Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits

Stephanie L Padilla1,2, Jill S Carmody1,2 & Lori M Zeltser2,3

Hypothalamic neuron circuits regulating energy balance are highly plastic and develop in response to nutrient and hormonal cues. To identify processes that might be susceptible to gestational influences in mice, we characterized the ontogeny of proopiomelanocortin (POMC) and neuropeptide Y (NPY) cell populations, which exert opposing influences on food intake and body weight. These analyses revealed that Pomc is broadly expressed in immature hypothalamic neurons and that half of embryonic Pomc-expressing precursors subsequently adopt a non-POMC fate in adult mice. Moreover, nearly one quarter of the mature NPY+ cell population shares a common progenitor with POMC+ cells.

The rapid increase in the prevalence of childhood obesity and the concomitant rise in obesity-related medical morbidities and costs lend urgency to the need for new insights into the causes and potential preventive measures for this disease1. Mounting evidence supports the idea that the maternal environment can impart a lasting effect on offspring at E13.5 (Fig. 1d). Subsequently, Npy+ cells reached a maximum at E13.5, after which its expression was extinguished in more than half of the population between E14.5 and E18.5 (Fig. 1f). We did not observe Npy expression in the ventricular zone; we first detected it in laterally situated cells in the rostral aspect of the presumptive ARH at E13.5 (Fig. 1d). Subsequently, Npy+ cells are predominately situated in the ventromedial ARH. We did not detect appreciable numbers of apoptotic cells by TUNEL staining (data not shown), which is consistent with the idea that Pomc expression is turned off in a large percentage of immature hypothalamic neurons1,2. These data argue that Pomc expression is not a cause of the terminal cell fate. Rather, the gradual extinction of Pomc and progressive onset of Npy represent an ongoing maturation process that extends throughout gestation. Supporting this idea, POMC and NPY neurons do not acquire their terminal peptidergic phenotype until the postnatal period in rodents, as reflected by the gene encoding cocaine- and amphetamine-regulated transcript and Agrp expression, respectively3,14.

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**Figure 1.** Pomc is transiently expressed in a broad population of hypothalamic neurons during embryonic development. (a–c) We injected wild-type dams once with 200 mg per kg body weight BrdU at E11.5 and killed the offspring at P9 for analysis. (a) BrdU Immunohistochemistry. (b,c) Combined FISH with immunohistochemistry with probes against Pomc (b) or Npy (c) in conjunction with an antibody against BrdU and counterstained for DAPI. Magnified view of the boxed areas in the composite are shown at the bottom; individual layers from the boxed regions are shown at the left. (d) FISH for Pomc (green) and Npy (red) in ventral hypothalamus from E11.5 to E18.5. 3V, third ventricle. (e) Confocal image of a cell containing both Npy and Pomc transcripts at E14.5. (f) We counted cells expressing Pomc and Npy as described in Supplementary Figure 3. Each group represents the average counts of at least five coronal sections per mouse, spanning the rostrocaudal extent of the presumptive ARH, with error bars representing means ± s.e.m. (n≥3 mice for each group). (g) Pomc and Npy expression, as assessed by PCR on cDNA generated from sorted GFP+ cells. We dissociated cells from transgenic Npy-hrGFP hypothalami and FACS-purified GFP+ cells at E14.5 and P9. –, no cDNA control; A, adult hypothalamus positive control; NG, Npy-hrGFP. Scale bars: a, 100 μm; b,c, 50 μm (low magnification); 10 μm (high magnification); d, 50 μm; e, 5 μm; all tissues are 10-μm cryosections.

*Pomc* and *Npy* are expressed in mutually exclusive cell populations in adults,[15] yet we detected *Pomc* and *Npy* colocalization at midgestation (Fig. 1e). To substantiate this unexpected finding that a subset of neurons expresses both *Pomc* and *Npy*, we compared the expression profiles of NPY neurons isolated from embryonic and postnatal stages. We used FACS to collect GFP+ cells from Npy-hrGFP embryos, which express humanized Renilla green fluorescent protein (hr-GFP) under the control of Npy promoter and enhancer elements.[16] We detected *Pomc* transcripts by PCR on sorted cells from E14.5 but not from P9 (Fig. 1g and Supplementary Fig. 3a,b). These observations support the idea that during gestation a subset of *Pomc*-expressing cells can differentiate into NPY neurons.

