**Cell Metabolism**

**Glucose-Dependent Insulinotropic Polypeptide Receptor-Expressing Cells in the Hypothalamus Regulate Food Intake**

**Graphical Abstract**

**Highlights**

- *Gipr* is expressed in the hypothalamus, as demonstrated using a new *Gipr-Cre* mouse model

- *Gipr* cells included somatostatin-positive neurons, glia, and vascular cells

- *Gipr* overlapped partially with *Glp1r* in human and mouse hypothalamus by RNAscope

- Activation of *Gipr* neurons using local AAV-delivered Gq-DREADDs reduced food intake

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**In Brief**

Adriaenssens et al. identified cells expressing receptors for the gut hormone GIP in human and mouse hypothalamus using a new *Gipr-Cre* mouse model and RNAscope. Local hypothalamic delivery of AAVs expressing Cre-dependent Gq-DREADDs revealed that activation of *Gipr*-positive neurons in mice reduced food consumption.
Glucose-Dependent Insulinotropic Polypeptide Receptor-Expressing Cells in the Hypothalamus Regulate Food Intake

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SUMMARY

Ambiguity regarding the role of glucose-dependent insulino-tropic polypeptide (GIP) in obesity arises from conflicting reports asserting that both GIP receptor (GIPR) agonism and antagonism are effective strategies for inhibiting weight gain. To enable identification and manipulation of Gipr-expressing (Gipr) cells, we created Gipr-Cre knockin mice. As GIPR-agonists have recently been reported to suppress food intake, we aimed to identify central mediators of this effect. Gipr cells were identified in the arcuate, dorsomedial, and paraventricular nuclei of the hypothalamus, as confirmed by RNAscope in mouse and human. Single-cell RNA-seq identified clusters of hypothalamic Gipr cells exhibiting transcriptomic signatures for vascular, glial, and neuronal cells, the latter expressing somatostatin but little pro-opiomelanocortin or agouti-related peptide. Activation of Gq-DREADDs in hypothalamic Gipr cells suppressed food intake in vivo, which was not obviously additive with concomitant GLP1R activation. These data identify hypothalamic GIPR as a target for the regulation of energy balance.

INTRODUCTION

Glucose-dependent insulino-tropic polypeptide (GIP) is a gut hormone released from enteroendocrine cells in the duodenum and jejunum (Buchan et al., 1978; Buffa et al., 1975) within minutes of ingesting a meal (Elliott et al., 1993). GIP binds its cognate receptor, GIP receptor (GIPR)—a class B G-protein-coupled receptor (GPCR) (Usdin et al., 1993). GIPR activation stimulates insulin release in pancreatic beta cells (Dupre et al., 1973), and together with its sister incretin, glucagon-like peptide-1 (GLP-1), GIP is an important glucostat to keep post-prandial blood glucose levels in check. It is GLP-1, however that has enjoyed the therapeutic limelight in efforts to design more effective type 2 diabetes treatments. Initial interest in GIP-based therapies waned following studies showing that the insulinotropic properties of GIP are attenuated in patients with type 2 diabetes (Nauck et al., 1993). The complex relationship between GIP and adiposity has further obscured our understanding of GIP’s therapeutic potential.

Studies showing that genetic or pharmacological blockade of GIPR protects against obesity have implicated GIP in promoting body weight gain (McClean et al., 2007; Miyawaki et al., 2002). These data are congruent with GIP’s role in facilitating triglyceride storage in adipose tissue (Eckel et al., 1979; Wasada et al., 1981). Though GIPR antagonists have inhibited weight gain in animal models (Boylan et al., 2015; Futurja et al., 2008; Killion et al., 2018; McClean et al., 2007), the therapeutic utility of GIPR antagonism in humans is yet to be determined. The recent realization that some peptides designed to be GIPR antagonists exhibit partial agonist activity (Sparre-Ulrich et al., 2015), confounding the interpretation of some of these studies, as well as evidence indicating that the lipogenic action of GIP may be mediated indirectly via insulin (Campbell et al., 2016; Ugleholdt et al., 2011), has led some to question the rationale behind blocking
GIP signaling as a route toward tackling obesity (Finan et al., 2016).

Augmenting GIPR signaling in combination with proven antidiabetic agents has yielded exciting results. In rodents and humans, GLP-1–GIP dual agonism significantly improved glycemic control and provided greater weight loss compared to treatment with a GLP-1 receptor agonist alone (Coskun et al., 2013; Ravn et al., 2013; Nørregaard et al., 2018). In mice, this additional weight loss could be attributable to a further reduction in food intake (Coskun et al., 2018; Finan et al., 2013; Frias et al., 2018; Nørregaard et al., 2018). It is tempting to suggest that the addition of GIPR activation underlies the superior performance of these combinatorial therapies (Coskun et al., 2018; DiMarchi, 2018), although GIPR-only agonists appear to either not or fairly modestly reduce body weight when given in isolation (Coskun et al., 2018; Mroz et al., 2019). While GLP-1 exhibits central inhibitory actions on food intake (Turton et al., 1996), comparatively little is known about the central activity of GIP on appetite or the expression profile of GIP receptors.

To create a map of central GIPr localization, brains of Gipr<sup>EYFP</sup> mice were serially sectioned and stained for EYFP. In line with in situ and radioligand binding data (Kaplan and Vigna, 1994; Paratore et al., 2011; Usdin et al., 1993), staining was fairly widespread within the CNS (Figure S1H), including key feeding centers of the hypothalamus, such as the arcuate (ARC), paraventricular (PVN), and dorsomedial hypothalamic (DMH) nuclei (Figure 1A). Active transcription of Gipr in the adult hypothalamus was confirmed by qPCR (Figure 1B).

To create a transcriptomic profile of Gipr cells in the hypothalamus, cell preparations from the hypothalami of Gipr<sup>EYFP</sup> mice were purified using fluorescence-activated cell sorting (FACS), and their transcriptomes were analyzed via single-cell RNA sequencing (scRNA-seq). Graph-based clustering analysis revealed that hypothalamic Gipr cells separate into six subpopulations (Figure 1C top). Cluster identities were assigned based on the expression patterns of cell-type-specific genes, including those found in the most enriched cluster markers (Figures 1C [bottom] and 1D, and Table S1), with mural cells (Kcnj8, Abcc9, Mustn1, and Mhy11), ependymocytes (Ccld153 and Hdc), vascular and leptomeningeal cells (VLMC) (Lum and Pdgfra), oligodendrocytes (Mal and Klf6), and neurons (Snap25 and Syt1) representing distinct clusters of Gipr cells.

As hypothalamic neurons are known to modulate feeding behavior, we analyzed the neuronal cluster in more detail. Gipr neurons expressed markers for both GABAergic (Slc32a1) and glutamatergic (Slc17a6) cells (Figure 1D). Genes encoding peptides previously implicated in energy homeostasis, namely Sst, Avp, Tac1, and Cartpt, were among the most highly expressed neurohormonal markers (Figure 1E). To examine the heterogeneity of Gipr-fluorescent neurons, we constructed a matrix showing the numbers of individual Gipr cells from the neuronal cluster coexpressing a selection of 20 genes implicated in neuroendocrine signaling pathways (Figure S2A). Sst was the primary neuroendocrine marker for Gipr neurons with 83% of Snap25-positive cells in the neuronal cluster expressing Sst. Avp and Pthih were also expressed in at least half of the Gipr neurons (58% and 50%), with Cartpt and Tac1 expressed in fewer than 50%. Pomc was expressed in less than 10% of Gipr neurons and only at low levels. Consistent with these scRNA-seq results, we observed an apparent enrichment in Sst and diminished Pomc message by qRT-PCR in independently isolated fluorescently labeled Gipr cells (Figure S2B).

