



Supplementary Information for

A hypothalamic pathway for Augmentor α controlled body weight regulation

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Material and Methods

Transgenic mice lines:

Mice were housed in Yale Animal Resource Center (YARC) controlled facility. Mice were fed ad libitum on a standard chow diet or 45% high fat diet (Research Diets- D12451) and checked daily by veterinary staff. All animals were housed on a 12/12-hour light/dark cycle. For the study, both male and female littermate mice were used unless stated otherwise. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC). Aug α whole body knockout mice with a Gfp insert on C57BL/6 background were generated through ingenious targeting laboratories. Aug α knockout mice with LacZ insert were generated in-house at Yale Genome Editing Center by using targeted ES cells purchased from the European Conditional Mouse Mutagenesis Program (www.eucomm.org). Aug α knockout mice were then crossed with AgRP-Cre/POMC-Cre/Ai14-tdT mice to label specific neurons for colocalization studies.

Metabolic assays:

For thinness kinetics, body weights were recorded every week for a year. Body composition of 4-7 months old mice was assessed using an EchoMRI system. Fat and lean mass values were plotted after normalization to body weight. For metabolic parameter, 3-6 months old mice were singly housed and acclimatized in metabolic chambers (TSE Systems) for 2 days, and then metabolic and locomotive parameters as indicated in results were recorded by the build-in automated instruments for another 2 days. Body weight was recorded at the beginning and body weight adjusted values were plotted as indicated in the metabolic cage dataset.

Glucose, Insulin and Norepinephrin measumementrts:

7-10 months old mice were deprived of food overnight for glucose tolerance test or 6h for insulin tolerance test. Glucose level of blood from food deprived mice was measured, and after that either glucose (2.5g/kg body weight) or Insulin (0.75U/kg body weight) was

administered through intraperitoneal injection. Blood glucose concentrations were measured at the time points as indicated in the data by drawing blood from the tail vein using TRUEtrack glucometer (Trividia Health). Norepinephrin level in adipose tissues and serum was measured using ELISA kit from Abnova.

Histopathology:

Brown and white adipose tissue were dissected from 4-7 months old mice. Mice were anesthetized using isoflourane and the adipose tissue depots were isolated from subcutaneous, gonadal, intraperitoneal and brown adipose depots. Adipose depots were postfixed in 4% formaldehyde overnight. Fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin staining (H&E) at Yale Histology Core.

Brain serial sections and Immunofluorescence microscopy:

Mice were euthanized with CO₂ and perfused with 4% paraformaldehyde (PFA). Dissected brain tissue was fixed overnight at 4°C in 4% PFA and embedded in 1.5% low melting agarose (in PBS). The hypothalamic area was sectioned serially into 4 wells of a 12-well plate into 50 µm coronal sections using a vibratome. Sections were stored in 1X PBS + 0.02% sodium azide solution. For staining, sections in one well were washed with 1X PBS and incubated with 5% normal donkey serum, 0.3% TWEEN 20 in PBS at RT for 30 mins, incubated with primary antibodies (1:100 to 1:1000 dilution) overnight at 4°C: Chicken anti-GFP (Aveslabs, GFP-1010), Rabbit Anti-RFP (Rockland, 600-401-379), Goat anti-AgRP (R&D, AF634), Rabbit Anti-POMC (Phoenix pharmaceuticals, H-029-30), Sheep Anti-Alk (R&D, AF4210), Anti-Rabbit- pAlk (Sigma, SAB4504604), Guinea pig Anti-CRH (Peninsula, T-5007) and secondary antibodies (1:200, Alexa Fluor 488 anti-Rabbit IgG, Alexa Fluor 594 anti-Rabbit IgG, and Alexa Fluor 594 anti-Mouse IgG, 1:400) for 1h. A Leica confocal system was used for fluorescence detection (LSM 800). For quantifications of Auga-Gfp neurons activation after fasting, all the sections on a slide were imaged for hypothalamic areas at 10X and the number of Gfp+ neurons within PVN and ARC were counted using Cell Counter Macro of Fiji. The data is plotted as bar graph. Similar quantifications were performed for c-Fos stainings. For pAlk quantifications, mean integrated intensity within PVN was measured using the Fiji software.

