

**Preproglucagon neurons in the nucleus of the solitary tract are the main source of brain GLP-1, mediate stress-induced hypophagia, and limit unusually large intakes of food**

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Running title: PPG neurons in the control of feeding

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**Abstract**

Centrally administered glucagon-like peptide-1 (GLP-1) suppresses food intake. Here we demonstrate that GLP-1-producing (PPG) neurons in the nucleus tractus solitarius (NTS) are the predominant source of endogenous GLP-1 within the brain. Selective ablation of NTS PPG neurons by viral expression of diphtheria toxin subunit A (DTA) substantially reduced active GLP-1 concentrations in brain and spinal cord. Contrary to expectations, this loss of central GLP-1 had no significant effect on *ad libitum* feeding of mice, affecting neither daily chow intake nor body weight or glucose tolerance. Only after bigger challenges to homeostasis were PPG neurons necessary for food intake control. PPG-ablated mice increased food intake following a prolonged fast and after a liquid diet preload. Consistent with our ablation data, acute inhibition of hM<sub>4</sub>Di-expressing PPG neurons did not affect *ad libitum* feeding, however, it increased post-fast refeeding intake and blocked stress-induced hypophagia. Additionally, chemogenetic PPG neuron activation through hM<sub>3</sub>Dq caused a strong acute anorectic effect. We conclude that PPG neurons are not involved in primary intake regulation, but form part of a secondary satiation/satiety circuit, activated by both psychogenic stress and large meals. Given their hypophagic capacity, PPG neurons might be an attractive drug target in obesity treatment.

**Keywords**

GLP-1, Preproglucagon, Food intake, DREADD, hM<sub>4</sub>Di, DTA, restraint stress, satiation, satiety, GCG neurons

## Introduction

Glucagon-like peptide-1 (GLP-1) is found in the CNS, with the highest levels reported in the hypothalamus, a major projection target of preproglucagon (PPG) neurons. PPG neurons are the presumptive main source of endogenous central GLP-1 and are expected to exert effects similar to those of exogenously delivered GLP-1. The most notable of these are reduced food intake and bodyweight loss. PPG neurons are in a prime position to fulfil this role since they innervate all areas identified as mediating GLP-1 effects in the CNS (1-4) and are sensitive to peripheral satiety signals, including gastric distension, leptin, and CCK (5-8). However, doubts have been raised whether PPG neurons are involved in the homeostatic regulation of food intake or whether they signal interoceptive stress and only regulate food intake under pathophysiological conditions (9-13). Three recent studies demonstrated that activation of PPG neurons *in vivo* using Gq-coupled DREADD or optogenetic stimulation reduces food intake and maintains glucose homeostasis (14-16). Whilst these studies confirmed that PPG neurons have the capacity to modulate food intake and glucose tolerance, they did not address the question of whether PPG neurons play a role in the regulation of appetite or blood glucose under physiological conditions.

Here we use a mouse line expressing Cre-recombinase (Cre) under the control of the glucagon promoter (17) to selectively target NTS PPG neurons. Pharmacogenetic activation, confirmed using *in vitro* Ca<sup>2+</sup> imaging, acutely reduced food intake, replicating previous studies, but failed to have a lasting impact on body weight. Selective ablation of these neurons significantly reduced active GLP-1 concentrations in hypothalamus, brainstem, and spinal cord but did not affect bodyweight or daily food intake. However, PPG-ablated mice ate more chow post-fast than control littermates and were less sensitive to the satiating effects of a liquid diet preload. Similarly, pharmacogenetic inhibition of NTS PPG neurons, confirmed *in vitro* using patch-clamp electrophysiology, had no effect in *ad libitum* fed mice, but significantly increased food intake after a long fast and prevented the hypophagic effect of acute restraint stress.

## Research Design and Methods

### Animal models

Adult male and female Glu-Cre/tdRFP (17), Glu-Cre/GCaMP3 (18; 19), and Glu-YFP (20) mice were group-housed whenever possible on a 12h light/dark cycle with chow and water available *ad libitum* unless otherwise stated. All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, with appropriate ethical approval.

Mice were anaesthetised with intramuscular ketamine (50mg/kg) and medetomidine (1mg/kg) or 1.5-2.5% isoflurane and injected with virus (200-250nl, bilaterally; Table 1) using a pressurized glass needle at the following coordinates from the calamus scriptorius: 500µm lateral, 100µm rostral, and 350µm ventral to transduce PPG neurons and were left for at least two weeks before experiments started.

### **Ca<sup>2+</sup> imaging and electrophysiology**

Coronal brainstem sections (200µm) were prepared as previously described (18).

Ca<sup>2+</sup> imaging was performed in widefield configuration using a 40x water immersion lens (18). Excitation and emission light were filtered at 470±20nm and 515±17nm, respectively (Chroma 59004) and images captured on a CCD camera (Q-click; QImaging). Camera and LED light source were controlled using Micro-Manager (21). Electrical activity of PPG neurons was recorded in cell-attached configuration as described previously (22). Currents were filtered at 1.0kHz and digitised at 3kHz. Recordings were analyzed with WinEDR Software (University of Strathclyde, Glasgow, United Kingdom).

### **Active GLP-1 assay**

Brains were rapidly extracted from the skull and lower brainstem, hypothalamus, cerebellum, olfactory bulbs and spinal cord were isolated, snap frozen and homogenised by pestle and mortar then trituration in 500µl ACSF supplemented with DPP-4 inhibitor (Millipore) through a 29G insulin syringe, before storage for 24hs at -80°C. To generate a crude protein lysate samples were thawed on ice and clarified twice by centrifugation at 500g for 10min and the supernatant collected. The active GLP-1 concentration was determined using a MSD kit (K150JWC-1; Meso Scale Diagnostics, Rockville, Maryland, USA). A Bradford protein assay was performed (Bio-Rad). Absorbance was determined using a Lab Systems Multiscan MS.

### **Immunohistochemistry**

Mice were transcardially perfuse-fixed with 4% PFA, brains sectioned at 30µm and immunostained for GCaMP3, EGFP, YFP, tdRFP, mCherry or cFOS (for details see Table 1) as previously described (18). Sections were incubated overnight at 4°C with primary antibodies in blocking solution followed by fluorophore-conjugated secondary antibody in blocking solution for 2h. Immunofluorescence was visualised on an upright microscope (Leica). Images were captured using a Retiga3000 camera (QImaging). Brightness and contrast were adjusted using Fiji software (23).

### **cFOS expression in PPG neurons**

Mice expressing hM<sub>3</sub>Dq in NTS PPG neurons were food-restricted for 3h prior to injection of vehicle or clozapine-N-oxide (CNO), which was administered 2mg/kg i.p. in 5ml/kg saline, 30mins before dark onset for all *in vivo* chemogenetic experiments. Mice were transcardially perfused 90mins after injection, tissue processed and immunostained for cFOS as described above.

Glu-YFP mice were trained to consume Vanilla Ensure. Following three days of stable Ensure intake, mice were randomly allocated into control (n=5) and Ensure-fed groups (n=5) and fasted for 3h. Control mice had no access to chow or Ensure, whereas Ensure-fed mice had access to Vanilla Ensure for 120 mins. At that point mice were transcardially perfused and tissues immunostained for cFOS and YFP.

### **Glucose Tolerance Test**

Mice were placed into new cages, given *ad libitum* access to water and fasted for 18h. Animals were injected with glucose (1g/kg i.p., 5ml/kg, Sigma). Blood glucose measurements were repeatedly taken from the tail over two hours and analyzed using a Roche Accu-Chek Glucose Meter.

### **Feeding paradigms**

Intake was measured using standard chow or Vanilla Ensure liquid diet. Mice were weighed daily and habituated to 5ml/kg i.p. saline injection and food intake measurements. On experimental days, food was removed 3h prior to dark onset (except for overnight fast/refeed paradigm) thus standardising the time all mice had their first meal.

#### *Repeated injections of CNO*

Using a between-subjects, repeated-measures design, body weight and food intake of control and hM<sub>3</sub>Dq-expressing mice were measured daily for five days. On the sixth day, twice daily injections of CNO were started.

#### *Normal chow intake*

The PPG-ablation study was a between-subjects design, while the activation study (hM<sub>3</sub>Dq) was a counterbalanced, within-subject design and the acute inhibition study (hM<sub>4</sub>Di) was a mixed-model design. Mice were transferred to individual cages and food removed 3h or 18h prior to dark onset. Chow was returned at dark onset and intake was measured manually in the following hours.

#### *Ensure preload*

This experiment was a between-subjects design. PPG-ablated and control mice were transferred to individual cages and food removed 3h prior to dark onset. Vanilla Ensure was provided for 15 mins at dark onset and intake measured manually, with this protocol repeated

for several days until intake was stable (Fig 6C). On test day, chow intake was measured for 1h immediately after Ensure access.

#### *Stress-induced hypophagia*

Stress-induced hypophagia was assessed using a mixed-model design. Control (EGFP-expressing) and hM<sub>4</sub>Di-expressing mice were transferred to individual cages without food 3h prior to dark onset. All mice received CNO 1h before dark onset. Thirty minutes before dark onset, mice were restrained in plastic bags with a breathing hole for 30 mins. At dark onset, they were returned to their cages and chow intake was measured in the following hours.

#### **Statistical analysis**

Statistical analysis was performed in GraphPad Prism7.0. Summary data are presented as mean±SEM. Statistical significance was tested using nonparametric tests, t-tests, three-way mixed-model ANOVA, two-way mixed-model or repeated measures ANOVA, and simple main effects as appropriate and as indicated in figure legends.

### **Results**

#### *Pharmacogenetic activation of PPG neurons robustly reduces food intake*

To assess the role of PPG neurons in feeding, we first confirmed and extended previous findings that PPG neurons have the capacity to reduce food intake (14; 15). We used transgenic mice expressing Cre under glucagon promoter control (Glu-Cre) and a Cre-dependent reporter (red fluorescent protein (RFP) or the Ca<sup>2+</sup> indicator GCaMP3 (18; 19)). GCaMP3-positive cells in this mouse are GLP-1-immunoreactive (19), and as an additional control we crossed this mouse with the Glu-YFP mouse that expresses YFP under glucagon promoter control. YFP-expressing neurons in the NTS of Glu-YFP mice have been shown to express preproglucagon mRNA by single-cell RT-PCR (6). The Glu-YFP/Glu-Cre cross revealed an almost complete overlap of these cell populations; more than 85% of YFP-expressing cells expressed RFP and less than 5% of RFP cells did not express YFP (Fig S1A). Previous reports used a Phox2b transgenic mouse to target PPG neurons, assuming Phox2b is expressed and active in PPG neurons (24; 25). However, targeting the lower brainstem with a lentivirus expressing GFP under the promoter responsive to Phox2b (PRSx8-AlstR-IRES-EGFP-LV; (26)) yielded no co-localisation between GFP and RFP (Fig S1B).

To selectively activate NTS PPG neurons *in vivo*, the excitatory DREADD hM<sub>3</sub>Dq was expressed by stereotaxic injection of AAV2-FLEX-hM<sub>3</sub>Dq:mcherry into Glu-Cre mice (Fig 1A). Activation of hM<sub>3</sub>Dq with CNO substantially increased [Ca<sup>2+</sup>]<sub>i</sub> in brainstem slices (Fig

1B). Similarly, in awake mice, CNO injection elicited cFOS immunoreactivity in PPG neurons (Fig 1C).

