Leptin action via hypothalamic nitric oxide synthase-1 neurons controls energy balance.

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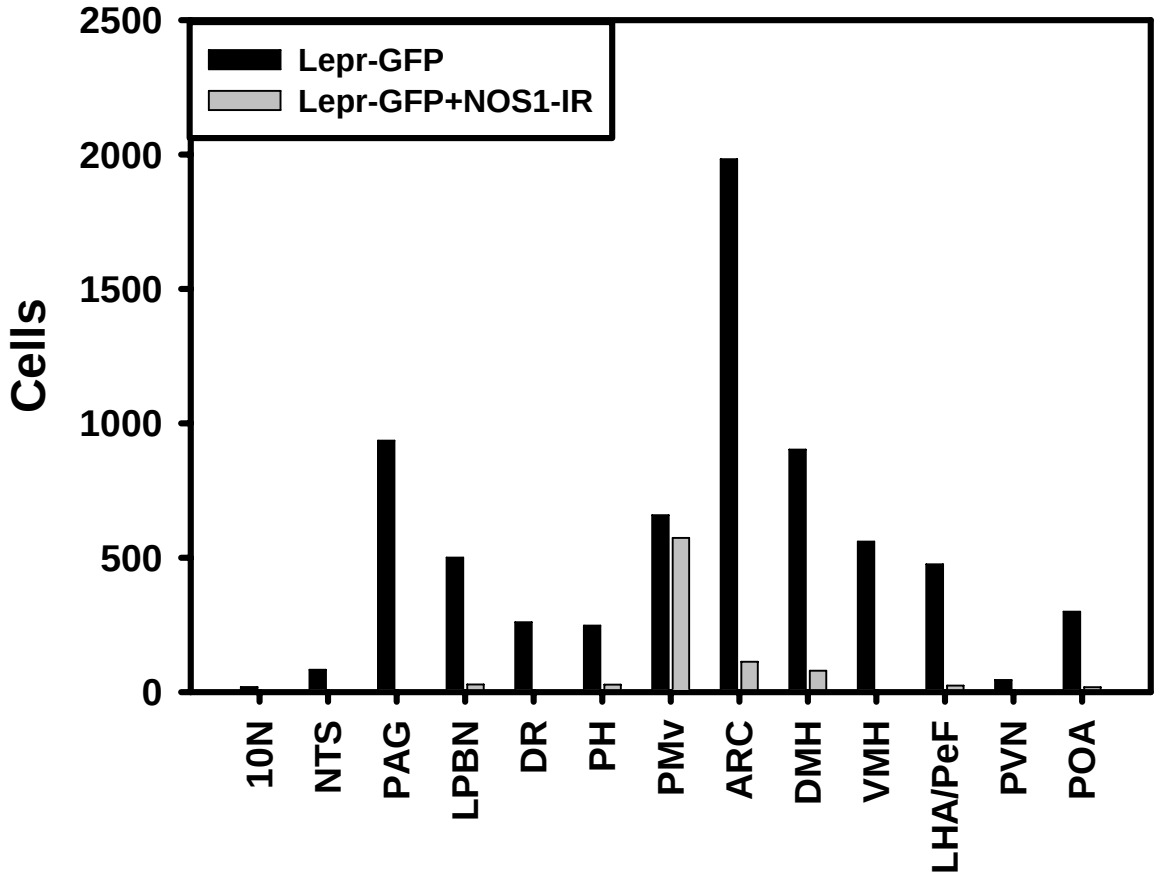
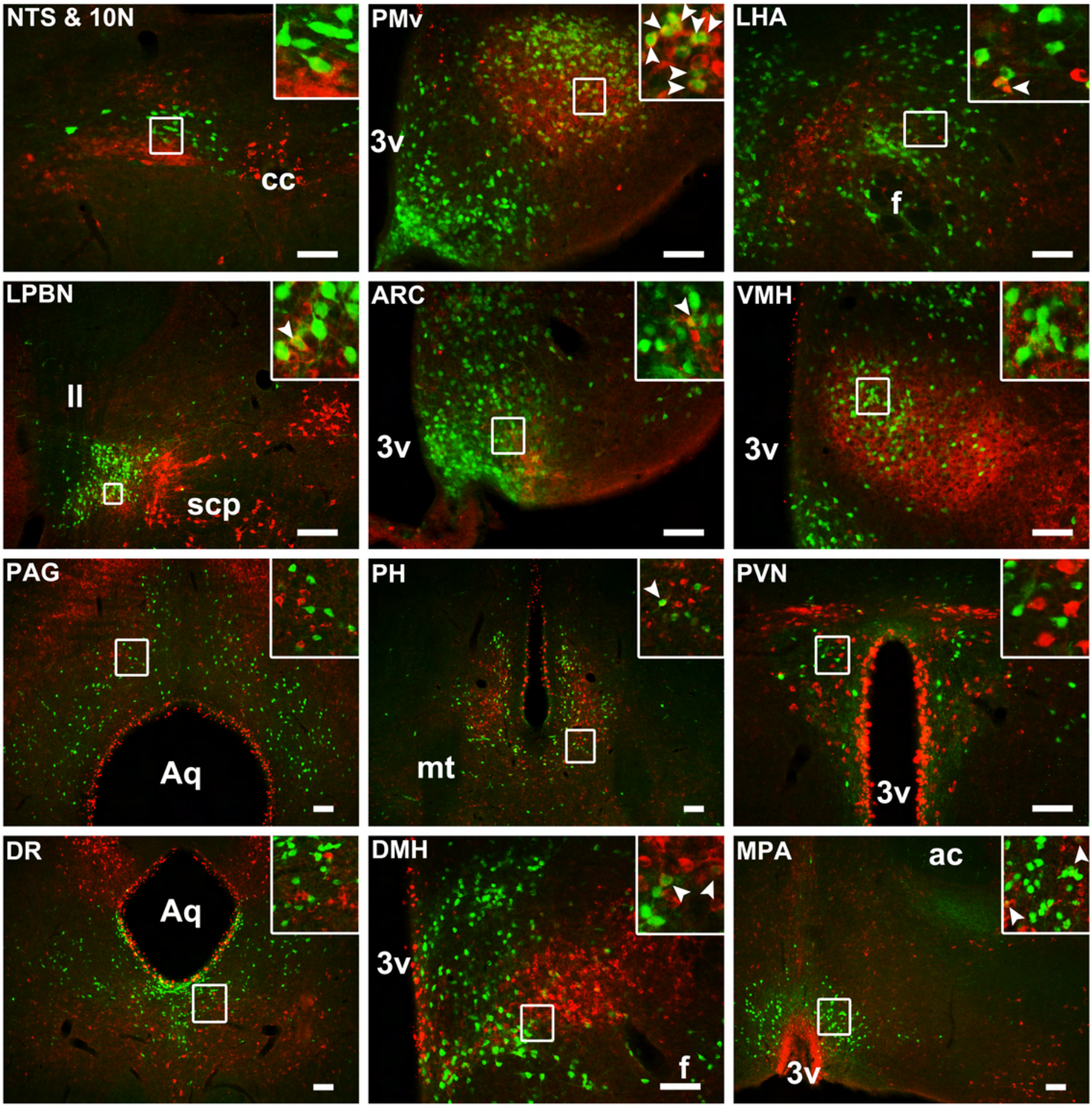
Supplemental Fig. 1A

