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

Stephanie L. Padilla¹², Jill S. Carmody¹² and Lori M. Zeltser²³*

¹ Institute of Human Nutrition, ² Naomi Berrie Diabetes Center, ³ Department of Pathology and Cell Biology

Columbia University, New York, NY 10032

*Correspondence: lz146@columbia.edu

**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 *ad libitum* access to standard chow diet (Lab Diet: PicoLab Rodent Diet 5053) and water. C57BL/6 and (ROSA)26Sor-EGFP reporter (R26-GFP) mice were obtained from the Jackson Laboratory and bred at the Russ Berrie Animal Facility ¹. **Pomc-Cre** ², **Pomc-GFP** ³ and **Npy-hrGFP** ⁴ transgenic animals were generously provided by Joel Elmquist and Bradford Lowell. All analyses were performed on mice which were **Pomc-Cre Tg** 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 **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’ ¹. Genotyping of **Npy-hrGFP** transgenic mice was performed using: NPY-ata-S-F: 5’-TATGTGGACGAGCAGAGATCCAGG-3’, and AA33 5’-GGTACGGTTCGTACTGGA-3’ ⁴. The Cre transgene was assessed with: 5’-GCGGTCTGGCAGTAATACCTATC-3’, 5’-GTGAAACAGCATTGCTGCTACTT-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.
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'-TGCTAGGTAAACAGCGATGG-3'/5'-CAACAACAAACAGCCGGAAATGG-3; (POMC) 5'-GTTAAGGCAGTGACTAAGAGGCC-3'/5'-CCTAACACAGGTAACTCAGAGGC-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. 3). Cy3 or GFP signals that did not have a corresponding DAPI-stained nucleus were excluded from our counts.

NPYP versus NPYX neurons were distinguished by assessing Npy and GFP expression in Pomc-Cre;R26-GFP animals. 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 (cyroprotection 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 $P<0.05$.

**References**

1. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21, 70-1 (1999).
2. Balthasar, N. et al. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* 42, 983-91 (2004).
3. Cowley, M.A. et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411, 480-4 (2001).
4. van den Pol, A.N. et al. Neuropeptide B and gastrin-releasing peptide excite arcuate nucleus peptidase Y neurons in a novel transgenic mouse expressing strong Renilla green fluorescent protein in NPY neurons. *J Neurosci* 29, 4622-39 (2009).
5. Wojtowicz, J.M. & Kee, N. BrdU assay for neurogenesis in rodents. *Nat Protoc* 1, 1399-405 (2006).
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

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** 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. 4

Supplementary Fig. 4  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 Pomc-Cre;R26-GFP animals at P9 (see Fig. 2f for corresponding PCR data).
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.