
300
200
100
0
0
30
60
90
120
Time (min)
e
WT Saline
WT dAG
Ghsr-/- Saline
Ghsr-/- dAG
Insulin (ng/mL)
0.8
0.6
0.4
0.2
0.0
***
**
0
10
20
30
40
50
60
Time (min)


a
Saline
dAG
Glucose (mg/dL)
300
200
100
0
140 150 210 220 230 240 250 300 310 320 330 340 350 400
Time(min)
b
Saline
dAG
GIR (mg/kg/min)
100
80
60
40
20
0
140 150 210 220 230 240 250 300 310 320 330 340 350 400
Time(min)
c
Saline
dAG
EGP (mg/kg/min)
40
30
20
10
0
-10
Basal
Final 30
Basal
Final 30
d
Saline
dAG
Δ Clearance (ml/kg/min)
20
15
10
5
0
-5
-10
*