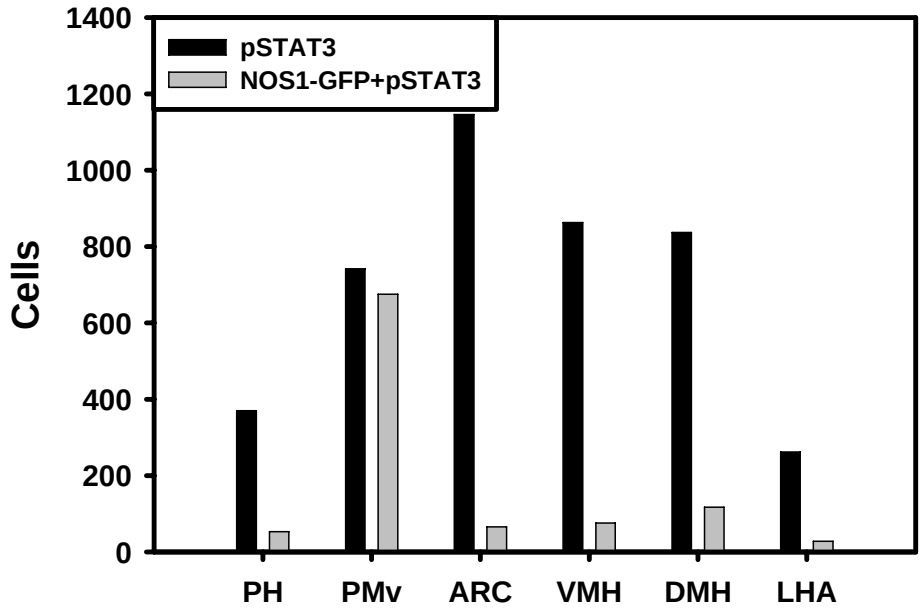


Supplemental Figure 1. Colocalization of LEPR-B/EGPF with NOS1-IR in the brain of *Lepr*^{EGFP} animals. Sections from *Lepr*^{EGFP} mice were processed for the detection of EGFP-IR and NOS1-IR. (A) Number of LEPR-B cells positive for EGFP and double-labeled EGFP-IR+NOS1-IR cells are plotted by region. (B) Representative images show EGFP-IR (green) and NOS1-IR (red). Insets show digital magnifications of indicated regions with arrowheads pointing to EGFP-IR+NOS1-IR colocalization. NTS, nucleus of the solitary tract; 10N, dorsal motor nucleus of vagus; cc, central canal; LPBN, lateral parabrachial nucleus; ll, lateral lemniscus; scp, superior cerebellar peduncle; PAG, periaqueductal gray; Aq, cerebral aqueduct; DR, dorsal raphe; 3v, 3rd cerebral ventricle; PH, posterior hypothalamus; mt, mamillothalamic tract; f, fornix; PVN, paraventricular hypothalamic nucleus; MPA, medial preoptic area; ac, anterior commissure. Scale bars=100µm

