L-Arginine promotes gut hormone release and reduces food intake in rodents.

Running title: L-arginine on food intake in rodents.

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Abstract

Aims: The amino acids generated by protein digestion may play a role in the weight loss driven by high protein diets. We investigated the anorectic effect of L-arginine (L-Arg) in rodents.

Materials and Methods: We investigated the effect of L-Arg on food intake, and the role of the anorectic gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), the G protein-coupled receptor family C group 6 member A (GPRC6A) and the vagus nerve in mediating these effects in rodents.

Results: Oral gavage of L-Arg reduced food intake in rodents, and chronically reduced cumulative food intake in diet-induced obese mice. Lack of the GPRC6A in mice or subdiaphragmatic vagal deafferentation in rats did not influence these anorectic effects. L-Arg stimulated GLP-1 and PYY release *in vitro* and *in vivo*. Pharmacological blockade of GLP-1 and PYY receptors did not influence the anorectic effect of L-Arg. L-Arg-mediated PYY release modulated net ion transport across the gut mucosa. Intracerebroventricular (ICV) and intraperitoneal (IP) administration of L-Arg suppressed food intake in rats.

Conclusions: L-Arg reduced food intake and stimulated gut hormone release in rodents. The anorectic effect of L-Arg is unlikely to be mediated by GLP-1 and PYY, does not require GPRC6A signalling and is not mediated via the vagus. ICV and IP administration of L-Arg suppressed food intake in rats, suggesting that L-Arg may act upon the brain to influence food intake. Further work is required to determine the mechanisms by which L-Arg suppresses food intake and its utility in the treatment of obesity.

Introduction

High protein diets (HPDs) promote satiety and weight loss (1, 2), but the exact mechanisms mediating these effects are unclear. However, evidence suggests that protein is sensed within the gastrointestinal (GI) tract, modulating appetite-regulating pathways (3).

Mechanisms proposed to mediate the effects of HPDs on food intake, include increased thermogenesis, intestinal gluconeogenesis and changes in gut hormone profiles (4). Such mechanisms may be instigated by the sensing of the amino acids produced by protein digestion. Rodents adapt their diet to balance amino acid intake (5). Different types of protein can result in different levels of satiety (6), perhaps reflecting their different amino acid compositions. The recent discovery of promiscuous L-amino acid-sensing G protein-coupled receptors and their expression in the GI tract has driven speculation that these receptors are involved in amino acid sensing and the regulation of food intake. These receptors include the calcium sensing receptor (CaSR), the T1R1-T1R3 umami taste receptor complex and the G protein-coupled receptor family C group 6 member A (GPRC6A) (7).

Amino acids may be sensed in the gut to promote the release of anorectic gut hormones (8). The aromatic amino acids L-phenylalanine and L-tryptophan induce the release of cholecystokinin (CCK) from isolated I-cells (9). The anorectic gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (3-36) are released from enteroendocrine L-cells in response to nutrients, including amino acids (10). Ingestion of a high protein meal alters circulating GLP-1 and PYY levels and promotes their release in both humans and rodents (11, 12). These hormones may act directly on appetite-regulating areas of the brain, but may also have indirect effects. The vagus nerve is one of the major extrinsic nerves with a key role in the gut-brain axis. Evidence suggests that vagal signalling is involved in the regulation of food intake, and may play a role in gut hormone-mediated satiety. Vagal afferents relay

mechanosensory and chemosensory signals from the gut to the nucleus of the solitary tract within the brainstem. In addition, specific gut hormones, in particular cholecystokinin (CCK), but including ghrelin, PYY(3-36) and GLP-1, have been reported to exert their effects on appetite and food intake via vagal afferents (13).

The ability of specific L-amino acids, including L-arginine (L-Arg), to stimulate GLP-1 and PYY release has been studied previously *in vitro* (14, 15). L-Arg, a conditionally essential amino acid, is derived from the diet, endogenous synthesis and protein turnover (16). L-Arg has a well characterised effect as a secretagogue promoting insulin release from pancreatic β-cells (17). Oral L-Arg can also stimulate insulin secretion by promoting GLP-1 release, improving glucose tolerance in mice (18). L-Arg is a potent agonist of GPRC6A (19), and it has been suggested that GPRC6A activity is necessary for some effects of L-Arg on glucose homeostasis (20). Furthermore, GPRC6A was required for ornithine–induced GLP-1 release from an *in vitro* model (21), suggesting that GPRC6A may play a role in L-Arg-mediated hormone release. In addition, L-Arg can stimulate growth hormone release from the pituitary, though the mechanism is unclear (22).

L-Arg thus has established effects on hormone release and metabolism. Recent evidence suggests that L-Arg may also be involved in the regulation of food intake (23). Therefore we investigated the effect of L-Arg on gut hormone release and energy homeostasis in rodents, and explored the potential mechanisms mediating its effects on gut function and food intake.

Materials and Methods

Animals

Male C57BL/6 mice, 8-10 weeks (Harlan, Bicester, UK) and male Wistar rats (200-250g) (Charles River, Margate, UK) were individually housed under controlled temperature (21-

23°C) and humidity on a 12h light: 12h darkness cycle. All animals had *ad libitum* access to standard chow RM1 (SDS, Witham, UK) and water, and were randomised by body weight unless stated otherwise. The GPRC6A knockout (GPRC6a-KO) model used in our studies was generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository. The deleted region completely covers the GPRC6a locus (24), and thus this model differs from others previously described (25, 26). The glucose homeostasis phenotype of the knockout model was assessed prior to performing feeding studies to address the conflicting reported phenotypes of other GPRC6a KO models (25, 27). All animal procedures were approved and performed under the UK Home Office Animals (Scientific Procedures) Act 1986.

Feeding studies

Animals were randomised by body weight and acclimatised to the procedures prior to all studies. Due to the basic nature of L-Arg solution, L-Arg monohydrochloride (L-Arg.HCl) neutral salt was used in all experiments. For fasted studies, animals were fasted for 16h overnight before receiving water or L-Arg.HCl (Sigma, Poole, UK), at doses stated (Supplementary Table 1), in the early light phase by either oral gavage (OG) or intraperitoneal (IP) injection. For *ad libitum* fed studies, the same administration procedure was used without fasting in either the early light phase or at the onset dark phase. For the study investigating the role of gut hormones in mediating the effect of L-Arg on food intake; fasted or *ad libitum* fed mice were given simultaneous IP administration of 400nmolkg^{-1} exendin9-39 (GLP-1R antagonist) and BIIE0246 (Y_2 receptor antagonist) at 5.26μmolkg^{-1} 15 min prior to the OG of water or 24mmolkg^{-1} L-Arg. Animals were returned to their cages following administration with pre-weighed amounts of standard chow diet and food intake measured 1, 2, 4, 8 and 24h following administration. GPRC6a-KO mice feeding studies used a crossover design in which GPRC6a-KO and wild-type (WT) mice received both control and

L-Arg treatments in random order on separate occasions separated by at least three days; accordingly, food intake was compared and analysed using a paired analysis approach. A summary of all feeding studies, including doses, species and time of day of the study, is provided in the Supplementary Table 1.