Local and Peripheral Signals Regulate Gipr Neurons

To identify regulatory cell surface receptors present in Gipr neurons, we analyzed the expression of GPCRs in the neuronal cluster. Grm5 and Gabbr1 were the most highly expressed GPCRs in Gipr neurons, which also expressed ionotropic receptors for glutamate and GABA (Gria2, Gria3, Grin2b, and Gabbr1; data not shown). Other neurotransmitters likely to contribute to Gipr neuron regulation include opioids (via Oprk1 and Oprf1), acetylcholine (via Chrm1 and Chrm3), histamine (Hrh3), and serotonin (Htr1b, Htr1d, and Htr2c). Gipr neurons also expressed...
receptors for peptide neuroendocrine regulators, including SST (Sstr2 and Sstr1), calcitonin (Calcr), and PACAP (Calcrl) and expressed receptors known to govern energy balance, including Cnr1, Mchr1, Hchr2, Tac1r, Ghsr, Cckbr, and Htr2c (Figure 2A).

The functional activity of several receptors was interrogated at the single-cell level using calcium imaging in cultured hypothalamic neurons from GiprGCaMP3 mice. The receptors selected were Cckbr, Ghsr, and Htr2c, the expression of which we confirmed by qRT-PCR in additional hypothalamic FACS sorts (Figure S2C). Given the finding that some Gipr neurons expressed Cartpt and Pomc, we also examined Lepr, which has previously been shown to induce calcium influx in POMC neurons (Heeley et al., 2018; Smith et al., 2018). Of the stimuli tested, glutamate increased calcium in the majority (40/59) of neurons, while only subsets responded to CCK (5/34) and the GHSR agonist hexarelin (7/73). Only 1/15 neurons responded to leptin and no cells responded to 5HT (Figures 2B and 2C).

**Activation of Hypothalamic Gipr Cells Decreases Food Intake**

To assess the effect of acute chemogenetic manipulation of Gipr cell activity on food intake, Gipr-Cre mice received hypothalamic injections of Cre-inducible AAVs expressing the Gq-coupled DREADD, hM3D (AAV-hSyn-DIO-hM3D(Gq)-mCherry) (Armbruster et al., 2007), designed to preferentially target neurons (Hammond et al., 2017; Kügler et al., 2003), to produce GiprhypDq

Figure 1. Gipr-Expressing Cells in the Brain

(A) Micrograph of GFP staining in brain from heterozygous GiprEYFP mice (see also Figure S1).
(B) Relative expression of Gipr in whole hypothalamic homogenates in WT mice (n = 3). Data are plotted as 2^ΔCt compared to Actb with the bar representing mean ± SD.
(C) Gipr cells were isolated from single-cell digests of hypothalami from two heterozygous GiprEYFP mice via FACS, and their transcriptomes were analyzed by scRNA-seq followed by clustering analysis. tSNE visualization of hypothalamic Gipr cells indicates that there are six clusters (top). Cell types were assigned according to expression of a combination of marker genes (bottom) (see also Table S1).
(D) t-SNE plots of the expression of selected markers for neurons (Snap25), GABAergic neurons (Slc32a1), glutamatergic neurons (Slc17a6), oligodendrocytes (Ma), mural cells (Abcc9 and Mustn1), VLMCs (Lum), and ependymocytes (Ccdc153).
(E) Violin plots representing expression of genes encoding secreted products within the neuronal cluster.
mice (Figures S3A and S3B). Food intake effects of Gipr-cell D₃ activation were assessed in a crossover study (Figure S3C). In chow-fed GiprhypD₃ mice, activation of D₃ receptors following injection of clozapine-N-oxide (CNO) significantly suppressed both light- and dark-phase food intake in ad lib-fed and fasted animals (Figures 3A–3C). Similarly, CNO injected at the onset of the dark phase in GiprhypD₃ mice fed an HFD for 2–4 weeks significantly reduced food intake, but no significant effect was observed when CNO was injected at the start of the light phase (Figures 3D–3F). No effect on food intake was observed in control (non-AAV-injected) mice following administration of CNO (Figure S3D).

**Gipr and Glp1r Are Co-expressed in a Subset of Cells in Humans and Mice**

To investigate potential overlap between Gipr and GLP-1 receptor (Glp1r) expression, we performed RNAscope analysis of mouse and human hypothalamus. Consistent with the cellular localization identified using GiprEYFP mice, RNAscope revealed Gipr-positive cells in mouse ARC and DMH. Glp1r-positive cells were also observed in these nuclei, with some cells exhibiting both Gipr and Glp1r expression (Figures 4A–4C). In human hypothalamic sections, we similarly observed cells positive for GIPR, GLP1R, or both receptors together (Figures 4D and 4E). It was noticeable that in both mouse and human, the probes for Glp1r or GLP1R detected a higher density of transcripts per cell than the Gipr or GIPR probes. In mice, this is in accordance with lower expression of Gipr measured by qPCR compared to Glp1r in FACS-purified hypothalamic Gipr cells (Figure S2C) as well as homogenates of whole hypothalamus (Figure S4A). In human, GIPR signal was also observed in periventricular cells in the ependymal region (Figure S4B).

**Co-activation of Gipr and Glp1r Cells Does Not Further Reduce Acute Food Intake**

To investigate potential additive effects of simultaneously activating hypothalamic Gipr- and Glp1r-positive cells on acute food intake, we first injected AAVs carrying hSyn-DIO-hM3D(Gq)-mCherry into the hypothalamus of mice expressing Cre under the control of both the Gipr and Glp1r promoters.
Gipr or Glp1r hypDq) or the Glp1r promoter alone (Glp1r hypDq). CNO injected at the start of the dark phase significantly reduced 2-h food intake in both fasted Gipr/Glp1r hypDq and Glp1r hypDq mice, although this did not reach statistical significance for the latter (Figure 4F). The CNO-dependent reduction in food intake of 52% ± 9% (n = 7) in Gipr/Glp1r hypDq mice was, however, similar in magnitude to the previously observed 50% ± 8% (n = 14) reduction in Glp1r hypDq mice (Figure 3C).

In a second approach, we tested the effect of peripherally administered Exendin-4 (Ex-4) in combination with Gipr cell Dq activation on food intake. A sub-maximal dose of Ex-4 was chosen from dose-response trials (Figure S4C). While Ex-4, CNO, and the combination of Ex-4 with CNO all reduced 2-h food intake in Glp1r hypDq mice, we were unable to detect a significant difference between treatments (Figure 4G) on acute food intake.

DISCUSSION

In this study, we generated a new Gipr-Cre mouse model, identifying Gipr-expressing cells in the hypothalamus, and enabling their transcriptomic and functional characterization. We show that (1) Gipr is expressed in neuronal and non-neuronal cell types in key feeding centers of the brain, (2) hypothalamic Gipr neurons express a diverse range of neuroendocrine hormones and hormonal or neurotransmitter receptors, (3) direct activation of hypothalamic Gipr cells potently suppresses food intake, and (4) Gipr is co-expressed with Glp1r in a subset of hypothalamic cells in humans and mice.