Neurodifferentiation:

The cells were harvested and dissociated from P0 mice to establish a primary neuronal stem cells culture. Briefly, P0 mice were sacrificed, and forebrains were isolated quickly. Using fine forceps, meninges were peeled off; the hippocampi were dissected precisely under a dissecting microscope. The hippocampi were chopped into fine pieces using a sterile scalpel blade and were collected into a 15 ml tube containing a papain-based solution(30). Tissue was incubated at 37°C for 15 min and triturated by a fire-polished glass pipette 5-10 times to dissociate the cells. Cells were centrifuged at 300 x g for 5 min at room temperature. After washing with 1X PBS twice, cells were plated onto low attachment dishes to propagate neural stem cells population. After 4 days, the neurospheres were plated onto the laminin and poly-D-lysine coated glass coverslips in neurobasal medium supplemented with B27, glutamine and antibiotic with or without recombinant mouse AUG- α protein. After 7 days, the coverslips were stained for MAP2, and images are acquired using LSM 800 (Leica).

In situ hybridization:

RNA probes were generated from mouse hypothalamic tissue cDNA as template (Alk, ENSMUST00000086639.6) and in vitro transcribed as per manufacturer's instructions using similar probes as reported earlier(31). Probes were purified by phenol/chloroform extraction, quantified and quality controlled and stored at -80°C till hybridization. Slide-mounted cryo-sections at 30 μ m thickness were processed for *in situ*. Briefly, brains were fixed overnight at 4°C in 4%PFA diluted in 1X PBS, equilibrated at 4°C in 30% sucrose in 1X PBS overnight. Fixed brains were then embedded in OCT, sliced on a cryostat (Leica Biosystem). Slides were stored at -80°C until processed for in situ hybridization. Sections were first postfixed in 4% PFA in 1X PBS for 15 min at RT, washed with 1X PBS, treated with proteinase K and submerged in hybridization buffer (5X SSC, 50% formamide, 1%SDS, 200 mg/ml of aBSA, 500 mg/ml of yeast tRNA and 50 mg/ml of heparin) supplemented with 1000 ng/ml appropriate digoxigenin-labeled probe at 70°C overnight. Sections were washed two times 45 min at 70°C in 2X SSC, 50% formamide, 1% SDS, followed by washing in 100 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% Tween,

blocked with 10% sheep inactivated serum (Sigma-Aldrich) and incubated overnight at 4°C with an anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5000, Roche). Sections were then rinsed in 100 mM Tris-Cl pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween before being overlaid with BCIP/NBT substrate (Sigma Aldrich). Revelation was done at RT in the dark until the desired signal is reached. Finally, sections were rinsed in 1X PBS, post-fixed with 4% PFA in 1X PBS, washed in water and mounted with paramount medium. The slides were scanned on Aperio CS2 (Leica Biosystems).

RNA isolation and qPCR:

To quantify the expression of thermogenic genes, white adipose tissues from 7-11 months old littermate mice were snap frozen in liquid nitrogen. Tissues were homogenized in Trizol and RNA was extracted with phenol/chloroform, digested with DNase for 15 min followed by cleaned up using PureLink RNA mini kit (Ambion) as per the manufacturer's protocol. cDNA was synthesized using iScript cDNA synthesis kits (Bio-Rad). qPCR reactions were performed in the CFX96 Real-Time PCR Detection System (BioRad) using SYBR supermix (Bio-RAD). Post-amplification melting curve analysis was performed to check for nonspecific products. For normalization, threshold cycles (Ct-values) were normalized to *Actin* within each sample to obtain sample-specific DCt values (= Ct *gene of interest* – Ct *housekeeping gene*). 2^{-DDCt} values were calculated to obtain fold expression levels. Values were presented as fold change over littermate control.

Adipocyte size

Adipocyte size analyses was performed using adiposoft plugin of image J software on images from H&E stained slides of white adipose tissues from eight months old mice fed on standard chow.