Next we used a genetic lineage tracing strategy[17] to visualize the mature POMC neuronal population, defined by *Pomc* expression in adults, in relation to the broad immature *Pomc*-expressing population in the embryo (Fig. 2). In this strategy, we used mice with Cre recombinase driven by *Pomc* regulatory elements to direct the excision of a *loxP*-flanked stop codon (*Pomc-Cre*) upstream of a Gfp reporter knocked into the constitutively active ROSA26 locus (R26-GFP). In this way, cells that express *Pomc* from gestation are permanently marked.[18] To assess *Pomc* transcriptional activity in conjunction with a GFP reporter, we developed a technique to combine images of direct GFP fluorescence with FISH (Supplementary Figs. 4 and 5). When we performed this assay on adult tissue from *Pomc-GFP* transgenic mice, 95% of *Pomc*-GFP+ neurons also expressed *Pomc* (Fig. 2a), validating the sensitivity of this technique.[2] In contrast, only half of the GFP+ cells in *Pomc-Cre*R26-GFP mice expressed *Pomc* (Fig. 2b) and Supplementary Fig. 6). *Pomc*-negative GFP+ cells in *Pomc-Cre*R26-GFP adults probably represent cells that turned off *Pomc* expression at some point after E13.5. GFP+ cell counts in *Pomc-Cre*R26-GFP mice were consistently twice as high as those generated by *Pomc FISH* or direct fluorescence in *Pomc-GFP*–transgenic mice (Fig. 2d).

Given our finding that *Npy* and *Pomc* colocalize in a subset of embryonic neurons, we considered whether some of the *Pomc*-negative GFP+ neurons in *Pomc-Cre*R26-GFP adults are NPY neurons. We detected Npy expression in 17% ± 2% of GFP+ neurons in adult *Pomc-Cre*R26-GFP mice (Fig. 2b). We used two strategies to independently verify this observation. First, confocal images of immunohistochemistry on Npy-GFP;*Pomc-Cre*R26-LacZ mice confirmed that 25% of NPY (GFP+) neurons also express the *Pomc-Cre* lineage trace (β-galactosidase immunohistochemistry) (Fig. 2c,e). Second, RT-PCR on FACS-purified GFP+ cells from *Pomc-Cre*R26-GFP mice showed that some cells marked by the lineage trace express *Npy* (Fig. 2f and Supplementary Fig. 3c).

These data provide evidence that a subpopulation of NPY neurons are derived from progenitors that are distinct from other ARH NPY neurons, raising the possibility that they serve different functions within the hypothalamic feeding circuit and, thus, may underlie the known heterogeneous electrophysiological properties of NPY neurons.[19] Although the origins of NPY subpopulations may differ, their subsequent differentiation converges on an orexigenic, GABAergic phenotype, as we found that all ARH NPY neurons express Agrp and the gene encoding glutamic acid decarboxylase-67, a rate-limiting enzyme in GABA synthesis.[2] A key area for future research is to use labeling techniques that distinguish between *Pomc*-derived and *Pomc*-nonderived NPY neurons in conjunction with analyses of gene expression profiles, neuronal architecture and electrophysiological properties. Classification of functionally distinct subsets of neurons derived from a *Pomc* lineage is crucial for elucidating how hormonal and nutrient signals are sensed by ARH neurons and relayed to downstream targets that regulate body weight and energy homeostasis.