Centrally expressed Gipr has previously been implicated in promoting neurogenesis and synaptic plasticity (Faire et al., 2011; Nyberg et al., 2005; Paratore et al., 2011). Our finding that Gipr cells are present in the ARC, DMH, and PVH suggests that GIPR signaling may also integrate with well-characterized hypothalamic circuits regulating energy balance (Waterson and Horvat, 2015). While it could be argued that EYFP or GCaMP3 labeling in Gipr-Cre mice could result in part from lineage tracing, we observed a similar location of Gipr-positive cells using RNAscope. The activation of hSyn-DIO-mH3D(Gq)_mCherry in the DREADD experiments provides further proof that Cre, and by implication, Gipr, is actively transcribed in adult hypothalamic cells.

To identify whether Gipr cells could be assigned to known neural networks, we performed scRNA-seq, which revealed substantial heterogeneity of Gipr cells. Although some genes are likely to have exhibited altered expression during the time taken for cell dissociation and separation, the results allowed us to cluster Gipr cells into several populations, including neurons, mural cells, ependymocytes, VLMCs, and oligodendrocytes, each characterized by a distinct profile of marker genes. Gipr neurons near-ubiquitously expressed Sst, with many also expressing Avp and Pthlh and fewer expressing Cartpt, Tac1 and Pomc. The broad expression of Sst in Gipr neurons is striking, and the co-expression of Pthlh in 50% of Gipr neurons suggests that they may predominantly belong to the Pthlh clade of SST neurons identified in recent studies (Campbell et al., 2017).

Collective stimulation of Gipr cells in the hypothalamus via chemogenetic activation resulted in acute anorexia. While the decrease in food intake upon acute activation of hypothalamic Gipr cells appears to be at odds with the protection of Gipr KO animals from diet-induced obesity, it is likely that the resistance to weight gain exhibited by Gipr KO mice is at least in part due to
Suppression of food intake in Gipr<sup>proDm</sup> mice was robust in the dark phase, when appetite-promoting signaling is at its highest in rodents (Yannielli et al., 2007). Suppression of food intake mediated by hypothalamic Gipr cells would be compatible with expression of established anorectic neuropeptides, in particular AVP and CART (Pei et al., 2014), (Farzi et al., 2018), which were expressed in over 40% of Gipr neurons. While it may be tempting to speculate that the melanocortin axis may play a role in Gipr cell-mediated anorexia, Pomc was only expressed in a minority of Gipr neurons and at relatively low levels (Figure 2E). Still, it is surprising that the majority of Gipr neurons expressed Sst, as activation of all five Sst-positive neuronal clades (defined by co-expression of Th, Nts, Agrp, Unc13c, or Pthlh) with D<sub>a</sub> in the ARC has been shown to be orexigenic (Campbell et al., 2017). By contrast, our results suggest that specific activation of Sst-positive subpopulations expressing Gipr (being Pthlh positive, but Agrp negative) results in anorexia. Although we are not able to link the clear anorexigenic effects seen with D<sub>a</sub> activation to a defined cell population, the use of hSyn-promoter AAV8 would suggest neuronal targeting (Hammond et al., 2017), though further experiments are required to determine exactly which Gipr cell types underlie the observed inhibition of food intake.

The synergy between pharmacological GIPR and GLP1R agonism on appetite (Coskun et al., 2018; Finan et al., 2013; Norregaard et al., 2018) suggests that the GIPR signaling cascade may underlie the observed inhibition of food intake. In contrast to the published results (Campbell et al., 2016), we report that only transiently expressed Gipr<sup>proDm</sup> mice were injected bilaterally with AAV-DIO-hM3D-mCherry into the hypothalamus to produce GLP1R agonist activation in the adult hypothalamus. Single- and double-labeled cells were counted and scored (C), (Dii), and (Eii). Bars represent the mean ± SD. Food intake was compared using a repeated measures 2-way ANOVA with a Sidak’s post-hoc test. **p < 0.01, ***p < 0.001.

Figure 4. Partial Cellular Overlap of Gipr and Glp1r Expression, but Limited Effect of GLP1R-Co-activation on Gipr-Expressing Cell-Mediated Acute Anorexia

(A–E) Coronal sections of mouse (A–C) and human (D–E) hypothalamus were co-labeled for Gipr or GIPR and Glp1r or GLP1R mRNA using RNAscope. Areas corresponding to the ARC and DMH in mouse and PVH/DMH, lateral hypothalamic nucleus (LH), and mediobasal hypothalamus (MBH) in human were assessed for Gipr or GIPR and Glp1r or GLP1R expression (B), (D), and (E). Single- and double-labeled cells were counted and scored (C), (Dii), and (Eii). Bars represent the mean ± SD (see also Figure S4).

(F) Gipr-Cre x Glp1r-Cre and Glp1r-Cre-only mice were injected bilaterally with AAV-DIO-hM3D-mCherry into the hypothalamus to produce Glp1r<sup>proDm</sup> and Glp1r<sup>proDm</sup> mice, respectively. CNO (1 mg/kg) or vehicle was injected i.p. following a 10-h daytime fast at the onset of the dark phase before measuring food intake 2 h post-activation (see also Figures S3C and S3D). Food intake was compared using a repeated measures 2-way ANOVA with a Sidak’s post-hoc test. *p < 0.01, Glp1r<sup>proDm</sup> n = 7, Glp1r<sup>proDm</sup> n = 4.

(G) Heterozygous Gipr-Cre mice were injected bilaterally with AAV-DIO-hM3D-mCherry into the hypothalamus to produce Gipr<sup>proDm</sup> mice. Following a 10-h daytime fast Exendin-4 (Ex-4) (1.5 nmol/kg) or saline was injected s.c. 1 h prior to the onset of the dark phase. CNO (0.3 mg/kg) or vehicle was injected i.p. at the onset of the dark phase, food was presented, and food intake measurements were taken 2 h post-activation (see also Figures S3E, S4C, and S4D). Bars represent mean ± SD. Food intake was compared using a repeated measures 2-way ANOVA with a Sidak’s post-hoc test. *p < 0.05, **p < 0.01, Gipr<sup>proDm</sup> n = 12.

In summary, we have characterized previously unrecognized populations of hypothalamic cells that express Gipr in rodents and humans and demonstrated that their acute stimulation potently reduces food intake, identifying the central hypothalamic GIP signaling axis as an additional contributor to the control of energy homeostasis.

**Limitations of Study**

We report a new Gipr-Cre knockin mouse model to characterize and manipulate hypothalamic cells. While a knockin model is less likely to result in aberrant Cre expression than transgenic models employing randomly integrated constructs in which a gene promoter (often of limited length) drives a transgene, we cannot exclude that some cells might report Cre activity even in the absence of physiologically relevant Gipr expression. This could also result through lineage tracing, where cells are reported that only transiently expressed Gipr, although the finding that DIO-AAVs were activated in the adult hypothalamus indicates ongoing Cre expression, mirroring the detection of Gipr mRNA by RNAscope. We did not observe Ca<sup>2+</sup> responses to GIP in primary cultured neurons, however, GIPR is predominantly G<sub>a</sub> coupled, and therefore, we would not expect that its activation would result in increased intracellular calcium levels. Similarly, we do not see acute Ca<sup>2+</sup> elevation in Glp1r-positive neurons in response to GLP-1, which also predominantly activates G<sub>a</sub> rather than G<sub>s</sub> signaling. It should also be noted that activation of neurons with D<sub>a</sub> does not replicate native G<sub>a</sub>-coupled activation of GIPR. However, transgenic overexpression of GIP elicited a marked reduction in energy intake (Kim et al., 2012), and ICV administration of GIP reduced food intake (NamKoong et al., 2017). These data, combined with a recent report using a potent GIPR agonist (Mroz et al., 2019), demonstrate the potential for native GIPR signaling to impact feeding behavior. Further work should address the differences of G<sub>a</sub> versus G<sub>s</sub> activation in Gipr and Glp1r neurons and the role of non-neuronal cells in GIPR signaling in the brain. As we were unable to detect an additive effect of GLP1R and hypothalamic Gipr cell activation on food intake, future work should also address the role of Gipr cells beyond the hypothalamus as well as the metabolic outcomes of chronic rather than acute Gipr cell activation.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Human Subjects
  - Animals
  - Primary Culture of Hypothalamic Neurons
- METHOD DETAILS
  - Body Composition Analysis
  - Immunohistochemistry
  - Flow Cytometry
  - Single Cell RNA Sequencing
  - Quantitative RT-PCR
  - Calcium Imaging
  - Viral Injections
  - Food Intake Measurements
  - RNAscope
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Data Analysis
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cmet.2019.07.013.