Energy expenditure studies

Mice were individually housed in a 24-chamber open-circuit comprehensive laboratory animal monitoring system (CLAMS) (Columbus Instruments, OH, USA) and acclimatised for 24h to generate stable reference data. They were then fasted for 16h overnight and were subsequently orally gavaged with water or 24mmolkg^{-1} L-Arg (n=12/group) at 0900h (early light phase). Animals continued to be fasted for the subsequent 8h, to examine the effects of L-Arg on energy expenditure independent of effects on food intake, before food was returned at 1700h. Metabolic parameters (VO_2 and VCO_2) and respiratory exchange ratio (RER) were measured every 24min for 24h following treatment administration, and values normalised to body weight (28).

Chronic feeding studies in mice

Male mice 6-8 weeks of age were group-housed (5/cage) with *ad libitum* access to water and 60% high fat diet (Research Diets, New Brunswick, USA) for 8 weeks. Animals were then individually housed and given one week to acclimatise before the study started, remaining on the high fat diet. Mice were orally gavaged with water or 16mmolkg^{-1} L-Arg (n=9/group) twice daily throughout the dark phase at 1900h and then 0100h for 5 nights. Body weight and food intake were measured daily at the beginning of dark phase and at 1h following the first daily gavage.

Subdiaphragmatic vagal deafferentation (SDA) surgery in rats

SDA was carried out in rats as previously described (29, 30), as it results in more accurate deafferentation and lower morbidity compared to mice. The effect of oral administration of water or 16mmolkg^{-1} L-Arg (n=9-10, crossover) on food intake was then studied in overnight fasted animals during the early light phase.

Murine colonic crypt isolation and hormone secretion assays

Primary mice colonic crypt isolation and secretion studies were performed using an adaptation of an established method previously described (31, 32). Gut hormone secretion was expressed as a fraction of the total peptide (secreted plus intracellular) measured in each well over 2h.

In vitro **mucosal studies**

Ileal or colonic mucosa from WT male mice (>15 weeks old), was voltage-clamped at 0mV in Ussing chambers as described previously (33). Vectorial ion transport was measured continuously as short-circuit current (I_{sc}) ($\mu A/cm^2$) and provided an acute readout for endogenous PYY release. Once stable I_{sc} levels were achieved, vehicle or the Y_1 receptor (Y_1R) antagonist BIBO3304 (BIBO; 300 nM) and L- or D-Arg were added to the apical or basolateral reservoirs bathing mucosae. L-Arg (1mM) responses were measured 15-20min after vasoactive intestinal peptide (VIP, 10nM), an optimal secretory stimulus for revealing subsequent $G_{\alpha i}$-coupled epithelial responses (33). Epithelial Y agonism results from $G_{\alpha i}$-coupled attenuation of cAMP levels, with consequent sustained decreases in Cl^- ion secretion and I_{sc} levels (34), thus PYY (10nM) was added after L-Arg as a control.

In vivo gut hormone studies

Rats were fasted overnight, before receiving an OG of either water or 16mmolkg^{-1} L-Arg (n=6-8) in the early light phase. They were immediately returned to their cages, euthanized by decapitation at 30 or 90min following administration and plasma samples collected as previously described (29).

Intra-ileal administration studies

These procedures were performed in anaesthetised animals as previously described (32). Rats received an injection of either saline or 1M L-Arg (n=4-5) in a volume of 2.5ml into the upper ileum; blood samples were collected at -15, 0, 15, 30, 45 and 60min post-administration via the jugular cannula. Mice were injected with either saline or 1M L-Arg (n=4-5) in a volume of 500µl into the upper ileum, and were euthanized 30 min post-administration and blood collected.

Gut hormone radioimmunoassay (RIA)

GLP-1 and PYY were measured using previously established in-house specific and sensitive RIAs (35, 36). The GLP-1 antibody has 100% cross-reactivity with all amidated forms of GLP-1, but does not cross-react with glycine extended forms. The PYY antibody has 100% cross-reactivity with PYY(1-36) and PYY(3-36). The intra-assay coefficients of variation for GLP-1 and PYY assays were 8.7% and 6.0% respectively.

ICV cannulation and administration

ICV injections were carried out as previously described (37). Rats recovered from surgery for 7 days before being injected with 5µl of either vehicle saline control or 4µM L-Arg (n=8-9) over 1 min using a 28-gauge stainless steel injector in the early light phase.

Statistical analyses

Acute feeding studies data and area under the curve (AUC) data are expressed as mean ± the standard error of the mean (SEM) and were analysed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. CLAMS data were analysed using two-way ANOVA and Bonferroni *post hoc* test, chronic and SDA feeding study data using multiple Student's t-test, GPRC6a-KO feeding data using two-way ANOVA with Sidak's *post hoc* analysis, *in vitro* and *in vivo* gut hormone data using one-way ANOVA with Dunnett's and two-way ANOVA with Bonferroni's *post hoc* test, respectively. Mucosal data measuring the maximal changes in I_{sc}, are expressed as the mean ± SEM per unit area (cm^2), and single comparisons performed by Student's unpaired t-test. All analyses utilised Graphpad Prism software (Prism 6.03, GraphPad Software Inc, CA, USA).

Results

The effect of oral L-Arg on food intake, body weight and energy expenditure in rodents

Oral administration of L-Arg significantly reduced food intake in both rats and mice (Figure 1). In rats, oral administration of L-Arg suppressed food intake in fasted animals in the early light phase (Figure 1A) and in *ad libitum* fed animals in the early dark phase (Figure 1B). Similarly, oral administration of L-Arg in fasted mice reduced food intake in a dose-dependent manner (Figure 1C). Oral administration of L-Arg in *ad libitum* fed mice significantly reduced cumulative food intake 24h following administration in the light and dark phases (Figures 1D and 1E). These anorectic effects were not secondary to abnormal behavioural side effects in rats (Supplementary Figure 1).

