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Functionally distinct POMC-expressing neuron subpopulations in hypothalamus revealed by intersectional targeting

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Pro-opiomelanocortin (POMC)-expressing neurons in the arcuate nucleus of the hypothalamus represent key regulators of metabolic homeostasis. Electrophysiological and single-cell sequencing experiments have revealed a remarkable degree of heterogeneity of these neurons. However, the exact molecular basis and functional consequences of this heterogeneity have not yet been addressed. Here, we have developed new mouse models in which intersectional Cre/Dre-dependent recombination allowed for successful labeling, translational profiling and functional characterization of distinct POMC neurons expressing the leptin receptor (Lepr) and glucagon like peptide 1 receptor (Glp1r). Our experiments reveal that POMCLepr+ and POMCGlp1r+ neurons represent largely nonoverlapping subpopulations with distinct basic electrophysiological properties. They exhibit a specific anatomical distribution within the arcuate nucleus and differentially express receptors for energy-state communicating hormones and neurotransmitters. Finally, we identify a differential ability of these subpopulations to suppress feeding. Collectively, we reveal a notably distinct functional microarchitecture of critical metabolism-regulatory neurons.

The melanocortin circuitry comprising agouti-related peptide (AgRP)-expressing neurons and POMC-expressing neurons in the arcuate nucleus of the hypothalamus (ARC) represents a prototypic homeostatic neurocircuit of metabolic homeostasis. These targets of insulin, leptin and glucagon like peptide (Glp1) integrate multiple inputs to compute the energy state of the organism and adapt feeding behavior. The POMC pro-peptide is processed to α-melanocyte-stimulating hormone, which is an activator of the melanocortin 4 receptor to suppress food intake. Conversely, AgRP released from AgRP neurons acts as an inverse agonist on the melanocortin 4 receptor, thereby promoting feeding. In addition, the melanocortin circuitry is an integrative regulator of numerous physiological functions. For instance, insulin action in AgRP neurons is required to efficiently suppress hepatic glucose production, and abrogation of insulin and leptin receptors from POMC neurons causes diabetes in mice. Further, acute chemogenetic activation or inhibition of Cre-expressing neurons allows for successful labeling, translational profiling and functional characterization of distinct POMC neurons expressing the leptin receptor (Lepr) and glucagon like peptide 1 receptor (Glp1r). Our experiments reveal that POMCLepr+ and POMCGlp1r+ neurons represent largely nonoverlapping subpopulations with distinct basic electrophysiological properties. They exhibit a specific anatomical distribution within the arcuate nucleus and differentially express receptors for energy-state communicating hormones and neurotransmitters. Finally, we identify a differential ability of these subpopulations to suppress feeding. Collectively, we reveal a notably distinct functional microarchitecture of critical metabolism-regulatory neurons.
an additional, complementary recombinase system that builds on expression of Dre-recombinase, an alternative and complementary approach has been defined. We have dedicated our efforts toward further developing a tool-box of transgenic mice that offers the opportunity to genetically dissect heterogeneous neuron populations, through combinatorial

**Fig. 1** | The POMC<sup>Δlox</sup> driver line successfully targets POMC neurons. 

**a.** Schematic showing POMC<sup>Δlox</sup>-dependent recombination in the ROSA26rSrZsGreen reporter line. Excision of rox-flanked stop cassette leads to ZsGreen expression in POMC neurons.

**b.** ZsGreen expression across the rostral, mid and caudal sections of the ARC in POMC<sup>Δlox</sup> ROSA26rSrZsGreen mice at 15 weeks of age.

**c.** Dispersed ZsGreen expression in the intermediate and anterior lobes of the pituitary in POMC<sup>Δlox</sup> ROSA26rSrZsGreen mice. C1, C2 and C3 depict magnifications of the posterior, intermediate and anterior pituitary, respectively. Scale bar, 150 μm (whole image) and 50 μm (magnified images).

**d.** RNA ISH against Pomc/ZsGreen (top) and Agrp/ZsGreen (bottom) in POMC<sup>Δlox</sup> ROSA26rSrZsGreen mice. Magnifications of the boxes are displayed on the right of each image. Scale bars, 50 μm (whole image) and 20 μm (magnified images). 3V, third ventricle.

**e.** Percentage of ZsGreen-positive cells coexpressing or lacking expression of either Pomc or Agrp, quantified from RNA ISH (d). Data are represented as the mean ± s.e.m. (Pomc: 97.57 ± 0.92; Agrp: 1.88 ± 0.36; non-Pomc: 2.43 ± 0.92; non-Agrp: 98.12 ± 0.36; n = 4 mice; a minimum of 13 sections were analyzed for each group).
Cre-dependent and Dre-dependent recombination, allowing for successful labeling, three-dimensional (3D) imaging, translational profiling and functional characterization of POMC neurons expressing Lepr or Glp1r, uncovering a new organismal and functional microarchitecture of critical metabolism-regulatory neurons.

Results

Generation of POMC<sup>Cre</sup>-transgenic mice. We generated mice, which express the Dre-recombinase under control of the POMC promoter (POMC<sup>Cre</sup> mice; Extended Data Fig. 1a). Assessment of Dre-dependent recombination via visualization of Dre-dependent reporter expression revealed ZsGreen-positive cells in the ARC and in the anterior and intermediate lobes of the pituitary for five of seven transgenic lines (Fig. 1a–c). In particular, one line effectively labeled POMC cells in the pituitary and in the ARC (Fig. 1b,c). Double RNA in situ hybridization (ISH) revealed that 97.6% of ZsGreen-positive neurons in the ARC expressed Pomc, while only 1.9% expressed Agrp (Fig. 1d,e). Correspondingly, the proportion of ZsGreen-labeled non-Pomc-expressing cells was 2.4% and that of ZsGreen-labeled non-Agrp-expressing cells was 98.1% (Fig. 1d,e). We observed no POMC<sup>Cre</sup>-dependent labeling of ZsGreen-positive neurons in the nucleus tractus solitarius (NTS; Extended Data Fig. 1b). Assessment of the bacterial artificial chromosome (BAC) transgene copy number revealed stable transgenerational copy numbers, indicating a single genomic integration (Extended Data Fig. 1c). Longitudinal assessment of Dre-dependent recombination revealed a continued increase of Dre-dependent recombination in POMC neurons from 3–15 weeks of age (Extended Data Fig. 1d). Thus, the later onset of Dre-dependent recombination in our model allows selective marking of bona fide POMC neurons without substantial recombination in the functionally antagonistic AgRP neurons as present in POMC<sup>Cre</sup>-transgenic mice<sup>11</sup>.

Next, we investigated whether insertion of the BAC transgene in this POMC<sup>Cre</sup> line resulted in any metabolic phenotype. However, POMC<sup>Cre</sup> mice did not exhibit any differences in body weight, food intake, locomotor activity, energy expenditure or glucose tolerance compared to their littermate controls (Extended Data Fig. 1e–n).

Combinatorial recombinase-dependent marking of heterogeneous POMC neuron populations. Leptin and Glp1 target POMC neurons to mediate at least part of their metabolism-regulatory functions<sup>4,19</sup>. Single-cell sequencing had revealed no overlap between Glp1r and Lepr expression in POMC neurons<sup>21</sup>. We therefore further examined whether Lepr and Glp1r mRNA levels were indeed not coexpressed in POMC neurons. We used double fluorescence RNA ISH against Lepr and Glp1r mRNAs in POMC neurons of wild-type mice, which revealed that while 10.2% of POMC neurons expressed mRNAs of both receptors, the larger proportion of POMC neurons expressed one receptor in the absence of the other (Fig. 2a,b).

To genetically mark heterogeneous POMC cell populations through Cre/Dre-dependent, intersectional recombination, we established reporter mouse models carrying a cDNA encoding the fluorescent marker protein NLS-ZsGreen in the ROSA26 locus<sup>20</sup>. The expression of ZsGreen is prevented by a loxP-flanked (lSl) and an additional rox-flanked (rSr) transcriptional STOP cassette (Fig. 2c). POMC<sup>Dre</sup> mice were crossed to Lepr<sup>Cre</sup> or Glp1r<sup>Cre</sup> mice<sup>21</sup>. Resulting double transgenic POMC<sup>Cre</sup> Lepr<sup>Cre</sup> or POMC<sup>Cre</sup> Glp1r<sup>Cre</sup> mice were bred with homozygous ROSA26lSlrSrZsGreen<sup>−/−</sup> mice to yield four different genotypes. This produced mice heterozygous for the reporter (ROSA26lSlrSrZsGreen<sup>+/−</sup>) in the absence of POMC<sup>Cre</sup> and of the respective Cre transgene, carrying only the POMC<sup>Cre</sup> transgene, carrying only the respective Cre transgene or the combination of both; that is, POMC<sup>Cre</sup> Lepr<sup>Cre</sup> or POMC<sup>Cre</sup> Glp1r<sup>Cre</sup> (Extended Data Fig. 2b). Assessment of ZsGreen expression in these mice revealed that ZsGreen immunofluorescence was absent in the ARC of mice carrying neither recombaine transgene or which only carried the POMC<sup>Cre</sup> or the respective Cre transgene (Lepr<sup>Cre</sup> or Glp1r<sup>Cre</sup>; Fig. 2d). In contrast, only triple transgenic mice exhibited ZsGreen expression in the ARC (Fig. 2d). Quantification of ZsGreen-positive neurons expressing Pomc revealed that 99.2% of ZsGreen-labeled cells in POMC<sup>Cre</sup> Lepr<sup>Cre</sup> mice, and 94.6% of ZsGreen-labeled cells in POMC<sup>Cre</sup> Glp1r<sup>Cre</sup> mice expressed Pomc (Fig. 2e). Further, light-sheet fluorescence microscopy (LSFM) on cleared brain tissue revealed exclusive expression of ZsGreen-positive cells in the ARC of POMC<sup>Cre</sup> Lepr<sup>Cre</sup> ROSA26lSlrSrZsGreen<sup>−/−</sup> mice (Extended Data Fig. 2c), indicating successful intersectional transgenic marking of selective POMC neuron subpopulations.

Distinct anatomical distribution of POMC<sup>Lepr</sup>- and POMC<sup>Glp1r</sup>-expressing neurons in the ARC. To obtain a holistic 3D representation of the neuronal subpopulations, we used the tissue-clearing technique uDISCO<sup>22</sup> in combination with LSFM. The 3D images obtained via LSFM from each individual mouse were registered onto a reference atlas, that is, a grayscale Nissl volume of reconstructed brain (Allen Brain 25-µm reference atlas), for subsequent quantitative image analysis. Image registration algorithms vary based on the transformation models they use to relate the target image space to the reference image space. Thus, we used the VINCI software for this purpose<sup>23</sup>. The neuronal coordinates were subsequently extracted and plotted as an isosurface density plot using a kernel mesh fit onto the neuronal population (Fig. 3a–f). Statistical analysis of these neuronal distributions showed significant differences in the localization patterns of Lepr-expressing POMC neurons in comparison to those with Glp1r expression (Fig. 3g). To compare this distribution pattern of POMC cells endogenously expressing Pomc...
Lepr or Glp1r as assessed by RNA ISH (Fig. 2a), we created coronal cross sections from the 3D coordinates of transgenically labeled POMCLepr+ and POMCGlp1r+ neurons (Extended Data Fig. 2d) and compared them to the distributions of POMC neurons endogenously expressing Lepr and Glp1r in a corresponding anatomical localization (Extended Data Fig. 2e), revealing a similar, differential
distribution pattern of both subpopulations in the two experimen-
tal approaches.

Similar projection patterns of POMCLepr+ and POMCGlp1r+ neu-
rons. To investigate, whether the Lepr- or Glp1r-expressing POMC
subpopulations could target distinct projection sites, we used mice expressing tdTomato upon Cre/Dre-dependent recombination (Extended Data Fig. 3a). POMCLepr+ and POMCGlp1r+ animal samples allowed for visualization of axonal projections and dendrites of POMCLepr+ and POMCGlp1r+ neurons (Extended Data Fig. 3e). Sections from well-defined projection areas of melanocortin neu-
rons in the bed nucleus of the striae terminais (BNST), peri-aqueductal gray (PAG), dorsomedial nucleus of the hypothalamus (DMH) and the paraventricular nucleus of the hypothalamus (PVH) were examined for immunoreactive fiber density by the quantification of POMC expression and transgenic tdTomato label-
ing (Extended Data Fig. 3b,c). tdTomato immunoreactive fiber den-
sity in the investigated areas was consistently higher in sections of POMCLepr+/tdTomato+ /POMCGlp1r+ mice compared to those of POMCLepr+/tdTomato−/POMCGlp1r− animals (Extended Data Fig. 3d), as explained by the larger population of genetically marked POMCLepr+ compared to POMCGlp1r+ neurons.

We also analyzed the projection densities in POMC target regions via whole-brain tdTomato immunostaining and LSPM-based image acquisition followed by data processing based on co-registration onto a unified anatomical atlas. This allowed for assessment of projec-
tion densities in the different regions of interest (ROIs; Fig. 3h and Extended Data Fig. 4). At the same time, projection intensities could be normalized to the number of transgenically labeled POMC neurons of the individual animals, revealing a similar projection intensity in the different regions taking into account the differential population sizes of both POMC subpopulations (Fig. 3i).

DREADD-dependent activation of POMCLepr+ and POMCGlp1r+ neurons differentially regulates food intake. To investigate the role of distinct POMC neuron populations in energy homeosta-
sis in vivo, we generated mice allowing for combinatorial Cre/
Dre-dependent expression of the activator DREADD (Designer Receptors Exclusively Activated by Designer Drugs) receptor hM3Dq and ZsGreen (Fig. 4a and Extended Data Fig. 5a,b). Male POMCLepr+ /ZsGreen+ mice were positive for Lepr expression (93.4%), supporting specific targeting of POMCLepr+ neurons (Fig. 4c). Furthermore, the proportion of ZsGreen-expressing cells that coexpressed POMC and Lepr mRNA was assessed, revealing the expression of ZsGreen (and thus hM3Dq) in 47.3% of the cells expressing Lepr and POMc (Fig. 4d).