Having confirmed that CNO activates PPG neurons both *in vitro* and *in vivo*, we explored whether *in vivo* activation suppresses feeding. First, we confirmed that viral over-expression of hM<sub>3</sub>Dq in PPG neurons had no intrinsic effect on food intake, nor did administration of CNO in mice transduced with a control virus (Fig S2). Subsequently, *ad libitum* dark onset food intake was measured in mice expressing hM<sub>3</sub>Dq in NTS PPG neurons in a counterbalanced cross-over design. CNO-injected mice consumed substantially less chow than when injected with saline in both the first and second hour (Fig 1D) and cumulative intake was suppressed over the first four hours after dark onset (Fig 1E). The effect of CNO disappeared over 21h with cumulative daily intake unaffected by PPG activation (Fig 1E).

To determine whether this was simply due to washout of CNO, we injected another cohort of hM<sub>3</sub>Dq-expressing and control mice with CNO every 12 hours (at dark and light onset) for four days. Twice-daily PPG activation initially suppressed feeding, with significantly lower 24h intake following the first injections, however intake suppression was not sustained (Fig 1F).

#### *NTS PPG neurons are the main source of GLP-1 in brain*

Whilst the above results demonstrate the capacity of PPG neurons to reduce feeding, they do not prove that brain GLP-1 is derived from these neurons. To address this, we used an AAV Cre-dependently encoding diphtheria toxin subunit A (DTA). Unilateral targeting of the NTS in Glu-Cre mice with AAV8-mCherry-FLEX-DTA selectively ablated PPG neurons, with complete disappearance of cell bodies within 14 days, whilst contralateral PPG neurons remained intact (Fig 2A). Bilateral ablation of NTS PPG neurons dramatically reduced active GLP-1 levels in brainstem, hypothalamus, and spinal cord (Fig. 2B), demonstrating that NTS PPG neurons are the main source of GLP-1 in these areas.

Active GLP-1 concentrations in brainstem, hypothalamus, and spinal cord were 4-fold, 6-fold, and 29-fold larger, respectively, than those from concurrent systemic blood samples, indicating that blood contained within the brain samples was not the source of the GLP-1. Consistent with this, amounts of active GLP-1 in cerebellum, which does not receive projections from PPG neurons (3), were negligible, and neither blood nor cerebellar concentrations were affected by PPG neuron ablation. Similarly, the GLP-1 concentration in the olfactory bulb, a confirmed location of additional PPG neurons (27; 28), was very low and not affected by DTA ablation in the brainstem.

*Ablation of NTS PPG neurons does not impact bodyweight, daily food intake, or glucose tolerance*

Although activation of PPG neurons robustly suppressed short-term feeding, sufficiency does not prove necessity. We therefore explored physiological conditions under which PPG neurons might regulate food intake. Bodyweight and daily food intake were recorded over two months in Glu-Cre mice injected bilaterally with AAV8-mCherry-FLEX-DTA or AAV1/2-FLEX-Perceval as control. Ablation of NTS PPG neurons did not affect bodyweight (Fig 3A) or daily food intake when fed *ad libitum* (Fig. 3B), suggesting no significant impact on long-term energy balance under these conditions.

PPG neurons were recently shown to be sufficient to improve glucose tolerance (16). We therefore investigated whether NTS PPG ablation affects the response to an intraperitoneal glucose load (Fig 3C,D). Before ablation, there was no difference in glucose tolerance, with males having poorer glucose tolerance than females (Fig S3). Seven weeks after PPG ablation, sex differences were still evident, but loss of NTS PPG neurons did not affect glucose tolerance (Fig 3C,D).

*Ad libitum food intake is unaffected by ablation or acute inhibition of NTS PPG neurons*

Having found no evidence that NTS PPG neurons regulate long-term energy balance, we next asked whether PPG neurons are necessary for short-term regulation of feeding. PPG ablation had no effect on cumulative (Fig 4A) or non-cumulative chow intake (Fig 4B) over 4h after dark onset, suggesting that although activation of PPG neurons is sufficient to reduce food intake, they are not necessary to regulate *ad libitum* feeding.

Although ablations were inflicted in adult mice, compensatory responses could account for the lack of effect on long-term energy balance. We therefore assessed the effect of acute inhibition of PPG neurons using the inhibitory DREADD hM<sub>4</sub>Di.

To assess whether hM<sub>4</sub>Di stimulation inhibits PPG neurons *in vitro*, virally transduced PPG neurons were identified by mCherry fluorescence in brainstem slices and electrical activity recorded in the cell-attached configuration. PPG neurons were spontaneously firing at  $1.57 \pm 0.22$  Hz, consistent with previous observations (6; 18). CNO superfusion reduced firing frequency to  $0.66 \pm 0.20$  Hz (Fig 4C).

hM<sub>4</sub>Di expression did not affect bodyweight or food intake in the absence of CNO (Fig S4) and daily food intake was unaffected after a single dose of CNO at dark onset (Fig S4C). As

with ablation, acute inhibition of PPG neurons did not affect dark onset feeding (Fig 4D,E). The data from both ablation and acute inhibition therefore suggest PPG neurons are not necessary for the regulation of long-term or short-term food intake in *ad libitum* fed mice.

#### *PPG neurons limit fasting-induced refeeding*

Having found no major role of NTS PPG neurons in *ad libitum* feeding, we next investigated whether PPG neurons need abnormally large meals to become engaged as suggested for rats (9; 10). To encourage significant refeeding, we fasted mice for 18h prior to intake measurements from dark onset. Control animals ate  $0.6\pm 0.05$ g chow in the first hour compared to  $0.4\pm 0.06$ g when food-restricted only briefly (Fig 5C and Fig 4A, respectively), whilst PPG-ablated animals ate  $0.9\pm 0.11$ g (Fig 5C). This elevated post-fast chow intake was evident over 4h (Fig 5A), but did not last overnight, although there was a trend towards increased intake (Fig 5B).

Similarly, CNO-injected hM<sub>4</sub>Di-expressing mice ate  $0.23\pm 0.1$ g more chow in the first hour of refeeding compared to when injected with saline (Fig 5F). This hypophagic effect was not sustained beyond 1h (Fig 5D) and there was no impact on 21h chow intake (Fig 5E).

#### *PPG neurons limit chow intake following a liquid diet preload*

Results from these two independent, complementary experiments suggest that PPG neurons may be recruited following unusually large intakes to limit subsequent feeding. To further investigate this hypothesis, we encouraged high intakes by provision of a highly-palatable liquid diet (Vanilla Ensure). Mice were habituated to dark onset Ensure access for eight days, during which 30min intake stabilised at  $1.7\pm 0.1$ g from day four. On day nine mice were randomly allocated to Ensure or fasted groups and subsequent analysis of cFOS immunoreactivity revealed that 45% of PPG neurons were activated following Ensure intake, compared to 10% activation in fasted controls (Fig 6A,B).

To determine whether ablation of PPG neurons affects feeding under these conditions, mice were habituated to consume a 15min Ensure preload at dark onset which stabilised at  $1.4\pm 0.04$ g after four sessions (Fig 6C). Ensure intake between PPG-ablated and control mice did not differ at the beginning (Fig 6C) or end (Fig 6D) of the habituation. Following the final Ensure session, chow intake was lower in the control group than usually seen 1h into dark phase, with control mice eating roughly half their normal intake (Fig 6E,  $0.21\pm 0.06$ g vs  $0.4\pm 0.06$ g in Fig 4B). In contrast, PPG-ablated mice ate significantly more than control mice,

with  $0.61 \pm 0.1$ g of chow eaten 1h after Ensure preload (Fig 6E). These data support the hypothesis that unusually large intakes recruit NTS PPG neurons to limit subsequent feeding.

#### *Stress-induced hypophagia requires PPG neurons*

We next explored whether there are other conditions under which PPG neurons are necessary for anorectic effects. Previous work suggests that central GLP-1 contributes to the intake suppression following acute stress in rats (29). To investigate whether PPG neurons contribute to the hypophagic response to restraint stress, we acutely inhibited PPG neurons *in vivo* by activating the hM<sub>4</sub>Di receptor. Control and PPG-inhibited mice were exposed to 30mins restraint stress and subsequent food intake was measured. Restraint stress significantly suppressed cumulative feeding over 4h in control mice, whereas PPG-inhibited mice displayed no stress-induced hypophagia (Fig 7A). In the absence of stress, CNO had no effect on food intake in both control and PPG-inhibited mice as compared to vehicle (Fig 7A). Stress-induced hypophagia was seen in the first hour (Fig 7B), during which intake in control mice was reduced by  $50 \pm 8\%$ , whereas intake was unaffected by stress in the PPG-inhibited group. There was no clear suppression of chow intake in hour 2. These data demonstrate that PPG neurons are required for the hypophagic response to acute restraint stress.

#### **Discussion**

We demonstrate here that NTS PPG neurons are the main source of brain GLP-1. Active GLP-1 was reduced by  $>60\%$  in brainstem, and almost 80% in hypothalamus and spinal cord following ablation of NTS PPG neurons. In contrast, circulating GLP-1 levels, which were substantially lower than in brain, were unaffected by ablation and levels in cerebellum, which receives no projections from PPG neurons (3), were negligible, and likely reflect only the vascular supply throughout the brain. Remaining GLP-1 levels in the sampled CNS regions after ablation likely reflect the IRT and ventral midline PPG cells, which were left intact in our study. These neurons make up 44% (29) and 32% (1) of all PPG neurons in the brainstem in rat and mouse, respectively. Outside the brainstem, small populations of PPG neurons have been described in olfactory bulb (27; 28), piriform cortex (3), and lumbar-sacral spinal cord (1). Of these, the olfactory bulb neurons project only locally (28), and the spinal neurons do not project to the brain (1), excluding these populations as the source of remaining GLP-1 after ablation of NTS PPG neurons. Approximately 50% of PPG neurons have axons

projecting to the autonomic control areas of the spinal cord (1). The current study adds to the significance of these projections by demonstrating a high GLP-1 content in spinal cord, the majority of which is supplied by NTS PPG neurons. This suggests a significant physiological role for spinal GLP-1 release, an intriguing path for future research considering the role of GLP-1 in sympathetic outflow (30-33).

In addition to providing unequivocal evidence that PPG neurons are the main source of brain GLP-1, these results validate that our genetic approach targets the cells that provide GLP-1 in the CNS. In contrast, injection of a lentivirus that expresses GFP in Phox2b-expressing cells (PR<sub>Sx8</sub>-AlstR-EGFP-LV; (26)), failed to show any co-localisation with Cre-expressing PPG neurons, indicating that the transcription factor Phox2b is not expressed in adult PPG neurons. Transgenic mice expressing Cre under the control of Phox2b have been used previously to target PPG neurons (24; 25). Whilst our results do not exclude the possibility that PPG neurons express Phox2b during development, they clearly show that using adult Phox2b-Cre mice combined with Cre-dependent viruses is not a valid approach to target PPG neurons. This substantiates previous concerns about that approach (34; 35).