Following observations that L-Arg administration could result in a sustained reduction in food intake in rodents, we investigated whether this anorectic effect could be sustained chronically and reduce body weight in a diet-induced obese (DIO) mouse model, a commonly

used model of obesity. Repeated L-Arg administration significantly reduced food intake at day 1 and day 2 in DIO mice compared to vehicle-treated animals (Figure 1F), although this effect was insufficient to significantly change body weight over the time period studied (Figure 1G).

To investigate the effect of L-Arg on energy expenditure, mice were placed in CLAMS metabolic cages. Oral administration of 24mmolkg^{-1} L-Arg had no significant effect on VO_2, VCO_2 or RER in mice during the 8h following administration. Interestingly, oral administration of 24mmolkg^{-1} L-Arg still reduced food intake when food was returned 8h after administration in mice placed in CLAMS cages, demonstrating a delayed and sustained anorectic effect (Figure 1H). Returning food did not significantly alter VO_2 or VCO_2 between both treatment groups (Figures 1I and 1J). However, the RER was significantly lower in L-Arg treated animals following the return of food (Figure 1K).

GPRC6A is not required for the anorectic effect of L-Arg in mice

Oral administration of 16 or 24mmolkg^{-1} L-Arg significantly reduced food intake in both WT and GPRC6a-KO mice to a similar magnitude 0-1h following administration, suggesting GPRC6A is not necessary for the anorectic effect of L-Arg (Figures 2A and 2B). Oral L-Arg also improved glucose tolerance in both WT and GPRC6a-KO mice (Supplementary Figure 2).

The anorectic effect of L-Arg is not mediated via the vagus

Oral gavage of 16mmolkg^{-1} L-Arg significantly reduced food intake in both sham operated and SDA operated rats 0-1h following administration, suggesting the vagus is not necessary for the anorectic effect of L-Arg (Figure 2C).

The effect of L-Arg on gut hormone release

L-Arg stimulated GLP-1 and PYY release from murine primary colonic L-cells (Figures 3A and 3B). Exposure to 100mM L-Arg for 2h stimulated PYY release from colonic cultures isolated from GPRC6a-KO, although the GLP-1 response to L-Arg appeared to be attenuated in GPRC6a-KO compared to the WT (Supplementary Figure 3). In keeping with observations *in vitro*, oral administration of 16mmolkg^{-1} L-Arg elevated plasma GLP-1 and PYY release in rats. Plasma GLP-1 levels were significantly elevated at 30 and 90min following administration compared to control (Figure 3C). PYY levels were significantly elevated at 30min post-administration (Figure 3D). Furthermore, direct upper ileal administration of 1M L-Arg elevated plasma GLP-1 (P<0.05) and PYY (P=0.07) levels in anaesthetised mice and rats (Figures 3E and 3F).

The effects of L-Arg-stimulated gut hormone release

To investigate whether the anorectic effect of L-Arg is mediated by increases in gut hormone levels, GLP-1 and Y_2 receptors were simultaneously antagonised in mice receiving an oral gavage of 24mmolkg^{-1} L-Arg. The GLP-1 receptor antagonist exendin9-39 and the Y_2 receptor antagonist BIIE0246 were administered IP at doses established to block the anorectic effects of exogenous exendin-4 and PYY respectively (Supplementary Figure 4). Oral gavage of 24mmolkg^{-1} L-Arg significantly reduced food intake both in fasted mice in the early light phase and fed mice in the early dark phase, whether they were co-administered saline control or a mixture of 5.26μmolkg^{-1} BIIE0246 and 400nmolkg^{-1} exendin9-39 (Figures 4A and 4B).

In line with the hormone release measured *in vitro* and *in vivo*, mucosal Isc measurements showed that L-Arg (1mM) altered ion transport acutely within 15-30min, while D-Arg was inactive (Figure 3C-G). Apical treatment of ileal and colonic mucosae with L-Arg increased I_{sc} initially (potentially a GLP-1-mediated effect). It then decreased I_{sc} more slowly and,

importantly, this response component was Y_1R-dependent and therefore most likely PYY-mediated (Figure 4C-I).

The effect of central and IP administration of L-Arg on food intake in rodents

IP administration of 4 and 8mmolkg^{-1}` L-Arg significantly reduced food intake in rats 0-1h following administration compared to saline controls (Figure 5A). Similarly, in mice, 12mmolkg^{-1} L-Arg significantly reduced food intake 0-1h following administration. Food intake was significantly lower in mice treated with 12mmolkg^{-1} L-Arg 4-8h post administration. The cumulative food intake at 8h post-administration was significantly lower in both 8 and 12mmolkg^{-1} L-Arg groups compared to the saline control (Figure 5B). Furthermore, ICV administration of L-Arg significantly reduced food intake in rats at 0-1h post-administration only, but had no effect on 0-24h cumulative food intake (Figure 5C).

Discussion

We investigated the anorectic properties of L-Arg in rodents and the potential mechanisms by which these effects are mediated. Our data demonstrates L-Arg reduces food intake in both mice and rats without causing behavioural side effects, but does not affect energy expenditure in mice at the dose investigated. Repeated L-Arg administration reduced cumulative food intake at days one and two in DIO mice, but without significant effect on body weight over the time studied. The anorectic effects of L-Arg were not dependent on the amino acid sensing receptor GPRC6A or on vagal signalling. L-Arg significantly stimulated GLP-1 and PYY release acutely *in vitro* and *in vivo*; however, its anorectic effects appear unlikely to be mediated by changes in these gut hormones.

The anorectic effect of L-Arg has been previously demonstrated in a rat model. Jordi *et al* demonstrated a significant reduction in food intake following oral gavage of 6.7mmolkg^{-1} L-Arg in rats (23). In our initial dose finding studies, doses of 4 and 6mmolkg^{-1} of the non-salt

L-Arg solution suppressed food intake in rats. However, equivalent doses of the L-Arg.HCl salt had no effect on food intake and a dose of 8mmolkg^{-1} or higher was required to significantly reduce food intake in rats. This suggests that the non-salt L-Arg may influence food intake at least in part because of its basicity. In order to avoid any possible pH-dependent effect, the neutral L-Arg.HCl salt was used in all of our reported experiments. While it is possible that L-Arg.HCl solution has non-specific osmotic effects on food intake, pilot studies using isomolar concentrations of sodium chloride demonstrated no effect of this concentration of sodium choloride on food intake, and found that L-Arg reduced food intake compared to iso-osmotic controls (data not shown).