In this study, we have shown that *Pomc* is transiently expressed by the vast majority of cells in the developing ventral hypothalamus...
Figure 2 NPY neurons derived from a Pomc-expressing lineage persist to adulthood. (a,b) Images of direct GFP fluorescence obtained before FISH processing in Pomcg-GFP (a, left) or Pomc-Cre;R26-GFP (b, left) adults, followed by FISH with Pomc alone (a, center) or Pomc (green) plus Npy (red) probes (b, center) or composite images (a, b, right) (technique described in Supplementary Fig. 5). In b, cells expressing Npy and the Pomc-Cre lineage marker, GFP, are indicated with arrowheads. Because of its perinuclear localization, the FISH signal appears as a ring around the GFP signal. (c) Confocal images of β-galactosidase immunohistochemistry (red) in conjunction with direct GFP fluorescence (green) in Pomc-Cre;R26-LacZ;Npy-GFP adults. (d) Cells counts across the rostral-caudal axis of the ARH for Pomc transcript (Pomc-FISH) or direct fluorescence of GFP from Pomc-GFP adults or from Pomc-Cre;R26-GFP (Pomc-Cre) adults. (e) High-magnification images of the region boxed in c. (f) We dissociated hypothalamic cells from transgenic Pomc-Cre;R26-GFP mice and FACS-purified GFP+ cells at P9. Pomic and Npy expression, as assessed by PCR on cDNA generated from sorted GFP+ cells. Adjacent lane doublets separated by the line were run simultaneously on the same gel but not in neighboring lanes; they have been juxtaposed for the purposes of this figure. PCG, Pomc-Cre;R26-GFP. Scale bars: 100 μm (a-c), 10 μm (e). Error bars represent means ± s.e.m. (n ≥ 42 sections for each group from n ≥ 6 mice). **P < 0.0001.

(Fig. 1d,f). During gestation, Pomc transcription is extinguished in more than half of these cells, some of which subsequently differentiate into NPY neurons and some of which adopt alternative terminal fates. Consistent with our FISH analyses, when we used Pomc-Cre;R26-GFP mice to trace Pomc-derived lineages in the adult hypothalamus, we found that half of the GFP-labeled neurons are non-POMC neurons. This Pomc-Cre driver was developed as a tool to investigate the physiological consequences of POMC-specific loss or gain of gene function. As we demonstrated that recombination of floxed alleles is not limited to mature POMC neurons and includes a substantial portion of mature NPY neurons, previous functional studies using this Cre driver should be reanalyzed. Indeed, some functions ascribed to POMC neurons might be mediated by non-POMC neurons that expressed Pomc at one time in development.

The observation that several functionally distinct cell types arise from a Pomc-expressing lineage raises the possibility that factors that influence cell fate decisions within the immature population during gestation could permanently affect the neuronal composition of circuits regulating energy homeostasis. Dietary manipulations during gestation have been associated with increased Pomc expression at postnatal stages, supporting the idea that maternal signals can influence the differentiation of the Pomc-expressing lineage. Two major areas for future research are to determine whether specific maternal nutrient or hormonal signals influence the differentiation or the architecture of neuronal lineages in hypothalamic circuits and, if so, to assess how these changes affect metabolic phenotypes in the offspring.

All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Columbia University Health Sciences Division.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS
S.L.P. performed experiments, analyzed data and wrote the paper; J.S.C. generated reagents. This work was supported by F31DK079372 (S.L.P.), US Institute of Human Nutrition Training Grant 2T32DK007647-21 (J.S.C.), American Diabetes Association Grant 7-07RA-195 (L.M.Z.), Columbia Diabetes and Endocrinology Research Center Pilot and Feasibility Award P30 DK63608-07 (L.M.Z.) and NY Obesity Research Center Pilot and Feasibility Grant P30 DK26687-26 (L.M.Z.).

COMPETING FINANCIAL INTERESTS
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Pomc-expressing progenitors give rise to antagonistic populations in hypothalamic feeding circuits

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Supplemental Material

Supplementary Methods

Animals
Animals were housed in temperature controlled rooms at 21 °C and subject to a 12 h light-dark cycle. Mice had \textit{ad libitum} access to standard chow diet (Lab Diet: PicoLab Rodent Diet 5053) and water. C57BL/6 and (\textit{ROSA})\textsuperscript{26}Sor-EGFP reporter (R26-GFP) mice were obtained from the Jackson Laboratory and bred at the Russ Berrie Animal Facility. \textit{Pomc-Cre} \textsuperscript{2}, \textit{Pomc-GFP} \textsuperscript{3} and \textit{Npy-hrGFP} \textsuperscript{4} transgenic animals were generously provided by Joel Elmquist, Malcom Low and Bradford Lowell respectively. All analyses were performed on mice which were \textit{Pomc-Cre Tg}\textsuperscript{*} and homozygous for the R26-GFP allele. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Columbia University Health Sciences Division.