Single-cell RNA-seq data analysis

Pre-processed mouse hypothalamus development scRNA-seq data were downloaded from Gene Expression Omnibus (GEO; accession number: GSE132730). According to previous protocols, we used subset matrices of expression for cluster number 24 (32),

which correspond to parvocellular corticotropin-releasing hormone (Crh) and thyrotropin-releasing hormone (Trh) neurons of the paraventricular nucleus of the hypothalamus on postnatal days 10 and 23. Data were processed using the R program environment as previously described (22). Briefly, Seurat R package (v4.0.4; (33)) was used for analysis and visualisation of cellular markers. We plotted a heatmap of Pearson's residual values of the genes selected from marker representative for Trh-positive and Crh-positive populations. Differential expression was assessed using the Wilcoxon test, and sorted by correlation with the Trh and Crh genes. Matrices of log-normalised expression values with pseudocount one were used to perform intersection-set analysis with the UpSetR R package (v1.4; (34)), and to examine Pearson correlation statistics, which were visualised using the ggstatsplot R package (v0.8; doi 10.21105/joss.03167).

Statistical analysis:

All mouse data are expressed as mean \pm standard error of the mean (SEM). Statistical significance was tested by two tailed unpaired student's t test, and one- or two-way ANOVA with Bonferoni corrections for multiple comparison test as indicated in figure legend. All figures and mouse statistical analyses were generated using Prism 8 (GraphPad). In all figures, statistical significance is represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