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AUTHOR CONTRIBUTIONS

A.E.A., E.K.B., J.T., F.R., F.M.G., and C.B. designed research studies. A.E.A., E.K.B., T.D., T.S., M.G., J.P.-W., W.P., and C.B. conducted experiments. A.E.A., D.C., and B.Y.L. performed data/bioinformatics analysis. I.Z. and E.K.B., T.D., T.S., M.G., J.P.-W., W.P., and C.B. conducted experiments. A.E.A., E.K.B., J.T., F.R., F.M.G., and C.B. designed research studies. A.E.A., E.K.B., J.T., F.R., F.M.G., and C.B. wrote the manuscript.

DECLARATION OF INTERESTS

F.M.G. is a paid consultant for Kalyoee, New York. The Gribble-Reimann lab hosts projects that receive funding from MedImmune/AstraZeneca (F.M.G./F.R.). The F.R./F.M.G. laboratory has recently agreed a collaboration with Lilly on future work in the mechanism of GIPR activation. J.P.-W. joined Novo Nordisk (DK) and E.K.B. joined Absolute Antibody (UK) after completing their contributions to this manuscript while working at IMS. There are no other conflicts of interest to declare.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| Goat Polyclonal anti-GLP-1 | Santa Cruz Biotechnology | Cat # sc-7782; RRID: AB_2107325 |
| Guinea Pig Polyclonal anti-insulin | Abcam | Cat # 7842; RRID: AB_306130 |
| Goat Polyclonal anti-GFP | Abcam | Cat # 5450; RRID: AB_304897 |
| Rabbit Polyclonal anti-DsRed | Takara Bio | Cat # 632496; RRID: AB_10013483 |
| Alexa 488 secondary anti-goat | Thermo Fisher Scientific | Cat # A32814; RRID: AB_2762838 |
| Alexa 555 secondary anti-rabbit | Thermo Fisher Scientific | Cat # A32794; RRID: AB_2762834 |
| Alexa 633 secondary anti-guinea pig | Thermo Fisher Scientific | Cat # A-21105; RRID: AB_2535757 |
| Biotinylated donkey anti-goat IgG | Millipore | Cat # AP180B; RRID: AB_11214009 |
| **Bacterial and Virus Strains** | | |
| AAV-hSyn-DIO-hM3D(Gq)-mCherry | Addgene | Cat # 44361-AAV8 |
| **Biological Samples** | | |
| Human hypothalamic brain blocks | Cambridge Brain Bank | [https://www.cuh.nhs.uk/for-public/cambridge-brain-bank](https://www.cuh.nhs.uk/for-public/cambridge-brain-bank) |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Papain | Worthingon/ Lorne Labs | Cat # LK003178 |
| SuperScript III Reverse Transcriptase | Thermo Fisher Scientific | Cat # 18080093 |
| SuperScript II Reverse Transcriptase | Thermo Fisher Scientific | Cat # 18064014 |
| TaqMan Fast Universal PCR Master Mix | Thermo Fisher Scientific | Cat # 4364103 |
| Glutamate | Sigma | Cat # G1251 |
| CCK (octapeptide, sulfated) | Tocris | Cat # 1166 |
| Hexarelin | LKT Laboratories | Cat # H1893 |
| Leptin | R&D Systems | Cat # 498-OB-01M |
| 5-HT | Sigma | Cat # H9523 |
| Clozapine-N-Oxide | Sigma | Cat # C0832 |
| Exendin-4 | Tocris | Cat # 1933 |
| **Critical Commercial Assays** | | |
| RNeasy Micro Kit | QIAGEN | Cat # 74004 |
| RNeasy Plus Micro Kit | QIAGEN | Cat # 74034 |
| 10x Genomics Chromium Single Cell Library Kit v2 | 10x Genomics | Cat # 120234 |
| RNAscope® 2.5 LS Multiplex Reagent Kit | Advanced Cell Diagnostics | Cat # 322800 |
| RNAscope® LS 2.5 Probe- Mm-Glp | Advanced Cell Diagnostics | Cat # 319128 |
| RNAscope® 2.5 LS Probe- Mm-Glp1r | Advanced Cell Diagnostics | Cat # 418858 |
| RNAscope® 3-plex LS Multiplex Control Positive Probe- Mm polr2A, ppib, ubc | Advanced Cell Diagnostics | Cat # 320888 |
| RNAscope® 3-plex LS Multiplex Negative Control Probe- dapB | Advanced Cell Diagnostics | Cat # 320878 |
| RNAscope® 2.5 LS Duplex Reagent Kit | Advanced Cell Diagnostics | Cat # 322440 |
| RNAscope® LS 2.5 Probe- Hs-GLP1R | Advanced Cell Diagnostics | Cat # 519828 |
| RNAscope® 2.5 LS Probe- Hs-GIPR | Advanced Cell Diagnostics | Cat # 471348 |
| RNAscope® 2.5 LS Positive Control Probe- Hs-PPIB | Advanced Cell Diagnostics | Cat # 313908 |
| RNAscope® 2.5 LS Duplex Negative Control Probe- DapB, DapB | Advanced Cell Diagnostics | Cat # 320758 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| scRNAseq data from hypothalamic Gipr-expressing cells isolated from Gipr-Cre mice | NCBI GEO | GSE134726 |

Experimental Models: Organisms/Strains

| Model Name | Source/Description | RO# |
|------------|--------------------|-----|
| Gipr-Cre Mice | This paper | N/A |
| Glp1r-Cre Mice | Richards et al. (2014) | N/A |
| ROSA26-EYFP Cre Reporter Mice | Derived from JAX:B6.129X1-Gt(ROSA)26Sortm1(EYFP)Coy/J | N/A |
| ROSA26-GCaMP3 Cre Reporter Mice | Gift, presumed derived from JAX:B6; 129S-Gt(ROSA)26Sortm38(CAG-GCaMP3)Het/J | N/A |

Oligonucleotides

| TaqMan Gene Expression Assay | Source | Cat # |
|-------------------------------|--------|-------|
| Gipr                          | Thermo Fisher Scientific | 4448892 |
| Sst                           | Thermo Fisher Scientific | 4448892 |
| Avp                           | Thermo Fisher Scientific | 4448892 |
| Porc                          | Thermo Fisher Scientific | 4448892 |
| Agrp                          | Thermo Fisher Scientific | 4448892 |
| Htr2c                         | Thermo Fisher Scientific | 4448892 |
| Cckbr                         | Thermo Fisher Scientific | 4448892 |
| Ghsr                          | Thermo Fisher Scientific | 4448892 |
| Glp1r                         | Thermo Fisher Scientific | 4448892 |
| iCre FAM/TAMRA primers/probe:  | This paper | N/A |
| probe: 5’(6FAM)TGAAGGACATCTCCC GCACCG(TAM)3’, Fwd: 5’CAATGTGGA TCAGCATTCTCC3’, Rev: 3’GCCGAAATTGCGCAGAATCG3’ | |