Supplemental Fig. 1B

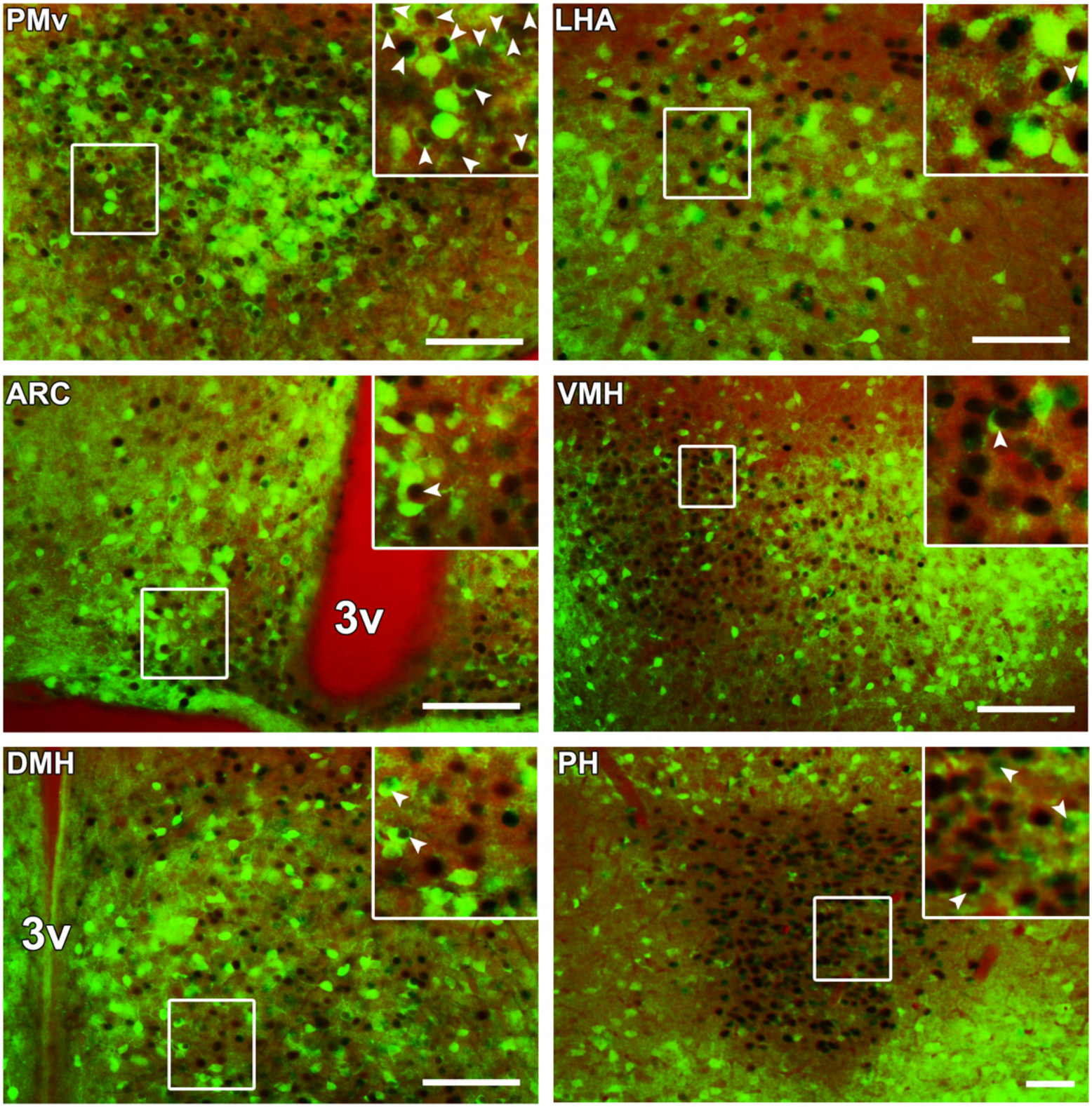


Supplemental Fig. 2A

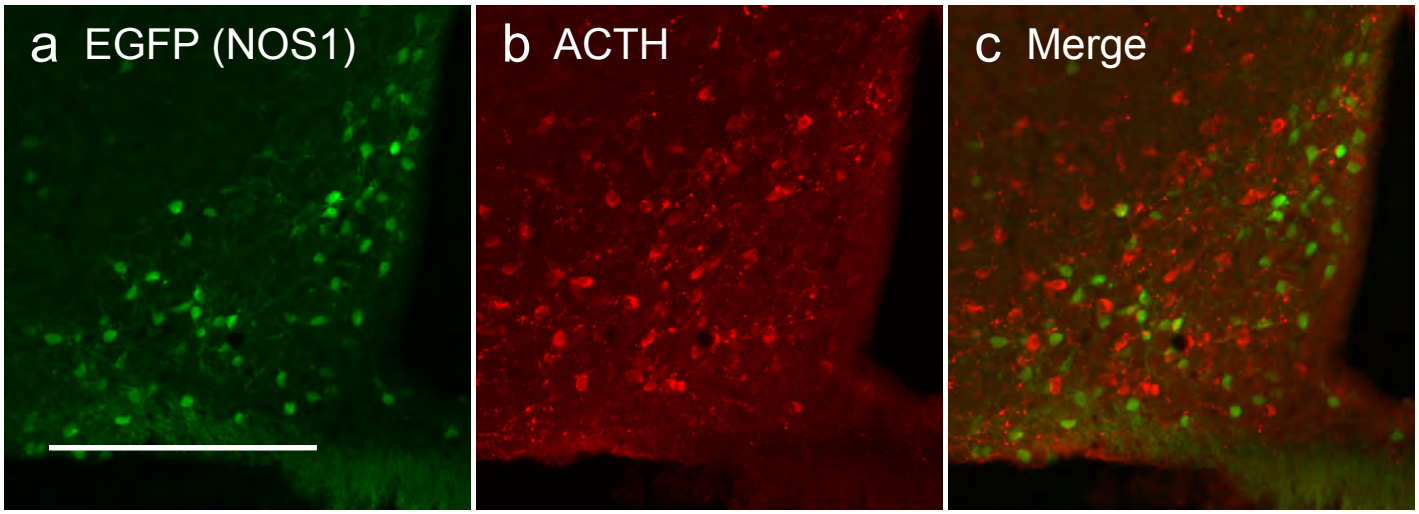


Supplemental Figure 2. Distribution of LEPR-B^{NOS1} neurons in the hypothalamus. *Nos1*^{EGFP} mice were treated with leptin (IP, 5mg/kg) for 1 hour before perfusion and processing for the detection of EGFP-IR and pSTAT3-IR. Number of LEPR-B cells positive for pSTAT3 and double-labeled EGFP-IR+pSTAT3-IR cells are plotted by region. (B) Representative images show EGFP-IR (green, cytoplasmic) and pSTAT3-IR (black, nuclear). Insets show digital magnifications of indicated areas with arrowheads pointing to EGFP-IR+pSTAT3-IR colocalization. Scale bars=100µm.