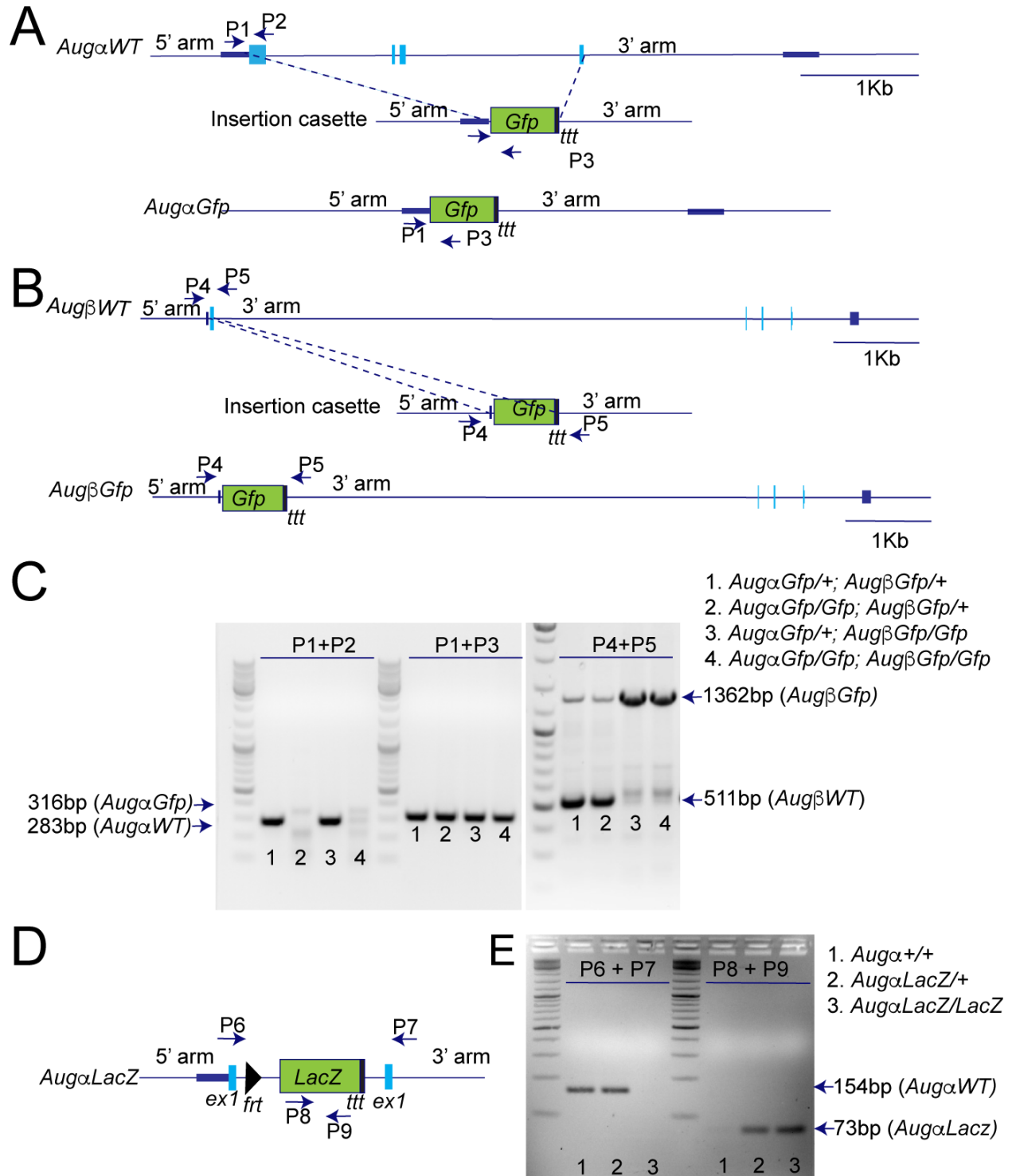


Fig. S1

Fig. S1. Schematic of *Augα* and *Augβ* knockout mice generation.

A. Schematic of $Aug\alpha$ WT genome locus containing exon1 to exon 4 that were edited using a Gfp expression cassette. Genotyping primers are annotated as P1, P2, P3.

B. Schematic of $Aug\beta$ WT genome locus containing exon1 that was edited using a Gfp expression cassette. Genotyping primers are annotated as P4, P5.

C. Gel picture showing the genotyping of $Aug\alpha$ and $Aug\beta$ mice using primer pairs P1+P2, P1+P3 and P4+P5.

D-E. Schematic of $Aug\alpha$ -LacZ knockout mice. (D), DNA gel showing genotype of mice by using primer P6+P7 (WT) and P8+P9 (LacZ).