Genotyping
Genotyping at the \textit{ROSA26} locus was performed using the following three-primer set: oIMR 0883: 5’-AAAGTCGCTCTGAGTTGTTAT-3’; oIMR 0315 5’-GCGAAGAGTTTGTCCTCAACC-3’; oIMR 0316 5’-GGAGCGGGAGAAATGGATATG-3’. Genotyping of \textit{Npy-hrGFP} transgenic mice was performed using: NPY-ata-S-F: 5’-TATGTGGACGCGGGCAAGAGATCCAGG-3’, NPY-ata-S-R: 5’-CCCAGCTCACATATTTATCTAGAG-3’ and AA33 5’-GGTGCGGTGGCCTGCATGGAG-3’ \textsuperscript{4}. The \textit{Cre} transgene was assessed with: 5’-GCGGTCTCGCGTACGCTGGCA-3’, 5’-GTGAAACAGCATTGCTGACTT-3’.

Tissue processing
P9 and adult mice were anesthetized and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB). Brains were post-fixed at 4°C overnight and cryoprotected with 30% sucrose for 48 h. For embryonic tissue, dams were anesthetized with
Avertin and embryos were dissected in cold PBS and fixed at 4 °C overnight and cryoprotected with 30% sucrose for 24 h. Tissue was embedded in O.C.T (Tissue Tek) and frozen at −80°C. 10 µm-thick coronal sections were collected across the rostral–caudal extent of the ARH on Superfrost Plus slides (Fisher).

**Fluorescent in situ hybridization**

Frozen sections were processed as described in the TSA Plus Cy3 System manual (Perkin Elmer). Antisense digoxigenin- or fluorescein-labeled riboprobes were generated from plasmids containing PCR fragments of *Npy* and *Pomc* using the following primers sets: (NPY) 5’-TGCTAGGTAACAAGCGAATGG-3'/5’-CAACAACAACAAAGGGAAATGG-3; (POMC) 5’-GTTAAGAGCAGTGACTAAGAGGCG-3'/5’-CCTAACACAGGTAACTCTAAGAGGC-3’.

**Imaging and quantification**

Fluorescent microscopy was performed using a Nikon Eclipse 80i equipped with a Retiga EXi camera and X cite 120 fluorescent illumination system. TIFF files were acquired using Q Capture Pro software (Qimaging) and analyzed using Adobe Photoshop. Because our studies involved comparisons of images of the same tissue captured at different times, we used a diamidino-2-phenylindole (DAPI) stain to establish a reference focal plane. Images were separated into independent RGB channels using Photoshop, and Cy3 or GFP signals were compared to signals in the DAPI channel (**Supplementary Fig. 4**). Cy3 or GFP signals that did not have a corresponding DAPI-stained nucleus were excluded from our counts.

GFP fluorescence is lost during the high temperature hybridization step; thus, we pre-imaged direct GFP fluorescence in conjunction with DAPI. Following FISH, the tissue was re-stained with DAPI and imaged. Using Photoshop, pre- and post-FISH images were aligned using the common DAPI stain as a reference to generate a composite image (**Supplementary Fig. 4**).

**Combined BrdU immunohistochemistry and FISH**

To label proliferating cells, dams were given a single intraperitoneal (i.p.) injection of 5-Bromo-2-deoxyuridine (BrdU) (200 mg kg⁻¹ Sigma) on E11.5, E12.5, E13.5, E14.5 and E15.5. Offspring were sacrificed at P9 and processed as described above (cryoprotection in sucrose was reduced to 24 h). Detection of BrdU-labeled DNA by IHC requires DNA denaturation, which can be accomplished in many ways ⁵. We compared two pretreatment methods: standard 2.0 N HCl at 37 °C for 1 h, versus 50% formamide at 68 °C overnight. We found that both methods yield similar numbers of BrdU-positive cells with fluorescent IHC (**Supplementary Fig. 1a,b**). Because the same formamide treatment is also used in FISH hybridization step, we used this method to combine BrdU IHC with FISH.
Day one of the FISH protocol was followed directly, except the permeabilization step was
performed with 0.1% Triton X-100 (in PBS, 30 min) instead of proteinase K. Following the
overnight hybridization plus denaturation step, sections were blocked for one hour in 2% normal
horse serum; 0.1% Triton X-100 (Block Buffer) at room temperature. Sections were then incubated
overnight at 4 °C with the combination sheep anti-DIG-POD and rat anti-BrdU antibody (1:400,
Novus Biologicals) in Block Buffer. The following day, sections were treated with tyramide Cy3 for
10 min room temperature (described in Perkin Elmer TSA amplification kit), washed 3X in PBS and
then incubated with goat anti-rat Alexa-488 (1:500, Invitrogen) for one hour at room temperature.
IHC with goat anti-β-Gal (1:4,000, Biogenesis) was performed without tyramide amplification.
Slides were counterstained with DAPI (Invitrogen) and mounted with VectaShield (Vector Labs).