Supplemental Fig. 2B

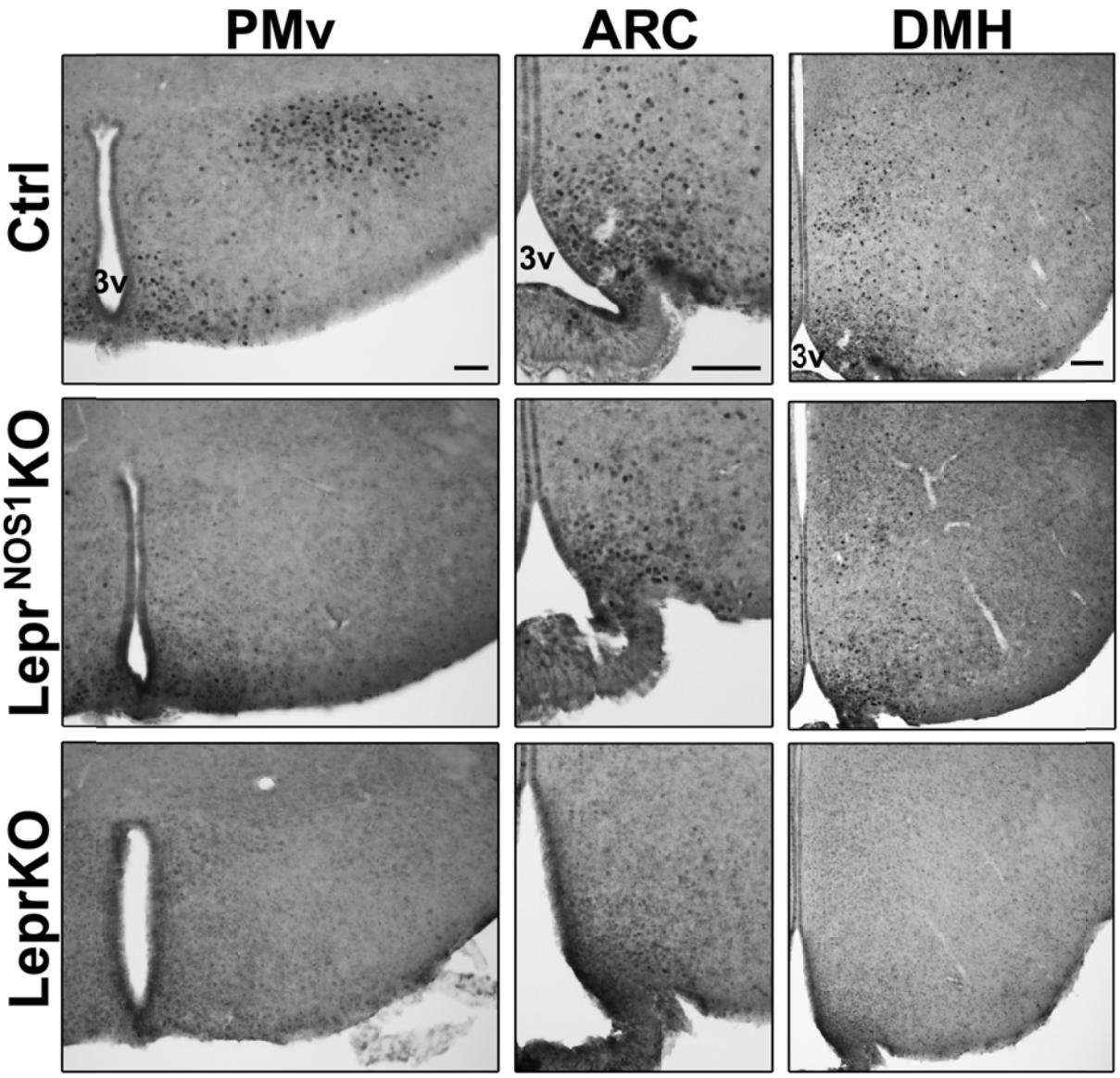


Supplemental Fig. 3



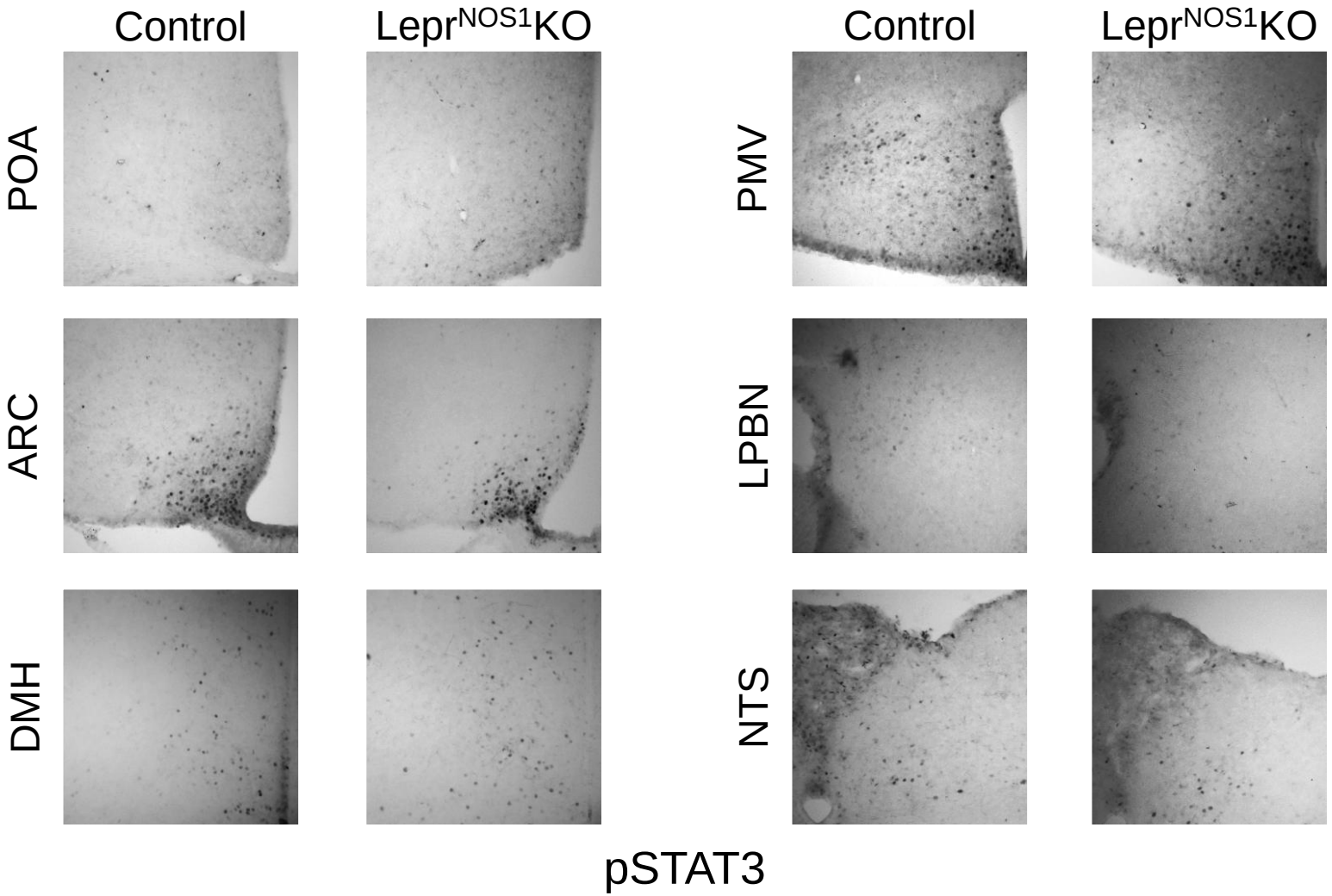
Supplemental Figure 3. Lack of colocalization between ARC NOS1 and POMC neurons in *Nos1*^{EGFP} mice. Representative images from the ARC of *Nos1*^{EGFP} mice demonstrate EGFP-IR (A; green), ACTH-IR (B; red) and merged (C) images. Scale bar=200 μm.