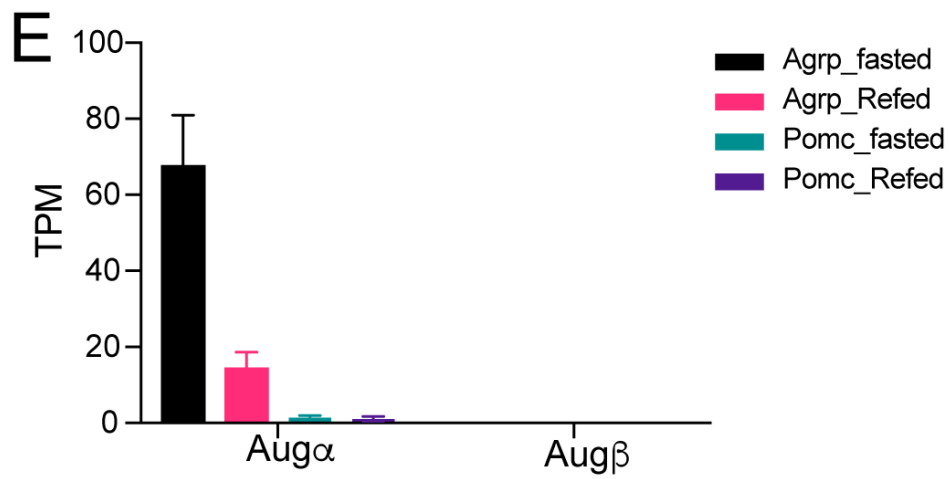
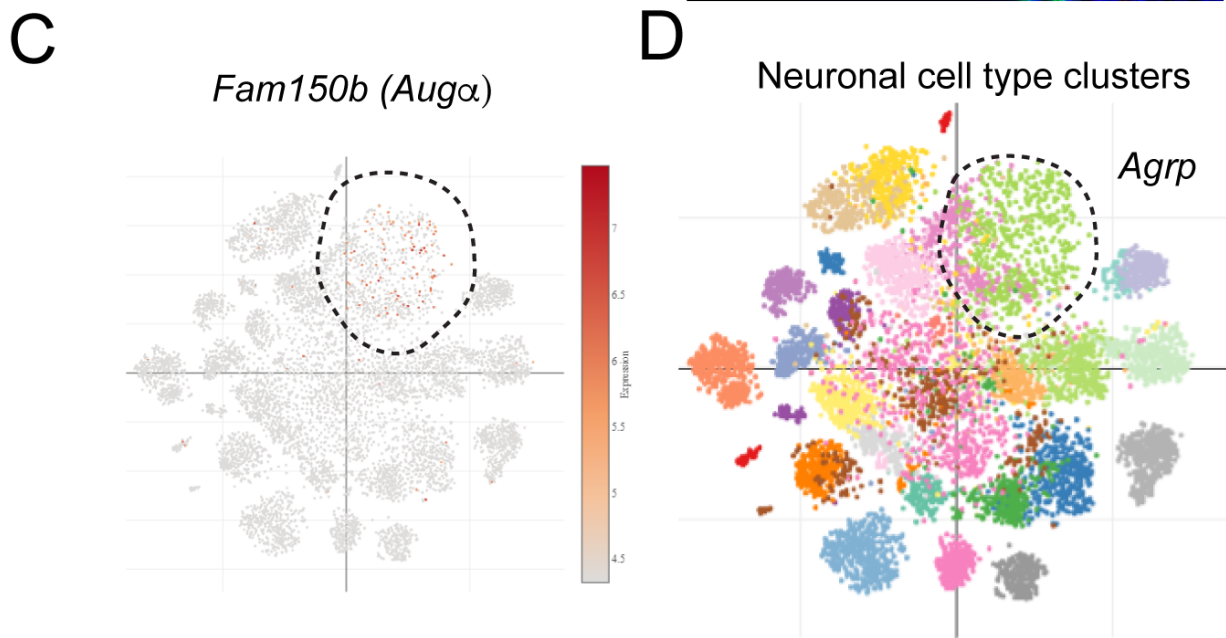
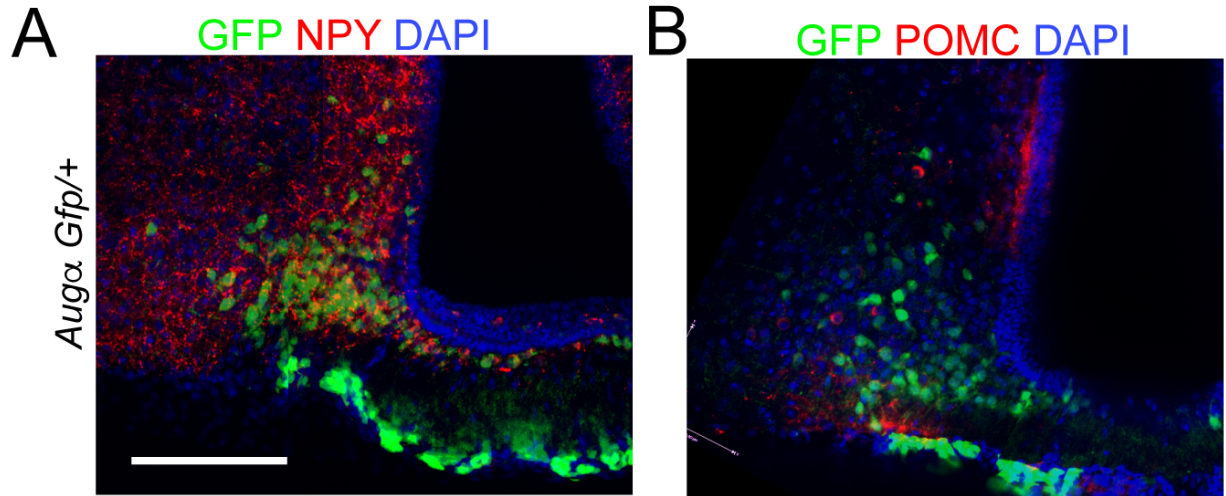


Fig. S2

Fig. S2. Aug α expression in AgRP positive neurons.

A-B. Immunostaining of coronal sections of Aug α -gfp/+ brains for NPY, and POMC.

C-D. t-SNE plots of Aug α expression in AgRP neurons, from single cell RNAseq data(16).

E. Bar plot showing Aug α and Aug β expression in AgRP and POMC neurons during fasting and refeeding state, from single cell RNAseq data(17).