FACS and PCR analysis
Hypothalamic tissue was dissociated as described by the Papain Dissociation System manual
(Worthington). GFP+ cells were collected using a BD FACS Aria Cell Sorter by the Herbert Irving
Cancer Institute facility, total RNA was isolated (Invitrogen) and cDNA was reversed transcribed
(Invitrogen). PCR was performed using the Npy and Pomc primers sets described above.

Statistical analysis
Significance was calculated using 2-tailed Student’s t-tests, and defined as P<0.05.

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Supplemental Figures:

Supplementary Fig. 1

**Supplementary Fig. 1** Overnight incubation at 70 °C in 50% formamide can substitute for HCl pre-treatment to detect BrdU by IHC. (a,b) IHC detection of BrdU label retained at P9 in adjacent hypothalamic sections resulting from a single BrdU injection at E11.5. Similar numbers of BrdU-labeled cells were detected using the standard HCl pre-treatment at 37 °C for 1 h (a) and 50% formamide overnight at 70 °C (b). 3V, third ventricle. Scale bar: 50 µM
Supplementary Fig. 2

A population of laterally situated cells in the ARH is born at E13.5. IHC on P9 hypothalamic sections to detect BrdU-labeled cells resulting from an E13.5 injection. 3V, third ventricle. Scale bar: 100 µM
Supplementary Fig. 3  

Supplementary Fig. 3  FACS isolation of GFP positive populations from dissociated ventral hypothalamic tissue. (a,b) Cells collected from Npy-hrGFP animals at E14.5 and P9 respectively (see Figure 1g for corresponding PCR data). (c) Cells collected from Pomp-Cre;R26-GFP animals at P9 (see Fig. 2f for corresponding PCR data).
Supplementary Fig. 4

Supplementary Fig. 4 Using DAPI stain as a criterion for cell counts. (Left) *Pomc* FISH image alone. (Center) The fluorescent signal from *Pomc* FISH (red) was imaged in conjunction with a DAPI counterstain (gray). To facilitate our analyses, the channel containing the DAPI image was copied to a separate layer, converted to grayscale and made semi-transparent in Photoshop. (Right) DAPI image alone. Fluorescent signals that did not overlap with DAPI stain (marked by arrows) were excluded from the cell counts. 3V, third ventricle. Scale bar: 100 µM
Supplementary Fig. 5

Technique to analyze GFP expression in conjunction with FISH at the single cell level. (Left) Section from a Pomc-Cre;R26-GFP adult hypothalamus was pre-imaged for direct GFP fluorescence (green) in conjunction with a DAPI counterstain (gray). (Right) FISH with a Pomc probe (red) was subsequently performed on the same section and imaged in conjunction with DAPI (gray). (Center) Using the DAPI stains as a guide, images in (A) and (C) were aligned and merged in Photoshop. 3V, third ventricle. Scale bar: 100 µM.
Supplementary Fig. 6

Supplementary Fig. 6 Only half of the GFP-labeled cells marked by a Pomc-Cre;R26-GFP genetic trace express Pomc in adults. Sectioned tissue from adult Pomc-Cre;R26-GFP animals was pre-imaged for direct GFP (green) fluorescence (Left), processed using FISH with a Pomc (red) probe (Right), and aligned on the basis of DAPI stain as described in (Supplementary Fig. 5) (Center). Scale bar: 100 µM; tissue 10 µm cryo-sections.