Supplemental Fig. 4A



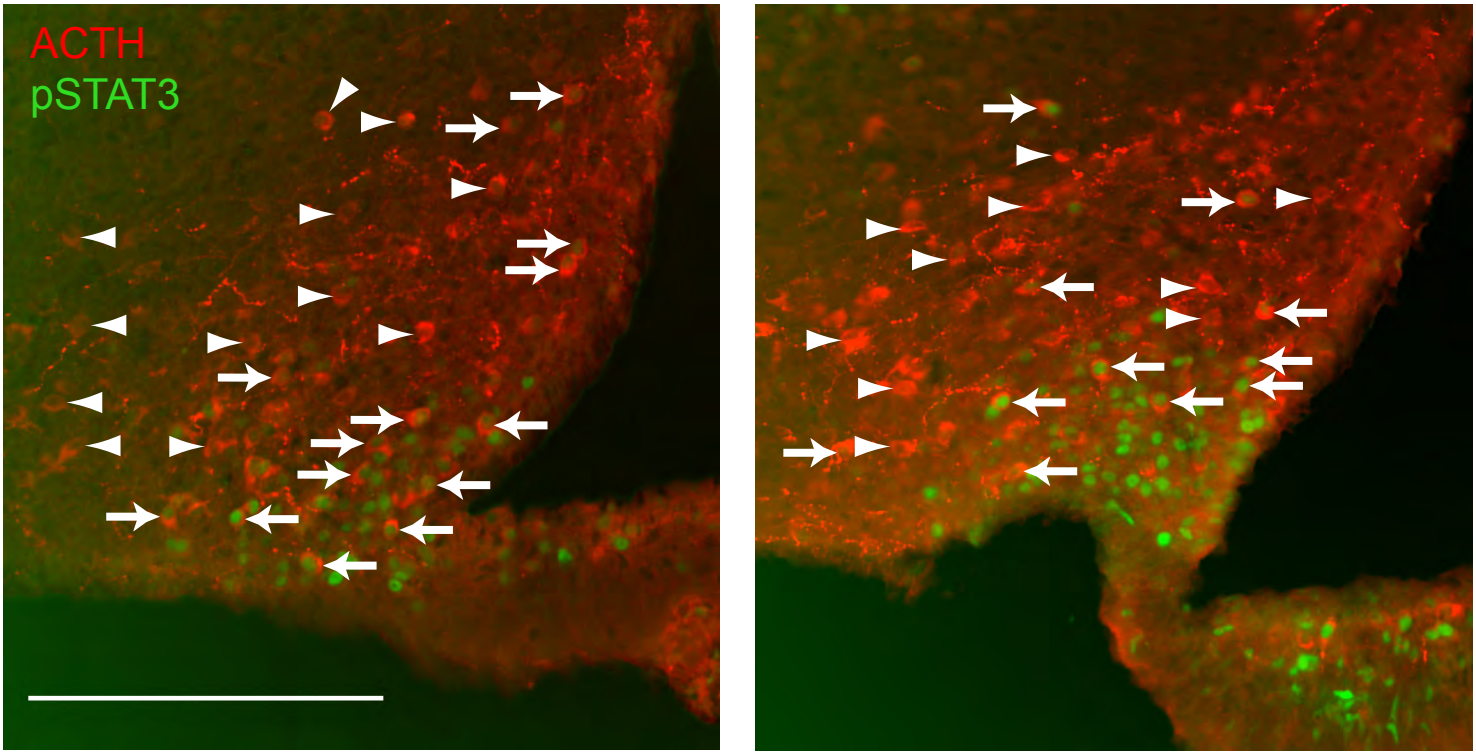
Supplemental Figure 4(A). Leptin-stimulated pSTAT3-IR in Control and *Lepr*^{NOS1}*KO* mice. Control (Ctrl, top row), *Lepr*^{NOS1}*KO* (middle row) and *LeprKO* (bottom row) mice were treated with leptin (IP, 5mg/kg) for 1 hour before perfusion and processing for immunohistochemical detection of pSTAT3-IR (black). Shown are representative images from PMv (left columns), ARC (middle columns) and DMH (right columns).

Supplemental Fig. 4B



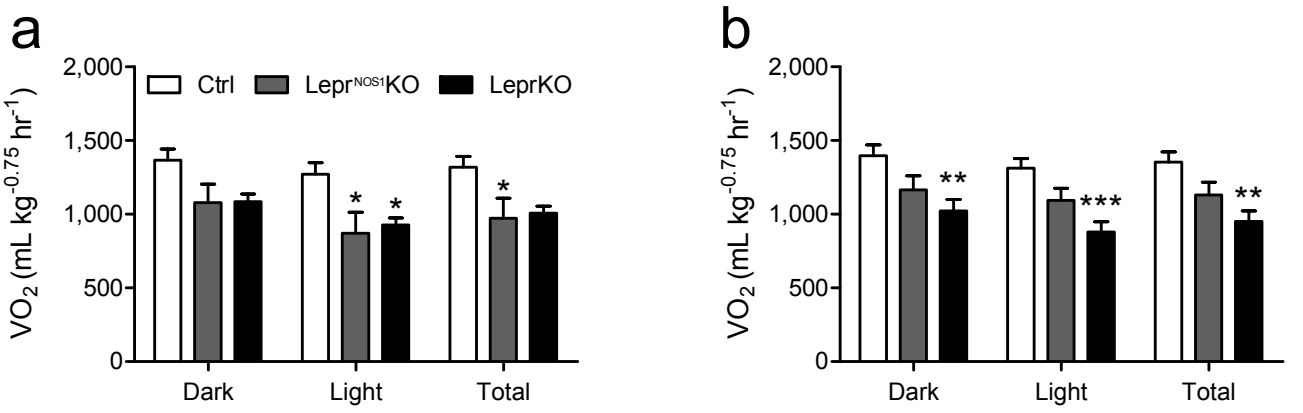
Supplemental Figure 4(B). Leptin-stimulated pSTAT3-IR in Control and *Lepr^{NOS1}KO* mice. Control and *Lepr^{NOS1}KO* mice, as indicated, were treated with leptin (IP, 5mg/kg) for 2 hours before perfusion and processing for pSTAT3-IR (black). Representative 20x images show preoptic area (POA), ARC, DMH, PMv, LPBN, and NTS.