Recent studies have pharmacogenetically and optogenetically activated PPG neurons, using a mouse model produced independently from ours but using an equivalent strategy (14-16). Based on those results and our observations, NTS PPG neurons are sufficient to suppress feeding in mice. Here we also demonstrate that repeated, twice daily chemogenetic activation of NTS PPG neurons leads to a transient decrease in food intake reminiscent of results reported for both CCK- and DBH-expressing NTS neurons (36). In both cases there was a strong short-term reduction in intake, but either no effect (CCK cells) or only a small effect (DBH cells) on 24h food intake. A similar lack of long-term effects has been observed with ICV GLP-1 infusions in rat (37) and it seems likely that compensatory mechanisms are activated to maintain energy balance and avoid excessive weight loss, although we cannot exclude that continued activation downregulates DREADD receptors.

Gaykema et al. (14) argued that chemogenetic activation using hM<sub>3</sub>Dq is comparable to activation by physiological stimuli, such as leptin and CCK. However, with <50% of PPG neurons activated, our cFOS-expression study demonstrated that even a large volume of Ensure did not activate PPG neurons to the same degree as hM<sub>3</sub>Dq-activation (98%; (14)).

Similarly, acute stress in rats activated ~74% of GLP-1-producing neurons (29), suggesting that chemogenetic activation of PPG neurons is a supraphysiological stimulus.

Prompted by these limitations we attempted to determine the physiological role of PPG neurons in food intake. Although Liu et al. (15) did address the role of endogenous GLP-1 in food intake by optogenetically inhibiting NTS<sup>PPG</sup>→PVN projections, the specific physiological conditions under which PPG neurons influence food intake were not investigated. Whilst chemogenetic activation strongly suppressed feeding, ablation of NTS PPG neurons had no effect on 24h intake or bodyweight in mice with *ad libitum* access to food. Not only was long-term energy balance unaltered by loss of NTS PPG neurons, neither ablation nor acute inhibition affected dark onset food intake when mice were fed *ad libitum*. Similarly, Liu et al. failed to observe persistent increases in food intake over 7 daily injections of 0.3mg/kg CNO (15), although this dose schedule may not provide lasting inhibition of the PPG neurons (38). This suggests that PPG neurons do not produce an obligatory meal termination signal required for normal satiation, a conclusion supported by both experimental approaches used here. Loss of NTS PPG neurons also failed to affect glucose tolerance, consistent with previous reports that central GLP-1R are not necessary for glucose control in mouse (39). However, both ablation and acute inactivation left a proportion of PPG neurons, in the IRT, intact. It is conceivable that only a small fraction of PPG neurons is needed to maintain satiation and that complete loss of PPG neurons could reveal a role in *ad libitum* feeding. This reliance on a few neurons only was demonstrated for orexin neurons, where >90% loss was necessary to reveal the cataplexy phenotype (40). Whilst this is difficult to categorically exclude, our results suggest that PPG neurons are not normally recruited in response to *ad libitum* feeding, but point to a role for PPG neurons in suppressing food intake in response to stronger physiological stimuli, such as a large meal or acute stress. In support, global and central knockdown of GLP-1R has little impact on food intake and body weight in mice (39; 41).

In contrast, studies in rat have provided some evidence for a role of endogenous GLP-1 in the control of normal feeding and glucose control (35; 42; 43). Infusion of exendin-9 into the ventricular system or knockdown of GLP-1 receptors in discrete nuclei increase food intake and shRNA-mediated knockdown of PPG in the brainstem led to increased bodyweight gain and hyperphagia (35; 44). It is unclear if these different observations reflect species

differences and, in that case, which rodent species is the best model of human physiology (35; 45).

Several studies in rat support the idea that GLP-1-producing neurons are mainly recruited following intake of a large meal. An unusually large meal is required to activate GLP-1-producing neurons in the NTS (10), gastric distension activates GLP-1 neurons (7), and the ensuing decrease in food intake is mediated by central GLP-1 signalling (46). In support of these findings, we demonstrate here that NTS PPG neurons are necessary for satiation/satiety following intake of a large meal, either encouraged by food-depriving mice for 18 hours or by providing them with access to a highly palatable diet. Seemingly contradictory findings by previous studies in rat suggest that GLP-1 neurons are inhibited by negative energy balance following an overnight fast, rendering them less sensitive to stimulation with CCK or acute stress (29; 47). However, we demonstrate here that a large meal following food deprivation is sufficient to recruit NTS PPG neurons to limit overeating as was previously suggested in rat (10).

In addition to playing a role in satiation/satiety following a large meal, we found that hypophagia induced by acute restraint stress was dependent on PPG neuron activity. In support of a role for central GLP-1 in stress regulation, central infusion of GLP-1 increases plasma ACTH and corticosterone levels and elicits anxiety-like behaviour in rats (48). Conversely, 3rd ventricular exendin-9 attenuated the rise in corticosterone after psychogenic stress, demonstrating HPA axis activation by exogenous as well as endogenous GLP-1. However, chemogenetic activation of PPG neurons in mouse did not affect stress hormone levels or anxiety-related behaviour (14), suggesting the restraint stress-induced hypophagia investigated in our study may not involve activation of the HPA axis, but employs different pathways. In *ad libitum* fed rats, restraint stress induces cFOS-expression in GLP-1-immunoreactive neurons and restraint stress-induced hypophagia can be reversed by lateral ventricular exendin-9 (29). Those findings are in line with the effects of direct inhibition of PPG neurons that we report here and suggest that these neurons play a central role in restraint stress-induced hypophagia in both mouse and rat. This places PPG neurons at the centre of behavioural decisions to maintain energy intake vs avoiding danger and stress.

### *Conclusions*

We report that NTS PPG neurons of the lower brainstem are the main source of the GLP-1 found in the CNS, that these neurons have the capacity to significantly suppress food intake, and describe conditions under which they are necessary for the control of feeding. Our results suggest that PPG neurons may not control *ad libitum* food intake but are essential for short term limitation of feeding following unusually large intakes, and in mediating stress-induced hypophagia. We thus conclude that PPG neurons likely form part of a secondary satiation/satiety circuit, activated by both psychogenic stress and presumptive gastric distension from unusually large intake. PPG neurons thus constitute a regulator with scope for substantial hypophagia, without being involved in day-to-day energy balance, and as such they may be an attractive target for pharmaceutical intervention to reduce body weight.

### **Acknowledgments**

This study was supported by grants MR/J013293/2 and MR/N02589X/1 from the MRC and BHF grant FS/14/43/30960 (PhD studentship DRC) to S.T., and a UCL Graduate Research Studentship and a Bogue Fellowship to M.K.H.

We thank Bill Wisden, Guy Rutter, Sergey Kasparov, Naoshige Uchida and Bryan Roth for plasmids and viruses as listed in Table 1. We thank Alexander Gourine, UCL, for advice regarding the stereotaxic brainstem injections, Ian Edwards, UCL, for assistance with the extraction of spinal cords, and we thank Linda Rinaman, Florida State University, for critical comments on the manuscript.

S.T. 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.

### **Author Contributions**

Conceptualization, S.T., J.E.R and M.K.H.; Methodology, S.T., J.E.R., M.K.H., D.I.B. and D.L.W., Investigation, M.K.H., J.E.R. D.R.C., S.T. and D.I.B.; Resources, F.R. and F.M.G.; Writing-Original Draft, M.K.H. J.E.R. and S.T.; Writing – Review & Editing, S.T., J.E.R., M.K.H., D.I.B., F.R., F.M.G., D.L.W. and D.R.C.; Supervision, S.T., J.E.R. and D.L.W. Funding Acquisition, S.T., M.K.H.

### **Duality of Interest**

The authors declare no competing interests.

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Table 1. Sources of virus and antibody preparations used.

Virus/Antibody Titer/dilution	Application	Source	References
AAV2-FLEX- hM <sub>3</sub> Dq:mCherry 6.1x10 <sup>12</sup>	Activation of Cre- expressing PPG neurons	UNC Vectorcore	pAAV-hSyn-DIO- hM3D(Gq)-mCherry was a gift from Bryan Roth (49)
AAV8-FLEX- hM <sub>3</sub> Dq:mCherry 4x10 <sup>12</sup>	Activation of Cre- expressing PPG neurons	VVF, ZNZ, Zurich	pAAV-hSyn-DIO- hM3D(Gq)-mCherry was a gift from Bryan Roth (49)
AAV8-mCherry- FLEX-DTA 3.3x10 <sup>12</sup>	Ablation of Cre- expressing PPG neurons	UNC Vectorcore	pAAV-mCherry-flex-dtA was a gift from Naoshige Uchida.
AAV2-FLEX- hM <sub>4</sub> Di:mCherry 6.4x10 <sup>12</sup>	Inhibition of Cre- expressing PPG neurons	VVF, ZNZ, Zurich	pAAV-hSyn-DIO- hM4D(Gi)-mCherry was a gift from Bryan Roth (49)
AAV1/2-FLEX- Perceval Titer not determined	Control for viral transduction	Made in house.	pAAV-FLEX-empty was a gift from Bill Wisden (50) pShuttleCMV-Perceval was a gift from Guy Rutter
AAV8-FLEX- EGFP; 8x10 <sup>12</sup>	Control for viral transduction	VVF, ZNZ, Zurich	pAAV-hSyn-DIO-EGFP was a gift from Bryan Roth
PRStx8-AlstR- EGFP-LV 1x10 <sup>10</sup>	Identification of Phox2b neurons	Sergei Kasparov	(26)
Chicken anti-GFP; Alexa488 goat	GCaMP3, EGFP, YFP	Abcam AB13970; Invitrogen #A-	(18)

anti-chicken; 1:1000		11039	
Rabbit anti-dsRed; Cy3 sheep anti-rabbit; 1:1000	mCherry, tdRFP	Takara Bio #632496; Sigma #C2306	
Rabbit anti-cFOS 1:500; Alexa488-goat anti-rabbit 1:1000	cFOS	Merck #ABE457; Invitrogen #A-11008	

### Figure Legends

#### Figure 1. Pharmacogenetic activation of PPG neurons robustly reduces food intake.

(A) Expression of hM3Dq:mCherry (magenta) in PPG neurons (detected with an anti-GFP antibody, green) three weeks after stereotaxic injection of AAV2-hM<sub>3</sub>Dq:mCherry into the NTS of Glu-Cre/GCaMP3 mice. Scale bar: 100  $\mu$ m. (B) Increase in  $[Ca^{2+}]_i$  in GCaMP3-expressing PPG neurons in response to superfusion of *ex vivo* brainstem slices with 1  $\mu$ M CNO. Data are displayed as traces (top panel) and a heat map (bottom panel) representing the fractional change in fluorescence from baseline. Right panel: Representative pseudocoloured cell responding to 1  $\mu$ M CNO with an increase in  $[Ca^{2+}]_i$ . Scale: 10  $\mu$ m, n=11 cells. (C) Expression of the immediate early gene cFOS (green) in PPG neurons expressing hM<sub>3</sub>Dq:mCherry (magenta) following i.p. injection of 2 mg/kg CNO (top panels) or saline (bottom panels). White arrows: Representative cFOS-positive hM<sub>3</sub>Dq-expressing cells. White stars: Representative cFOS-negative hM<sub>3</sub>Dq-positive cells. Scale bars: 100  $\mu$ m (middle panel) and 20  $\mu$ m (right panel). (D-E) Non-cumulative (D) and cumulative (E) food intake in the first four hours of dark phase following injection of CNO (2 mg/kg i.p.) or saline. CNO was delivered 30 mins prior to dark onset. Data given as mean $\pm$ SEM, n=9 mice. D: No significant time  $\times$  drug interaction ( $F(2, 16) = 2.897$ ,  $p=0.0844$ ), but a significant main effect of drug treatment ( $F(1,8)=17.31$ ,  $p=0.0032$ ); E: Significant time  $\times$  drug interaction ( $F(2,16)=5.626$ ,  $p=0.0141$ ). 1h  $p=0.0005$ , 2h  $p<0.0001$ , 4h  $p<0.0001$  (Sidak's multiple comparisons test). Inset:  $p=0.20$  (paired t-test). (F) Daily chow intake in hM<sub>3</sub>Dq-expressing (n=7) and control (n=6) Glu-Cre mice in response to twice daily i.p. injection of 2 mg/kg CNO (indicated with black arrows). Significant time  $\times$  virus interaction ( $F(11, 121) = 2.06$ ,

$p=0.0283$ ); day 6  $p=0.0119$  (Sidak's multiple comparisons test). Data given as mean $\pm$ SEM. \* $p<0.05$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ .

**Figure 2. NTS PPG neurons are the main source of brain GLP-1.**

(A) Expression of mCherry and GCaMP3 (as a marker for PPG neurons) four, seven, and 14 days after unilateral stereotaxic injection of AAV8-mCherry-FLEX-DTA into the NTS of a Glu-Cre/GCaMP3 mouse (schematic on left). White arrows indicate GCaMP3-positive PPG neurons remaining. Scale bars: 100  $\mu\text{m}$  (top panels) and 20  $\mu\text{m}$  (inset).

(B) Protein levels of active GLP-1 (normalised to total protein) detected in several brain regions after bilateral stereotaxic injection of AAV8-mCherry-FLEX-DTA or a control virus (AAV1/2-FLEX-Perceval). Brainstem:  $p=0.0317$ , Hypothalamus:  $p=0.0079$ , (Mann-Whitney U Test); Spinal cord:  $p=0.0004$  (unpaired t-test). Data given as mean $\pm$ SEM,  $n=5$  in each group. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

**Figure 3. Ablation of NTS PPG neurons has no impact on body weight, food intake, or glucose tolerance.**

(A-B) Bodyweight change (A) and daily chow intake (B) following stereotaxic injection of AAV8-mCherry-FLEX-DTA or control virus. Bodyweight was measured every 2-3 days over two months. Mean $\pm$ SEM,  $n=7$  (control),  $n=6$  (DTA). A: No significant time  $\times$  virus interaction ( $F(1, 12)=0.08578$ ,  $p=0.7746$ ) and no significant main effect of virus ( $F(1, 12)=0.08578$ ,  $p=0.7746$ ); B:  $p=0.45$  (unpaired t-test). (C-D) Blood glucose in response to an i.p. injection of glucose (1 g/kg) at  $t=0$  seven weeks after stereotaxic injection of DTA or control virus in six male (C) and six female (D) Glu-Cre mice. Area under the curve (AUC) of the i.p. glucose tolerance test for each group is given on the right of each graph. Data given as mean $\pm$ SEM. Glucose concentrations: There was a significant time  $\times$  virus interaction for males ( $F(5, 20)=3.83$ ,  $p=0.014$ ), but no significant difference between DTA and control mice at any timepoint. There was no significant time  $\times$  virus interaction for females ( $F(5, 20)=0.19$ ,  $p=0.96$ ) and no significant main effect of virus ( $F(1, 4)=1.08$ ,  $p=0.36$ ). AUC: No effect of virus for males ( $p>0.99$ , Mann-Whitney U test) or females ( $p=0.34$ , unpaired t-test).

**Figure 4. *Ad libitum* food intake is unaffected by ablation or acute inhibition of NTS PPG neurons**

(A-B) Cumulative (A) and non-cumulative (B) food intake of control and PPG-ablated (DTA) mice in the first four hours of the dark phase. Data given as mean $\pm$ SEM, n=7 (control), n=6 (DTA). n.s.: not significant. A: No significant time  $\times$  virus interaction ( $F(2, 22)=0.5406$ ,  $p=0.5900$ ) and no significant main effect of virus ( $F(1, 11)=0.012$ ,  $p=0.91$ ); B: No significant time  $\times$  virus interaction ( $F(2, 22)=0.834$ ,  $p=0.4476$ ) and no significant main effect of virus ( $F(1, 11)=0.1472$ ,  $p=0.7085$ ). (C) Cre-dependent expression of hM<sub>4</sub>Di and EGFP as control in the NTS of Glu-Cre/GCaMP3 mice. Bottom left: Schematic of bilateral injections. Bottom right: Representative voltage-clamp recording (top) and summary data (bottom) of hM<sub>4</sub>Di-expressing PPG neurons in *ex vivo* slice preparation superfused with CNO (1  $\mu$ M). Data given as mean $\pm$ SEM, n=5. \* $p<0.05$  (paired t-test).

(D-E) Cumulative (D) and non-cumulative (E) food intake of hM<sub>4</sub>Di-expressing mice injected with saline or CNO (2 mg/kg, i.p., 30 mins prior to dark onset) in the first four hours of the dark phase. Data given as mean $\pm$ SEM, n=12. D: No significant main effect of virus ( $F(1,21)=1.19$ ,  $p=0.29$ ) or drug ( $F(1,21)=0.002$ ,  $p=0.96$ ); E: No significant main effect of virus ( $F(1,20)=0.11$ ,  $p=0.75$ ) or drug ( $F(1,20)=0.045$ ,  $p=0.83$ ). Data from mice expressing control virus (Fig S4) included in analysis. n.s.: not significant.

**Figure 5. Ablation or acute inhibition of PPG neurons increases food intake only after a large meal.**

(A-C) Cumulative (A-B) and non-cumulative (C) food intake of control and PPG-ablated mice in the first four hours (A,C) and 21 hours (B) after onset of the dark phase following 18 hours of food-deprivation prior to the onset of dark. Data given as mean $\pm$ SEM, n=7 (control), n=6 (DTA). A: No significant virus  $\times$  time interaction ( $F(2, 22) = 1.81$ ,  $p=0.19$ ), but a significant main effect of virus ( $F(1,11)=8.0$ ,  $p=0.016$ ); B:  $p=0.056$  (unpaired t-test); C: No significant virus  $\times$  time interaction ( $F(2, 22)=0.75$ ,  $p=0.49$ ), but a significant main effect of virus ( $F(1,11)=6.1$ ,  $p=0.031$ ).

(D-F) Cumulative (D-E) and non-cumulative (F) food intake of hM<sub>4</sub>Di-expressing mice injected with saline or CNO (2 mg/kg, i.p., 30 mins prior to dark onset) in the first four hours of the dark phase following 18 hours of food-deprivation prior to the onset of dark. Data given as mean $\pm$ SEM, n=12. D: There was a significant virus  $\times$  drug interaction at hour 1 ( $F(1, 21)=4.733$ ,  $p=0.0411$ ),  $p=0.038$  (CNO vs Saline, Sidak's multiple comparisons test); E: No significant virus  $\times$  drug interaction ( $F(1, 21)=1.245$ ,  $p=0.28$ ) and no significant main effect of drug ( $F(1, 21)=1.67$ ,  $p=0.21$ ) or virus ( $F(1, 21)=0.084$ ,  $p=0.77$ ); F: There was a significant virus  $\times$  drug interaction at hour 1 ( $F(1, 21)=4.733$ ,  $p=0.0411$ ),  $p=0.038$  (CNO vs

Saline, Sidak's multiple comparisons test). Data from mice expressing control virus included in analysis. \*:  $p < 0.05$ , n.s.: not significant.

**Figure 6. Intake of large volumes of highly palatable diet activates PPG neurons.**

(A) Expression of the immediate early gene cFOS (green) in PPG neurons following 30 mins access to Vanilla Ensure or no access to food (Control). White arrows: Representative cFOS-positive PPG neurons. Scale bar: 100  $\mu\text{m}$ . (B) Percentage of PPG neurons expressing cFOS 90 mins after 30 mins access to Vanilla Ensure or no access to food. Data given as mean $\pm$ SEM, n=3 (Control), n=3 (Ensure).  $p=0.0079$  (Mann-Whitney UTest). (C) Ensure intake during 15-min access at dark onset over several days of habituation in control and PPG-ablated (DTA) mice. Data given as mean $\pm$ SEM, n=7 (Control), n=7 (DTA). No interaction virus  $\times$  time ( $F(3, 36)=0.592$ ,  $p=0.62$ ) and no significant main effect of virus ( $F(1, 12)=1.135$ ,  $p=0.31$ ) or time ( $F(3, 36)=0.19$ ,  $p=0.90$ ). (D) Ensure intake during 15-min access period on the test day in control and PPG-ablated (DTA) mice. Data given as mean $\pm$ SEM, n=7 (Control), n=7 (DTA).  $p=0.70$  (unpaired t-test). (E) Chow intake of control and PPG-ablated (DTA) mice for one hour following 15-min access to Vanilla Ensure. Data given as mean $\pm$ SEM, n=7 (Control), n=7 (DTA).  $p=0.0052$  (unpaired t-test). \*\*:  $p < 0.01$ ; n.s.: not significant.

**Figure 7. Stress-induced hypophagia requires PPG neurons.**

(A-B) Cumulative (A) and non-cumulative (B) chow intake of mice expressing EGFP or hM<sub>4</sub>Di injected with 2 mg/kg CNO i.p. 60 mins prior to dark onset and left undisturbed or exposed to 30 mins restraint stress 30 mins prior to dark onset. Also included in (A) is data from the same EGFP- and hM<sub>4</sub>Di-expressing mice injected with saline and left undisturbed. Data given as mean $\pm$ SEM, n=9 (Control), n=12 (hM<sub>4</sub>Di). A: Significant main effect of stress for control ( $F(1, 8)=14.14$ ,  $p=0.0055$ ), but not hM<sub>4</sub>Di: ( $F(1, 11)=0.8684$ ,  $p=0.37$ ). No effect of CNO on food intake in both control ( $p=0.5$ ) and PPG-inhibited mice ( $p=0.98$ ) as compared to saline vehicle in the absence of stress. B: Hour 0-1: Significant effect of stress in the EGFP group ( $p=0.0008$ ), but not in the hM<sub>4</sub>Di group ( $p=0.25$ ) (Sidak's multiple comparisons test). Hour 1-2: No significant virus  $\times$  stress interaction ( $F(1, 19)=0.091$ ,  $p=0.77$ ) and no main effect of virus ( $F(1, 19)=0.49$ ,  $p=0.49$ ) or stress ( $F(1, 19)=1.16$ ,  $p=0.29$ ). \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; n.s.: not significant.