To investigate the efficiency of CNO-dependent cell activation, the ratio of Fos-positive cells over ZsGreen and POMc double-positive neurons was assessed in saline-injected and CNO-injected POMCLepr+ /ZsGreen+ animals. While only 6.9% of hM3Dq-expressing POMC neurons were positive for Fos mRNA expression in saline-injected animals, this proportion increased to 94.0% after CNO injection (Fig. 4e). Similarly, POMCLepr+ /ZsGreen+ animals exhibited CNO-dependent activation of POMCLepr+ neurons in males (Fig. 4c–e and Extended Data Fig. 5c). Moreover, CNO-only injection elicited increased Fos expression in POMCLepr+ /ZsGreen+ and POMCGlp1r+ /ZsGreen+ animals but not in ROSA26SlrShrM3Dq−/− animals lacking Dre and Cre expression or expressing either recombine alone (Extended Data Fig. 5d,e). Moreover, more than 90% of the labeled POMC neurons in POMCLepr+ /ZsGreen+ and POMCGlp1r+ /ZsGreen+ mice were Lepr positive and 93.5% expressed Glp1r (Extended Data Fig. 5f,g). Finally, CNO application similarly activated POMCLepr+ and POMCGlp1r+ neurons in female triple transgenic animals as observed in males (Extended Data Fig. 5h–j).

Next, we assessed parameters of energy homeostasis upon treat-
ing POMCLepr+ /ZsGreen+ or POMCGlp1r+ /ZsGreen+ mice at the beginning of the natural feeding cycle, reduced food intake starting at 3 h after CNO injection (Fig. 4f). This effect cumu-
lated into a statistically nonsignificant 15.6% suppression of feeding over the duration of the dark cycle (Fig. 4f). However, chemogenetic activation of male POMCLepr+ /ZsGreen+ mice resulted in an earlier (2.5 h after CNO injection) and even stronger feeding suppression, accounting for a significant 32.3% reduction in food intake over the duration of the dark cycle (Fig. 4g). In contrast, chemogenetic activation of POMCLepr+ or POMCGlp1r+ neurons in male mice did not affect energy expenditure or locomotor activity, while mildly shifting the respiratory exchange ratio toward fatty acid metabolism (Extended Data Fig. 6c–h). Interestingly, chemogenetic activation of POMCLepr+ and POMCGlp1r+ neurons in female mice did not suppress feeding.

Given the different kinetics in feeding suppression upon activation of POMCGlp1r+ neurons compared POMCLepr+ neurons in male

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Fig. 3 | POMCLepr+ and POMCGlp1r+ show distinct spatial distribution throughout the ARC. a–c. Representative 3D reconstruction of the entire POMC population labeled in POMCLepr+ /ZsGreen+ mice (a), and 3D reconstruction of POMC subpopulations in POMCLepr+/ZsGreen+ (b) or POMCGlp1r+/ZsGreen+ (c) mice at 15 weeks of age. Scans were obtained using the LSPM at x8 total magnification. n = 9 (a), n = 7 (b) and n = 8 (c). d. Isosurface density plots of the entire POMC population (d), and the POMCLepr+ (e) and POMCGlp1r+ (f) subpopulations. Gray shaded areas in e and f depict the entire POMC population. g. Statistical representation of the differences in distribution between the POMCLepr+ and POMCGlp1r+ subpopulations using a two-tailed t-test. P values are plotted as spheres within the space occupied by the POMC neurons (background). The size and color of the spheres indicate the significance values in ranges of yellow to red (POMCLepr+ and green to blue (POMCGlp1r+). h. Representative images of 3D projection densities in POMCLepr+ /ZsGreen+ mice in the PVH, PAG, DMH, BNST and NTS. i. Quantification of 3D projection densities shown in h, normalized to the number of neurons. Data are represented as mean ± s.e.m., from n = 3 mice per group. POMCLepr+: BNST: 56,068,002.46 ± 4,157,623.02, DMH: 33,195,450.61 ± 2,618,432.01, PAG: 10,481,156.64 ± 819,984.06, PVH: 18,370,352.69 ± 1149,637.53, NTS: 45,834,835.18 ± 6,188,416.90. POMCGlp1r+: BNST: 63,188,837.7 ± 2,376,156.35, DMH: 37,050,145.65 ± 478,713.82, PAG: 11,722,976.37 ± 369,033.55, PVH: 20,851,215.3 ± 337,219.38, NTS: 5,294,0731.54 ± 984,858.12, BNST, Glp1r+ versus Lepr+: P = 0.249747, t = 1.345, df = 4, DMH, Glp1r+ versus Lepr+: P = 0.632027, t = 1.448, df = 4. PAG, Glp1r+ versus Lepr+: P = 0.632027, t = 1.381, df = 4. PVH, Glp1r+ versus Lepr+: P = 0.432578, t = 2.071, df = 4. NTS, Glp1r+ versus Lepr+: P = 0.632027, t = 0.9654, df = 4, unpaired Student’s t-test, Holm–Sidák correction.
mice, we asked whether both neuronal subpopulations might differ in their neurotransmitter characteristics. Therefore, we compared the expression of the vesicular GABA transporter (Vgat or Slc32a1) and of the vesicular glutamate transporter (Vglut2 or Slc17a6) in POMC<sup>Lepr</sup> and POMC<sup>Glp1r</sup> neurons (Extended Data Fig. 7a). Quantification of the proportion of GABAergic and glutamatergic POMC neurons in both mouse lines revealed a slightly higher proportion of glutamatergic POMC<sup>Lepr</sup> neurons. However, non-POMC
neurons appeared to exhibit a stronger signal for Vglut2 expression compared to POMC neurons (Extended Data Fig. 7b).

Since POMC is also expressed not only in the ARC, but also in the anterior and intermediate lobes of the pituitary, we compared the detectability of ZsGreen-positive cells in the pituitary of POMCCre Leptr-/-/Rosa26SlIrSrzsGreen+/- or POMCCre Glp1r-/-/Rosa26SlIrSrzsGreen+/- animals (Extended Data Fig. 8a,b). Although ZsGreen expression was detectable in the majority of cells in the intermediate lobe of both POMCCre Leptr-/-/Rosa26SlIrSrzsGreen+/- and POMCCre Glp1r-/-/Rosa26SlIrSrzsGreen+/- animals, we did not detect ZsGreen-positive cells in the anterior lobe of the pituitary in either mouse line (Extended Data Fig. 8a,b). Activation of each subpopulation did not alter circulating corticosterone concentrations in the serum of either mouse line (Extended Data Fig. 8c,d).

Distinct translational signatures of POMCpr+ and POMCprb+ neurons. To obtain detailed transcriptional/translational profiles of POMCpr+ and POMCprb+ neurons, we generated mice enabling combinatorial Cre/Dre-dependent expression of the L10a ribosomal protein fused to enhanced green fluorescent protein (EGFP; Fig. 5a and Extended Data Fig. 9a,b). Hypothalami of resulting triple transgenic animals (POMCCre Leptr-/-/Rosa26SlIrSrEgfpfpl10a-/- and POMCCre Glp1r-/-/Rosa26SlIrSrEgfpfpl10a-/- mice) were used for RNA extraction. Biological replicates for the individual lines provided input RNA samples, which were subjected to anti-EGFP immunopurification of EGFP-tagged ribosomes of POMCpr+ or POMCprb+ neurons. Having confirmed the specific expression of the Egfpfpl10a fusion protein using the Rosa26SlIrSrEgfpfpl10a mouse line (Extended Data Fig. 5c) and validated the successful, specific pulldown of RNA of subpopulations of POMC neurons, our aim was to compare the ribosome-associated transcriptome of POMCpr+ neurons with POMCprb+ neurons. Thus, RNA-seq was performed on input and immunopurification (IP) RNA samples of POMCCre Leptr-/-/Rosa26SlIrSrEgfpfpl10a-/- and POMCCre Glp1r-/-/Rosa26SlIrSrEgfpfpl10a-/- mice. In total, reads mapped to 17,239 genes. Principal-component analyses for the highest expressed genes showed clear separation of input from IP samples, as well as for POMCCre Leptr-/-/Rosa26SlIrSrEgfpfpl10a-/- IP from POMCCre Glp1r-/-/Rosa26SlIrSrEgfpfpl10a-/- IP samples (Extended Data Fig. 9a). Next, the expression level of each IP sample was normalized to its own input sample. The normalized expression analysis of all samples from both triple transgenic mouse groups was used to visualize the differences in expression of all detected genes in both subpopulations. Almost an equal number of genes were expressed at higher levels in the POMCpr+ compared to POMCprb+ neurons and vice versa (Fig. 5b).

As expected, POMC transcript reads were drastically (>1,000-fold) enriched in both POMCpr+ and POMCprb+ neurons compared to the global hypothalamic background (Fig. 5c). POMCpr+ neurons exhibited a stronger enrichment for POMC mRNA compared to POMCprb+ neurons, indicating a potentially differential POMC mRNA expression (Fig. 5c). Similarly, we found clear overrepresentation for the reads of Lepr in IP samples from POMCpr+ neurons and an enrichment of Glp1r expression in IP samples from POMCprb+ neurons (Fig. 5c).

To identify which classes of transcripts differentiate POMCpr+ and POMCprb+ neurons, we subjected the differentially enriched genes of each subpopulation to a Gene Ontology (GO)-term analysis (Extended Data Fig. 9e–g). This analysis revealed that, specifically, transcripts in the GO terms neuropeptide signaling, regulation of response to nutrient levels and dendrite cytoplasm differed between both types of neurons (Extended Data Fig. 9e–g).

To further investigate which neuropeptides and neuropeptide receptors are differentially expressed by POMCpr+ and POMCprb+ neurons, the relative expression levels for all genes listed in the GO term ‘neuropeptide-signaling pathway’ (GO:0007218) were analyzed. While opioid receptors (Oprd1 and Oprm1) and enrichment of neuropeptide Y (Npy) was higher in POMCprb+ neurons, enrichment for prodynorphin (Pdyn), Cocaine and amphetamine regulated transcript protein (Carpt), Neurinemed-U receptor 2 (Nm2u2), and NPY-receptor (Npy1r) was higher in POMCpr+ neurons (Fig. 5d).

Next, we aimed to investigate whether and to which extent the genes enriched in either subpopulation identified by targeted ribosomal profiling might define clusters of POMC neurons as revealed by clustering in single-cell RNA-seq. The first data source was droplet single-cell RNA-seq of 20,921 cells from the ARC/median eminence1. Here, we filtered cells expressing POMC (4,248/20,921 cells) and clustered them using the R Seurat package1. This yielded the identification of 11 clusters of POMC-expressing neurons (Fig. 5e and Extended Data Fig. 9b). The second data source was a single-cell mRNA-seq dataset of 163 genetically marked POMC-expressing neu...
rons of mice yielding four clusters of POMC neurons\(^{12}\) (Fig. 5c). In Fig. 5e, we filtered the volcano plot of Fig. 5b for the markers of each cluster from the aforementioned datasets. For each of these clusters, we determined the log ratio of the marker genes differentially expressed in our ribosomal profiling between POMC\(^{Lepr^+}\) and POMC\(^{Glp1r^+}\) neurons (log ratio = \(\log_2 (n_{Glp1r}/n_{Lepr})\)). We
Fig. 5 | POMCLepr and POMCGlp1r neurons exhibit distinct translational profiles. a. Illustration of experimental mice and schematic diagram showing Dre- and Cre-dependent targeted expression of EGFPL10a in either POMCLepr or POMCGlp1r neurons. Excision ofloxP-flanked and rox-flanked stop cassettes through recombination of both Dre and Cre drivers led to EGFPL10a expression in the targeted subpopulation. b. Volcano plot of differentially ribotag-enriched transcripts in POMCLepr and POMCGlp1r neurons. Significantly differentially enriched transcripts (P ≤ 0.05) are indicated in the colored region. Yellow and cyan depict a significantly higher enrichment in POMCLepr and POMCGlp1r neurons, respectively. P values adjusted for multiple comparisons were calculated by DESeq2 (1.26.0). c. Expression ofPomc, Lepr andGlp1r in input (IN) and IP samples of each subpopulation. Statistics were analyzed using unpaired two-tailed Welch’s t-test. Pomc: Lepr IN: 39.44 ± 19.39, Lepr IP: 4,740.55 ± 618.41, IN vs IP, t = 7.6, P = 0.0047. Glp1r IN: 14.14.55 ± 0.49, Glp1r IP: 1005.42 ± 40.19, IN vs IP, t = 24.7, P = 0.0016. Lepr: Lepr IN: 0.92 ± 0.14, Lepr IP: 11.12 ± 2.05, IN vs IP, t = 4.95, P = 0.0155. Glp1r IN: 0.62 ± 0.07, Glp1r IP: 1.50 ± 0.40, IN vs IP, t = 2.18, P = 0.000153, Glp1r IN: 2.97 ± 0.38, Lepr IP: 8.80 ± 1.82, IN vs IP, t = 3.15, P = 0.0158. Glp1r IN: 3.23.55 ± 0.35, Glp1r IP: 29.17 ± 2.83, IN vs IP, t = 9.08, P = 0.0010. *P ≤ 0.05, **P ≤ 0.01. d. Significantly differentially enriched genes (P ≤ 0.05) of POMCLepr and POMCGlp1r neurons, belonging to the GO term ‘neuropeptide-signaling pathways’. Vertical colored area separates higher enrichment in POMCLepr neurons (left) from higher enrichment in POMCGlp1r neurons (right). e. Overlap analysis of the publicly available single-cell RNA-seq data from mouse hypothalami with our dataset. Here, the volcano plot in b is filtered for the markers reported for each cluster. For POMCLeprGlp1r ROSA26SlrSrEGFPL10a, n = 4 samples of pooled hypothalami from N = 24 mice; for POMCGlp1rGlp1r ROSA26SlrSrEGFPL10a, n = 3 samples of pooled hypothalami from N = 36 mice. Data are represented as the mean ± s.e.m.
**Fig. 6 | Differential expression of endogenous mRNAs for identified candidates in POMC\textsuperscript{Lepr\textsuperscript{+}} and POMC\textsuperscript{Glpi\textsuperscript{+}} neurons.**

**a.** Representative microscopic images of RNA ISH against Pomc, Glp1r and Lepr together with differentially expressed neuropeptidergic signaling candidate RNAs identified in the ribotag experiments, Carpt, Npy1r, Oprm1 and Nmur2 in C57BL/6N mice at 12 weeks of age. First image shows ISH in the ARC with nuclear counterstain (blue, DAPI). Magnifications of the boxes (right) are shown with the indicated stainings. Pomc-positive neurons are outlined in white. Yellow and cyan arrows indicate Lepr-positive or Glp1r-positive POMC neurons, respectively. Scale bars represent 100 μm in the merged image and 25 μm in the magnifications.