Supplemental Fig. 4C



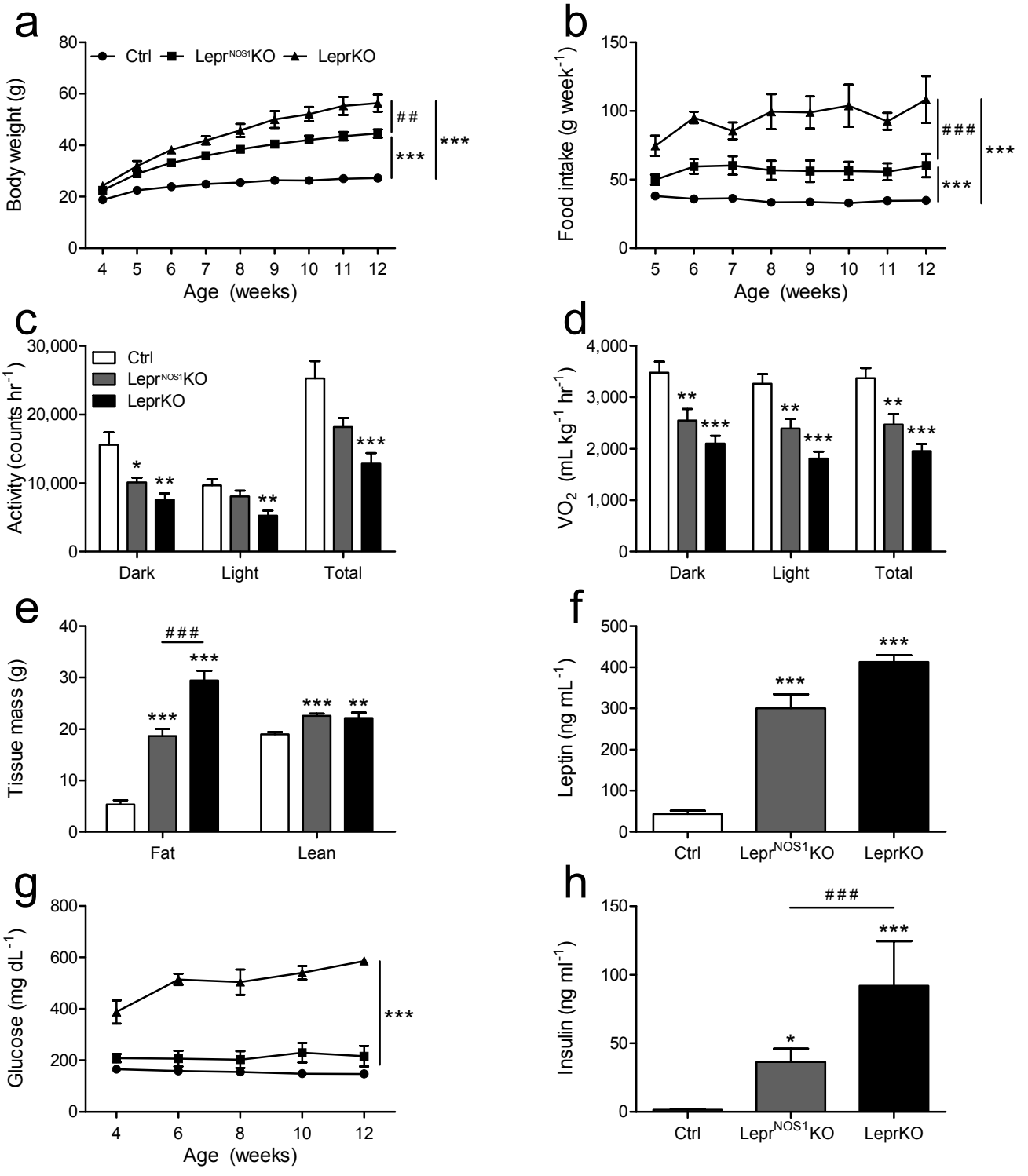
Supplemental Figure 4(C). Leptin-stimulated pSTAT3-IR in Control and *Lepr*^{NOS1}*KO* mice. Control and *Lepr*^{NOS1}*KO* mice, as indicated, were treated with leptin (IP, 5mg/kg) for 1 hour before perfusion and processing for the detection of pSTAT3-IR (green) and ACTH-IR (POMC neurons; red). Representative images of the ARC demonstrate that while not all POMC neurons contain LEPR-B, even in wild-type animals [5,6], similar numbers contain leptin-stimulated pSTAT3-IR in Control and *Lepr*^{NOS1}*KO* mice. Arrows indicate ACTH-IR (POMC)+pSTAT3-IR neurons; arrowheads indicate ACTH-IR (POMC) only. Scale bars=500 μm.