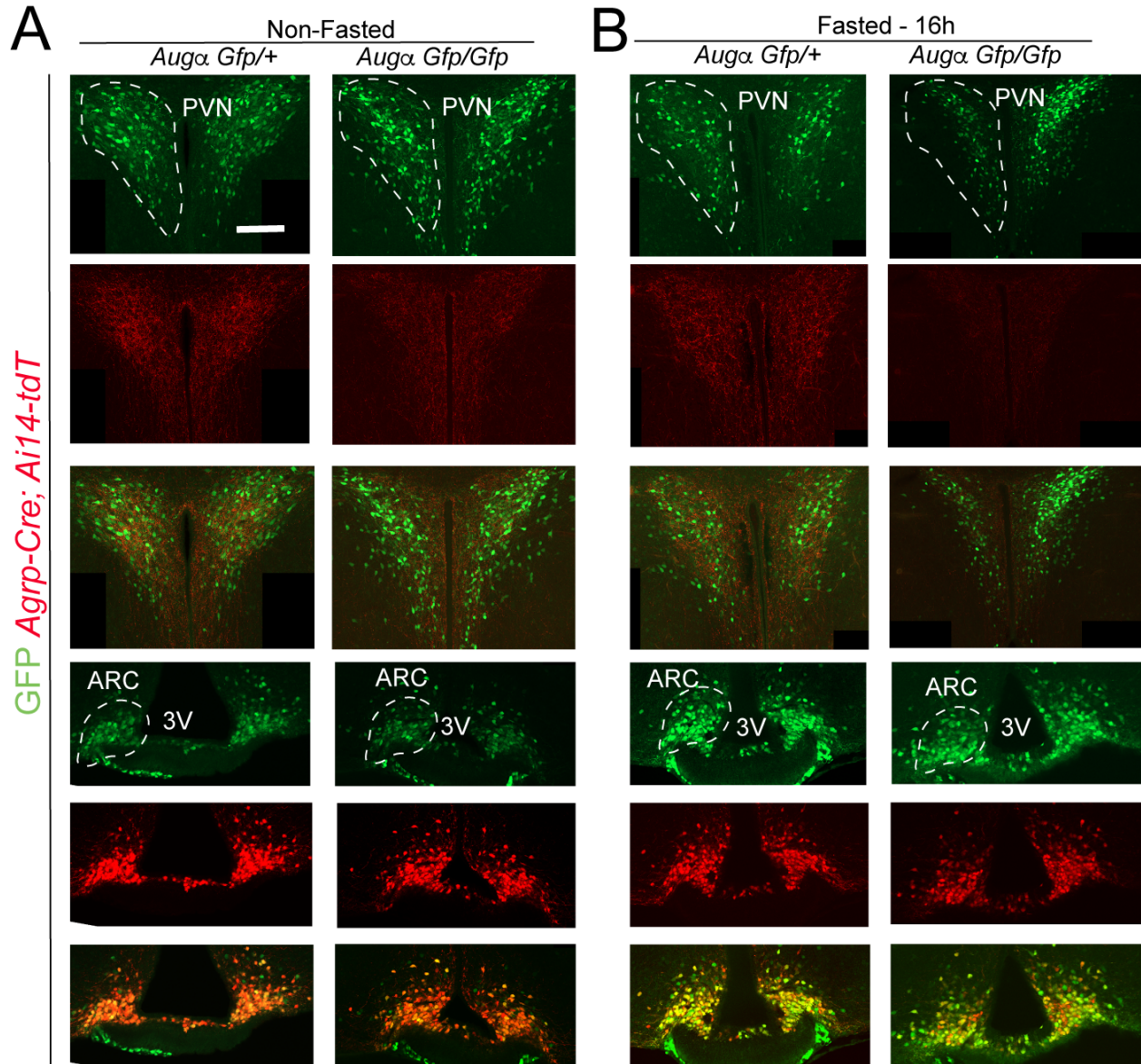


Fig. S3

Fig. S3. *Augα* expression is increased upon fasting.