**b.** Violin plots showing the quantified intensity of Pomc mRNA across the rostrocaudal axis of the ARC in POMC\textsuperscript{Lepr\textsuperscript{+}} or POMC\textsuperscript{Glpi\textsuperscript{+}} neurons as assessed from RNA ISH (Fig. 2a). Data are from \( n = 4 \) mice, with a minimum of four sections analyzed per animal. \( P = 0.0029. \) a.u., arbitrary units.

**c.** Violin plots showing quantified expression of the RNA-seq candidates measured as integrated density in POMC\textsuperscript{Lepr\textsuperscript{+}} or POMC\textsuperscript{Glpi\textsuperscript{+}} neurons as assessed from RNA ISH (a). Carpt: Lepr\textsuperscript{+}, Q: 2.977, Q: 6.729, Q: 13.244, Glp1r\textsuperscript{+}, Q: 2.405, Q: 4.741, Q: 9.198, Lepr\textsuperscript{+} versus Glp1r\textsuperscript{+}, \( U=9,252, P<0.0001; \) Opm1: Lepr\textsuperscript{+}, Q: 1.609, Q: 2.470, Q: 3.887, Glp1r\textsuperscript{+}, Q: 1.154, Q: 1.802, Q: 3.069, Lepr\textsuperscript{+} versus Glp1r\textsuperscript{+}, \( U=22,352, P<0.0001. \) P values were calculated using the unpaired Mann–Whitney (MW) U-test. * \( P \leq 0.05, ** P \leq 0.01, *** P \leq 0.001, **** P \leq 0.0001. \)
considered a cluster to be enriched for POMC<sup>Qtrt</sup> marker genes with a log ratio ≥ 2 and vice versa enriched for POMC<sup>Lepr</sup> marker genes with a log ratio ≤ –2. Applying this threshold to our clustering of POMC-positive neurons from the ARC/median eminence dataset identified clusters 2 and 10 as enriched for POMC<sup>Lepr</sup> marker genes and cluster 3 as enriched for POMC<sup>Qtrt</sup> marker genes (Fig. 5e). Similarly, we defined clusters 3 and 4 of the dataset of fluorescence-activated cell sorting (FACS)-purified POMC neurons enriched for POMC<sup>Lepr</sup> and clusters 1 and 2 enriched for POMC<sup>Qtrt</sup> marker genes (Fig. 5e). Collectively, our translational profiles of POMC<sup>Lepr</sup> and POMC<sup>Qtrt</sup> neurons allowed for successful independent identification of molecularly defined POMC clusters, which had been defined based on single-cell RNA-seq.

**Verification of endogenously expressed, differentially regulated genes in POMC<sup>Lepr</sup> and POMC<sup>Qtrt</sup> neurons.** We then aimed to validate the mRNA expression profiles of genes characterized as differentially enriched in genetically marked POMC<sup>Lepr</sup> and POMC<sup>Qtrt</sup> neurons via RNA ISH upon co-detection of endogenously expressed Lepr and Glp1r mRNA in wild-type mice (Fig. 6a). These analyses confirmed increased expression of POMC<sup>Qtrt</sup> in neurons to POMC<sup>Lepr</sup> neurons compared to POMC<sup>Qtrt</sup> neurons (Fig. 6b). Similarly, Cartpt expression was increased in POMC<sup>Qtrt</sup> neurons compared to POMC<sup>Lepr</sup> neurons (Fig. 6c). When we investigated the expression of exemplary genes encoding receptors for neuropeptides and energy-sensing signals, we confirmed the differential enrichment of Npy1r, Oprm1 and Nmn2r in POMC<sup>Lepr</sup> and POMC<sup>Qtrt</sup> neurons (Fig. 6c), as previously indicated by our RNA-seq results (Fig. 5d).

**POMC<sup>Lepr</sup> and POMC<sup>Qtrt</sup> neurons have distinct intrinsic electrophysiological properties.** Next, we performed perforated patch-clamp recordings in genetically marked Lepr- and Glp1r-expressing POMC neurons in POMC<sup>lepr<sup>Cre</sup></sup> ROSA26SlirSlrZsGreen<sup>+</sup> or POMC<sup>Glp1r<sup>Cre</sup></sup> ROSA26SlirSlrZsGreen<sup>+</sup> mice. Both neuronal populations have distinct functional properties and input–output relationships. While there were no differences in certain general properties including whole-cell capacitance, spontaneous firing rate and in the proportion of spontaneously active or silent neurons (Extended Data Fig. 10a,b), we found differences in key intrinsic electrophysiological properties (Fig. 7a–i). POMC<sup>Qtrt</sup> neurons were more depolarized (Fig. 7a), had a higher input resistance (Fig. 7b,c) and exhibited a higher excitability (Fig. 7d–h). During prolonged depolarization, they responded with pronounced phasic activity, while the POMC<sup>Lepr</sup> neurons were more tonically active (Fig. 7f,m,n).

During ascending and subsequently descending current ramps (Fig. 7d,e), POMC<sup>Qtrt</sup> neurons tended to have a lower threshold
current (Extended Data Fig. 10c) and generated more action potentials during ascending ramps than the POMC<sup>Lepr</sup> neurons. During descending ramps, we observed the opposite; POMC<sup>Lepr</sup> neurons generated more action potentials than POMC<sup>Glp1r</sup> neurons. This resulted in a higher spike-number ratio between the ascending and descending ramps in POMC<sup>Glp1r</sup> neurons compared to POMC<sup>Lepr</sup> neurons, which indicates a voltage-dependent adaptation (Fig. 7e). These data suggest a phasic, adaptive excitability of POMC Glp1r<sup>+</sup> neurons in response to sustained excitatory input, which cannot be explained simply by the differences in input resistance (Fig. 7b,c).

On a finer scale, we measured frequency–current relationships, post-inhibitory rebound, spike-frequency adaptation (SFA), and sag potentials during hyperpolarization from POMC<sup>Glp1r</sup> and POMC<sup>Lepr</sup> neurons, and found higher excitability in POMC<sup>Glp1r</sup> neurons, which was reflected by a ‘steeper’ frequency–current relationship (Fig. 7f–h).

We also observed a stronger post-inhibitory rebound excitation in POMC Glp1r<sup>+</sup> (Fig. 7i,j). It is remarkable that we found profound post-inhibitory rebound excitation in POMC Glp1r<sup>+</sup> neurons, while they generated smaller sag potentials than POMC Lepr<sup>+</sup> neurons (Fig. 7k, l).
During depolarizing current pulses, POMC<sup>Lepr<sup>−</sup></sup> cells underwent stronger SFA after the initial excitation (Fig. 7m,n), as reflected in a significantly higher SFA ratio (Fig. 7n). The high SFA ratio of POMC<sup>Lepr<sup>−</sup></sup> neurons was accompanied by a marked afterhyperpolarization that followed depolarizations, for example, after trains of action potentials (Fig. 7o,p). This is mechanistically plausible since both SFA and afterhyperpolarization can be mediated by Ca<sup>2+</sup>-dependent K<sup>+</sup> currents, which have been found in POMC neurons<sup>26,27</sup>. In contrast, in POMC<sup>epr<sup>+</sup></sup> neurons, which have a lower SFA and a clear tonic activity component during depolarizations, a sustained train of action potentials was followed by an afterdepolarization outlasting the excitatory input (Fig. 7o–q). A spike waveform analysis (Fig. 7r–t) showed a lower action potential threshold (Fig. 7r,s), a faster depolarization rate (Fig. 7r,t) and a trend toward a faster repolarization rate (Extended Data Fig. 10d) in action potentials of POMC<sup>epr<sup>+</sup></sup> neurons.

Since most active membrane properties are mediated by sets of ionic conductances, that is, multiple ion channel types, it is challenging to attribute these complex physiological properties causally to translational profiles. Nevertheless, we have identified cell-type-specific differences in the expression of ion channels (or subunits) that are considered regulators of some physiological properties that differ between POMC<sup>epr<sup>+</sup></sup> and POMC<sup>Lepr<sup>−</sup></sup> neurons (Extended Data Fig. 10e–j). The phasic, adaptive excitability and the accompanying greater rebound properties of the POMC<sup>epr<sup>+</sup></sup> neurons are in line with their comparatively higher expression of a modulatory β-subunit (Cacnb4; Extended Data Fig. 10i), which can lower the activation threshold and increase the conductance of voltage-gated Ca<sup>2+</sup> channels<sup>28</sup>. The stronger SFA and afterhyperpolarization of POMC<sup>Lepr<sup>−</sup></sup> cells compared to POMC<sup>epr<sup>+</sup></sup> neurons are consistent with the relatively high expression of the regulatory β4-subunit (Kcnmb4; Extended Data Fig. 10j), which decreases the conductance of big-conductance Ca<sup>2+</sup>–activated potassium channels<sup>28</sup>. Additionally, M-currents expressed by the KCNQ gene family can contribute to SFA and are upregulated in POMC<sup>epr<sup>+</sup></sup> cells (Extended Data Fig. 10j)<sup>28</sup>. In agreement with the relatively depolarized membrane potential of POMC<sup>Lepr<sup>−</sup></sup> cells and their relatively small sag potentials during hyperpolarization, we found low expression of Na<sup>+</sup><sup>+</sup>/K<sup>+</sup> ATPases (Atp1a1) and hyperpolarization-activated cyclic-nucleotide-gated cation channel 1 (Hcn1), respectively (Extended Data Fig. 10, h)<sup>30</sup>. In summary, we have revealed cell-type-specific electrophysiological differences between POMC<sup>epr<sup>+</sup></sup> and POMC<sup>Lepr<sup>−</sup></sup> neurons, which are paralleled by the cell-type-specific expression of ion channels and receptors for energy homeostasis-related signals.

**Fig. 8 | Leptin and Glp1 differentially modulate POMC<sup>epr<sup>+</sup></sup> and POMC<sup>Lepr<sup>−</sup></sup> neurons and exhibit differences in NPY-induced currents.** a–h. Effect of leptin on POMC<sup>epr<sup>+</sup></sup> neurons (a–d) and Glp1 on POMC<sup>Lepr<sup>−</sup></sup> neurons (e–h). Rate histograms and respective original recordings illustrating the effect of leptin on POMC<sup>epr<sup>+</sup></sup> neurons (a–c) and the effect Glp1 on POMC<sup>epr<sup>+</sup></sup> neurons (e–g). Each figure shows a single example each of a peptide-excited, a peptide-inhibited and a nonresponsive neuron. d,h. Numbers of peptide-responsive neurons in the respective cell types. i–n. Effect of Glp1 on POMC<sup>epr<sup>+</sup></sup> neurons (i–k) and leptin on POMC<sup>Lepr<sup>−</sup></sup> neurons (l–n). i,j,m. Rate histograms and respective original recordings showing single examples of neurons that were nonresponsive or inhibited by the respective peptides. The asterisk in the rate histogram (j) reflects action potentials that were elicited by current protocols. k,n. Current-clamp recordings, in which action-potential-induced synaptic release is suppressed by TTX (1 μM). Top: original recordings. Bottom left: summary and quantification of all recordings. Red lines indicate recordings with significant changes in membrane potentials. The population responses were compared by using one-way ANOVA with Tukey’s post hoc test (K: POMC<sup>epr<sup>+</sup></sup>, n = 14, control (ctrl) vs Glp1; P = 0.0692, Glp1 vs wash; P = 0.238, ctrl vs wash; P = 0.0218, F = 6.40; n: POMC<sup>epr<sup>+</sup></sup>, n = 12, ctrl vs leptin; P = 0.0067, leptin vs wash; P = 0.008, ctrl vs wash; P = 0.722; F = 8.72; **P < 0.01). Box plots were generated according to Tukey’s test, where ‘+’ illustrates the mean. Bottom right: numbers of peptide-responsive neurons in the respective cell types. o,p. Effect of NPY on POMC<sup>epr<sup>+</sup></sup> neurons and POMC<sup>Lepr<sup>−</sup></sup> neurons. o. Voltage-clamp recordings. NPY induced inward currents in POMC<sup>Lepr<sup>−</sup></sup> neurons (n = 14; yellow) and POMC<sup>epr<sup>+</sup></sup> neurons (n = 14; blue), shown as the mean ± s.e.m. p. Electrical charge that flowed during 10 min of NPY application (POMC<sup>epr<sup>+</sup></sup>, n = 14; minima = 2.73, Q1 = 4.03, Q3 = 5.68, Q = 9.19, maxima = 12.24, mean ± s.e.m. = 6.54 ± 0.80 nC; POMC<sup>Lepr<sup>−</sup></sup>, n = 14, minima = 0.78, Q1 = 2.34, Q3 = 3.274, Q = 4.82, maxima = 7.32, mean ± s.e.m. = 3.53 ± 0.48 nC; t = 0.0034, t = 3.32, df = 26, two-tailed unpaired Student’s t-test. **P < 0.01**. In the box plots, a ‘+’ sign show the mean and the horizontal line is the median. The whiskers were calculated according to the Tukey method. In all recordings, synaptic input was pharmacologically blocked (Methods). Peptides were bath applied at the indicated concentrations: leptin (100 nM), Glp1 (300 nM) and NPY (100 nM). Responsiveness of individual neurons was defined by the 3-σ criterion (Methods). n, number of cells recorded. exc, excited; inh, inhibited; NR, not responsive. Q3, Q2, and Q1 represent the lower quartile, median and upper quartile, respectively.

Specific regulation of POMC<sup>epr<sup>+</sup></sup> and POMC<sup>Lepr<sup>−</sup></sup> neurons by energy-state-sensing signals. Most neurons showed significant but mostly subtle modulatory effects to stimulation with the cognate ligands of their defining receptors, that is, leptin (Fig. 8a–d) and Glp1 (Fig. 8e–h). The modulatory effects were often differential and not homogeneous within one cell type, and we also observed modulation upon stimulation with the non-cognate ligands (Fig. 8i–n).