We found that acute administration of L-Arg had no significant effect on VO_2 and VCO_2 in mice. However, this study demonstrated that oral gavage of 24mmolkg^{-1} L-Arg significantly reduced food intake in mice when food was returned 8h later. This demonstrates a prolonged anorectic effect that is not necessarily observable if food is returned immediately. It also suggests L-Arg can have long-term effects on food intake when food is not immediately available following administration, which may be exploitable by weight loss promoting agents. Furthermore, RER was significantly lower in the L-Arg treated cohort once food was returned. This effect likely reflects the significantly lower food intake in the L-Arg treated animals. Different experimental conditions may alter the pharmacokinetic profile of L-Arg, and further work is required to determine whether such long-term effects occur in other contexts. It is also possible that the effects observed reflect the action of L-Arg metabolites or other molecules of which it is a precursor, such as nitric oxide and glutamate. However, simple availability of L-Arg does not regulate the levels of many of these downstream agents. Chronic L-Arg supplementation in mice on a low protein diet has been reported to reduce epididymal fat, while increasing food intake (41). L-Arg may facilitate increased protein synthesis in animals deficient in protein, but its effects might be expected to be very different

in rodents with a normal protein intake. We found repeated administration of L-Arg reduced cumulative food intake but did not significantly influence weight gain in DIO mice. A longer period of administration and perhaps a higher dose might result in a significant effect on body weight. Our data do not exclude the possibility of small changes to body weight that we were unable to detect in response to chronic L-Arg administration. It is also possible that L-Arg promotes the absorption of other nutrients or causes small decreases in energy expenditure that were not detectable by the CLAMS which would explain the lack of difference in body weight. Further work is required to establish the chronic effects of L-Arg administration on energy homeostasis.

L-Arg can influence hormone release from other endocrine tissues including the pancreas (17) and the pituitary (22). We therefore investigated the role of anorectic gut hormones in mediating the effect of L-Arg on food intake. L-Arg stimulated the release of GLP-1 and PYY *in vitro* and *in vivo*. Our data suggested that the anorectic effects of L-Arg are not mediated via gut hormone release, although a potential role for other gut hormones cannot be excluded. Mucosal studies, however, complemented the hormonal release we observed and indicated that L-Arg caused acute endogenous PYY release with consequent rapid inhibition of local epithelial ion transport that was Y_1R-mediated. This mechanism is similar to that described for other amino acids acting via the CaSR in mouse colon (38). PYY and GLP-1 inhibit gastric emptying, the former most likely acting via Y1 and Y2 receptors (39); PYY(1-36) binds to both receptors with similar affinity, while the truncated form which reduces appetite has higher affinity for the Y2 receptor. Furthermore, L-Arg reduces gastric emptying in man, apparently via changes in basal levels of nitric oxide (40, 41). In addition, both GLP-1 and PYY inhibit gastric motility in rats (42, 43). Increased plasma GLP-1 and PYY following L-Arg administration may slow gastric emptying acutely, but our evidence suggests that these rapid changes in hormone levels may not be responsible for the

subsequent reduction in food intake, which was not blocked by antagonising either GLP-1 or PYY-Y2 receptors. However, both PYY(1-36) and PYY(3-36) likely influence upper gastrointestinal transit, including gastric emptying, in mice (39), and it is therefore possible that the effects of L-Arg on PYY(1-36) release alter gastric emptying via the Y1 receptor sufficiently to account for some of the observed anorectic effect in mice.

Basic amino acids including L-Arg are potent activators of GPRC6A (19), which is highly expressed in the GI tract and is involved in a number of important physiological pathways (44). The effects of L-Arg were examined in mice lacking the GPRC6A at both 24mmolkg^{-1}, which was previously shown to reduce food intake in mice, and at a lower dose of 16mmolkg^{-1}, in case the effects of higher doses were mediated by different, perhaps non-physiological mechanisms. However, at both doses, oral administration of L-Arg significantly reduced food intake in GPRC6a-KO mice. Previously, small interfering RNA-induced depletion of endogenous GPRC6A has been shown to abolish L-ornithine-stimulated GLP-1 release from GLUTag cells (21). GPRC6A ablation did not appear to block L-Arg-induced GLP-1 and PYY release from a primary cultured murine colonic epithelium, although the effect on GLP-1 release was attenuated. These data suggest that GPRC6A is not necessary for the anorectic effects of L-Arg, and that it plays at most a minor role in its effects on gut hormone release. L-Arg also activates both T1R1-T1R3 and CaSR receptors, albeit to a lesser extent than GPRC6A (7). The involvement of these receptors cannot be ruled out. L-Arg-induced GLP-1 and PYY release from isolated rat small intestinal loops was attenuated by a CaSR antagonist, suggesting it is in part mediated by CaSR (15).

Other mechanisms may be involved in mediating the effects of L-Arg. Evidence suggests L-Arg stimulates the release of insulin from pancreatic β-cells by causing membrane depolarization, and that this effect is not mediated by calcium or ATP sensitive potassium channels, but as a consequence of electrogenic transport of L-Arg into the β-cell via specific

amino acid transporters (45). Amino acid transporter systems may also be involved in amino acid sensing in the gut (14). *In vitro* studies suggest that L-cells exhibit action potential-driven calcium influx in response to nutrients including amino acids, leading to acute hormone release. The sodium-coupled neutral amino acid transporter 2 (SNAT2) has been implicated in nutrient sensing and gut hormone release. SNAT2 acts as a secondary active transporter by coupling the transfer of amino acids against their concentration gradient to the simultaneous inward movement of sodium ions down its electrochemical gradient. This sodium-dependent transport mechanism has been shown to increase intracellular calcium levels and to consequently stimulate the release of gut hormones (46). Of note, L-glutamine has been shown to stimulate GLP-1 release from intestinal L-cells via a SNAT2-mediated mechanism (14). In addition, the CaSR has been shown to mediate the pharmacological effects of specific amino acids on gut hormone release from cell lines and *ex-vivo* tissue (15, 47).

There is evidence that the vagus nerve responds to nutrient load and is involved in protein-induced satiety (3). Proteins and amino acids activate neurons within the nucleus of the solitary tract via visceral vagus mediated signals. In addition, GLP-1, Y_2 and CCK receptors are expressed on vagal afferents which are proposed to play a key role in gut-brain mediated responses in satiety and food intake regulation (48). We examined the effect of L-Arg on food intake in rats that had undergone SDA surgery and observed no difference on the effects of L-Arg on food intake, in accord with previous work suggesting the anorectic effect of L-Arg is not vagally-mediated (23).