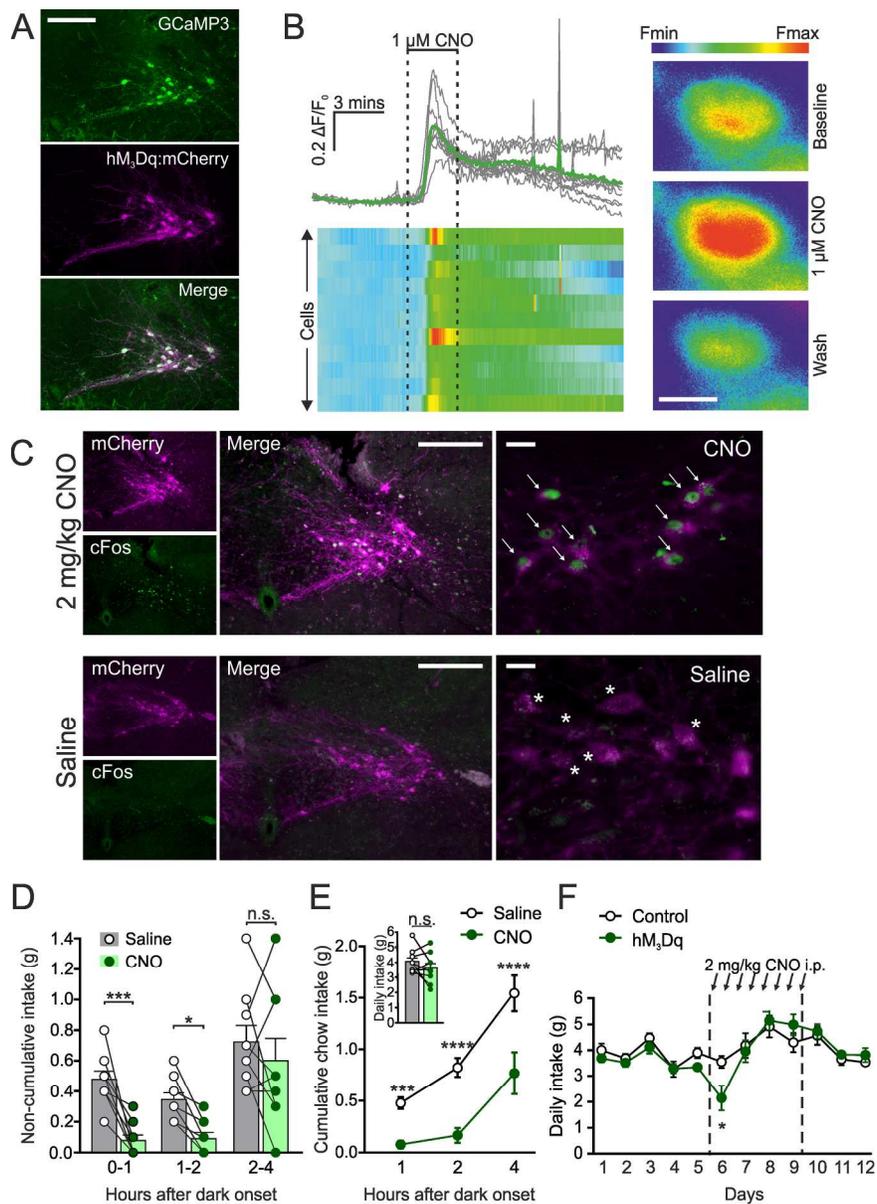


Figure 1. Pharmacogenetic activation of PPG neurons robustly reduces food intake.!! † (A) Expression of hM3Dq:mCherry (magenta) in PPG neurons (detected with an anti-GFP antibody, green) three weeks after stereotaxic injection of AAV2-hM3Dq:mCherry into the NTS of Glu-Cre/GCaMP3 mice. Scale bar: 100  $\mu$ m. (B) Increase in  $[Ca^{2+}]_i$  in GCaMP3-expressing PPG neurons in response to superfusion of ex vivo brainstem slices with 1  $\mu$ M CNO. Data are displayed as traces (top panel) and a heat map (bottom panel) representing the fractional change in fluorescence from baseline. Right panel: Representative pseudocoloured cell responding to 1  $\mu$ M CNO with an increase in  $[Ca^{2+}]_i$ . Scale: 10  $\mu$ m, n=11 cells. (C) Expression of the immediate early gene cFOS (green) in PPG neurons expressing hM3Dq:mCherry (magenta) following i.p. injection of 2 mg/kg CNO (top panels) or saline (bottom panels). White arrows: Representative cFOS-positive hM3Dq-expressing cells. White stars: Representative cFOS-negative hM3Dq-positive cells. Scale bars: 100  $\mu$ m (middle panel) and 20  $\mu$ m (right panel). (D-E) Non-cumulative (D) and cumulative (E) food intake in the first four hours of dark phase following injection of CNO (2 mg/kg i.p.) or saline. CNO was delivered 30 mins prior to dark onset. Data given as mean $\pm$ SEM, n=9 mice. D: No significant time  $\times$  drug

interaction ( $F(2, 16) = 2.897, p=0.0844$ ), but a significant main effect of drug treatment ( $F(1,8)=17.31, p=0.0032$ ); E: Significant time  $\times$  drug interaction ( $F(2,16)=5.626, p=0.0141$ ). 1h  $p=0.0005$ , 2h  $p<0.0001$ , 4h  $p<0.0001$  (Sidak's multiple comparisons test). Inset:  $p=0.20$  (paired t-test). (F) Daily chow intake in hM3Dq-expressing ( $n=7$ ) and control ( $n=6$ ) Glu-Cre mice in response to twice daily i.p. injection of 2 mg/kg CNO (indicated with black arrows). Significant time  $\times$  virus interaction ( $F(11, 121) = 2.06, p=0.0283$ ); day 6  $p=0.0119$  (Sidak's multiple comparisons test). Data given as mean $\pm$ SEM. \* $p<0.05$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ .!! †

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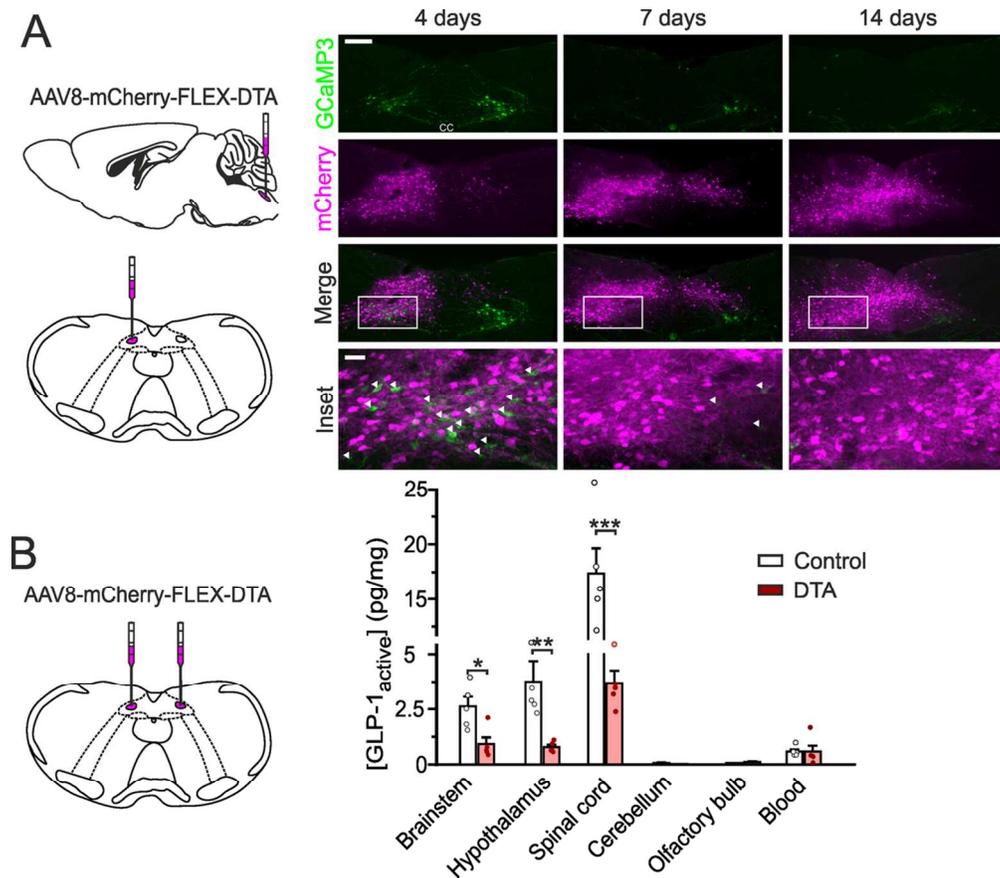


Figure 2. NTS PPG neurons are the main source of brain GLP-1.!! † (A) Expression of mCherry and GCaMP3 (as a marker for PPG neurons) four, seven, and 14 days after unilateral stereotaxic injection of AAV8-mCherry-FLEX-DTA into the NTS of a Glu-Cre/GCaMP3 mouse (schematic on left). White arrows indicate GCaMP3-positive PPG neurons remaining. Scale bars: 100  $\mu$ m (top panels) and 20  $\mu$ m (inset). !! † (B) Protein levels of active GLP-1 (normalised to total protein) detected in several brain regions after bilateral stereotaxic injection of AAV8-mCherry-FLEX-DTA or a control virus (AAV1/2-FLEX-Perceval). Brainstem:  $p=0.0317$ , Hypothalamus:  $p=0.0079$ , (Mann-Whitney U Test); Spinal cord:  $p=0.0004$  (unpaired t-test). Data given as mean $\pm$ SEM,  $n=5$  in each group. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .!! †

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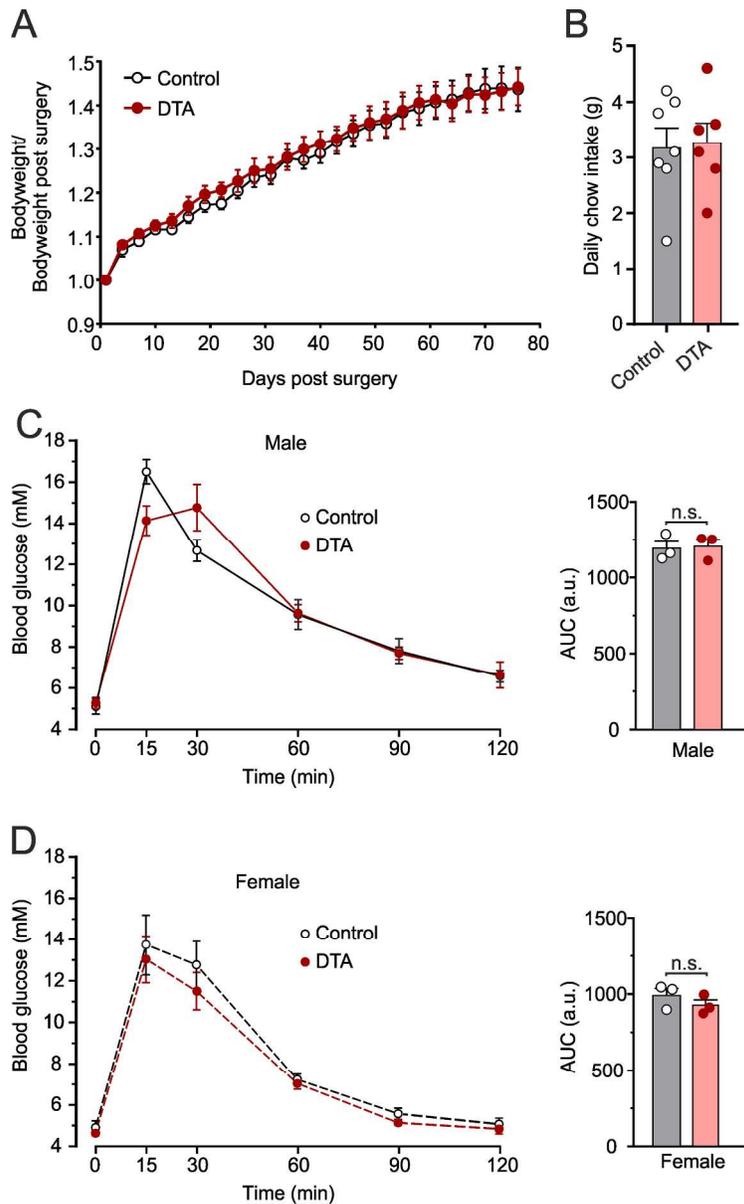