Leptin (100 nM) excited 40% and inhibited 20% of POMC<sup>epr<sup>+</sup></sup> neurons, while the remaining 40% of these cells were not responsive (Fig. 8a–d). In 60% of POMC<sup>Lepr<sup>−</sup></sup> neurons, leptin did not affect the neuronal activity, while, despite the absence of Lepr expression, it inhibited 40% of these cells (Fig. 8i,m). Thus, in the presence of GABA receptor and glutamate receptor blockers, leptin modulated both neuronal populations differentially.

Correspondingly, Glp1 (300 nM) excited most (63%) POMC<sup>Lepr<sup>−</sup></sup> neurons, whereas only 13% of the neurons were inhibited, and 22% were not affected by Glp1 (Fig. 8e–h). POMC<sup>epr<sup>+</sup></sup> neurons, which largely do not express the Glp1r, were mostly unaffected by Glp1 (81%), and only 18% of these neurons were inhibited (Fig. 8i, j).

While the distinctive responses of both cell types to the two ligands indicate that classification of these neurons according to Lepr and Glp1r expression defines different responses to two satiety-communicating signals, the relatively high variability of the effects also raises the question of whether interactions within the energy homeostasis-regulating network contribute to the modulatory effects. Consequently, we performed recordings where, in addition to blocking GABA and glutamate receptors, action-potential-dependent signaling was suppressed by tetrodotoxin (TTX; 1 μM). Around 80% of POMC<sup>epr<sup>+</sup></sup> neurons remained unaffected by Glp1, while 14% of neurons depolarized and 6% hyperpolarized (Fig. 8k). The proportion of POMC<sup>epr<sup>+</sup></sup> neurons that responded to Glp1 is consistent with the proportion of these cell types expressing both Lepr and Glp1r, although lower than that previously reported to respond to Glp1, where 37% of alternatively identified POMC<sup>epr<sup>+</sup></sup> neurons responded to a higher Glp1 dose (1 μM)<sup>31</sup>. Approximately 91% of POMC<sup>Lepr<sup>−</sup></sup> neurons responded with a reversible hyperpolarization, while only 9% of neurons were unaffected by leptin (Fig. 8n). At first sight, these data might imply that leptin has direct effects on POMC<sup>Lepr<sup>−</sup></sup> neurons (and Glp1 directly affects POMC<sup>epr<sup>+</sup></sup>). Alternatively, the results could indicate modulation of spontaneous release<sup>32</sup>. Moreover, these experiments indicate that the modulation of POMC neurons might lead to stronger cross-interactions between POMC<sup>epr<sup>+</sup></sup> and POMC<sup>Lepr<sup>−</sup></sup> neurons than originally expected.

Furthermore, consistent with a higher level of the Npy1r in POMC<sup>epr<sup>+</sup></sup> neurons (Figs. 5d and 6c), NPY (100 nM) elicited larger...
outward currents in POMC<sup>Lepr</sup> cells compared to POMC<sup>Glp1r</sup> neurons (Fig. 8o,p). Collectively, differentially expressed neuropeptide receptors, as identified via translational profiling, accurately predicted differential pharmacological responses of these cell types.

**Discussion**

Recent developments in single-cell RNA-seq have largely extended our view on the heterogeneity of molecularly defined neurocircuits, including those in control of energy and glucose homeostasis<sup>1</sup>.
However, defining the underlying molecular heterogeneity of distinct neurocircuits, their functional organization and output remains a largely unmet territory in modern neuroscience. Here, we provide new mouse models, which allow for functional interrogation of heterogeneous populations based on combinatorial Cre and Dre-dependent recombination in vivo. Recombinase-based gene targeting has become a valuable tool not only in neuroscience allowing for the modulation of specific cell types mostly via Cre-loxP-mediated recombination\(^\text{[14]}\). Alternative recombination systems such as FLP/FRT-mediated recombination provide complementary tools, but are limited through a lower efficiency of recombination in mice despite that improved versions have been developed (FLPe and FLPo)\(^\text{[15]}\). While FLPe has been used for successful combinatorial recombination-based targeting of heterogeneous dopamine neurons using viral approaches, only a few examples have used FLp-based recombination for intersectional targeting in transgenic mice\(^\text{[15]}\).

An alternative recombine system has been identified, that is, Dre-rox-mediated recombination\(^\text{[16]}\). Previous studies had indicated the feasibility of combinatorial Cre/Dre-recombine usage for targeting specific neuronal subpopulations\(^\text{[16]}\). Here, we have systematically expanded the repertoire of tools and validated mice, enabling use of this system to define specific heterogeneous cell types. First, we show that Dre-based recombination allows for efficient targeting of rox-marked transgenes in single-allele configuration. Moreover, we show that Dre-dependent recombination is specific for rox-flanked DNA segments, as we have observed no cross-reactivity with loxP-flanked alleles in several lines of transgenic mice. While it had previously been reported that Dre-mediated recombination may result in background recombination of loxP-flanked DNA in a ROSA26SlTomato reporter strain\(^\text{[16]}\), our extensive experiments clearly validate the usage of Cre/Dre-mediated combinatorial recombinases for complementary analyses of heterogeneous populations in addition to FLp-based approaches.

Nevertheless, Dre-dependent recombination appears to be less efficient compared to Cre-dependent recombination. Of the numerous, independent POMC\(^\text{Cre}\) transgenic mouse lines employing the exact same POMC BAC construct to express Dre as previously used to express Cre in these cells, the efficiency of Dre-mediated recombination is lower than Cre-mediated recombination, since the maximum rate of recombination was lower and increased over a longer period of development\(^\text{[17]}\). However, this represents a crucial advantage, when it allows Dre-mediated recombination to bypass narrow developmental periods, where a promoter is temporarily active during development in a subset of cells, and which thus unfaithfully marks cells upon very efficient recombinase targeting. This has been exemplified for POMC cells, where efficient POMC\(^\text{Cre}\)-mediated recombination during development targets cells, which later during development acquire a functionally antagonistic phenotype, that is, a ROX-dependent POMC\(^\text{Dre}\) target cell with reduced expression of Glp1r and increased expression of Lepr receptors compared to the POMC\(^\text{Cre}\)-expressing cell types\(^\text{[18]}\).

In addition, our experiments provide numerous new insights into the functional organization of hypothalamic POMC neurocircuits. First, the role of leptin in the regulation of POMC neurons has been subject to controversial findings over the last few years. Deletion of leptin receptors from POMC neurons throughout development causes mild obesity and hyperphagia, while the same intervention during adulthood has no effect on body weight or energy homeostasis\(^\text{[19]}\). On the other hand, POMC deficiency or selective ablation of hypothalamic POMC neurons in mice\(^\text{[20]}\) and POMC deficiency in humans causes massive obesity\(^\text{[21]}\), and re-expression of POMC in Lepr-expressing cells is sufficient to restore this effect in mice\(^\text{[22]}\), providing evidence for a role of POMC\(^\text{ Cre}\) neurons in the control of energy homeostasis. However, selective chemogenetic stimulation of POMC\(^\text{ Cre}\) neurons in our study only induces a minor suppression of food intake. Collectively, these data support a predominantly developmental role of Lepr-expressing POMC neurons in the control of energy homeostasis, but at the same time indicate that additional regulators other than the direct action of leptin contribute to control their activity. Recent experiments using Ca\(^{2+}\) imaging of POMC neuron activity in mice, have revealed that POMC neurons rapidly change their activity in response to sensory food perception, independent of changes in circulating energy-state-sensing hormones, and that this regulation contributes to the priming of liver ER adaptation upon sensory food perception\(^\text{[23]}\). Thus, top-down control of POMC\(^\text{ Cre}\) neurons or additional energy-state-sensing signals may also represent functionally relevant regulators of POMC\(^\text{ Cre}\) neuron activity.

In contrast, chemogenetic activation of POMC\(^\text{Cre}\) and POMC\(^\text{Cre}\) neurons in female mice had no effect on food intake. ISH analysis showed that these neurons were also efficiently activated in females (Extended Data Fig. 5j). Thus, it is likely that the regulation of food intake in males and females is controlled differently, which is corroborated by previous reports of higher expression of Pomc mRNA and higher firing rates of POMC neurons in female mice compared to males\(^\text{[24]}\).

Another possibility for why selective activation of POMC\(^\text{Cre}\) neurons may have a more pronounced food intake-suppressing effect than that previously reported using a global POMC population stimulation is evidenced by our electrophysiological studies. Typically, we observed differential and nonhomogeneous modulatory effects within one neuronal type, even in the presence of glutamate receptor and GABA receptor blockers. These findings raise the question whether factors other than glutamatergic and GABAergic interactions within this regulatory network might contribute to the modulation. One reason why this is a crucial question is that we have observed modulatory effects of ligands whose associated receptors are not expressed in the respective cell types. POMC\(^\text{Cre}\) neurons, for example, are inhibited during leptin application, even with glutamate and GABA receptor blockers present, and when action-potential-dependent synaptic release is suppressed (Fig. 8n). Since we mainly did not detect the corresponding receptors in the majority of these neurons, this could indicate that leptin induces the release of inhibiting mediators from presynaptic neurons in a non-action-potential-dependent manner. These mediators might be released by modulation of spontaneous release\(^\text{[25]}\). In primary spinal afferents, activation of TRPV1 channels
facilitates asynchronous synaptic release presumably by presynaptic Ca \(^{2+}\) influx \(^{41}\). Thus, it is plausible to consider that leptin mediates activation of TRP channels as previously described \(^{41}\) and thereby induces or increases the spontaneous release of neuronal mediators via increased Ca \(^{2+}\) influx.

In line with the notion of alternative regulators of POMC \(^{4+}\) and POMC \(^{Glp1r}\text{-}\) neuronal activity, our translational profiling has allowed the identification of numerous potentially differential regulators of these neuronal classes besides leptin and Glp1p. Here, POMC \(^{4+}\) neurons in particular express numerous receptors for additional energy-state-sensing signals, which have already been validated to suppress feeding and even reduce body weight in obesity. Interestingly, our study reveals the differential expression of Npy1r and Npy5r in distinct POMC neuron populations. Given that the primary source of NPY for POMC neurons in the ARC is neighboring AgRP neurons coexpressing NPY, these findings may point to the possibility that POMC \(^{4+}\) neurons and POMC \(^{Glp1r}\text{-}\) neurons are under differential inhibitory control by AgRP neurons. Indeed, we have also electrophysiologically validated that NPY induces a larger (inhibitory) outward current in POMC Lepr \(^{+}\) neurons (Fig. 8o,p). Moreover, the surprising identification of the expression of NPY in POMC \(^{Glp1r}\text{-}\) neurons also warrants further study.

In summary, we have employed new models to begin to shed light on the anatomical, electrophysiological, molecular and functional heterogeneity of critical metabolism-regulatory POMC neurons. The detailed insights may also aid the development of new, rationalized strategies for the therapeutic manipulation of the melanocortin circuitry.

Online content
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**Methods**

**Animals and animal care.** All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln). Permission for breeding and experiments on mice was issued by the Department for Environment and Consumer Protection—Veterinary Section in Cologne ((§11) 571.35.2.9 07/18, 84-02.04.2017.A058). Mice were housed in individually ventilated cages at 22–24°C using a 12-h light-dark cycle. Animals had access to water and food ad libitum. Unless otherwise stated, animals were fed a normal chow diet (NCD; Teklad Global Rodent 2018, Harlan) containing 53.5% carbohydrate, 18.5% protein and 35.5% fat (12% of calories from fat). The high-fat diet (HFD; C1057, Altran) consisted of 32.7% carbohydrate, 20% protein and 35.5% fat (55.2% of calories from fat).

**Generation of genetically modified mouse strains.** *Driver lines.* The Leptr<sup>+</sup> and Gip1<sup>+</sup> mice have been previously described<sup>1</sup>. Leptr<sup>−/−</sup> mice and the Gip1<sup>−/−</sup> mice were kindly provided by M. G. Myers and F. Reimann, respectively. The POMC<sup>−/−</sup>-BAC construct was generated by inserting the sequence encoding Dre recombinase together with a kanamycin/ neomycin resistance cassette from the pTEDre plasmid into the start codon of the POMC gene of pRIP1-124K7 BAC via Red/ET recombination. Primers containing POMC-specific homology arms, 5′-ccctccactccagttgctctgggcagacactgctacagcagggacacgttgagtaaagggagaggaa-ga-3′ and 3′POMC Dre: 5′-tccctccaatcttgtttgcctctgcagagactaggcctgacacggtggaaggccaccatgggtaagaagaaga-3′, were used for experiments.

**Generation of ROSA26 transgenic mouse lines.** Generation of ROSA26SlrSrZsGreen (ROSA26-CAGS-lox-STOP-lox-STOP-ox-ZsGreen) mice was described in a previous study. These mice have been crossed to a ubiquitously expressed Delter-Cre line to obtain R26rSrZsGreen mice<sup>20</sup>. ROSA26SlrSrtdTomato (ROSA POMC<sup>−/−</sup> line, the breeding scheme consisted of mating heterozygous POMC<sup>−/−</sup> mice with protocols approved by local government authorities (Bezirksregierung Köln). Permission for breeding and experiments on mice was issued by the Department for Environment and Consumer Protection—Veterinary Section in Cologne ((§11) 571.35.2.9 07/18, 84-02.04.2017.A058). Mice were housed in individually ventilated cages at 22–24 °C using a 12-h light/dark cycle. Animals had access to water and food ad libitum. Unless otherwise stated, animals were fed a normal chow diet (NCD; Teklad Global Rodent 2018, Harlan) containing 53.5% carbohydrate, 18.5% protein and 35.5% fat (12% of calories from fat). The high-fat diet (HFD; C1057, Altran) consisted of 32.7% carbohydrate, 20% protein and 35.5% fat (55.2% of calories from fat).

**Immunohistochemistry.** Indirect immunofluorescence staining was performed as described previously<sup>30</sup>. Littermates of both sexes were used for experiments as indicated in the figure legends.

**Experimental details.** Glucose tolerance tests. Glucose tolerance tests were performed at 13 weeks of age with 16-h fasted mice. Body weights of mice and their basal blood glucose using a glucometer and glucose strips (Contour Next, Bayer HealthCare) were determined before the start of the experiment. Mice were injected with 20% glucose, and blood glucose was measured at 15, 30, 60 and 120 min after injection.