IP or ICV administration of L-Arg significantly reduced food intake in rats. These findings raise the possibility that a post-absorptive mechanism may be involved in mediating the anorectic effects of L-Arg. Jordi and colleagues suggested that the anorectic effect of L-Arg is mediated centrally via the area postrema (AP) in the brain stem. Oral administration of L-

Arg solution increased c-fos positive cells in AP, and the anorectic effect of L-Arg was abolished in animals that had undergone AP lesioning surgery (23), although, as mentioned earlier, these studies were performed using the non-salt, basic L-Arg solution. We have previously reported an increase in c-fos positive cells in the AP following oral L-cysteine administration, suggesting that there may be similar mechanisms by which amino acids influence food intake (29). Branched chain amino acids have also been shown to reduce food intake in rodents centrally (49). Recent studies suggest that a member of the soluble carrier family of proteins, SLC38A9, may play a role in central L-Arg sensing via mammalian target of rapamycin complex 1 (mTORC1)-dependent mechanisms (50). Further studies are required to investigate the putative role of post-absorptive mechanisms in the anorectic effects of L-Arg.

In summary, our data further demonstrate the anorectic properties of L-Arg and explore the potential mechanisms involved. The doses of L-Arg administered orally were pharmacological, with the amounts administered acutely being of a similar order of magnitude to the levels that a rodent would consume daily on a 45% HPD. However, our results may also represent pharmacological activation of a physiological nutrient-sensing system. Our chronic administration study suggests that L-Arg may not reduce body weight following repeated dosing. However, further work is required to establish the mechanisms involved in mediating the anorectic effects of L-Arg and to explore whether altering the dose and timing of chronic administration might result in significant effects on body weight, and thus suggest therapeutic potential in obesity.

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Legends to Figures

Figure 1. The effect of oral administration of L-Arg on food intake, body weight and energy expenditure in rodents. The effect of OG of control (water), 8, and 16 mmolkg^{-1} L-Arg on food intake in male rats (**A**) following an overnight fast (n=9-10, ##*P*<0.01 vs L-Arg (8mmolkg^{-1}), ***P<0.001 vs water control) and (**B**) *ad libitum* fed at the beginning of dark phase (n=12-16, *P<0.05, **P<0.01, ***P<0.01 vs. water control, #P<0.05 vs. 8 mmolkg^{-1} L-Arg) at 0-1, 1-2, 2-4, 4-8, and 0-24 h following administration. (**C**) The effect of OG of control (water), 8, 16 and 24 mmolkg^{-1} L-Arg on food intake in male mice following an overnight fast during 0-1, 1-2, 2-4, 4-8, and 0-24 h following administration (n=8-9, *P<0.05, ***P <0.001 vs. water control; #P<0.05, ##P<0.01 vs. 8mmolkg^{-1} L-Arg; $$P$<0.05 vs. 16 mmolkg^{-1} L-Arg). The effect of OG of control (water) or 24 mmolkg^{-1} L-Arg in *ad libitum* fed mice during the early light phase (**D**) (n=10 per group), and (**E**) early dark phase (n=10 per group) during 0-1, 1-2, 2-4, 4-8, and 0-24 h post administration. *P<0.05, **P<0.01 ***P<0.001 vs. control.

The effect of repeated OG administration of L-Arg on food intake (**F**) and body weight (**G**) in DIO mice. The effect of three times daily OG administration of control (water) (black circles, solid line) or 16 mmolkg-^{1} L-Arg (white circles, dotted line) on cumulative food intake and body weight change in DIO mice during a period of 5 days. n = 9 per group. *P<0.05, ***P<0.001 vs. vehicle.

The effect of OG administration of control (water) (black circles, solid line) or 24 mmolkg^{-1} L-Arg (white circles, dotted line) on cumulative food intake (**H**), O_2 consumption (**I**) and CO_2 production (**J**) and RER (**K**) in mice injected at early light phase and placed in CLAMS cages. The OG was performed at 09:00 and food was returned at 17:00 as indicated by dotted line. Recordings were taken over a period of 24 h and at subsequent 24 min intervals

following administration. The shaded areas represent the dark phase from 19:00. n=12 per group. *P<0.05, **P<0.01, ***P<0.001 vs water control. All data is presented as mean ± SEM.

Figure 2. The effect of L-Arg on food intake in GPRC6a-KO mice and rats following SDA surgery. The effect of OG administration of (**A**) control (water) or 16 mmolkg^{-1} L-Arg, and (**B**) control (water) or 24 mmolkg^{-1} L-Arg on 0-1 h food intake in WT and GPRC6a-KO mice with *ad libitum* access to food injected at the beginning of dark phase. *P<0.05, **P<0.01 vs. water control (n = 4, crossover). (**C**) The effect of OG of control (water) and 16 mmolkg^{-1} L-Arg on 0-1 h food intake in male rats that underwent sham or SDA surgery (n=9-10, crossover). ***P<0.001 vs. control. All data is presented as mean ± SEM.

Figure 3. The effect of L-Arg on gut hormone release. The effect of L-Arg on (**A**) GLP-1 and (**B**) PYY release from primary mice colonic L-cells incubated with 1, 10 and 100 mM L-Arg and IBMX-forskolin mix (10 μM, each) for 2 h. The release is shown as percentages of total hormone contained for each well in the experiment. Data presented as mean + SEM. n = 9 plates from 9 mice. *P<0.05, **P<0.01, ***P<0.001 vs. control. The effect of OG administration of control (water) and 16 mmolkg^{-1} L-Arg on (**C**) GLP-1, and (**D**) PYY in overnight fasted male rats at 30 and 90 min following administration. Data presented as mean + SEM. n = 6-8. *P<0.05, ***P<0.001 vs. water control, ##P<0.01 vs. 12 mmolkg^{-1} L-Arg. The effect of intra-ileal administration of saline and 1M L-Arg on plasma GLP-1 and PYY concentrations in overnight fasted (**E**) anaesthetised mice and (**F**) rats. Blood samples were taken from mice at 30 min, and from rats at 0, 15, 30, 45 and 60 min following administration. Mice results are expressed as mean ± SEM. Rat results are expressed as AUC mean + SEM. n=4-5 per group. * P<0.05 vs. control.

Figure 4. The effect of L-Arg-mediated gut hormone release on food intake and gut function. The effect of IP administration of a mixture of 400 nmolkg^{-1} exendin 9-39 and 5.26 μmolkg^{-1} BIIE0246 on the anorectic effect of orally gavaged 24 mmolkg^{-1} L-Arg in (**A**) fasted mice during early light phase (n=10) and (**B**) *ad libitum* fed mice during dark phase (n=10) in the 0-1 h period post administration. Data presented as mean ± SEM. **P*<0.05, ****P*<0.001 vs. vehicle control group.