Supplemental Fig. 5



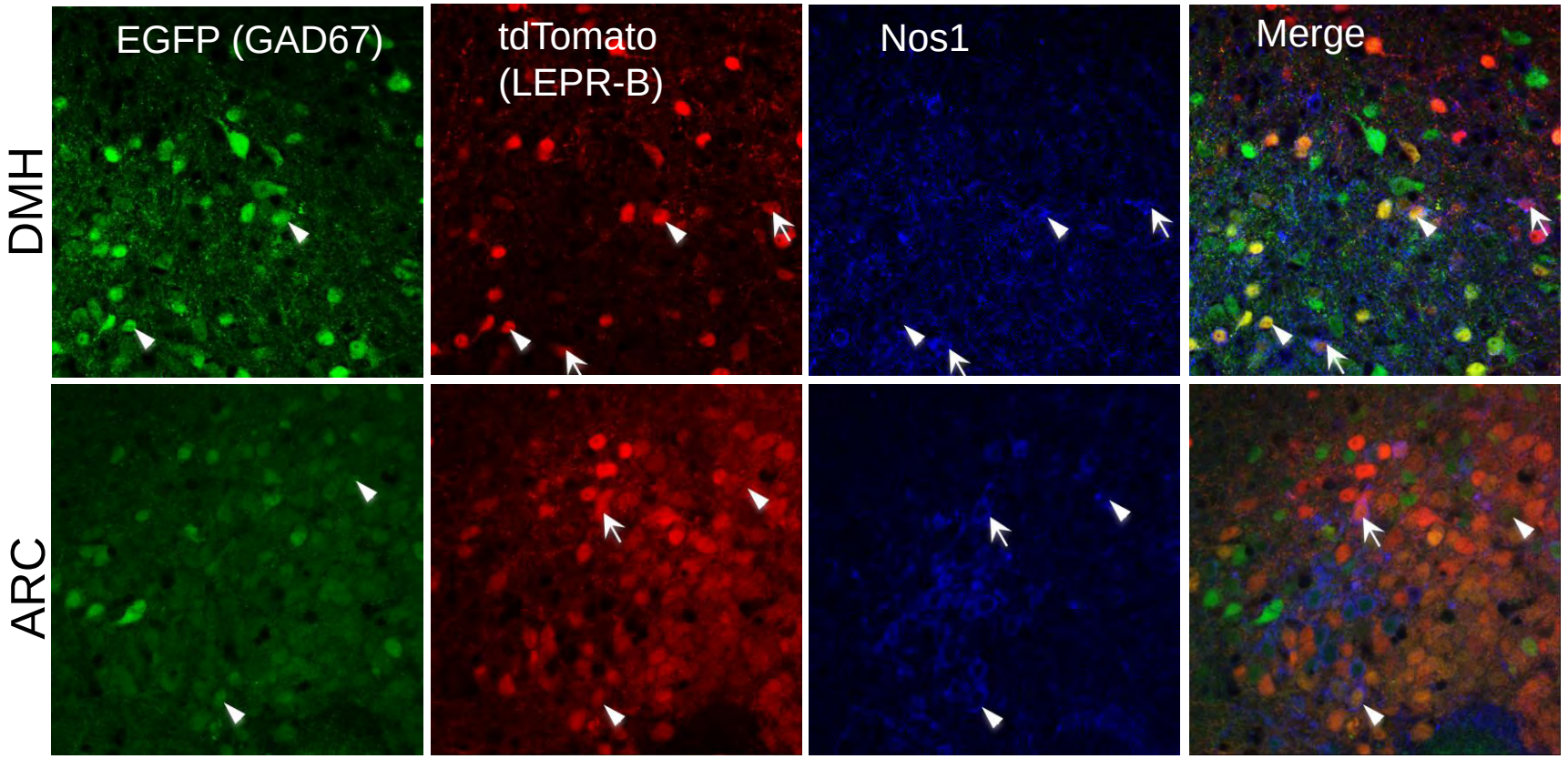
Supplemental Figure 5. Leptin action via LEPR-B^{NOS1} neurons regulates energy expenditure. 12-14 week old male (a) and female (b) mice were subjected to CLAMS analysis to measure VO_2. Data are shown for dark cycle (Dark), light cycle (Light) and averaged over 24 hours (Total). Corresponding VO_2 figures: Figure 2D and Supplemental Figure 5A (males), Supplemental Figure 6D and Supplemental Figure 5B (females) each represent the same data, the former in each pair showing absolute values and the latter showing values normalized to (body weight)$^{0.75}$. * P<0.05 versus Ctrl; ** P<0.01 versus Ctrl; *** P<0.001 versus Ctrl.

Supplemental Fig. 6



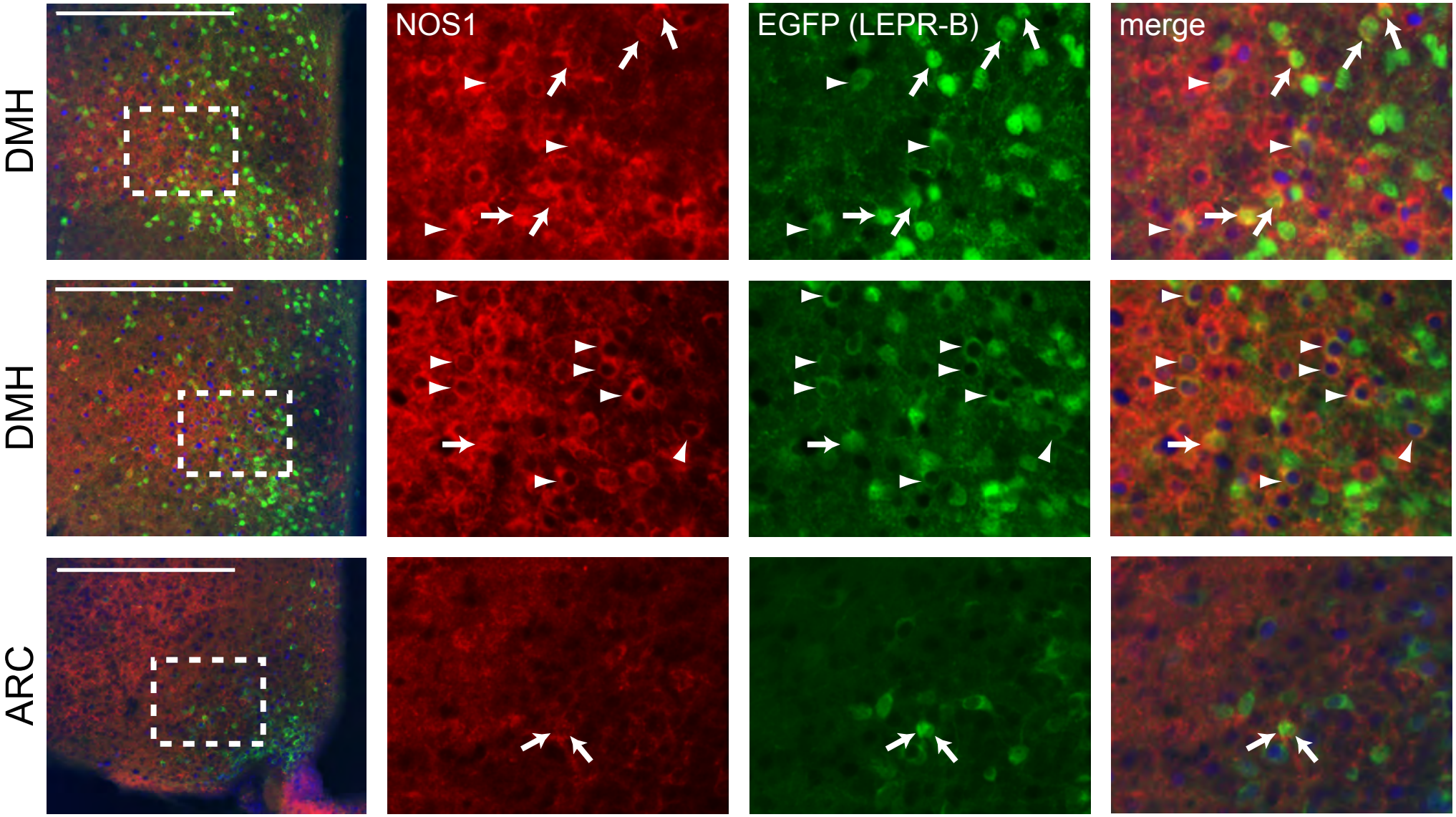
Supplemental Figure 6. Leptin action via LEPR-B^{NOS1} neurons regulates energy balance and adiposity (females). (A) Body weight and (B) food intake were measured weekly from 4-12 weeks of age for female control (Ctrl), Lepr^{NOS1}KO and LeprKO mice. 12-14 week old female mice were subjected to CLAMS analysis to determine (C) ambulatory locomotor activity and (D) VO_2. Data are shown for dark cycle (Dark), light cycle (Light) and averaged over 24 hours (Total). (E) Body composition analysis of 12-14 week-old animals. (F) Serum leptin concentrations for 12 week-old animals. (G) Biweekly blood glucose concentrations from 4-12 weeks of age for female Ctrl, Lepr^{NOS1}KO and LeprKO mice. (H) Serum insulin concentrations for 8 week-old animals. Graphed data represent average values SEM. * P<0.05 versus Ctrl; ** P<0.01 versus Ctrl; *** P<0.001 versus Ctrl; ##P<0.01; ###P<0.001.