**Nuclear magnetic resonance.** Lean and fat mass were determined via nuclear magnetic resonance (NMR Analyzer Minispec mq 7.5, Bruker Optik) in live mice. Alternatively, body composition was analyzed by computed tomography (CT) in isoflurane-anesthetized mice (Dräger and Pyramal Healthcare). For data acquisition on an IVIS Spectrum CT scanner (Caliper Life Sciences), we used IVIS LivingImage Software V4.3.1. Quantification of lean and fat mass contents were determined with a modification of the previously described VINCI software package 4.6.10.

**Indirect calorimetry.** Metabolic phenotyping and food intake were measured by an automated indirect Master open-circuit, calorimeter system (TSE Systems). Mice were allowed to acclimate to the experimental setup for 4 days before the start of each experiment. Food and water were available ad libitum. Data acquisition was carried out by TSE Phenomaster versions 6.2.5 and above.

**Study design of DREADD animals.** For POMC<sup>−/−</sup>Lep<sup>−/−</sup>Gip1<sup>−/−</sup> ROSA26SlrSrEGFPL10a and POMC<sup>−/−</sup>Lep<sup>−/−</sup>Gip1<sup>−/−</sup> ROSA26SlrSrHm3Dq mice and corresponding genotype controls were characterized at 13–15 weeks of age with ad libitum access to NCD. Saline (0.9%) or CNO injections (3 mg per kg body weight) were administered intraperitoneally. Furthermore, for experiments of food intake (as described under 'Indirect calorimetry'), mice were injected with saline at 18:00 and 23:00, followed by a 1-d recovery period and subsequent CNO injections at 18:00 and 23:00 on the next day. For measurements of energy expenditure, respiratory exchange ratio and locomotion (as described under 'Indirect calorimetry'), mice were injected with saline at 17:00, 22:00 and 07:00 followed by CNO at 17:00, 22:00 and 07:00 on the next day. Perifusions at 22–26 weeks of age, mice were fasted for 2 h and injected with saline or CNO 1 h before perfusion. For the corticosterone enzyme-linked immunosorbent assay (ELISA) was obtained from mice fasted for 2 h and injected with CNO 1 h before blood collection. Littermates of both sexes were used for experiments as indicated in text and figures.

**CNO administration.** CNO (Abcam, ab141704) powder was dissolved in dimethyl sulfoxide (DMSO; 100 mg ml<sup>−1</sup>) and diluted at a ratio of 1:333 in 0.9% NaCl (saline).

**Study design of bacTRAP (EGFPL10a) mice.** For POMC<sup>−/−</sup>Lep<sup>−/−</sup>Gip1<sup>−/−</sup> ROSA26SlrSrEGFPL10a and POMC<sup>−/−</sup>Lep<sup>−/−</sup>Gip1<sup>−/−</sup> ROSA26SlrSrHm3Dq animals, 6 triple transgenic bacTRAP mice (3 females and 3 males) and 12 triple transgenic bacTRAP mice (6 females and 6 males) were pooled for each replicate, respectively, accounting for three to four replicates per POMC subpopulation. Mice were killed at 12 weeks of age in a random-fed state by decapitation. Whole hypothalami were obtained using a mouse brain slicer matrix and snap frozen in liquid nitrogen until translating ribosome affinity purification (TRAP).

**Perfusion and tissue fixation.** With the exception of DREADD animals, all mice were perfused in a random-fed state. Mice were deeply anesthetized and perfused transcardially with 1x PBS followed by ice-cold 4% paraformaldehyde (PFA; in 1x PBS; pH 7.4). The brain was removed from the skull and post-fixed in 4% PFA at 4°C for 1–2 h. After approximately 24 h, and then moved to 20% sucrose solution (in 1x PBS) at 4°C. The brains were cut at 20 μm on a sliding microtome (Leica Microsystems, SM2010R) equipped with a stage for dry ice. For immunohistochemistry, sections were either collected on slides or in bins containing anti-freeze solution (30% ethylene glycol and 20% glycerol in PBS), and subsequently stored at −20°C until further processing. For RNA ISH, sections were mounted on SuperFrost Plus Gold slides (Thermo Fisher Scientific, 11976299) and subsequently stored at −80°C until further processing.

**Immunohistochemistry.** For immunofluorescence stainings against ZsGreen, POMC, tdTomato and EGFP, all incubation steps were performed at room temperature unless otherwise stated.

Floating sections were washed once for 10 min in PBS, incubated for 10 min in 0.3% glycine, washed again for 5 min in PBS and incubated for 10 min in 0.03% SDS/PBS. Subsequently, sections were blocked for 1 h in 3% donkey serum in PBS containing 0.25% Triton X. Next, sections were incubated overnight at 4°C in primary antibody diluted in Signal (Cell Signaling, 8114). Primary antibodies were used for experiments as indicated in the figure legends.
and dilutions were used: rabbit anti-ZsGreen (Takara Bio Clontech, 632474; 1:100), rabbit anti-POMC (Phoenix, H-029-3; 1:1,000), rat anti-mCherry (for tdTomato; Thermo Fisher Scientific, M11217; 1:1,000) and chicken anti-GFP (Abcam, ab137640; 1:1,000). The following mouse primary antibodies were incubated three times for 10 min in PBS containing 0.1% Triton X and incubated for 1 h in secondary antibody in PBS containing 0.25% Triton X. Secondary antibodies and dilutions were donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher, A21206; 1:500), donkey anti-rat Alexa Fluor 594 (Jackson ImmunoResearch, 712-385-153; 1:500) or goat anti-rabbit Alexa Fluor 594 (Thermo Fisher Scientific, A11012; 1:500) and goat anti-chicken Alexa Fluor 488 (Thermo Fisher Scientific, A11039; 1:500), for 1 h at room temperature. After three washing steps for 10 min in PBS containing 0.1% Triton X, sections were mounted in Vectashield DAPI-containing mounting medium (Vector Laboratories, VEC-H-1200) and stored at 4°C in the dark until imaging.

**Corticosterone ELISA.** Concentrations were determined using a commercial Corticosterone ELISA kit from CrystalChem as described in the user's manual (80556).

**Imaging and quantification of immunohistochemistry.** Images were captured using a Leica TCS SP-8 X microscope, equipped with a ×40/1.30 objective with the acquisition software (Leica ASX V.3.5.5.19976). Next, z-stacks were taken with optical sections of 0.9 μm. Laser intensities were kept constant throughout all related conditions. Images were imported into FIJI (National Institutes of Health, version 2.0.0-rc–11.3504) in which maximum intensities were projected. For representative images, adjustments in brightness and contrast for each channel were kept constant throughout all related conditions, whereas for quantifications of POMC and tdTomato signals, all channels were kept unmodified and one to four sections were quantified per mouse and area. Images were converted to 8-bit, and the threshold for signal detection for each channel was determined by visual judgment and consistently applied to all images. ROIs were defined around corresponding anatomical locations and raw integrated densities measured within ROIs for POMC and tdTomato signals.

**RNA in situ hybridization.** The fluorescence ISH technique (RNASequencing) was used to detect mRNA of Pomp, Lepr, Fos, Cartpt, Vglut2, Vglut, P2, Agrp, G proteincoupled receptor type 1, and Nmur2. All reagents were purchased from Advanced Cell Diagnostics (ACD) if not otherwise stated. The Pomp probe (314808) contained ten oligonucleotide pairs targeting region 19–995 (NM_008895.3) of the Pomp transcript; the Agrp probe (400711) contained 16 oligonucleotide pairs targeting region 980–1655 (JQ071441.1) of the Agrp transcript; the P2 probe (315841) contained 20 oligonucleotide pairs targeting region 1135–2162 (NM_010934.4) of the P2 transcript; and the Vglut2 probe (319171) contained 20 oligonucleotide pairs targeting region 894–2037 (NM_009508.2) of the Vglut2 transcript.

**Imaging and quantification.** An automated workflow for co-registering each single-cell ROI showing Pomp signal and five or more signals of the Lepr or Gpr1 probe. Cell counting of ROSA26ZsRZsGreen and ROSA26SlrSlrM13D3G mice was performed manually, defining single-cell ROIs showing Pomp and ZsGreen signal, and considering five or more probe signals per ROI as positive. For statistical analysis, the distribution of POMC immunopositive signals was measured through a coronal section, comparable anatomical locations within the ARC were analyzed correspondingly by the Halo software and three mice per replicate were merged. For quantification of Cartpt, Npy1r, Oprm1 and Nmur2 in C57BL/6N mice, integrated density was assessed via FIJI in previously defined single-cell ROIs showing Pomp signal and five or more signals of the Lepr or Gpr1 probe. Cell counting of ROSA26ZsRZsGreen and ROSA26SlrSlrM13D3G mice was performed manually, defining single-cell ROIs showing Pomp and ZsGreen signal, and considering five or more probe signals per ROI as positive.

**Gene copy number assay.** For determining the Pomp copy number, 20 ng of genomic DNA extracted via standard isopropanol precipitation from tail biopsies was used. Selected gene segments were amplified using TaqMan Universal PCR-Master Mix (Thermo Fisher Scientific, 4305719) according to the manufacturer's instructions. Non-exon-spanning primers (P1: 5′-TCACGGAGCAACAAACTGTCG-3′; P2: 5′-AGACACCCTCTATACGGC-3′) and probes (5′-FAM-TCAAGGGTCCTCCTAGTTACGCTG-TAMRA-3′) were designed for amplification of Pomp exon 1, while the gene expression assay for Soc3 (Thermo Fisher Scientific, Mm00545913_s1, 4331182) was purchased. The expression of Pomp exon 1 was normalized to Soc3 mRNA. Results were calculated by the ΔCt comparative method (2−ΔΔCt), in which fold changes were calculated by defining wild-type animals with two Pomp−/− male and female mice were perfused with 1x PBS (pH 7.4) for 10 min followed by 4% PFA/PBS for 10 min. The brains were post-fixed for 24 h in 4% PFA/PBS (pH 7.4) at 4°C. The brains were dehydrated in a gradient manner via incubations in tert-Butanol diluted in distilled water: 30%, 50%, 70%, 80%, 90% and 96% (vol) for 10–16 h at 4°C. The brains were subsequently incubated in dichromohane for 90–120 min at RT to remove lipids. Next, BABB-D4 was used as the reagent-matching solution for 12 h at RT to complete the clearing process (BABB: benzyl alcohol + benzyl benzoate at a 1:2 ratio, respectively; BABB-D4: a mixture of BABB and diphenyl ether (DPE) at a ratio 4:1 (vol/vol)). The cleared brains were imaged with a Leica TCS Ultramicroscope II with the acquisition software Leica Application Suite (LÁS) version 2.0.0, 5× Airyscan and 10× HiRes Airyscan. Whole-brain and magnified scans of the ARC were obtained using ×1.6 and ×8 total magnification, respectively. For the protection density analysis, cleared brains from POMC−/− and POMC−/− (ΔGpr1+−) and POMC−/− (ΔGpr1+−) ROSA26SlrSlrTomato+− mice were imaged using ×1.6 magnification with the same laser intensity across all samples.

**Whole-brain immunostaining.** Pretreatment before staining was as follows: the fixed brains were washed twice in PBS for 1 h, incubated in 50% methanol (in PBS) for 1 h, 80% methanol for 1 h and two times in 100% methanol for 1 h. The samples were bleached with 5% H2O2 in 20% DMSO/methanol (one vol 30% H2O2/one vol DMSO/four vol methanol) at 4°C overnight. Subsequently, samples were transferred in PBS containing methanol for 90–120 min at RT to remove lipids. Next, BABB-D4 was used as the reagent-matching solution for 12 h at RT to complete the clearing process (BABB: benzyl alcohol + benzyl benzoate at a 1:2 ratio, respectively; BABB-D4: a mixture of BABB and diphenyl ether (DPE) at a ratio 4:1 (vol/vol)). The cleared brains were imaged with a Leica TCS Ultramicroscope II with the acquisition software Leica Application Suite (LÁS) version 2.0.0, 5× Airyscan and 10× HiRes Airyscan.
brain atlas, using rigid-body correlation-coefficient and 12-parameter affine mutual information schemas in VINCI. Originally, VINCI was developed for co-registration of clinical data but can also be utilized in animal studies. The parameters were adapted for the multi-scale approach and prepandemic workflow (anticluster filtering) for microscopy data (VINCI versions 4.6.1 and 4.9.60). However, the size of the microscopy scans meant that further optimization was required; we needed to co-register 32-GB image files (8 × 108 voxels), while typical-use cases in positron-emission tomography, magnetic resonance imaging and computed tomography for animal and human data are in the order of 2 × 108 voxels. The processing of these large datasets was expedited through multi-threading, and the co-registration time on a dedicated system (56 cores) could be reduced from over 24 h to 0.7 h. The transformation matrix of the x4 image to the atlas was derived as the combination of the two transformations. Quality of registration was determined based on a visual inspection of the registration result (a match of the interhemispheric fissures and the anatomical surface at the base of the hypothalamus). The scans with the best co-registration in each group were identified and the x4 images from each mouse were aligned to the x4 scan of the reference mouse within each group. This was performed using another specially adapted 12-parameter affine mutual information schema combined with the known transformation of the reference mouse full-brain image to the atlas. The correlation-coefficient values for the reference brain for POMC, Lepr and Glp1r were 0.85, 0.75 and 0.90, respectively. The quality of registration for the Glp1r group was not optimal so the Glp1r scans were co-registered to the x4–x4 script instead to the reference mouse of the POMC group.

Extraction of neuronal coordinates using Arivis and three-dimensional scatterplots. The output NIFTI files of the co-registered x4 brain scans were uploaded into the Arivis Vision 4D software (V3.3.0). Individual neurons were identified using the Blob Finder feature in the software and their xyz coordinates were extracted. A Python script was used to visualize the 3D scatterplots for each neuron. We used the mutual information based on the 3D neuronal coordinates to demonstrate the spatial concentration, which was then color coded to visualize the regions within the brain with higher neuronal counts for each subpopulation.