Software and Algorithms

| Software | Website | RRID |
|----------|---------|------|
| CellRanger Analysis Pipeline v2.0 | https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/installation | |
| Seurat v2.3.4 R Package | http://seurat.r-forge.r-project.org/; RRID: SCR_007322 | |
| GraphPad Prism 7.0 | GraphPad Software | SCR_002798 |
| MetaFluor | Molecular Devices/ Cairn Research | http://www.moleculardevices.com/systems/metamorph-research-imaging/metalfluor-fluorescence-ratio-imaging-software; RRID: SCR_014294 |
| ZEN Blue | Zeiss | http://www.zeiss.com/microscopy/en_us/products/microscope-software/zen.html?introduction; RRID: SCR_013672 |
| HALO v2.3 | Indica Labs | http://www.indicalab.com/halo |
| HALO FISH v2.1.6 Analysis Module | Indica Labs | http://www.indicalab.com/halo |
| HALO ISH v2.2 Analysis Module | Indica Labs | http://www.indicalab.com/halo |
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Frank Reimann (fr222@cam.ac.uk). Gipr-Cre and Glp1r-Cre mice are available for collaborations upon reasonable request and will require an MTA before distribution.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Subjects
Anonymised human hypothalamic tissue samples were provided by the Cambridge Brain Bank. Subjects were approached in life for written consent for brain banking, and all tissue donations were collected and stored following legal and ethical guidelines (NHS reference number 11/0EE/0011). Hypothalamic tissue samples from two individuals were used in RNAscope analysis. Both individuals were female—one aged 95 and one aged 86 at the time of death.

Animals
All animal procedures were approved by the University of Cambridge Animal Welfare and Ethical Review Body and conformed to the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039). The work was performed under the UK Home Office Project Licenses 70/7824, PE50F6065, and PC02F3663. All mice were group-housed and maintained under SPF health/immune status in individually ventilated cages with standard bedding and enrichment unless otherwise stated. Mice were housed in a temperature (24°C) and humidity-controlled room on a 12 h light/dark cycle (lights on 6:00, lights out 18:00) with ad libitum access to water and standard laboratory chow diet (13.3% calories from fat, 22.4 % calories from protein, 64.3% calories from carbohydrate, 3.5 kcal/g; Scientific Animal Food Engineering) unless otherwise stated.

Generation of Mouse Models
Gipr-Cre knock-in mice were generated using CRISPR/Cas9 technology. In brief, initially the Gipr-coding sequence from the start codon in exon2 to the stop codon in exon 14 in the bacterial artificial chromosome RP23-384-i23 (Children’s Hospital Oakland Research Institute) was replaced with iCre-sequence (a generous gift from Rolf Sprengel, Max Planck Institute for Medical Research, Heidelberg, Germany) using Red/ET recombination technology (Genebridges). From this, a 2754bp sequence containing the iCre sequence flanked by 816bp and 879bp from the Gipr locus was amplified and cloned into pCR-BluntII-TOPO vector to be used as a donor for homologous recombination in C57B16/CBA-F1 embryos. One-cell stage fertilized mouse embryos were injected with 15ng/ul circular donor plasmid, 40ng/ul Cas9-protein (ToolGen), 0.61 pmols/ul guide RNAs targeting the wild-type Gipr gene (Dharmacon) and 50uM SCR7 inhibitor (Sigma). Positive recombinants were identified by PCR analysis specific for the recombined allele and correct recombination was confirmed by Sanger sequencing. Likely off-target genetic alterations were also sequenced and excluded, even though mice were subsequently crossed with C57B16/JN for >8 generations, which will further remove unwanted genetic modifications. Homozygous Gipr-Cre mice (= Gipr knock-out) were created by crossing heterozygous mice after >8 generations of back-crossing into C57B16.

Gipr-Cre mice were crossed with ROSA26-EYFP or ROSA26-GCaMP3 reporter strains to enable fluorescent detection and intra-cellular calcium level monitoring of cells expressing Gipr by cytosolic EYFP or GCaMP3 expression, respectively (Luche et al., 2007; Zanwala et al., 2012). Reporter strains were on a mixed C57B6J/N genetic background.

To produce mice expressing Cre in both Gipr and Glp1r expressing cells, Gipr-Cre and Glp1r-Cre (Richards et al., 2014) mice were crossed.

Primary Culture of Hypothalamic Neurons
Primary cultures of hypothalamic neurons were prepared from male and female 4 to 6-week-old GiprGCaMP3 mice as previously described (Heeley et al., 2018). For each preparation, tissue isolated from two mice was pooled. Mice were killed by cervical dislocation. Brains were extracted, placed into ice-cold Hibernate-A medium (Thermo Fisher Scientific) containing 0.25% GlutaMAX and 2% B27 (Sigma). The hypothalamus was microdissected and placed in extraction media on ice and cut into 1-mm chunks using a scalpel. Tissue was transferred to Hibernate-A minus calcium medium (BrainBits) containing papain (20 U/ml, Worthington) and 1% GlutaMAX (Sigma) pre-heated at 37°C and digested for 30 min at 37°C under agitation (Thermomixer, 500 rpm). After digestion, tissue extracts from 2 animals were pooled, transferred to a tube containing Hibernate-A with 3.5 U/ml DNase I (Sigma) and triturated using a fire-polished glass pipette. The trituration supernatant was gently loaded on top of a BSA gradient prepared in Hibernate-A medium, spun for 5 min at 300 rcf, and the pellet was resuspended in Neurobasal-A medium containing 0.3 mM FGF-Basic (PeproTech, Rocky Hill, NJ, United States of America), 0.25% GlutaMAX (Sigma), and 2% B27 (Sigma). 100 μl of resuspended cells were plated into cloning cylinders (Sigma) on glass bottom 35 mm dishes (MatTek Corporation), coated with poly-lysine (0.1 mg/ml, Sigma). Plates were placed in an incubator (37°C, 5% CO2) for 1 h. After 1 h, an additional 2 ml culture media was added and the cloning cylinders were removed.

METHOD DETAILS

Body Composition Analysis
Gipr-Cre mice heterozygous for iCre at the Gipr locus were crossed. At 6–7 weeks of age, the resulting male Gipr homozygous, heterozygous, and null offspring were placed on a 45% high fat diet (HFD; 45% calories from fat, 20 % calories from protein,
35% calories from carbohydrate, 4.7 kcal/g; Research Diets Inc.) for 17 weeks. Body weights were recorded twice per week. At the end of 17 weeks on HFD, mice were scanned using a time domain nuclear magnetic resonance (TD-NMR, Bruker Minispec, Bruker Optics, Inc.). The instrument was calibrated for these studies using a quality control check of internal voltages, temperature, magnets, and NMR parameters using a standard provided by the manufacturer.