Figure 3. Ablation of NTS PPG neurons has no impact on body weight, food intake, or glucose tolerance. !! † (A-B) Bodyweight change (A) and daily chow intake (B) following stereotaxic injection of AAV8-mCherry-FLEX-DTA or control virus. Mean $\pm$ SEM, n=7 (control), n=6 (DTA). A: No significant time  $\times$  virus interaction ( $F(1, 12)=0.08578$ ,  $p=0.7746$ ) and no significant main effect of virus ( $F(1, 12)=0.08578$ ,  $p=0.7746$ ); B:  $p=0.45$  (unpaired t-test). (C-D) Blood glucose in response to an i.p. injection of glucose (1 g/kg) at t=0 seven weeks after stereotaxic injection of -DTA or control virus in six male (C) and six female (D) Glu-Cre mice. Area under the curve (AUC) of the i.p. glucose tolerance test for each group is given on the right of each graph. Data given as mean $\pm$ SEM. Glucose concentrations: There was a significant time  $\times$  virus interaction for males ( $F(5, 20)=3.83$ ,  $p=0.014$ ), but no significant difference between DTA and control mice at any timepoint. There was no significant time  $\times$  virus interaction for females ( $F(5, 20)=0.19$ ,  $p=0.96$ ) and no significant main effect of virus ( $F(1, 4)=1.08$ ,  $p=0.36$ ). AUC: No effect of virus for males ( $p>0.99$ , Mann-Whitney U test) or females ( $p=0.34$ , unpaired t-test). !! †

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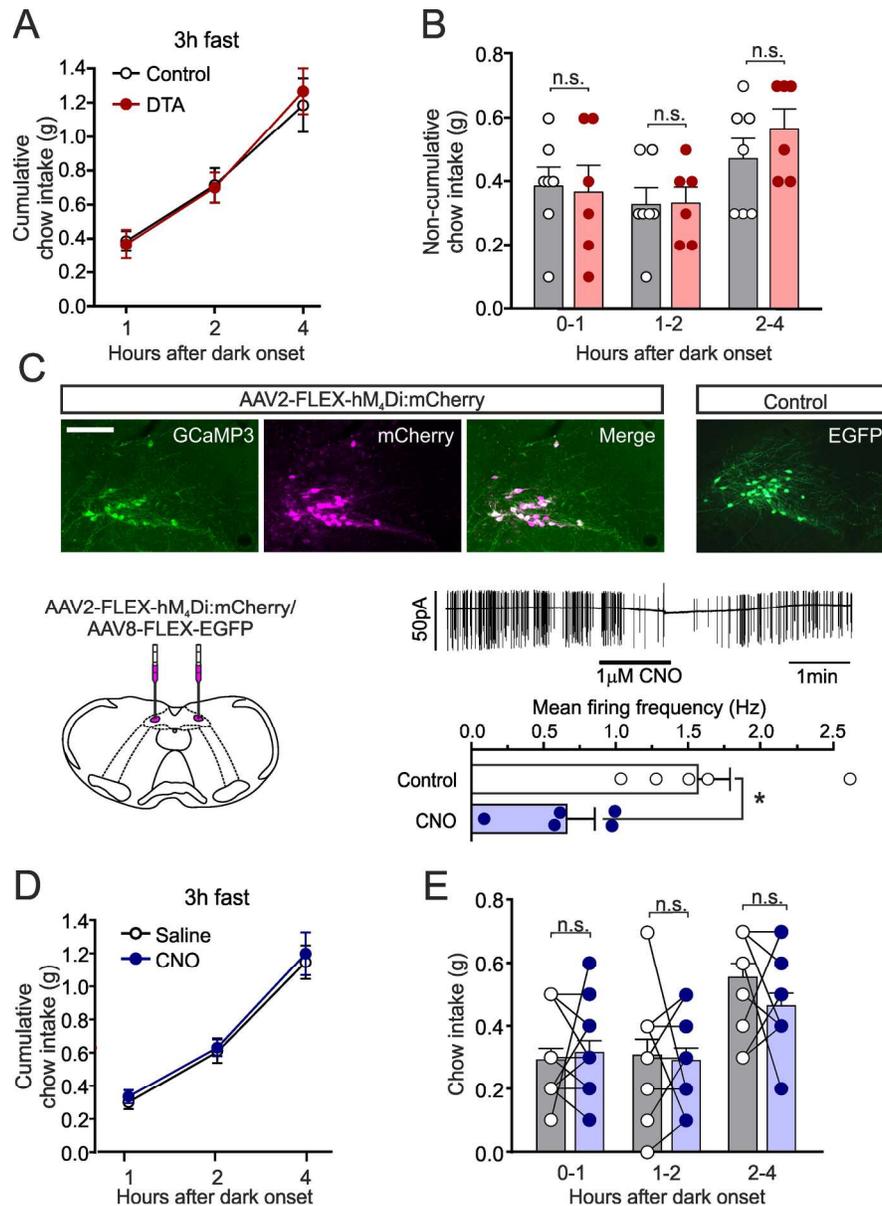


Figure 4. Ad libitum food intake is unaffected by ablation or acute inhibition of NTS PPG neurons (A-B) Cumulative (A) and non-cumulative (B) food intake of control and PPG-ablated (DTA) mice in the first four hours of the dark phase. Data given as mean±SEM, n=7 (control), n=6 (DTA). n.s.: not significant. A: No significant time × virus interaction ( $F(2, 22)=0.5406$ ,  $p=0.5900$ ) and no significant main effect of virus ( $F(2, 11)=0.012$ ,  $p=0.91$ ); B: No significant time × virus interaction ( $F(2, 22)=0.834$ ,  $p=0.4476$ ) and no significant main effect of virus ( $F(1, 11)=0.1472$ ,  $p=0.7085$ ). (C) Cre-dependent expression of hM4Di and EGFP as control in the NTS of Glu-Cre/GCaMP3 mice. Bottom left: Schematic of bilateral injections. Bottom right: Representative voltage-clamp recording (top) and summary data (bottom) of hM4Di-expressing PPG neurons in ex vivo slice preparation superfused with CNO (1 μM). Data given as mean±SEM, n=5. \* $p<0.05$  (paired t-test). (D-E) Cumulative (D) and non-cumulative (E) food intake of hM4Di-expressing mice injected with saline or CNO (2 mg/kg, i.p., 30 mins prior to dark onset) in the first four hours of the dark phase. Data given as mean±SEM, n=12. D: No significant main effect of virus ( $F(1,21)=1.19$ ,  $p=0.29$ ) or drug ( $F(1,21)=0.002$ ,  $p=0.96$ ); E: No significant main effect of virus ( $F(1,20)=0.11$ ,  $p=0.75$ ) or drug

( $F(1,20)=0.045$ ,  $p=0.83$ ). Data from mice expressing control virus (Fig S4) included in analysis. n.s.: not significant. † †

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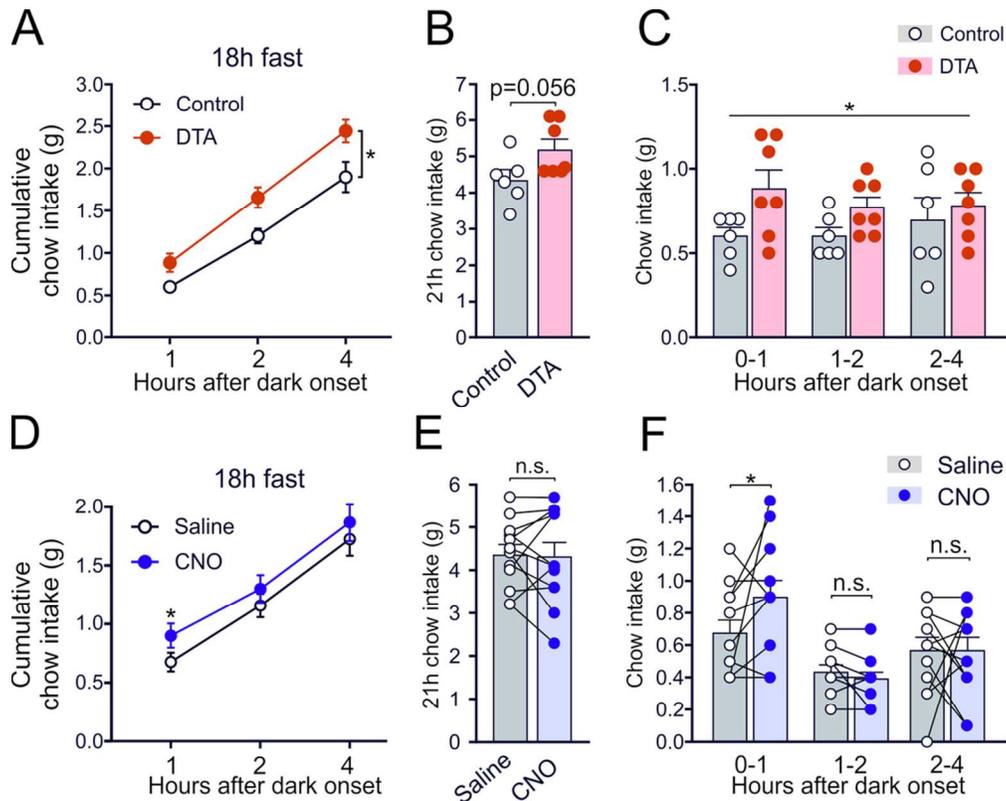


Figure 5. Ablation or acute inhibition of PPG neurons increases food intake only after a large meal. !! † (A-C) Cumulative (A-B) and non-cumulative (C) food intake of control and PPG-ablated mice in the first four hours (A,C) and 21 hours (B) after onset of the dark phase following 18 hours of food-deprivation prior to the onset of dark. Data given as mean±SEM, n=7 (control), n=6 (DTA). A: No significant virus × time interaction ( $F(2, 22) = 1.81, p=0.19$ ), but a significant main effect of virus ( $F(1,11)=8.0, p=0.016$ ); B:  $p=0.056$  (unpaired t-test); C: No significant virus × time interaction ( $F(2, 22)=0.75, p=0.49$ ), but a significant main effect of virus ( $F(1,11)=6.1, p=0.031$ ). !! † (D-F) Cumulative (D-E) and non-cumulative (F) food intake of hM4Di-expressing mice injected with saline or CNO (2 mg/kg, i.p., 30 mins prior to dark onset) in the first four hours of the dark phase following 18 hours of food-deprivation prior to the onset of dark. Data given as mean±SEM, n=12. D: There was a significant virus × drug interaction at hour 1 ( $F(1, 21)=4.733, p=0.0411$ ),  $p=0.038$  (CNO vs Saline, Sidak's multiple comparisons test); E: No significant virus × drug interaction ( $F(1, 21)=1.245, p=0.28$ ) and no significant main effect of drug ( $F(1, 21)=1.67, p=0.21$ ) or virus ( $F(1, 21)=0.084, p=0.77$ ); F: There was a significant virus × drug interaction at hour 1 ( $F(1, 21)=4.733, p=0.0411$ ),  $p=0.038$  (CNO vs Saline, Sidak's multiple comparisons test). Data from mice expressing control virus included in analysis. \*:  $p<0.05$ , n.s.: not significant.!! †