Data analysis of three-dimensional projections. Brain scans were co-registered to an annotated atlas (https://scalablebrainatlases.insc.org/mouse/ABA12z#downloads/) using VINCI. Thresholding was achieved using the threshold function on VINCI by applying values to the output .nii scans. Using the output .nii file from VINCI, a mask was created from the ROIs in 3D, and the intensity profile of each region was determined using the VOI define function on VINCI. Representative images in the areas of interest were rendered using the Arivis software.

Purification of mRNA from triple-negative EGFPView 10a mice. The TRAP technique was performed using a modified version of a previous study on hypothalami of mice described under ‘Study design of bacTRAP (EGFPView 10a) mice’. One day before TRAP, 375 µl of Dynal Protein G magnetic beads (Invitrogen) was washed three times (1 ml) and resuspended in 275 µl of IP wash buffer (20 mM HEPS (pH 7.4), 150 mM KCl, 5 mM MgCl2, and 1% NP-40). Then, 30 µg of two monoclonal anti-GFP antibodies (HtzGFP-19C8 and HtzGFP-19F7) were added to the supernatant at a final concentration of 1% and 30 mM, respectively. After incubation on ice for 5 min, the clarified lysate was centrifuged for 10 min at 13,000g to pellet insoluble material. Next, 30 µl of supernatant (input) was snap frozen in liquid nitrogen until RNA extraction as a comparison to the immunoprecipitated sample. Then, 200 µl of anti-GFP coated Dynal protein G magnetic beads was added to the supernatant, and the mixture was incubated at room temperature for 1 h. Beads were subsequently collected on a MagnaRack (Invitrogen), washed four times with high-salt polystyrene wash buffer (20 mM HEPS (pH 7.4), 350 mM KCl, 5 mM MgCl2, 1% NP-40, 0.5 mM dithiotreitol and 100 mg ml−1 cycloheximide). Input and IP beads were resuspended and incubated in RLT buffer (RNAsasy micro kit, Qiagen) for 5 mins at RT. Supernatant was removed from the IP beads and RNA extraction was performed according to Qiagen’s protocol, including in-column DNase digestion. RNA was resuspended in 10 µl nuclease-free water. RNA quantity and quality of input and IP were determined with a Qubit Fluorometer (Invitrogen) and Agilent 2100 Bioanalyzer.

RNA sequencing. Pre-amplification was carried out using the Ovation RNA-seq system (V2). Total RNA was used for first-strand cDNA synthesis, using both poly(T) and random primers, followed by second-strand synthesis and isothermal strand-displacement amplification. For library preparation, the Illumina Nextera XT DNA sample preparation protocol was used, with 1 ng cDNA input. After validation (Agilent 2200 TapeStation and quantification) the initial 50 ng library was used (V2.7.12). The probability density function of the 3D distribution of neurons had been previously aligned manually. The final distribution graph was plotted using the Prism software.

Gene Ontology analysis. The regulated GO terms were derived using the clusterProfiler R package and visualized by mapping the percentage of regulated genes to the total GO term gene count against significant gene counts and adjusted P values per term. We visualized the GO term as a heat map, where each row represents a differentially expressed gene belonging to the term and each column represents a different library. To remove the skewing by highly expressed genes, the values of each sample were converted to z-scores with respect to the individual genes.

Overlap analysis. We used publicly available single-cell RNA-seq data from mouse hypothalami to detect overlaps with markers identified in our Glp1r and Lepr datasets. The first data source used was single-cell RNA-seq of 20,921 cells from the accredate–median eminence complex of mice. We filtered cells expressing POMC (4,248/20,921 cells) and clustered them using the R Seurat package. The clustering result was visualized using the UMAP plot of Seurat. The second data source was from the single-cell RNA-seq dataset of 163 POMC-expressing neurons in mice yielding four clusters.

Electrophysiological experiments. Animals and brain slice preparation. Experiments were performed on brain slices from 12- to 15-week-old genetically marked (with ZsGreen) Glp1r- and Lepr-expressing POMC neurons using POMC- or Lepr promoter-coupled ZsGreen (POMC- or LeprZsGreen). Male and female mice. Animals were kept under standard laboratory conditions, with tap water and chow available ad libitum, on a 12 h light/dark cycle. The animals were lightly anesthetized with isoflurane (B506; AbbVie) and decapitated. Coronal slices (250–300 mm) containing the ARC were obtained with a vibratome (HM 650 V; Thermofisher). Brains were then cold (4 °C), carbogenated (95% O2 and 5% CO2), glycerol-based modified artificial cerebrospinal fluid (GaCSF). GaCSF contained (in mM): 244 glycerol, 2.5 KCl, 2 MgCl2, 2 CaCl2, 1.2 NaHPO4, 21 NaHCO3, and 5 glucose, adjusted to pH 7.2 with NaOH. If not mentioned otherwise, the brain slices were continuously superfused with carbogenated aCSF at a flow rate of 2.3 ml min−1. aCSF contained (in mM): 125 NaCl, 2.5 KCl, 2 MgCl2, 2 CaCl2, 1.2 NaHPO4, 10 HEPES, 21 NaHCO3 and 5 glucose, adjusted to pH 7.2 with NaOH. To block GABAergic and glutamatergic synaptic input, in all recordings, the aCSF contained 10 Mmicrotoolox (P1675; Sigma-Aldrich), 5 × 10−5 M CGP (CGP-54626 hydrochloride; BN0597; Biotechnne), 5 × 10−5 M DL-AP5 (DL-2-amino-5-phosphonopentanoic acid; BN1625; EM Sciences) and 10−5 M DL-AP7 (6-cyano-7-nitroquinoxaline-2,3-dione; C127; Sigma-Aldrich). To suppress action-potential-dependent synaptic release, blocked voltage-dependent Na+ channels were blocked by 10−6 M TTX (T-550, Alomone).

Electrophysiology. Current-clamp and voltage-clamp recordings of ZsGreen-expressing POMC neurons were performed at −32 °C in the perforated patch-clamp configuration. Neurons were visualized with a fixed-stage upright microscope (BX51WI, Olympus) using ×40 and ×60 water-immersion objectives (UPlanFL N/×0, 0.8 numerical aperture, 2 mm working distance; UPlanFL N/×60, 1.0 numerical aperture, 2 mm working distance, Olympus) with infrared differential interface contrast optics and fluorescence optics. ZsGreen-expressing POMC neurons were identified by their anatomical location in the ARC and by their ZsGreen fluorescence that was visualized with an X-Cite 120 illumination system (EXFO Photonic Solutions) in combination with a Chroma 4101 filter set (ex: HQ480/×40; em: Q505LP; em: HQ535/50m). Electrodes with tip resistances of between 4 and 7 MΩ were fashioned from borosilicate glass (0.86-
Electrophysiological properties. To analyze in detail the intrinsic electrophysiological properties of ZsGreen-expressing POMC neurons, a set of current-clamp protocols from a holding potential of −70 mV was applied. Cell input resistance was determined from a series of hyperpolarizing small current pulses (1 s, 2–10 pA increments) and the slope of the resulting I–V relation. Whole-cell capacitances were calculated from the membrane time constant (τ) and the input resistance (R); C = τ/R. To analyze the I<sub>c</sub>-dependent sag potentials, the neurons were hyperpolarized with five consecutively incrementing current pulses. The increments were adjusted so that the last pulse hyperpolarized the membrane to −120 mV. The sag potential of the difference between the lowest voltage reached at the beginning of the pulse and the membrane potential reached at the end of hyperpolarization. To analyze post-inhibitory rebound excitation, we used an ‘enhanced rebound protocol’, whereby the same current-step amplitudes were applied as those used for the sag-potential analysis, but this time as 2-s hyperpolarizing pre-pulses that were followed by a 1-s test pulse with the amplitude of the single increment. The maximum instantaneous frequencies during the rebound were determined and plotted over the membrane potentials of the pre-pulses. To analyze input–output relations, we applied a series of ascending and then descending current ramps (5 s each), where the ramp amplitudes were increased from 10 to 25 pA in 5-pA increments. Amplitudes were further increased if the 2 s/5 s ratio did not produce action potentials. Spike-number ratios were calculated by dividing the number of action potentials during the ascending ramp by the number of action potentials during the descending ramp. To further analyze excitability, that is, evoked action potential firing, a series of depolarizing current pulses (1 s; 5–50 pA in 5-pA increments) was applied. For each current pulse, the number of action potentials was determined, plotted over the current amplitude and linearly fit. Linear fits were performed for data points where action potentials were elicited. Only data points at which action potentials were triggered were considered for fit.

For SFA ratios, 10-s depolarizing stimuli were applied from a holding potential of −70 mV with initial instantaneous action potential frequencies between 30 and 40 Hz. Instantaneous action frequencies were plotted for the 10-s time periode and fit to a mono-exponential decay equation with Y<sub>0</sub> set to the initial instantaneous frequency: Y = (Y<sub>0</sub> − plateau) × exp(−K × T) + plateau, where ‘plateau’ is the asymptotic frequency, K is the inverse time constant and T is the time. The SFA ratio is determined by dividing the maximum initial instantaneous frequency by the plateau frequency of the fit. Action potential waveform parameters were obtained from action potentials with instantaneous frequencies ≤5 Hz. If necessary, hyperpolarizing bias currents were used to decrease spontaneous firing.

Peptide signaling. Leptin (100 nM; L7372, Sigma-Aldrich), and Glp1 (300 nM; H-9596, Bachem AG) were bath applied for 15 or 30 min with a perfusion rate of 2.5 ml s<sup>−1</sup>. Npy (100 nM; N5071, Sigma-Aldrich) was bath applied for 10 min. Leptin and Glp1 signaling. In line with previous studies, we found that the basic firing properties of POMC neurons and their responsiveness to leptin and Glp1 were not homogenous. Therefore, we used the ‘three times standard deviation’ (3σ) criterion, and a neuron was considered responsive if the change in firing frequency or membrane potential induced by leptin or Glp1 was three times larger than the standard deviation. Means and respective standard deviations of spontaneous action potential firing or membrane potential were calculated from a period of 120 s, divided into 12 bins, each 10 s long. Data were taken immediately before and at the end of the peptide application.

Voltage-clamp recording of peptide-induced currents. The NPY action on POMC neurons was analyzed under voltage clamp. Based on previous studies<sup>36,50</sup> and on the peptide–frequency action potential modulation, which we observed in current-clamp recordings, the holding potential was set to −55 mV to optimize the recording conditions to measure inward currents. NPY (100 nM) was bath applied for 10 min after a 5-min baseline recording. The recorded peptide-induced currents were baseline subtracted and the mean (±s.e.m) was calculated. To quantify differences in the peptide-induced currents, the area under the curve (electrical charge in nC) during the 10-min application of NPY was calculated.

Quantification and statistical analysis. Statistical analyses and reproducibility. Data analysis was performed on Figs. 3, 5, 7 and 8 can be found in ‘Statistical analysis of 3D data’, ‘RNA-sequencing analysis workflow’ and ‘Electrophysiological data analysis and statistics’, respectively. Primary data processing and organization were performed in Microsoft Excel (2010). Statistical analyses were performed using Prism software (GraphPad, V.5.0–V8.0). Statistical significance for two groups was determined by unpaired two-tailed Student’s t-test. In the case of unequal variance between the two groups, the unpaired Welch’s t-test or unequal variance Student’s t-test was used. For determining differences between more than two groups, one-way ANOVA was applied. Depending on the scientific question, one-way ANOVA was followed by post hoc test, by Dunnett’s post hoc test (comparing all groups to one control) or by Tukey’s post hoc test (comparing all groups among each other), as indicated in the figure legends. All remaining data (more than two groups, more than one independent factor) were analyzed with two-way ANOVA followed by Sidak’s or Tukey’s post hoc test (depending on the factors). P values are expressed as the mean, and the error bars indicate the s.e.m. unless specified otherwise. In the violin plots, solid lines represent median values, and dashed lines represent lower and upper quartiles. A detailed description of statistics, including individual data points, tests, P values and further statistical parameters, are provided in the figure legends and as source data. Statistical significance was defined as: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001.

No statistical methods were used to predetermined sample sizes, although sample sizes are similar to those reported in previous publications<sup>42,62</sup>. Data collection and analysis were carried out in a blinded format throughout the study, unless this was not possible due to the visual differences in cases of varying neuronal numbers resulting from genetic labeling as depicted in Fig. 3a–c. Data distribution was assumed to be normal, but this was not formally tested. Except for animals that died during an experiment, no data were excluded. For metabolic phenotyping, every mouse represents a replicate (n) and the number of replicates is mentioned for each experiment in the figure legend and/or source data. In this case, data were pooled from independent experiments of varying n numbers. For RNA-seq, samples of pooled hypothalami were collected from individual mice of several cohorts. For electrophysiological experiments, the sample numbers indicate the number of cells used in recordings. All measurements that did not require statistical analysis, such as representative images, were obtained from at least two animals and in most cases a minimum of three animals were used.

Statistical analysis of three-dimensional data. To carry out a statistical assessment of the differences in distribution patterns for the two subpopulations, the bounding box containing all the neuronal coordinates was divided into smaller cubes and two-sided Student’s t-test was calculated for each cube using a Python script (V.2.7.12). Due to the variance in the total neuronal counts between the Lepr and Glp1r groups, the number of neurons in each cube was corrected by introducing a density factor (number of Glp1r neurons/number of Lepr neurons). This was performed by multiplying the density factor to the total number of neurons in each group. Since the null distribution is two-sided, both null hypotheses were visualized within the same plot.

RNA-sequencing analysis workflow. The RNA-seq results of Glp1r and Lepr samples were processed using the nf-core/museq pipeline (V.1.4.2)<sup>51</sup>. This involves (1) aligning the raw reads to the reference genome GRCh38.p6 using STAR (2.6.1d)<sup>52</sup> and (2) transcript abundance estimation using Salmon (0.14.1)<sup>53</sup> with the reference transcriptome from Ensemble (release 97)<sup>54</sup>. To normalize the ribosomal pulldown to the hypothalamic background (input) per sample, we calculated a ratio of the Salmon gene counts (IP/input). The differentially expressed genes between Glp1r and Lepr groups were derived using DESeq2 (V.2.6.0)<sup>55</sup> where P values attained by the Wald test were corrected for multiple testing using the Benjamini–Hochberg method. This yielded genes that are regulated between the Glp1r and Lepr conditions and act as markers for these POMC populations.