**Immunohistochemistry**

Pancreatic and adipose tissues were fixed in 4% paraformaldehyde (PFA), dehydrated in 15% and 30% sucrose and frozen in OCT embedding media (VWR). Cryostat-cut sections (6-10 μm) were mounted directly onto poly-lysine covered glass slides (Thermo Fisher Scientific). Slides were incubated for 1 h in blocking solution containing 5% goat or donkey serum, 0.05% (v/v) Tween-20 and 1% (w/v) BSA. Slides were stained overnight at 4°C with primary antisera in the same blocking solution for proglucagon (1:100, Santa Cruz), insulin (1:100, Abcam), and/or GFP (1:1000, Abcam). Slides were washed with PBS, and incubated with appropriate secondary antisera (donkey or goat AlexaFluors 488 or 555 or 633, Thermo Fisher Scientific) diluted 1:300 for 1 h. Control sections were stained with secondary antisera alone. Sections were mounted with Hydromount (National Diagnostics) prior to confocal microscopy (TCS SP8, Leica).

Brain tissue was collected from perfusion fixed mice. Animals were anaesthetized with Euthatal solution (150 mg/kg in saline) and transcardiatically perfused with PBS followed by 4% PFA. Brains were extracted and post-fixed in 4% PFA, 30% sucrose for 48 h at 4°C. Brains were sectioned using a freezing sliding microtome into 5 subsets of 25 μm sections. For DAB-staining, slices were washed in PBS, then incubated with 0.5% (v/v) hydrogen peroxide for 15 minutes. Slices were washed, then blocked for 1 h in 5% donkey serum, 0.03% (v/v) Tween-20, then incubated with GFP antigen (1:1000, Abcam) in blocking solution overnight at 4°C. Slices were washed, then incubated in biotinylated donkey anti-goat IgG (1:400, Millipore) in 0.3% (v/v) PBS-Tween20. Sections were incubated with avidin-biotin complex (Vector Laboratories Inc.) and developed using DAB (Abcam). For immunofluorescent staining, slices were washed in PBS, then blocked for 1 h in 5% donkey serum, 0.03% (v/v) Tween-20, then incubated with GFP (1:1000, Abcam) and DsRed antisera (1:1000, Takara Bio) in blocking solution overnight at 4°C. Slices were washed, then incubated with appropriate secondary antisera (AlexaFluors 488 and 555, Thermo Fisher Scientific) diluted 1:300 for 1 h. Sections were then washed, mounted on slides and coverslipped with Vectashield (Vector Laboratories Inc.). Slides were imaged using an Axio Scan.Z1 sliced scanner (Zeiss).

**Flow Cytometry**

Single cell suspensions were prepared and pooled from the hypothalami of 2-3 Gipr<sub>EYFP</sub> or Gipr<sub>GCaMP3</sub> mice as described previously (Lam et al., 2017). Briefly, mice were sacrificed by cervical dislocation, and tissue from the hypothalamus located ventrally caudal of the optical nerve chiasm (~Bregma -0.3 to -2.92 mm) was dissected into Hibernate-A medium without calcium (BrainBits). The tissue was digested with 20 U/ml Papain (Worthington) for 30 min at 37°C, followed by trituration in Hibernate-A medium (Thermo Fisher Scientific) containing 0.005% (w/v) DNase 1 (Worthington). The cell suspension was filtered through a 40 μm mesh, and coverslipped with Vectashield (Vector Laboratories Inc.). Slides were imaged using an Axio Scan.Z1 sliced scanner (Zeiss).

**Single Cell RNA Sequencing**

3500 purified EYFP-positive cells from two 4 to 6-week old female Gipr<sub>EYFP</sub> mice were purified as described above and pooled for droplet encapsulation. cdRNA libraries from purified EYFP-positive cells were generated using the 10× Genomics Chromium Instrument and single-cell expression V2 reagents (10X Genomics). Pooled libraries were sequenced on an Illumina HiSeq 4000 instrument (26-bp first read, 76bp second read), yielding an average of 99000 reads per cell. Library preparation was performed by the Genomics and Transcriptomic Core at the Institute of Metabolic Science. The sequencing was performed at the Genomics Core, Cancer Research UK Cambridge Institute.

Sequencing reads were aligned to the mouse genome (mm10) using CellRanger analysis pipeline V2.0 (10× Genomics). Downstream analyses were performed using the Seurat v2.3.4 R package (Butler et al., 2018). Cells expressing fewer than 200 unique genes were filtered out from the analysis, leaving 2420 cells.

Gene expression measurements for each cell were normalised using a global-scaling method. 2571 highly variable genes were identified using Seurat with default settings. Dimensionality reduction was performed using principle component analysis (PCA) on these variable genes to identify statistically significant (p<0.05) PCs for downstream clustering analysis. Clustering was performed using the Seurat default graph-based clustering approach. The resultant six clusters were plotted using t-distributed stochastic neighbour embedding (t-SNE).

Marker genes were identified for all clusters using the Mann-Whitney U test, implemented by the FindAllMarkers function in the Seurat v2.3.4 R package. The top 20 gene markers were cross referenced against other bulk and scRNAseq databases (Campbell et al., 2017; Chen et al., 2017; He et al., 2016; Marques et al., 2016) to assign cell type identities for each cluster.

**Quantitative RT-PCR**

For quantitative RT-PCR conducted on purified hypothalamic Gipr-expressing cells, EYFP- or GCaMP3-positive cells were FACS-purified as described above, and collected into RLT lysis buffer (QIAGEN) before being frozen on dry ice. EYFP/GCaMP3-negative
cells were also collected. Total RNA was extracted using an RNeasy Plus Micro kit (Qiagen) according to the manufacturer’s protocol. DNaseI treatment was performed using gDNA spin columns (Qiagen). RNA was reverse transcribed using the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific).

For quantitative RT-PCR performed on homogenates of whole hypothalamic tissue, total RNA from the hypothalami isolated from 3 Gipr wildtype animals was extracted using an RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. RNA was treated with DNaseI (Invitrogen), and reverse transcribed using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific).

qPCR was performed with a QuantStudio 7 Real-Time PCR system (Applied Biosystems). The PCR reaction mix consisted of first-strand cDNA template, TaqMan™ gene expression primer/probe mix (Thermo Fisher Scientific), and PCR master mix (Thermo Fisher Scientific). Expression of the selected targets was compared to that of Actb measured on the same sample in parallel on the same plate, giving a CT difference (∆CT) for Actb minus the test gene. Statistics were performed on the ∆CT data and only converted to relative expression levels (2^∆CT) for presentation in the figures.

TaqMan™ primers/probes used are listed in the Key Resources Table.

**Calcium Imaging**

Imaging experiments were performed using Hamamatsu Orca-ER digital camera (Cairn Research) attached to an Olympus IX71 inverted fluorescent microscope with a 40× oil-immersion objective.

Cultured primary hypothalamic cells were imaged 2-16 h after dissociation. Cells were rinsed with standard bath solution (138 mmol/l NaCl, 4.5 mmol/l KCl, 4.2 mmol/l NaHCO3, 1.2 mmol/l NaH2PO4, 2.6 mmol/l CaCl2, 1.2 mmol/l MgCl2, 10 mmol/l HEPES and 10 mmol/l glucose, pH 7.4) and allowed to equilibrate for 15 min. Gipr cells were identified by their GCaMP3 fluorescence when excited with 488/8 nm and images were taken every 2 seconds using a 75-W xenon arc lamp. Emission was collected using a 510-nm long-pass filter and all images were collected on MetaFluor software (Molecular Devices). Calcium responses to 100 μmol glutamate (Sigma), 100 mmol/l CCK (Tocris), 100 mmol/l hexarelin (LKT Laboratories), 20 μmol/l 5HT (Sigma), and 10 nM leptin (R&D Systems) were recorded as increases in GCaMP3 emission. Responses to 30 mmol/l KCl were used as a positive control.