(**C**) Representative recordings from mouse colon mucosa showing a biphasic I_{sc} change to apical L-Arg (1mM, upper) compared with minor effects to apical D-Arg (1mM, lower). Basolateral VIP (10nM) pre-treatment increased I_{sc}, and subsequent control PYY (10nM, basolateral) anti-secretory responses are evident. Basal I_{sc} values (in μA) are shown to the left of each trace (exposed mucosal area, 0.14cm^2). Responses to apical L-Arg, D-Arg and control PYY responses in ileum (**D, F** & **H**) and colon (**E, G** & **I**) colon are shown after either vehicle (+DMSO, 0.03%) or Y_1R antagonist BIBO3304 (+BIBO, 300nM). Responses are the mean ± SEM from observation numbers in parenthesis. Only L-Arg 2˚ I_{sc} reductions were sensitive to BIBO treatment in (**D**) ileum and (**E**) colon mucosae. Note PYY responses in ileum (**H**) are due to Y_2 signalling (and thus are not significantly reduced by BIBO) while Y_1R signalling predominates in the mouse colon and is BIBO-sensitive (**I**). **P*<0.05, ***P*<0.01. All data is presented as mean ± SEM.

Figure 5. The effect of IP and ICV administration of L-Arg on food intake in rodents. The effect of IP administration of (**A**) control (saline), 4, and 8 mmolkg^{-1} L-Arg on food intake in fasted male rats (n=8-9, **P*<0.05, ***P*<0.01 vs. control) and (**B**) control (saline), 4, 8, and 12 mmolkg^{-1} L-Arg in fasted male mice (n=7-9, **P*<0.05, ***P*<0.01, ****P*<0.001 vs. control; $$*P*<0.01, $$$*P*<0.001 vs. 4 mmolkg^{-1} L-Arg; ##*P*<0.01, ###*P*<0.001 vs. 8 mmolkg^{-1} L-Arg) at 0-1, 1-2, 2-4, 4-8, and 0-8 h following administration during early light phase. (**C**) The effect of ICV administration of control (saline) and 4μM L-Arg on food intake in male

rats following an overnight fast during 0-1, 1-2, 2-4, 4-8, and 0-24 h following administration (n=8-9, *$p<0.05$ vs. control). All data is presented as mean ± SEM.

Figures

Figure 1.

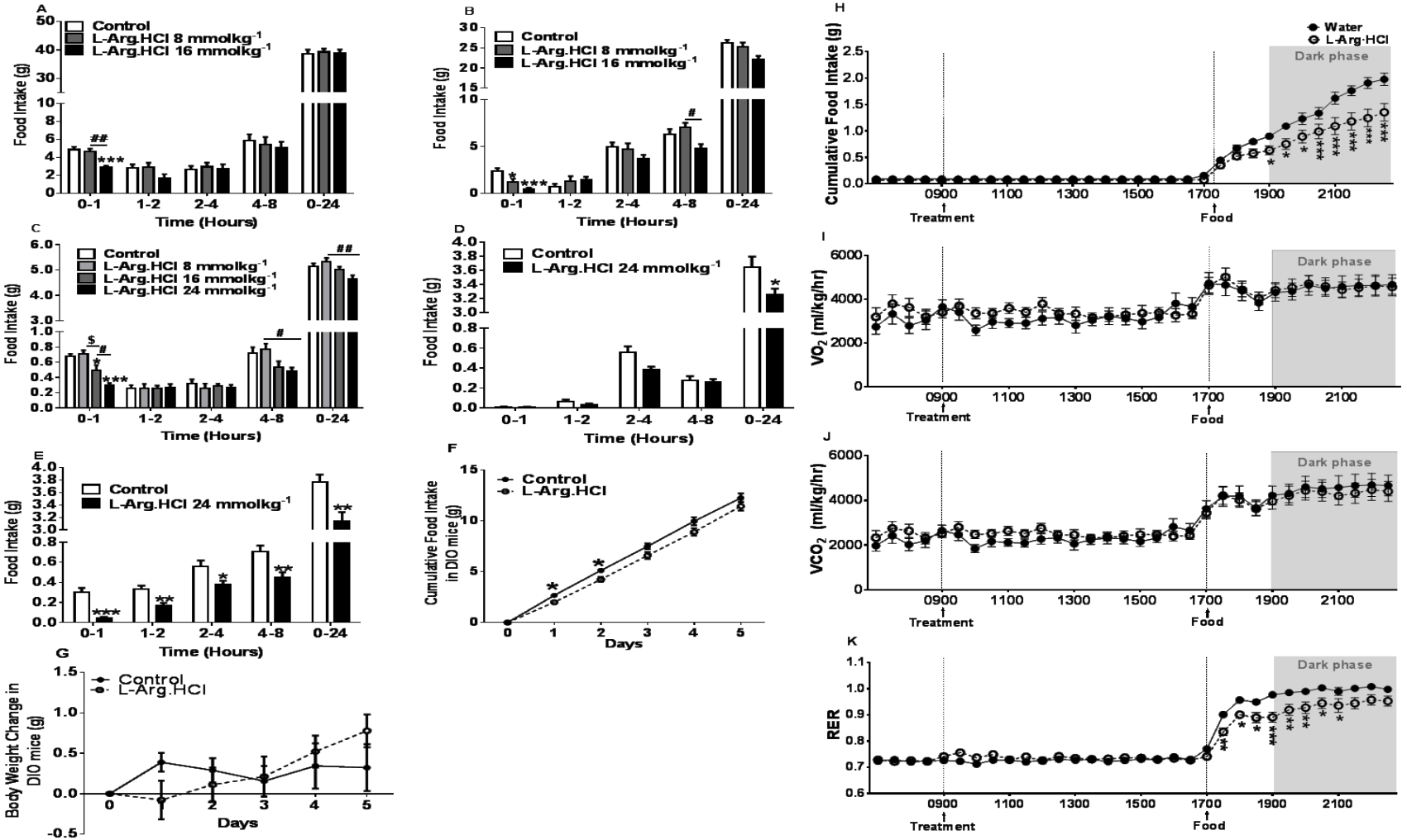


Figure 2.