Electrophysiological data analysis and statistics. Data analysis was performed with Spike2 (V.7.0; Cambridge Electronic Design), Igor Pro 6 (WaveMetrics) and GraphPad Prism (V.5.0.8.0). In violin plots, the solid line represents the median,
and dashed lines represent lower and upper quartiles. In the box plots, the ‘+’ sign depicts the mean, and the horizontal line represents the median. The whiskers were calculated according to Tukey's method. For pairwise comparisons of dependent and independent normal distributions, paired and unpaired t-tests were used, respectively. For pairwise comparisons of independent, non-normal distributions, a Mann–Whitney U-test was used. For multiple comparisons, one-way ANOVA with post hoc Tukey's test was performed. A significance level of 0.05 was accepted for all tests. In the figures, n values are given in parentheses. Exact P values are reported if P > 0.05.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Raw RNA-seq data have been deposited in the NCBI Gene Expression Omnibus under accession code GSE153575. Other raw data is available under reasonable request to the corresponding author.

**Code availability**

The code for RNA-seq analysis is available at https://github.com/bruening-lab/pomc-neurons-architecture-rnaseq/ and the code for isosurface density plots and the 3D statistical analysis is available at https://github.com/bruening-lab/Heterogeneity_Scripts/. Source data are provided with this paper.

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**Author contributions**

Conceptualization: J.C.B. and P. Kloppong; methodology: N.B., P. Kloppong, L.P. and J.C.B.; investigation: N.B., I.G., J.R., J.S., S.C., W.C., C.W., T.K. and T.S.-H.; formal analysis, N.B., I.G., P. Klemm, J.R., J.S., L.P. and S.C.; writing of original draft: J.C.B.; project administration: N.B., J.S., L.P. and J.C.B.; resources: S.V., F.T.W., P. Kloppong and J.C.B.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | See next page for caption.
The POMC<sup>chrome</sup> transgene shows stable integration into the genome and causes no metabolic phenotype as compared to wildtype controls. 

**a.** Schematic diagram of the targeting strategy for the POMCDre BAC construct. 

**b.** RNA in situ hybridization against ZsGreen in POMCDre ROSA26rSrZsGreen mice. Scale bars represent 100 μm. ap = area postrema, NTS = nucleus tractus solitarius, DMX = dorsal motor nucleus. 

**c.** Quantification of copy number PCR of <i>Pomc</i> exon 1, performed on genomic DNA from the first four generations (f1-f4) of the POMC<sup>chrome</sup> mouse line in comparison to wildtype (wt) animals. For wt, f1, f2, f3, f4; n = 4, 4, 3, 2, 3, respectively. For wt vs. f1 p = 0.0004, wt vs. f2 p = 0.0003, wt vs. f3 p = 0.0016, wt vs. f4 p = 0.0031. 

**d.** Quantification of ZsGreen-positive POMC neurons in the ARC of POMC<sup>chrome</sup> ROSA26rSrZsGreen mice at indicated time points. For age groups 5, 10, 15, 20, 25 and 31 weeks; n = 3, 3, 6, 4, 3, 3, respectively. 

**e-f.** Body weight curve of POMC<sup>chrome</sup> males (e, Control NCD n = 19, Control HFD n = 23, POMC<sup>chrome</sup> NCD n = 15, POMC<sup>chrome</sup> HFD n = 4) and females (f, Control NCD n = 12, Control HFD n = 8, POMC<sup>chrome</sup> NCD n = 19, POMC<sup>chrome</sup> HFD n = 7) versus control littersmates on NCD and HFD. 

**g-n.** Glucose tolerance test (g, h), body composition (i, j), food intake (k, l), and energy expenditure (m, n) of POMC<sup>chrome</sup> males and females on NCD. 

**n** indicates the number of mice. Data are represented as mean ± SEM. p-values were calculated by one-way-ANOVA (d) followed by Dunnettt’s (c), two-way-ANOVA (g, h, m, n) or unpaired two-tailed Student’s t-test followed by Holm-Sidak correction for multiple comparisons (i, j, k, l). *

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Further statistical details are given in Source data extended Fig. 1.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Schematic diagram of the B9-36 targeting, breeding scheme for experimental mice, whole brain images displaying ZsGreen expression and the comparison of coronal sections in 2D vs 3D. a, Schematic diagram of the B9-36 targeting and Southern blot strategy for transgene expression dependent on intersectional Cre- and Dre-recombinase activity. Filled triangles: loxP sites; Filled diamonds: rox sites; SAH: short arm of homology; LAH: long arm of homology; WSS: Westphal Stop Sequence; NEO: neomycin resistance gene; CAG: chicken-β-actin promotor; WPRE: woodchuck hepatitis post-transcriptional regulatory element; 2 A: self-cleaving T2A peptide sequence. b, Breeding scheme for obtaining experimental mice, depicting the triple transgenic mice and their littermate controls. c, Left to right: Whole brain imaging of Pomc^{loxP} Lepr^{loxP} ROSA26rSrIsiZSGreen^{+/−}, Pomc^{loxP} GLP1R^{loxP} ROSA26rSrIsiZSGreen^{+/−}, Pomc^{loxP} Lepr^{loxP} ROSA26rSrIsiZSGreen^{+/−} 15-week-old mice, respectively. ZsGreen signal specific to the POMC^{loxP} in the ARC is displayed in the boxed area within the last image on the right. No ectopic expression was observed in only-Cre+ or only-Dre+ mice as depicted in the images on the left. Whole-brain scans were acquired using the LSFM with total magnification of 1.6X. d, Bilateral, coronal view of the distribution pattern of POMC^{loxP} and POMC^{GLP1R} neurons in POMC^{loxP} Lepr^{loxP} ROSA26lSrIsrZsGreen and POMC^{GLP1R} Glp1r^{loxP} ROSA26lSrIsrZsGreen mice. Coronal cross-sections were generated by extracting the 3D coordinates of transgenically labelled POMC^{loxP} and POMC^{GLP1R} neurons in the given section. Data was merged from n = 3 mice/group. e, Unilateral, coronal view of the distribution pattern of POMC^{loxP} and POMC^{GLP1R} neurons labelled via RNA in situ hybridization. Data was merged from n = 3 mice/group.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Cre/Dre-dependent tdTomato transgenic lines used for projection analysis of POMCLepr+ and POMCGlp1r+ neurons in 2D sections. a, Illustration of experimental mice and schematic diagram showing Dre- and Cre-dependent targeted expression of tdTomato in either POMCLepr+ or POMCGlp1r+ neurons. Excision of loxP and rox-flanked stop cassettes through recombination of both Cre and Dre drivers leads to tdTomato expression in the targeted subpopulation. b, Representative microscopic images of immunofluorescent staining against tdTomato and POMC in 12-week-old POMCLepr+ ROSA26srSrlSltdTomato and POMCGlp1r+ ROSA26srSrlSltdTomato in the anterior bed nucleus of the stria terminalis (BNST), the periaqueductal gray (PAG), the dorsomedial hypothalamic area (DMH) and the paraventricular nucleus of the hypothalamus (PVH). Scale bar represents 150 μm. 3V = third ventricle, ac = anterior commissure, AQ = cerebral aqueduct. c-d, Quantification of POMC (c) and tdTomato fiber density (d) assessed as raw integrated density of immunofluorescent staining depicted in (b). c, n = 5 mice and a minimum of 3, 3, 2, 1 section(s) per mouse were analyzed for the BNST, PVH, DMH and PAG. d, n = 5 mice and a minimum of 3,3,2,1 sections per mouse were analyzed for the BNST, PVH, DMH and PAG. In d and e, data are represented as mean ± SEM. *p ≤ 0.05. Further statistical details are given in Source data extended Fig. 2.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Visualized 3D projection densities of POMC<sup>Glp1r</sup> + neurons. Representative images of 3D-rendered tdTomato fiber density in POMC<sup>Glp1r</sup> δ<sup>Rosa26</sup> SlrSlrSlrdTomato mice in the paraventricular nucleus of the hypothalamus (PVH), the periaqueductal gray (PAG), the dorsomedial hypothalamic area (DMH), the anterior bed nucleus of the stria terminalis (BNST) and the nucleus tractus solitarius (NTS).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Cre/Dre-dependent hM3Dq transgenic lines allow for specific neuronal activation solely in triple transgenic mice. a-b, Southern blots of R26lSlrSrhM3Dq mouse line with the ROSA26 (a) and NEO probe (b). The asterisk indicates the clone chosen for blastocyst injection. (c) Representative microscopic images of RNA in situ hybridization against Pomc, Glp1r, ZsGreen (in lieu of hM3Dq) and Fos in POMC\textsuperscript{Cam} Glp1r\textsuperscript{Cam} ROSA26lSlrSrhM3Dq mice injected with saline (top) or CNO (bottom). Images on the left show ISH in the ARC with nuclear counterstain (blue, DAPI). Magnifications of the dashed boxes are displayed on the right showing the indicated stainings. Pomc-positive neurons have been traced with a white outline. Scale bars represent 50\,\mu m in the merged image and 25\,\mu m in the magnifications. 3\,V = third ventricle. d-e, Percentage of Pomc-positive cells expressing Fos in the different CNO-injected genotype controls (d) and triple transgenic animals injected with saline or CNO (e) of POMC\textsuperscript{Cam} Lepr\textsuperscript{Cam} ROSA26lSlrSrhM3Dq and POMC\textsuperscript{Cam} Glp1r\textsuperscript{Cam} ROSA26lSlrSrhM3Dq mice. d, n = 4 mice, e, n = 5 and 6 mice for groups with and without CNO, respectively. f-g, Percentage of Lepr\textsuperscript{Cam} or Glp1r\textsuperscript{Cam} cells in (f) POMC\textsuperscript{Cam} Lepr\textsuperscript{Cam} ROSA26lSlrSrhM3Dq and (g) POMC\textsuperscript{Cam} Glp1r\textsuperscript{Cam} ROSA26lSlrSrhM3Dq male mice as quantified from RNA in situ hybridization. n = 3 mice. h-j, Percentage of ZsGreen-Pomc-positive cells expressing Lepr or Glp1r (h), percentage of Lepr/Glp1r-Pomc-positive cells expressing ZsGreen (i) and percentage of ZsGreen-Pomc-positive cells expressing Fos (j) in POMC\textsuperscript{Cam} Lepr\textsuperscript{Cam} ROSA26lSlrSrhM3Dq or POMC\textsuperscript{Cam} Glp1r\textsuperscript{Cam} ROSA26lSlrSrhM3Dq females (22–26 weeks old) injected with saline or CNO. j, POMC\textsuperscript{Cam}, saline n = 3, CNO n = 4, saline vs CNO p < 0.000001, POMC\textsuperscript{Cam}, saline n = 3, CNO n = 3, saline vs CNO p = 0.000002. n indicates number of mice. Data are represented as mean ± SEM. CNO = 3 mg/kg. Statistical analyses on (b) were assessed by two-way-ANOVA. All other statistical analyses were performed by unpaired two-tailed Student’s t-test with (h-j) or without (e-g) Holm-Sidak correction for multiple comparisons. ***p ≤ 0.001, ****p ≤ 0.0001. Further statistical details are given in Source data extended Fig. 3.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Metabolic phenotyping of Cre/Dre-dependent ROSA26IslSrM3Dq in males and food intake measurements in female mice.

a–b, Cumulative food intake over a time course of 24 hours in all saline-injected genotype controls (a) and pooled saline- or CNO-injected control animals vs. saline-injected POMC<sup>Lepr<sup>C<sup>+</sup></sup></sup> ROSA26IslSrM3Dq and POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sup> ROSA26IslSrM3Dq male mice (b) starting with the night cycle. Mice were injected with saline at 18:00 and 23:00, followed by one-day gap and subsequent CNO injections at 18:00 and 23:00 on the next day. Data of all genotype controls in (a) is shown as pooled saline-injected Control in (b), data of saline-injected triple transgenic animals (a) are shown in Fig. 4f and g as control group. (a) Cre-/Dre-, Cre-/Dre<sup>+</sup>, LeprCre<sup>+</sup>/Dre-, Glp1rCre<sup>+</sup>/Dre-, n = 8, 9, 4 and 5 mice respectively. (b) Control Saline n = 25 and Control CNO n = 26 mice.