Recordings were background subtracted and represented as the 488-nm fluorescence intensity. The average GCaMP3 fluorescence intensity was calculated over 10-second time windows for the entirety of the experiment. Responses to test reagents were expressed as fold-changes determined from the peak fluorescence during a 30 second window following the perfusion of the test reagent divided by the average of the baseline taken 30 seconds before and after test reagent application and wash off, respectively. Responses were considered real if they reached a fold defined as those in which the fluorescence change following stimulus addition was at least 1.2 and above-fold.

**Viral Injections**

All viral brain injections were performed on 8-10 week old male heterozygous Gipr-Cre, Gipr-Cre x Glp1r-Cre, or Glp1r-Cre mice, producing GiprHydDq, Gipr/Glp1HydDq, or Glp1HydDq animals, respectively. Surgical procedures were performed under isofluorane anesthesia, and all animals received Metacam prior to the surgery. Mice were stereotactically implanted with bilateral stainless steel guide cannulae (Plastics One) positioned 1 mm above the ARH (A/P: −1.1 mm, D/V: −4.9 mm, lateral: +0.4 mm from Bregma). Bevelled stainless steel injectors (33 gauge, Plastics One) extending 1 mm from the tip of the guide were used for injections, delivering 500 nl AAV-hSyn-DIO-hM3D(Gq)-mCherry at 75 nl/min (Addgene # 44361-AAV8, 4×10^{12} vg/mL). Mice were allowed a 2 week recovery period.

**Food Intake Measurements**

All food intake studies were performed in a crossover manner (Figure S3) on age-matched groups after 1 week recovery and 1 week daily handling acclimatization post-surgery.

For experiments assessing the effect of Gipr D3 activation in GiprHydDq mice, animals were singly housed the day before the experiment. Mice were administered 1 mg/kg clozapine-N-oxide (CNO; Sigma) or an equivalent volume of vehicle containing a matched concentration of DMSO (1%). For light phase activation measurements, mice were injected with either CNO or vehicle at 9:00 following a 2 h fast. Food was weighed 1 h, 2 h, 4 h, 8 h, and 24 h post-injection. For dark phase activation measurements, mice were injected with either CNO or vehicle at 18:00 (start of dark cycle) and food was weighed 2 h later. In dark phase activation measurements on fasted animals, mice were fasted for 10 h prior to the injection. This was a crossover design study, and a full trial was complete after mice had received both CNO and vehicle on each testing regime. At least 3 days elapsed between each injection.

For experiments assessing the effect of Gipr/Glp1r cell co-D3 activation, Gipr/Glp1HydDq and Glp1HydDq mice were singly housed following surgery. Mice were fasted for 10 h prior to D3 activation. Mice were administered 1 mg/kg CNO or an equivalent volume of vehicle containing a matched concentration of DMSO (1%) at 18:00 (start of dark cycle) and food was weighed 2 h later. A full trial was complete after mice had received both CNO and vehicle. At least 3 days elapsed between each testing paradigm.

For experiments assessing the effect of Gipr cell D3 activation in addition to Exendin-4 (Ex-4) treatment GiprHydDq mice were singly housed following surgery. Mice were fasted for 10 h prior to D3 activation. 1.5 nmol/kg Ex-4 (Tocris) or saline control was administered subcutaneously 1 h prior to the onset of the dark phase. Mice were administered 0.3 mg/kg CNO or an equivalent volume of vehicle containing DMSO (1%) at 18:00 (start of dark cycle) and food was weighed 2 h later. A full trial was complete after mice had received both CNO and vehicle on both the Ex-4 and saline control backgrounds. At least 3 days elapsed between each testing paradigm.
RNAscope

Mouse

Brain tissue was collected from three mice for RNAscope analysis. Animals were anaesthetized with Euthatal solution (150 mg/kg in saline) and transecardially perfused with PBS followed by 4% PFA. Brains were extracted, sectioned into 0.5 cm thick slices and post-fixed in 4% PFA for 4 h before being transferred to 30% sucrose for 48 h at 4°C and then frozen. Coronal sections were cut at 12 μm and stored at -80°C until required.

Simultaneous detection of mouse Gipr and Glp1r was performed on fixed, frozen sections using Advanced Cell Diagnostics (ACD) RNAscope® 2.5 LS Multiplex Reagent Kit, RNAscope® LS 2.5 Probe- Mm-Gipr, and RNAscope® 2.5 LS Probe- Mm-Glp1r (ACD). Positive [RNAscope® 3-plex LS Multiplex Control Positive Probe - Mm polr2A, ppiB, ubc; ACD] and negative [RNAscope® 3-plex LS Multiplex Negative Control Probe dapB; ACD] controls were performed in parallel. Slides were thawed at room temperature for 10 min before baking at 60°C for 45 min. The sections were then post-fixed in pre-chilled 4% PFA for 15 min at 4°C, washed in 3 changes of PBS for 5 min each before dehydration through 50%, 70 & 100% and 100% Ethanol for 5 min each. The slides were air-dried for 5 min before loading onto a Bond RX instrument (Leica Biosystems). Slides were prepared using the frozen slide delay prior to pre-treatments using Epitope Retrieval Solution 2 (Leica Biosystems) at 95°C for 5 min, and ACD Enzyme from the Multiplex Reagent kit at 40°C for 10 min. Probe hybridisation and signal amplification was performed according to manufacturer’s instructions. The following TSA plus fluorophores were used to detect corresponding RNAscope probes using the BondRx platform according to the ACD protocol: Fluorescein (Akoya Biosciences), and Cy5 (Akoya Biosciences) were Slides were then removed from the Bond Rx and mounted using Prolong Diamond (Thermo Fisher Scientific).

Slides were imaged on a Cell Discoverer 7 microscope (Zeiss). Z-stack images with 1.0 μm spacing were taken for a representative slice from each mouse corresponding to -1.34 to -1.84 mm A/P from Bregma using a 25x water immersion objective. Z-stacks were deconvolved and compressed into 2D images using extended depth of focus (EDF) with maximum projection processing (ZEN Blue, Zeiss). EDF images were read into HALO v2.2 (Indica Labs) as .CZI files for analysis. Gipr and Glp1r positive cells were detected using the HALO FISH v2.1.6 analysis module based on intensity thresholds set using negative controls for both the fluorescein and Cy5 channels. Cells detected as positive for Gipr or Glp1r were checked by eye, and were only included in final analysis if there were 2 or more spots corresponding to Gipr mRNA, and/or 3 or more spots corresponding to Glp1r mRNA.

Human

Simultaneous detection of Human GLP1R and GIPR was performed on FFPE sections using Advanced Cell Diagnostics (ACD) RNAscope® 2.5 LS Duplex Reagent Kit, RNAscope® LS 2.5 Probe- Hs-GLP1R and RNAscope® 2.5 LS Probe- Hs-GIPR- (ACD, Hayward, CA, USA). Positive [RNAscope® 2.5 LS Positive Control Probe_Hs-PPIB] and negative (RNAscope® 2.5 LS Duplex Negative Control Probe DapB, DapB) controls were performed in parallel (ACD, Hayward, CA, USA). Briefly, sections were baked for 1 h at 60°C before loading onto a Bond RX instrument (Leica Biosystems). Slides were deparaffinized and rehydrated on board before pre-treatments using Epitope Retrieval Solution 2 (Leica Biosystems) at 88°C for 10 minutes, and ACD Enzyme from the Duplex Reagent kit at 40°C for 10 minutes. Probe hybridisation and signal amplification was performed according to manufacturer’s instructions. Fast red detection of human GLP1R was performed on the Bond Rx using the Bond Polymer Refine Red Detection Kit (Leica Biosystems) according to ACD protocol. Slides were then removed from the Bond Rx and detection of the human GIPR signal was performed using the RNAscope® 2.5 LS Green Accessory Pack (ACD) according to kit instructions. Controls were detected using both the fast red and green detection kits. Slides were heated at 60°C for 1 h, dipped in Xylene and mounted using VectaMount Permanent Mounting Medium (Vector Laboratories).