A-B. Expression of GFP (*Augα*) and RFP (AgRP) within the ARC and PVN of the *Augα Gfp/+*; AgRP-Cre; Ai14-tdT and *Augα Gfp/Gfp*; AgRP-Cre; Ai14-tdT mice under non-fasted condition (A) and 16h of food deprivation (B). Scale bar = 40 μ m

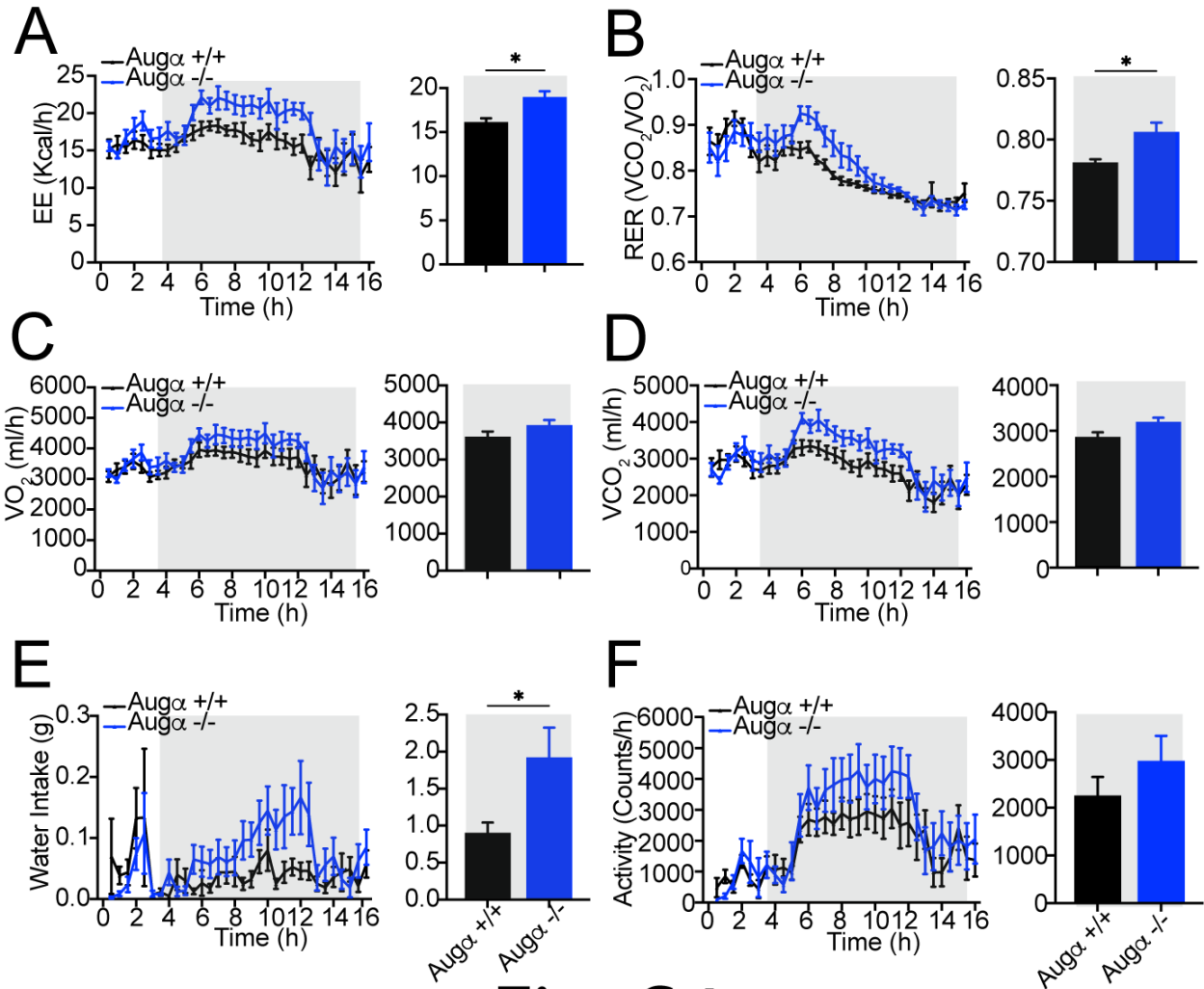


Fig. S4

Fig. S4. Metabolic dataset of food deprived mice.

A-F. Metabolic parameters; EE (A), RER (B), VO_2 (C), VCO_2 (D), Water Intake (E), Activity (F). Data presented as mean \pm SEM, unpaired student's t test is applied; $p < 0.05$, *; ** $p < 0.01$, $n \geq 7$.