c–h, Energy expenditure (c, d), RER (e, f) and locomotion (g, h) in POMC<sup>Lepr<sup>C<sup>+</sup></sup></sup> ROSA26IslSrM3Dq (c, e, g) and POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sup> ROSA26IslSrM3Dq male mice (d, f, h) vs. controls at 12–14 weeks of age. Mice were injected with saline at 17:00, 22:00 and 07:00 followed by CNO at 17:00, 22:00 and 07:00 on the next day. Scatter plots on the right show the average values during night and day as averaged from left graph. Values of control animals were pooled from all corresponding genotype controls. Sal = Saline. c and e, Control n = 29, POMC<sup>Lepr<sup>C<sup>+</sup></sup></sup> n = 13. d and f, Control n = 38, POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sup> n = 12. g, Control n = 31, POMC<sup>Lepr<sup>C<sup>+</sup></sup></sup> n = 14. h Control n = 41, POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sup> n = 12. E<sub>night</sub>: Night: Control<sub>S</sub> vs POMC<sup>Lepr<sup>C<sup>+</sup></sup></sup><sub>C<sub>NO</sub></sub>, p = 0.016, Day: Control<sub>S</sub> vs POMC<sup>Lepr<sup>C<sup>+</sup></sup></sub><sub>C<sub>NO</sub></sub>, p = 0.0014, POMC<sup>Lepr<sup>C<sup>+</sup></sup></sub><sub>C<sub>NO</sub></sub> vs POMC<sup>Lepr<sup>C<sup>+</sup></sup></sub><sub>C<sub>saline</sub></sub>, p = 0.0065, Day: Control<sub>S</sub> vs POMC<sup>Lepr<sup>C<sup>+</sup></sup></sub><sub>C<sub>NO</sub></sub>, p = 0.01. F<sub>night</sub>: Night: Control<sub>S</sub> vs POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sub><sub>C<sub>NO</sub></sub>, p = 0.001, Control<sub>C</sub> vs POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sub><sub>C<sub>saline</sub></sub>, p = 0.0049, Day: Control<sub>S</sub> vs POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sub><sub>C<sub>NO</sub></sub>, p = 0.035, POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sub><sub>C<sub>saline</sub></sub> vs POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sub><sub>C<sub>NO</sub></sub>, p = 0.028. i–j, Food intake over a time course of 24 hours in POMC<sup>Lepr<sup>C<sup>+</sup></sup></sup> ROSA26IslSrM3Dq (i) and POMC<sup>Glp1r<sup>C<sup>+</sup></sup></sup> ROSA26IslSrM3Dq female mice (j) starting with the night cycle. Mice were injected with saline at 18:00 and 23:00, followed by one day gap and subsequent CNO injections at 18:00 and 23:00 on the next day. Left: Cumulative food intake in mice injected with saline vs. CNO. Right: Total food intake during night and day. n = 9 mice/group (i), n = 10 mice/group (j). CNO = 3 mg/kg. Data are represented as mean ± SEM. All p-values were calculated by two-way-ANOVA. For cumulative food intake (i–j, left) two-way-ANOVA was used, for total food intake (i–j, right) two-way-ANOVA followed by Sidak’s post-hoc test. In c–h significance was calculated exclusively on scatter plots and ANOVA was followed by Tukey’s post-hoc test, where relevant. *p < 0.05, **p < 0.01, ***p < 0.001. Further statistical details are given in Source data extended Fig. 4.
Extended Data Fig. 7 | Vgat and Vglut2 expression in POMC^{Lepr}{\textsuperscript{cre}} and POMC^{Glp1r}{\textsuperscript{cre}} neurons. a, Representative microscopic images of RNA in situ hybridization against ZsGreen and Vgat or Vglut2 in POMC^{Lepr}{\textsuperscript{cre}} ROSA26iSlrSrM3Dq (left) and POMC^{Glp1r}{\textsuperscript{cre}} ROSA26iSlrSrM3Dq (right) male mice. Scale bars represent 100 \( \mu\)m in the merged image and 25 \( \mu\)m in the magnifications. b, Quantification of the percentage overlap of Vgat or Vglut2 with ZsGreen-expressing cells. For POMC^{Lepr} \( n = 4 \) and POMC^{Glp1r} \( n = 5 \) mice, respectively. Data are represented as mean \( \pm \) SEM. Statistical analyses on B were performed by two-way ANOVA with Sidak’s post-hoc test. **\( p \leq 0.01 \). Further statistical details are given in Source data extended Fig. 5.
Extended Data Fig. 8 | ZsGreen expression in pituitary of POMC<sup>Cre<sup> Lepr<sup>Cre<sup> ROSA26iSlrSrZsGreen and POMC<sup>Cre<sup> Glp1r<sup>Cre<sup> ROSA26iSlrSrZsGreen mice.

a, b, Representative microscopic images of RNA in situ hybridization against ZsGreen in pituitary of POMC<sup>Cre<sup> Lepr<sup>Cre<sup> ROSA26iSlrSrZsGreen (a) and POMC<sup>Cre<sup> Glp1r<sup>Cre<sup> ROSA26iSlrSrZsGreen (b) mice, respectively. Scale bars represent 150 μm in the merged image and 50 μm in the magnifications.

c, d, Corticosterone levels in sera of CNO treated POMC<sup>Cre<sup> Lepr<sup>Cre<sup> ROSA26iSlrSrZsGreenM3Dq (c) and POMC<sup>Cre<sup> Glp1r<sup>Cre<sup> ROSA26iSlrSrZsGreenM3Dq (d) male and female mice. Mice were fasted for 2 hours and injected with CNO 1 hour into the fast prior to serum collection. CNO = 3 mg/kg. C males; Controls n = 7, Cre<sup>−<sup>Dre<sup>− n = 11, C females; Controls n = 8, Cre<sup>−<sup>Dre<sup>− n = 8, D males; Controls n = 8, Cre<sup>−<sup>Dre<sup>− n = 7, D females; Controls n = 10, Cre<sup>−<sup>Dre<sup>− n = 11. Data are represented as mean ± SEM. Statistical analyses on (c–d) were performed by unpaired two-tailed Student’s t test with Holm-Sidak correction for multiple comparisons. Further statistical details are given in Source data extended Fig. 6.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Validation of the Dre-Cre dependent ROSA26TdTomrSrEGFPL10a transgenic line. a–b, Southern blots of ROSA26TdTomrSrEGFPL10a mouse line with the ROSA26 (a) and Neo probe (b). The asterisk indicates the clone chosen for blastocyst injection. c, Representative microscopic images of immunofluorescent staining against POMC and EGFP in the ARC of all resulting genotypes at 12 weeks of age. Scale bar represents 50 μm. 3V = third ventricle. d, Principal component analysis of all RNA input and IP samples. e–g, GO term representations of the differentially enriched genes between the POMC<sup>Lepr<sup>+</sup></sup> and POMC<sup>Glp1r<sup>+</sup></sup> clusters mapping the percentage of regulated genes to the total GO term gene count against significant gene count and adjusted p-value per term. h, UMAP plot of 773 POMC neurons identified in normal chow diet fed mice. Cells that are closer in position in the plot have a similar genetic profile. In (e–g) p-values were calculated using the DESeq2 1.26.0 pipeline with adjustments for multiple comparisons.
Extended Data Fig. 10 | Spontaneous firing frequency, cell capacitance and significantly differentially enriched genes encoding ion channels and sub-units potentially contributing to different electrophysiological profiles of POMC<sup>Lepr</sup> and POMC<sup>Glp1r</sup> neurons. Violin plots illustrating spontaneous firing frequencies (a), whole-cell capacitance (b), threshold currents for the generation of action potentials (c), and the repolarisation rate of action potentials (d) of POMC<sup>Lepr</sup> and POMC<sup>Glp1r</sup> neurons. e, Genes encoding sodium-potassium-ATPases. f, Genes encoding voltage-gated Na<sup>+</sup> channels and subunits modulating voltage-gated Na<sup>+</sup> channels. g, Genes encoding voltage-gated K<sup>+</sup> channels and subunits modulating voltage-gated K<sup>+</sup> channels. h, Gene Hcn1 hyperpolarization-activated cyclic-nucleotide-gated cation channels 1. i, Gene Cacnb4 encoding a subunit modulating L-Type voltage-gated Ca<sup>2+</sup> channels. j, Gene Kcnq5 and Kcnmb4 encoding a M-current K<sup>+</sup> channel and the beta-subunit 4 modulating large conductance Ca<sup>2+</sup> activated K<sup>+</sup> channels, respectively. Data are represented as mean ± SEM. (a-d) p-values were calculated using the unpaired Mann-Whitney-U test. e-j, Selected from differentially ribotag-enriched transcripts analyzed in Fig. 5b (p ≤ 0.05). For POMC<sup>Lepr</sup> ROSA26SlrSrEGFPL10a n = 4 hypothalami pooled from N = 24 mice, for POMC<sup>Glp1r</sup> ROSA26SlrSrEGFPL10a n = 3 hypothalami pooled from N = 36 mice. p-values were calculated by DESeq2 1.26.0. Statistical details are given in Source data extended Fig. 7.
**Reporting Summary**

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**Statistics**

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- **n/a** Confirmed

- [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

- [ ] A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

- [ ] The statistical test(s) used AND whether they are one- or two-sided

  - *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*

- [ ] A description of all covariates tested

- [ ] A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

- [ ] A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

- [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

  - Give P values as exact values whenever suitable.

- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

- [ ] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

*Our web collection on [statistics for biologists](https://www.nature.com/natureprotocol/2015/10-29-000045) contains articles on many of the points above.*

**Software and code**

Policy information about [availability of computer code](https://www.nature.com/natureprotocol/2020/20-04-20-000045)

**Data collection**

- **Image acquisition software**: Leica ASX v.3.5.5.19976, Imспект Pro v.5.0.222.0.
- Indirect calorimetry data acquisition was carried out using TSE Phenomaster versions 6.2.5 and above.
- For data acquisition on an IVIS Spectrum CT scanner (Caliper LifeScience, USA) we used IVIS LivingImage Software V4.3.1.

**Data analysis**

Details are provided in the Methods section. The main software used are as follows:

- **VINCI** (versions 4.61.0 and 4.96.0) was used for co-registration of the mouse brain scans acquired using light sheet fluorescent microscopy.
- **Arivis Vision 4D** (versions 2.12 and 3.3.0) was used for 3D rendering, visualization and neuronal coordinate extraction.
- Custom-written Python (version 2.7.12) script was used to create isosurface density plots and to carry out statistical analysis of the 3D data. The RNA-sequencing results were processed using the nf-core/rnaseq pipeline v1.4.2. Both aforementioned codes are available on Github.
- Confocal images were mainly analyzed by FIJI (NIH, version 2.0.0-rc-41/1.50d). For intensity quantification of endogenous Lepr, Glp1r and Pomc expression in C57BL/6N mice, the Halo software (Indica Labs, V.2.2.1870) was used.
- Data analysis was also performed using Spike2 (version 7; Cambridge Electronic Design Ltd., Cambridge, UK), Igor Pro 6 (Wavemetrics, Portland, OR, USA), and Graphpad Prism (versions 5.0-8.0; Graphpad Software Inc., La Jolla, CA, USA).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](https://www.nature.com/natureprotocol/2020/20-04-20-000045) for further information.
Raw RNA-Seq data have been deposited into the NCBI Gene Expression Omnibus (GEO) under the accession code: GSE153753. Raw data is available under reasonable request to the corresponding author.

The code for RNA-sequencing analysis is available at https://github.com/bruening-lab/pomc-neurons-architecture-rnaseq and the code for isosurface density plots as well as the 3D statistical analysis is available at https://github.com/bruening-lab/Heterogeneity_Scripts

Ensembl release 97 was used for reference genome, transcripts and GO-term analysis. In the comparison of the results of our RNA sequencing with publicly available datasets we used data published by Campbell et al. (2017), as well as the study by Yeo et al. (2017).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No statistical methods were used to pre-determine sample sizes and the sample sizes in the study are similar to those reported previously: Brandt et al. (2018), Vogt et al. (2015), Konner et al. (2007).

Data exclusions
Except for animals that died during the course of an experiment, no data were excluded.

Replication
For metabolic phenotyping, every mouse represents a replicate (n) and the number of replicates is mentioned for each experiment in the figure legend and/or supporting materials. In this case, data was pooled from independent experiments of varying n numbers. For RNA sequencing, samples of pooled hypothalami were collected from individual mice of several cohorts. For electrophysiological experiments, the sample numbers indicate the number of cells recorded from. All measurements that didn’t require statistical analysis such as representative images were obtained from at least 2 animals and in most cases a minimum of 3 animals were used.

Randomization
Mice used for experiments were assigned to their corresponding experimental group by genotype. In case of treatments within one genotype, mice were assigned randomly to the treatment groups.

Blinding
Data collection and analysis were carried out in a blinded format throughout the study, unless this was not possible due to the visual differences in cases of varying neuronal numbers resulting from genetic labeling as depicted in Figure 3A-C.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
|     | Eukaryotic cell lines |
| ☑   | Palaeontology and archaeology |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |
|     | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
|     | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

Antibodies

Primary antibodies:
rabbit anti-ZsGreen (Takara Bio Clontech #632474, 1:100), rabbit anti-POMC (1:1000, Phoenix, #H-029-30), rat anti-mCherry (1:1000)
Validation

Thermo Fisher Scientific, #M11217) and chicken anti-GFP (1:1000 Abcam, #ab13970)

Secondary antibodies:
donkey anti-rabbit-Alexa488 (ThermoFisher #A21206, 1:500), donkey anti-rat-594 (JacksonImmunoResearch, #712-585-153, 1:500) or goat anti-rabbit-594 (Thermo Fisher Scientific, #A11012, 1:500) and goat anti-chicken-488 (Thermo Fisher Scientific, #A11039, 1:500)

rabbit anti-ZsGreen (Takara Bio Clontech #632474)
https://www.takarabio.com/products/antibodies-and-elisa/fluorescent-protein-antibodies/green-fluorescent-protein-antibodies?catalog=632474

rabbit anti-POMC (Phoenix, #H-029-30)
https://www.phoenixpeptide.com/products/view/Antibodies/H-029-30

rat anti-mCherry (Thermo Fisher Scientific, #M11217)
https://www.thermofisher.com/antibody/product/mCherry-Antibody-clone-16D7-Monoclonal/M11217

canary anti-GFP (Abcam, #ab13970)
https://www.abcam.com/gfp-antibody-ab13970.html

donkey anti-rabbit-Alexa488 (ThermoFisher #A21206)
https://www.thermofisher.com/order/genome-database/details/antibody/A-21206.html

donkey anti-rat-594 (JacksonImmunoResearch, #712-585-153)
https://www.jacksonimmuno.com/catalog/products/712-585-153

goat anti-rabbit-594 (Thermo Fisher Scientific, #A11012)
https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11012

goat anti-chicken-488 (Thermo Fisher Scientific, #A11039)
https://www.thermofisher.com/antibody/product/Goat-anti-Chicken-IgY-H-L-Secondary-Antibody-Polyclonal/A-11039

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals
For all experiments, male and female mice, between the ages of 4 to 26 weeks from the C57BL/6N background were used. Mice were housed in individually ventilated cages (IVCs) at 22°C–24°C using a 12 hr light/dark cycle. Animals had access to water and food ad libitum. All animal experiments were performed in accordance with regulations of the relevant animal welfare acts and protocols approved by the respective regulatory authorities. LeprCre mice (Leshan et al. 2006) and the Glp1rCre (Richards et al. 2014) lines were kindly provided by Prof. MG. Myers and Prof. F. Reimann, respectively. The ROSA26rSrlSltdTomato was purchased from Jackson Laboratories. The POMCDre recombinase line and ROSA26lSrlSr СергM3Dq, ROSA26lSrlSrEGFPL10a mouse lines were newly generated in this study.

Wild animals
No wild animals were assessed in this study.

Field-collected samples
No field collected samples were assessed in this study.

Ethics oversight
All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln). Permissions for experiments and to maintain and breed mice was issued by the Department for Environment and Consumer Protection-Veterinary Section, Cologne, North Rhine-Westphalia, Germany.

Note that full information on the approval of the study protocol must also be provided in the manuscript.