Slides were imaged on a Slide Scanner Axio Scan.Z1 microscope (Zeiss). Images were taken in regions where positive cells were detected using a 40x air objective and sharpened using the Unsharp Masking processing in ZEN Blue (Zeiss). CZI files were read into HALO v2.3 (Indica Labs) for analysis. GIPR and GLP1R positive cells were detected using the HALO ISH v2.2 analysis module with a cell classifier trained to detect cells with classical neuronal morphology. Cells detected as positive for GIPR or GLP1R were checked by eye.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data Analysis

Data are presented as mean and SD. Statistical analysis was performed using Microsoft Excel and GraphPad Prism 7.0. For all statistical tests, an α risk of 5% was used. Multiple comparisons were made using a 2-way ANOVA or a repeated measures 2-way ANOVA with a post-hoc Tukey or Sidak test, as indicated in the figure legends. Single comparisons were made using either a paired or unpaired Student’s t tests where appropriate as indicated in the figure legends. N numbers represent the number of mice used in each study as indicated in the figure legends, with the exception of calcium imaging experiments, where n represents the number of cells imaged. For calcium imaging analysis, 78 Gipr-expressing cells from 16 separate preparations (representing cells isolated from 32 mice in total) were recorded.

DATA AND CODE AVAILABILITY

All raw scRNAseq data generated from Gipr-positive hypothalamic cells have been deposited into the NCBI GEO: database. The accession number for these data is NCBI GEO: GSE134726.
Supplemental Information

Glucose-Dependent Insulinotropic Polypeptide Receptor-Expressing Cells in the Hypothalamus Regulate Food Intake

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Figure S1: Gipr-Cre mice allow for the manipulation and identification of Gipr-expressing cells in vivo (Related to Figure 1):

A. Gipr-Cre mice heterozygous for Cre at the Gipr locus were crossed producing offspring null, heterozygous, or homozygous for Gipr. Body weight was measured in response to 17 weeks on high fat diet. Significance in weight gain was tested via 2-way ANOVA with a Tukey's post hoc test; 'a' indicates P < 0.05 for Gipr −/− vs Gipr +/+; 'b' indicates P < 0.05 Gipr −/− vs Gipr +/−; n = 7 (Gipr −/−), 8 (Gipr +/−), 10 (Gipr +/+). B. After 17 weeks on HFD, body composition was measured via NMR. Significance was determined using one-way ANOVA with a Tukey's post hoc test; n = 7 (Gipr −/−), 8 (Gipr +/−), 10 (Gipr +/+). C. Relative expression of iCre and Gipr in whole islets isolated from Gipr +/−, Gipr +/−, Gipr −/− mice (n=3 for each genotype). Data are plotted as 2ΔCt compared to Actb with bars representing mean ± SD. Statistical significance was assessed through one-way ANOVA. Pancreas tissue from GiprEYFP mice was stained for GFP (green) and glucagon (red, D), or insulin (white, E). Inguinal white (F) and interscapular brown (G) adipose tissue from GiprEYFP mice was stained for GFP (green). H. Coronal sections from heterozygous GiprEYFP and heterozygous GiprGCaMP3 mice were stained for GFP. Red circles represent the presence of GFP-immunoreactive somata. Drawings are based on the Paxinos Mouse Brain Atlas with the numerical values in the bottom right corner indicating the A/P location relative to Bregma. Pir, piriform cortex; MPOM, medial preoptic nucleus; SFO, subfornical organ; AD, anterodorsal thalamic nucleus; PVA/PV, paraventricular thalamic nucleus; PaAP/PaPo, paraventricular hypothalamic nucleus; MCPO, magnocellular preoptic nucleus; sChDL, suprachiasmatic nucleus; DG, dentate gyrus; ARC, arcuate nucleus; DM, dorsomedial hypothalamus; IF, interfascicular nucleus; AP, area postrema.
Figure S2. Expression of selected neuroendocrine targets in Gipr cells (Related to Figure 1):
A. Expression matrix showing the number of cells from the neuronal cluster expressing a selection of neuroendocrine genes. RNA was extracted from FACS purified hypothalamic Gipr-expressing cells from heterozygous GiprEYFP or GiprGCaMP3 mice and converted to cDNA. Gene expression of selected neuropeptides (B) and cell surface receptors (C) in Gipr-positive and Gipr-negative cells was measured by qPCR. Data are plotted as $2^{\Delta Ct}$ compared to Actb with bar representing mean + SD. n = 3 sorts, equivalent to 8 mice.
Figure S3: Targeting of AAV-DIO-hM3D-mCherry to Gipr cells; Food intake study design (Related to Figure 3,4):

Brains were harvested from Gipr<sup>hypDq</sup> mice. Coronal sections were stained for GFP and mCherry to assess the targeting of hM3D-mCherry into Gipr-expressing cells. A. Representative slice showing GFP (green) and mCherry (red) staining. B. Enlarged image of cells demarcated by white box in panel A. C. Illustration of crossover design used for experiments represented in Figure 3 and 4F. Each mouse served as its own control. D. Heterozygous Gipr-Cre mice that had not received injections of AAV-DIO-hM3D-mCherry were administered either CNO (1 mg/kg) or vehicle i.p following a 10hr daytime fast in a crossover design study. The amount of food consumed during the beginning of the dark phase was measured 2hr post-injection and compared using a paired t-test. n= 9. E. Illustration of crossover study design used for experiments represented in Figure 4G. Each mouse served as its own control.
Figure S4: Hypothalamic \textit{Gip} localisation; CNO/Ex-4 dose response (Related to Figure 4):

A. Relative expression of \textit{Gipr} and \textit{Glpr} in whole hypothalamic homogenates in WT mice (n=3). Data are plotted as $2^{\Delta Ct}$ compared to Actb with bars representing mean +/- SD. Expression levels of \textit{Gipr} and \textit{Glpr} were compared using an unpaired student's t test. ***P<0.001; n =3.

B. Human hypothalamus tissue samples were labelled for GIPR and GLP1R mRNA using RNAscope. Cells lining the third ventricle (3V), likely to be ependymal cells expressing GIPR.

C. Following a 10 h daytime fast heterozygous \textit{Gipr-Cre} and \textit{Gipr} WT mice were injected with Ex-4 (1.5, 3, or 10 nmol/kg) or saline s.c. 1hr prior to the onset of the dark phase. At the onset of the dark phase food was presented, and food intake measurements were taken 2hr later. This was a crossover design study where each mouse served as its own control. Food intake was compared using a repeated measures 2-way ANOVA with a Sidak’s post hoc test. *P<0.05, ***P<0.001; n = 12.

D. Following a 10hr daytime fast \textit{Gip}hypDq mice were injected with saline s.c. 1hr prior to the onset of the dark phase. CNO (0.1, 0.3, or 1 mg/kg) or vehicle was injected i.p. at the onset of the dark phase, food was presented, and food intake measurements were taken 2hr post-activation. This was a crossover design study where each mouse served as its own control. Food intake was compared using a repeated measures 2-way ANOVA with a Sidak’s post hoc test. *P<0.05; n = 12.