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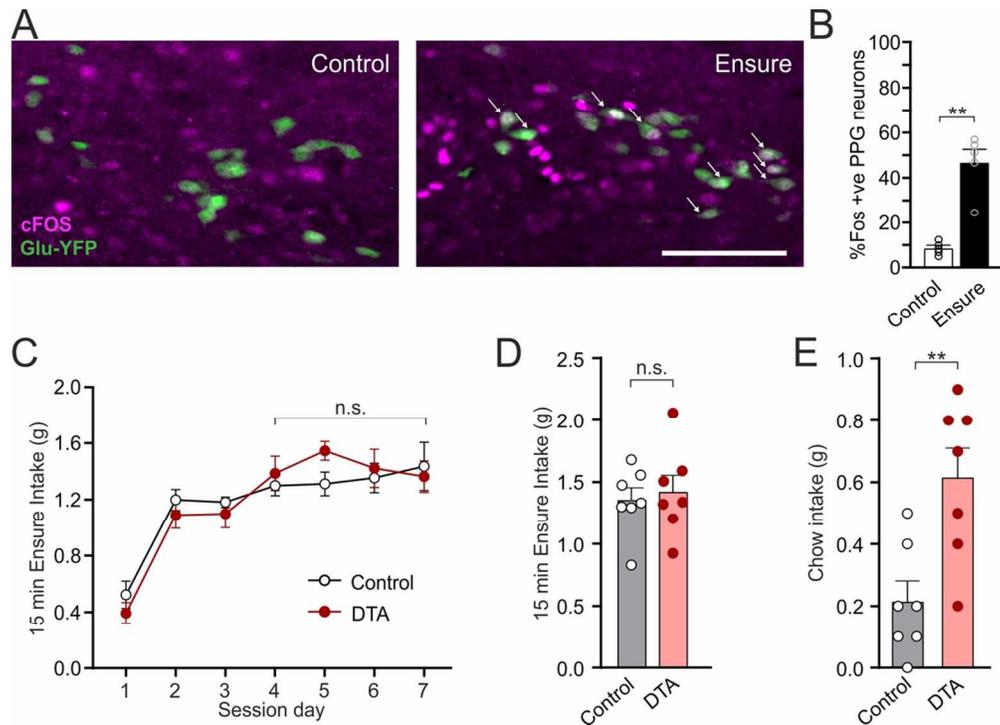


Figure 6. Intake of large volumes of highly palatable diet activates PPG neurons.!! † (A) Expression of the immediate early gene cFOS (green) in PPG neurons following 30 mins access to Vanilla Ensure or no access to food (Control). White arrows: Representative cFOS-positive PPG neurons. Scale bar: 100  $\mu$ m. (B) Percentage of PPG neurons expressing cFOS 90 mins after 30 mins access to Vanilla Ensure or no access to food. Data given as mean $\pm$ SEM, n=3 (Control), n=3 (Ensure).  $p=0.0079$  (Mann-Whitney UTest). (C) Ensure intake during 15-min access at dark onset over several days of habituation in control and PPG-ablated (DTA) mice. Data given as mean $\pm$ SEM, n=7 (Control), n=7 (DTA). No interaction virus  $\times$  time ( $F(3, 36)=0.592$ ,  $p=0.62$ ) and no significant main effect of virus ( $F(1, 12)=1.135$ ,  $p=0.31$ ) or time ( $F(3, 36)=0.19$ ,  $p=0.90$ ). (D) Ensure intake during 15-min access period on the test day in control and PPG-ablated (DTA) mice. Data given as mean $\pm$ SEM, n=7 (Control), n=7 (DTA).  $p=0.70$  (unpaired t-test). (E) Chow intake of control and PPG-ablated (DTA) mice for one hour following 15-min access to Vanilla Ensure. Data given as mean $\pm$ SEM, n=7 (Control), n=7 (DTA).  $p=0.0052$  (unpaired t-test). \*\*:  $p<0.01$ ; n.s.: not significant.!! †

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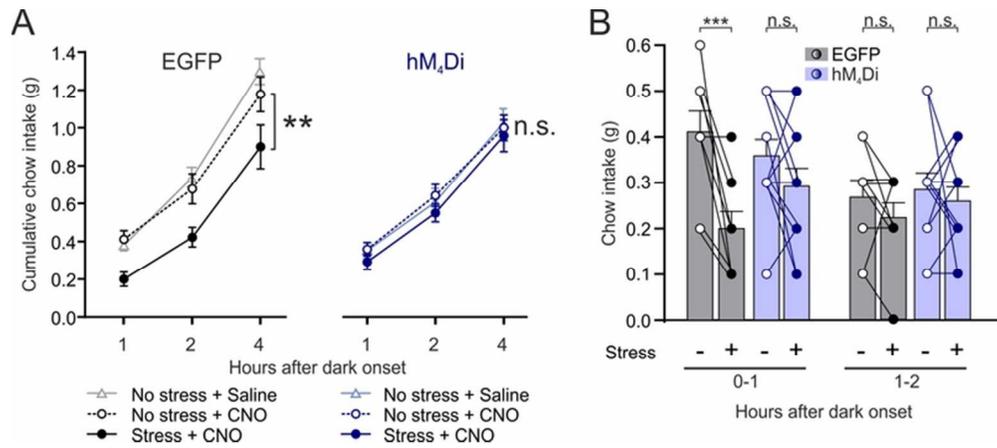
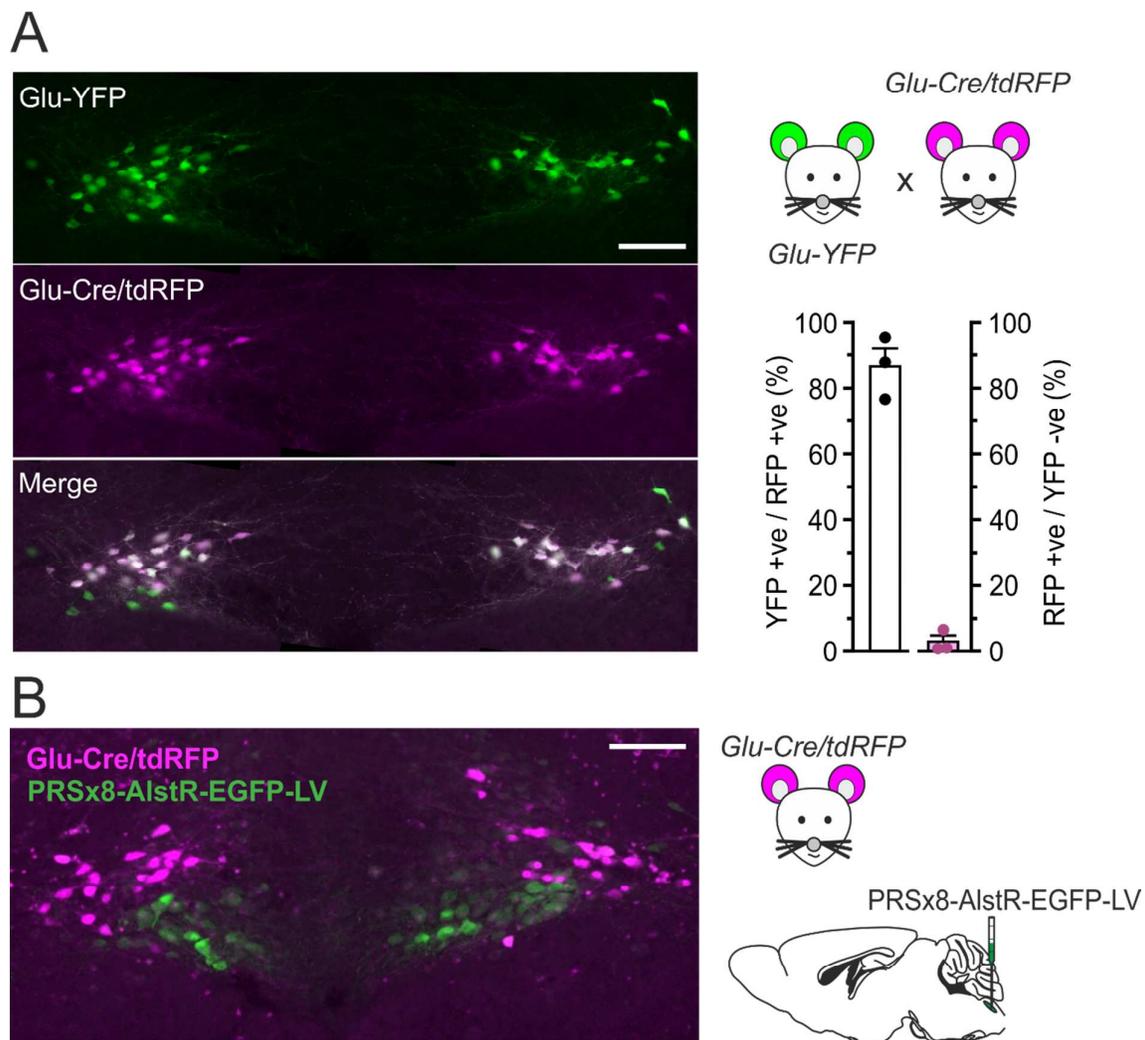