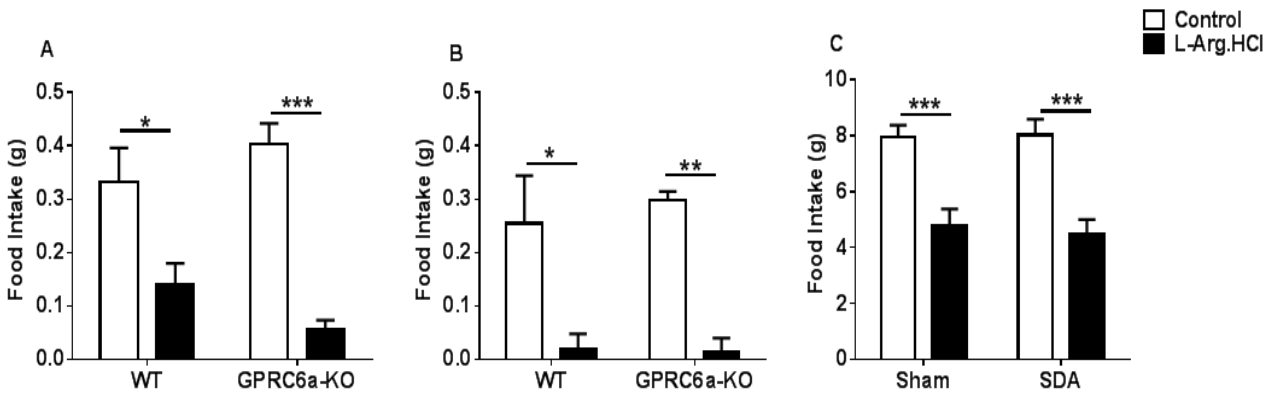

A
B
C
Control
L-Arg.HCl
Food Intake (g)
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
*
***
**
WT
GPRC6a-KO
Sham
SDA

Figure 3.

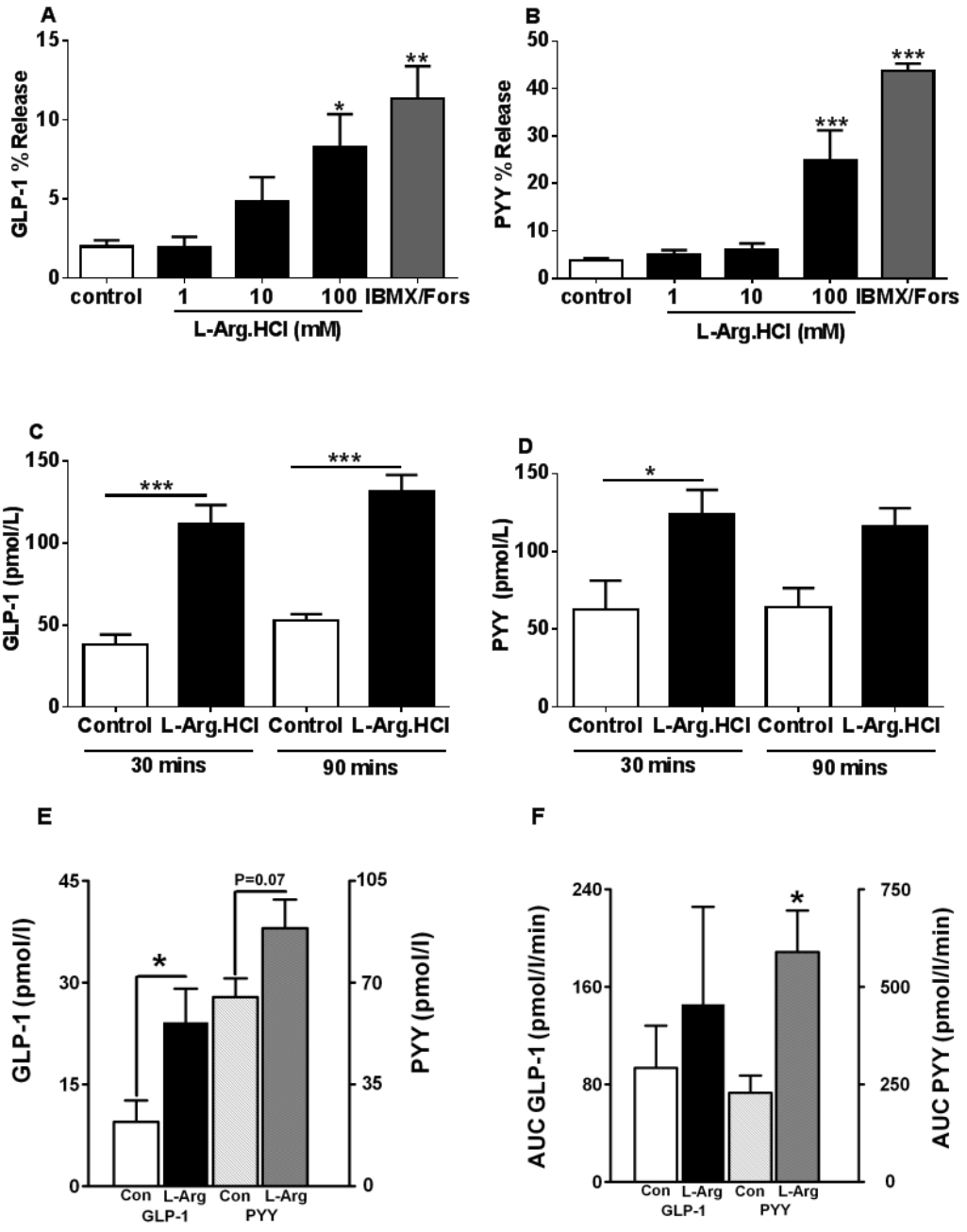

A
GLP-1 % Release
control
1
10
100
IBMX/Fors
L-Arg.HCl (mM)
B
PYY % Release
C
GLP-1 (pmol/L)
Control
L-Arg.HCl
30 mins
90 mins
D
PYY (pmol/L)
E
GLP-1 (pmol/l)
PYY (pmol/l)
P=0.07
Con
L-Arg
GLP-1
PYY
F
AUC GLP-1 (pmol/l/min)
AUC PYY (pmol/l/min)

Figure 4.