Supplemental Fig. 7A



Supplemental Figure 7(A). Expression of GAD67 in DMH and ARC LEPR-B neurons. Representative images show colocalization of GAD67 (EGFP-IR; green), LEPR-B (tdTomato; red) and NOS1-IR (blue) in *Lepr*^{tdTomato}*;Gad1*^{GFP} mice. Merged images are shown on the right. Top panels, DMH; bottom panels, lateral ARC. Arrows indicate LEPR-B+NOS1-IR only; arrowheads indicate LEPR-B+NOS-IR+GAD.

Supplemental Fig. 7B



Supplemental Figure 7(B). Leptin activation of DMH and ARC LEPR-B^{NOS1} neurons. Representative images show colocalization of NOS1-IR (red), LEPR-B (EGFP-IR, green) and c-fos-IR (blue nuclei in merged images at left and right). Boxed areas in left panels indicate location of digital magnifications in right panels. Arrows indicate LEPR-B+NOS1-IR only; arrowheads indicate LEPR-B+NOS1-IR+c-fos-IR. Scale bars=500 μm.

Supplemental Methods:

Animals. All mice were bred in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan. All animals and procedures used were in accordance with the guidelines and with the approval of the University Committee on the Care and Use of Animals. Animals were given *ad libitum* access to food and water. *ROSA*^{tdTomato} mice were purchased from Jackson Labs and bred to *Lepr*^{cre} and *GAD1-GFP*[1,2] mice to produce *Lepr*^{tdTomato}*;GAD1-GFP* animals. *Lepr*^{EGFP} mice have been described[3].

Perfusion and immunolabeling. Mice were anesthetized with an overdose of intraperitoneal (IP) pentobarbital and transcardially perfused with 10% neutral buffered formalin. Brains were sectioned coronally (30 µm) using a sliding microtome followed by immunohistochemical or immunofluoresent analysis as described. DAB was used for detection of pSTAT3 (Cell Signaling, rabbit, Cat# 9131; 1:500) and cFos (Santa Cruz, goat, Cat# sc-52-g; 1:1000). All other antigens were visualized via immunofluorescence using species-specific AlexaFluor-488, -568, or -647 secondary antibodies (Invitrogen, Cat# A11039, A10042, A21244; 1:200) and processed and imaged as previously described[4]. Antibodies used were GFP (Abcam, chicken, Cat# ab13970; 1:1000), NOS1 (ImmunoStar, rabbit, Cat# 24287; 1:5000), ACTH (NHPP, rabbit, 1:20). Normal donkey serum and biotinylated donkey anti-rabbit were purchased from Jackson ImmunoResearch. ABC Vectastain Elite kit was purchased from Vector Laboratories and Metal-Enhanced DAB kit was from Thermo Scientific. Endogenous fluorescence was utilized to visualize tdTomato.

Sections were mounted onto Superfrost Plus slides (Fisher Scientific) and coverslipped with ProLong Antifade mounting medium (Invitrogen). Microscopic images were obtained using an Olympus BX-51 brightfield microscope with DP30BW camera (Olympus). Cell counts were performed using Adobe Photoshop software.

References

1. Leshan, R.L.*, et al.* Direct innervation of GnRH neurons by metabolic- and sexual odorant-sensing leptin receptor neurons in the hypothalamic ventral premammillary nucleus. *J Neurosci.* **29**, 3138-3147 (2009).
2. Abe, H.*, et al.* Epithelial localization of green fluorescent protein-positive cells in epididymis of the GAD67-GFP knock-in mouse. *J Androl* **26**, 568-577 (2005).
3. Leinninger, G.M.*, et al.* Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. *Cell Metab* **10**, 89-98 (2009).
4. Munzberg, H.*, et al.* Appropriate inhibition of orexigenic hypothalamic arcuate nucleus neurons independently of leptin receptor/STAT3 signaling. *J Neurosci.* **27**, 69-74 (2007).
5. Sohn, J.W.*, et al.* Serotonin 2C receptor activates a distinct population of arcuate pro-opiomelanocortin neurons via TRPC channels. *Neuron* **71**, 488-497 (2011).
6. Williams, K.W.*, et al.* Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *J Neurosci* **30**, 2472-2479 (2010).