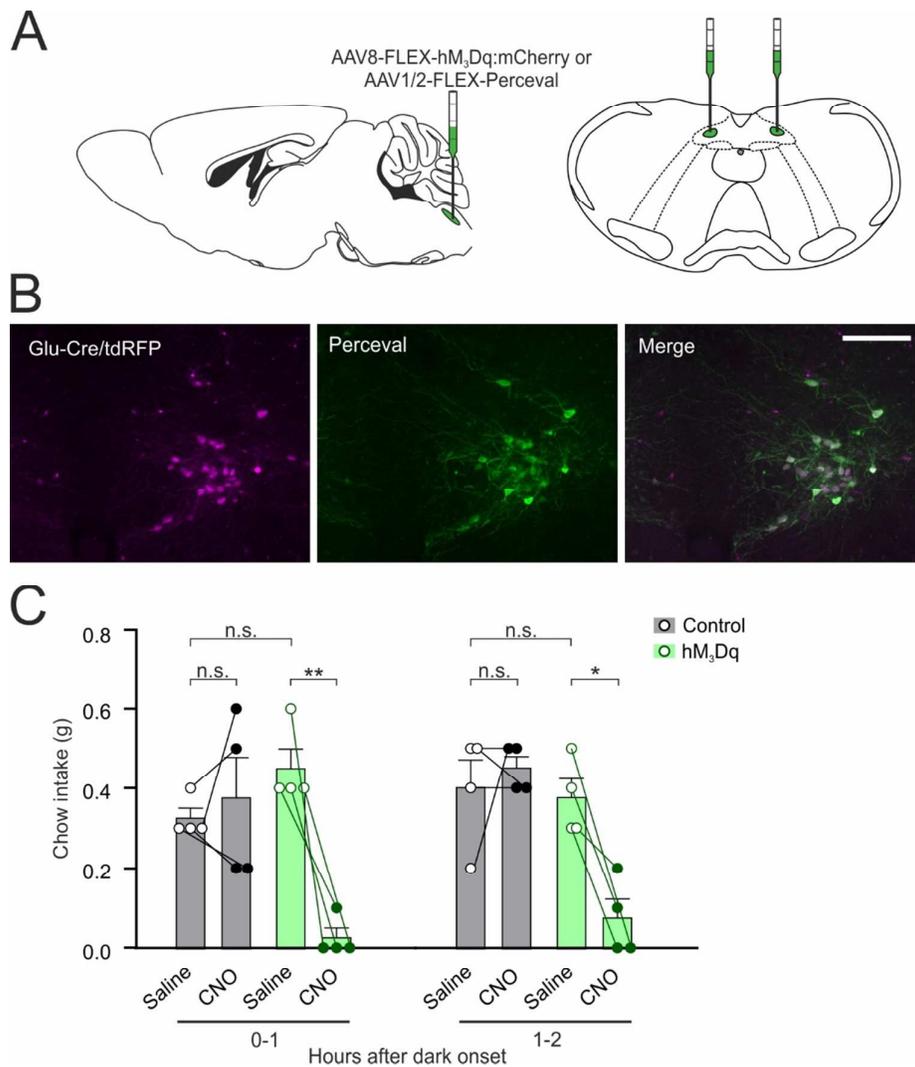
Figure 7. Stress-induced hypophagia requires PPG neurons.!! † (A-B) Cumulative (A) and non-cumulative (B) chow intake of mice expressing EGFP or hM4Di injected with 2 mg/kg CNO i.p. 60 mins prior to dark onset and left undisturbed or exposed to 30 mins restraint stress 30 mins prior to dark onset. Also included in (A) is data from the same EGFP- and hM4Di-expressing mice injected with saline and left undisturbed. Data given as mean±SEM, n=9 (Control), n=12 (hM4Di). A: Significant main effect of stress for control ( $F(1, 8)=14.14$ ,  $p=0.0055$ ), but not hM4Di: ( $F(1, 11)=0.8684$ ,  $p=0.37$ ). No effect of CNO on food intake in both control ( $p=0.5$ ) and PPG-inhibited mice ( $p=0.98$ ) as compared to saline vehicle in the absence of stress. B: Hour 0-1: Significant effect of stress in the EGFP group ( $p=0.0008$ ), but not in the hM4Di group ( $p=0.25$ ) (Sidak's multiple comparisons test). Hour 1-2: No significant virus × stress interaction ( $F(1, 19)=0.091$ ,  $p=0.77$ ) and no main effect of virus ( $F(1, 19)=0.49$ ,  $p=0.49$ ) or stress ( $F(1, 19)=1.16$ ,  $p=0.29$ ). \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ ; n.s.: not significant.!! †

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**Supplemental Material****Supplemental figures**

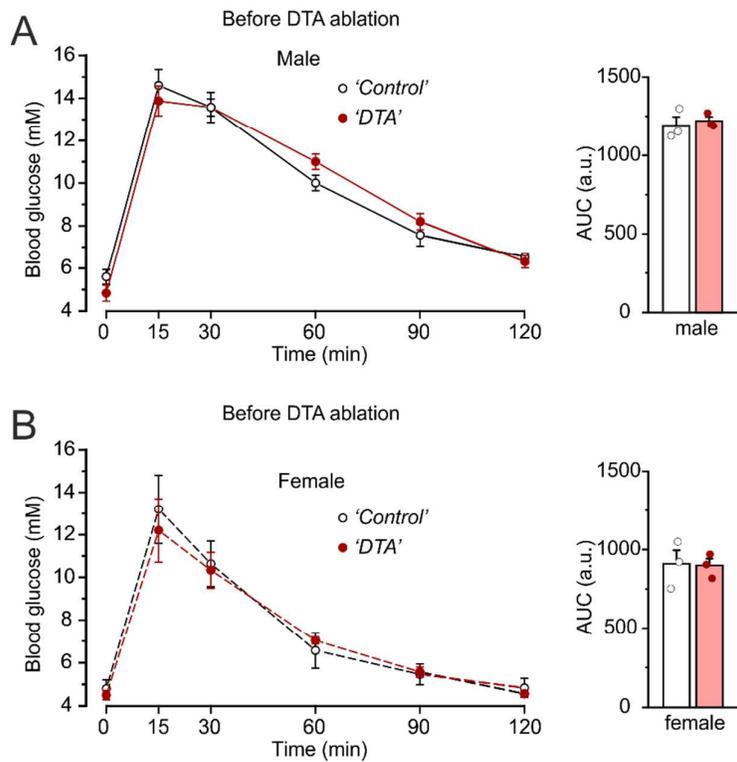
**Supplemental figure 1 – related to Fig 1. RFP-positive Glu-Cre cells overlap with Glu-YFP cells, but not Phox2b-expressing cells.**

(A) GFP- and RFP-immunoreactivity in the NTS of the result of crossbreeding Glu-YFP and Glu-Cre/tdRFP mice, demonstrating clear overlap between these neuronal populations. Scale: 100  $\mu$ m. Right panel: Percentage of RFP-positive cells also expressing YFP (left) as well as RFP-positive cells *not* expressing YFP (right) throughout the NTS. Data given as mean $\pm$ SEM, n=3 mice. (B) RFP-immunoreactivity and Phox2b-dependent expression of EGFP in the NTS of Glu-Cre/tdRFP mice injected with PRX8-AlstR-EGFP-LV. Scale: 100  $\mu$ m. N=3 mice.



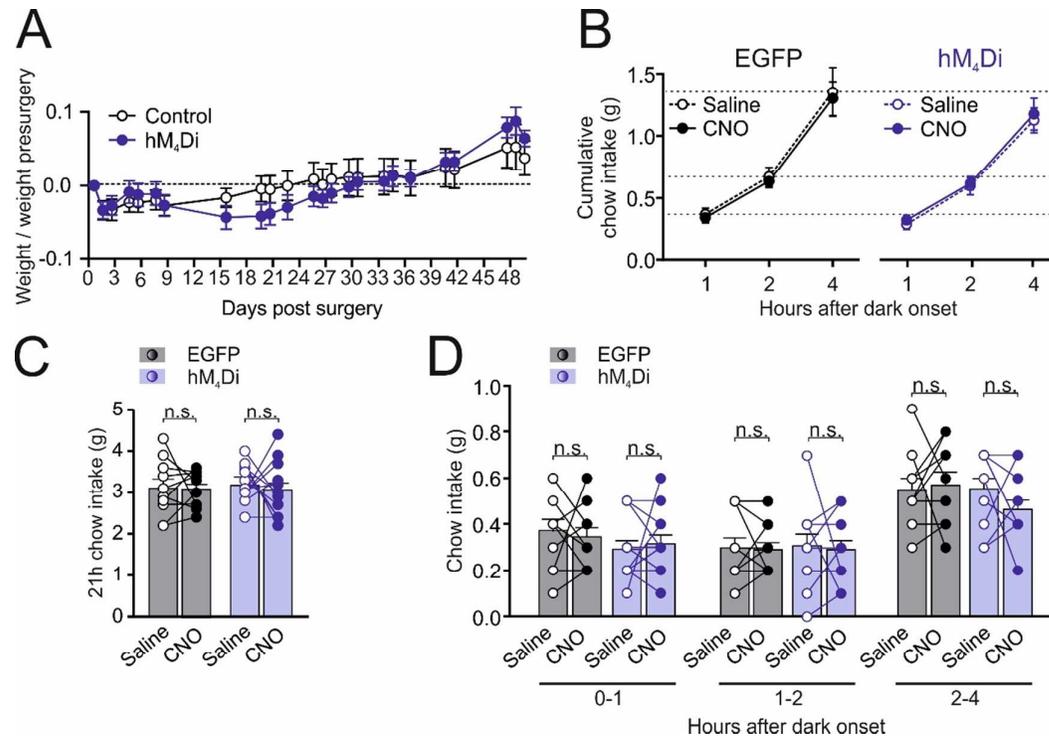
**Supplemental figure 2 – related to Fig 1. hM<sub>3</sub>Dq lacks intrinsic activity on food intake.**

(A) Schematic showing injection of AAV2-FLEX-hM<sub>3</sub>Dq:mCherry or AAV1/2-FLEX-Perceval into the NTS of Glu-Cre mice. (B) Expression of tdRFP and Perceval (detected with an anti-GFP antibody). Scale: 100  $\mu$ m. (C) Non-cumulative chow intake of Glu-Cre mice expressing Perceval or hM<sub>3</sub>Dq in the first two hours after dark onset following injection of 2 mg/kg CNO i.p. 30 mins prior to dark onset. Data given as mean $\pm$ SEM, n=4 (Control), n=4 (hM<sub>3</sub>Dq). There was a significant virus  $\times$  drug interaction at both hour 0-1 ( $F(1, 6)=17.19$ ,  $p=0.006$ ) and hour 1-2 ( $F(1, 6)=8.65$ ,  $p=0.026$ ) and a significant effect of CNO in the hM<sub>3</sub>Dq group in hour 0-1 ( $p=0.0038$ ) and in hour 1-2 ( $p=0.024$ ), but no effect of CNO in the control group in hour 0-1 ( $p=0.81$ ) or hour 1-2 ( $p=0.82$ ) (Sidak's multiple comparisons test). \*:  $p<0.05$ ; \*\*:  $p<0.01$ ; n.s.: not significant.



**Supplemental figure 3 – related to Fig 3. Glucose tolerance before PPG ablation.**

(A-B) Blood glucose in response to an i.p. injection of glucose (1 g/kg) at t=0 prior to stereotaxic injection of -DTA or control virus in six male (A) and six female (B) Glu-Cre mice. Area under the curve (AUC) of the i.p. glucose tolerance test for each group is given on the right of each graph. Data given as mean±SEM.



**Supplemental figure 4 – related to Fig 4. hM<sub>4</sub>Di lacks intrinsic activity on body weight and food intake.**

(A) Change in bodyweight following stereotaxic injection of AAV2-FLEX-hM<sub>4</sub>Di (n=13) or AAV2-FLEX-EGFP (n=11, control). There was a significant virus × time interaction ( $F(23, 506)=2.93$ ,  $p=0.0001$ ), but no significant difference between groups at any timepoint (Sidak's multiple comparisons test). (B-D) Cumulative (B-C) and non-cumulative (D) chow intake in the first four hours (B,D) and 21 hours (C) after dark onset. Mice were expressing either hM<sub>4</sub>Di or EGFP in NTS PPG neurons and were injected with 2 mg/kg CNO i.p. 30 mins prior to dark onset. Data given as mean±SEM, n=11 (control), n=12 (hM<sub>4</sub>Di). B: There was no significant drug × time interaction for either the control ( $F(2, 20)=0.086$ ,  $p=0.92$ ) or hM<sub>4</sub>Di group ( $F(2, 22)=0.036$ ,  $p=0.96$ ), and there was no significant main effect of CNO in the control ( $F(1, 10)=0.22$ ,  $p=0.65$ ) or in the hM<sub>4</sub>Di group ( $F(1, 11)=0.11$ ,  $p=0.74$ ). C: There was no significant drug × virus interaction ( $F(1, 20)=0.22$ ,  $p=0.65$ ) and no main effect of CNO ( $F(1, 20)=0.47$ ,  $p=0.5$ ) or virus ( $F(1, 20)=0.11$ ,  $p=0.75$ ). D: No significant drug × virus interactions (Hour 0-1: ( $F(1, 21)=0.18$ ,  $p=0.67$ ), hour 1-2: ( $F(1, 21)=0.011$ ,  $p=0.92$ ), and hours 2-4 ( $F(1, 19)=1.30$ ,  $p=0.27$ )). n.s.: not significant.