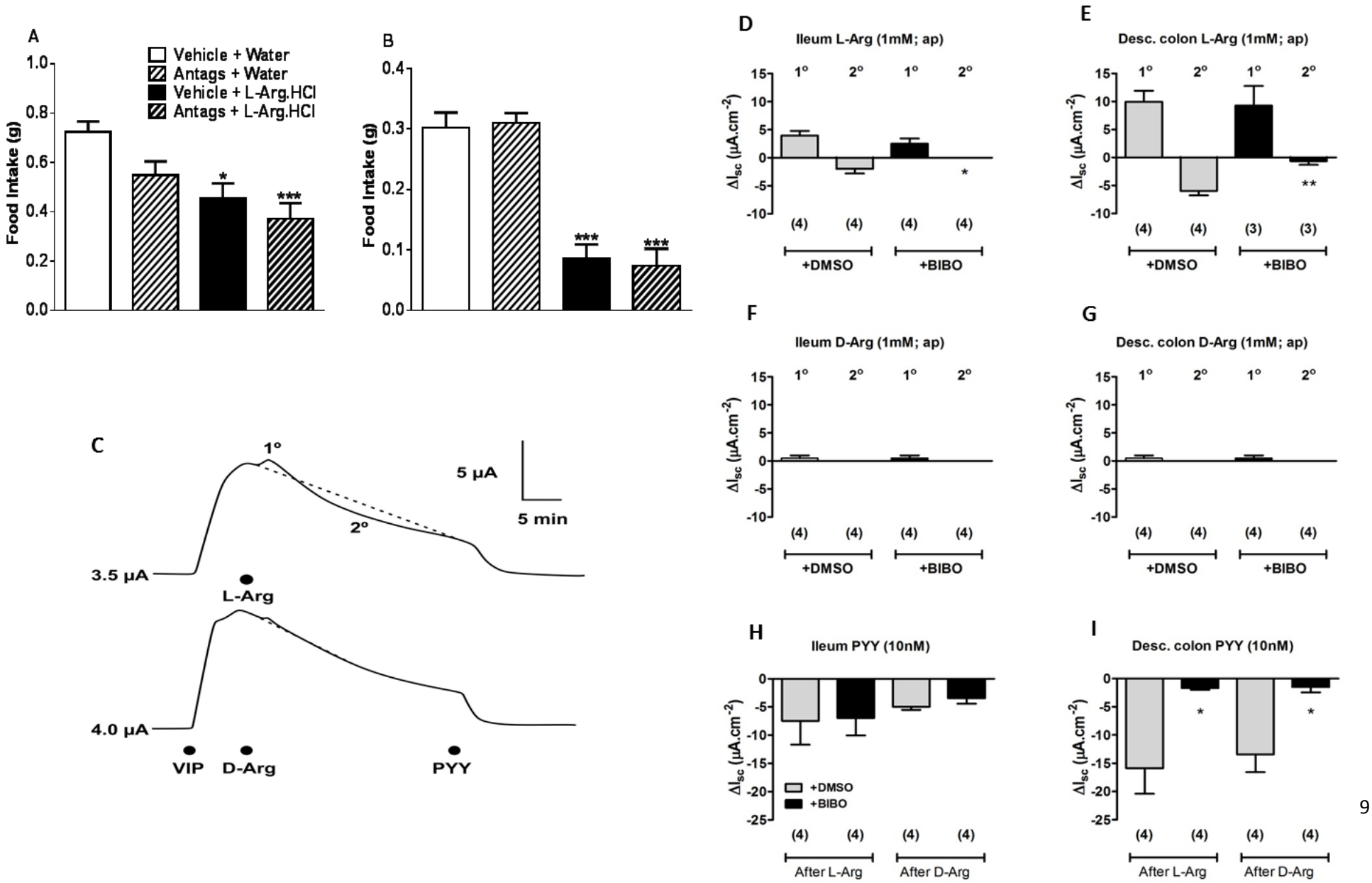
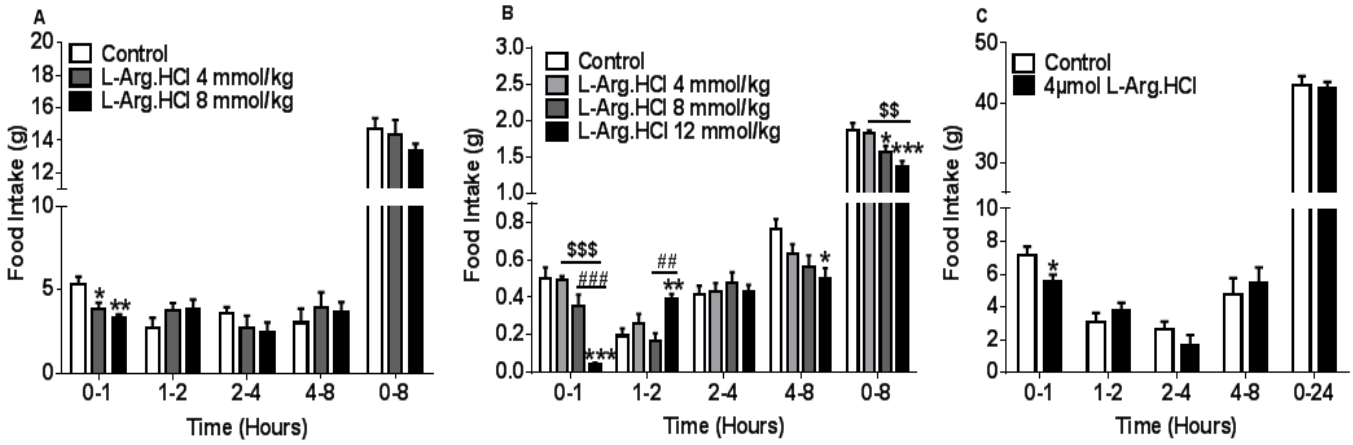

A
Vehicle + Water
Antags + Water
Vehicle + L-Arg.HCl
Antags + L-Arg.HCl
Food Intake (g)
B
Food Intake (g)
C
1°
2°
5 µA
5 min
3.5 µA
L-Arg
4.0 µA
VIP
D-Arg
PYY
D
Ileum L-Arg (1mM; ap)
E
Desc. colon L-Arg (1mM; ap)
F
Ileum D-Arg (1mM; ap)
G
Desc. colon D-Arg (1mM; ap)
H
Ileum PYY (10nM)
I
Desc. colon PYY (10nM)
+DMSO
+BIBO
After L-Arg
After D-Arg

Figure 5.


A
Control
L-Arg.HCl 4 mmol/kg
L-Arg.HCl 8 mmol/kg
Food Intake (g)
Time (Hours)
0-1
1-2
2-4
4-8
0-8
B
Control
L-Arg.HCl 4 mmol/kg
L-Arg.HCl 8 mmol/kg
L-Arg.HCl 12 mmol/kg
$$$
###
##
$$
Food Intake (g)
Time (Hours)
C
Control
4µmol L-Arg.HCl
Food Intake (g)
Time (Hours)
0-24