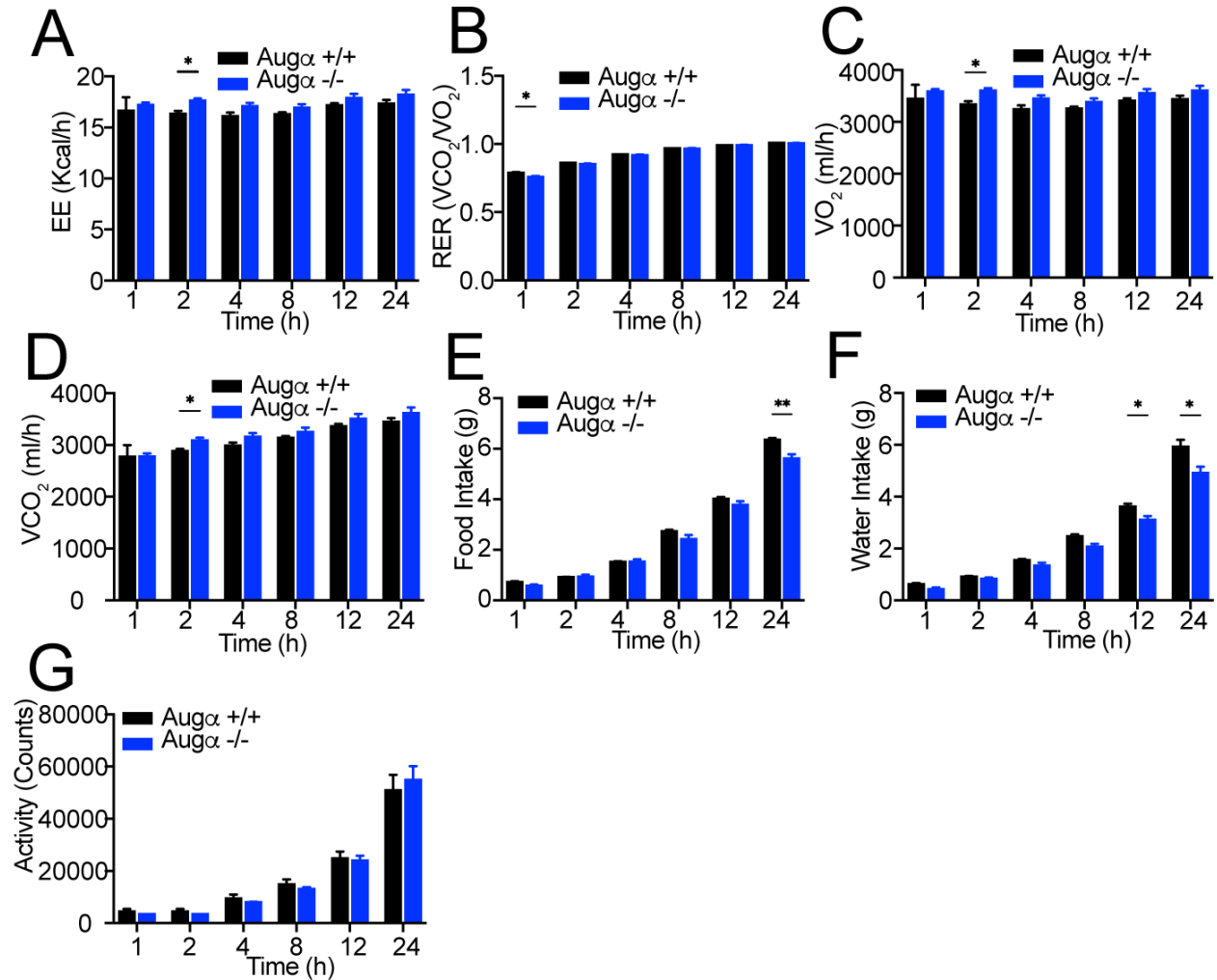


Fig. S5

Fig. S5. Metabolic dataset during refeeding after 16h fast.

A-D. Bar graph showing cumulative changes in the EE (A), R;ER (B), VO₂ (C), VCO₂ (D) food intake (E), water intake (F) and Activity (G) during refeeding after 16h fasting. Data presented as mean ± SEM, unpaired student's t test is applied, p < 0.05, *; p < 0.01, **; p < 0.0001, ****, n ≥ 7.

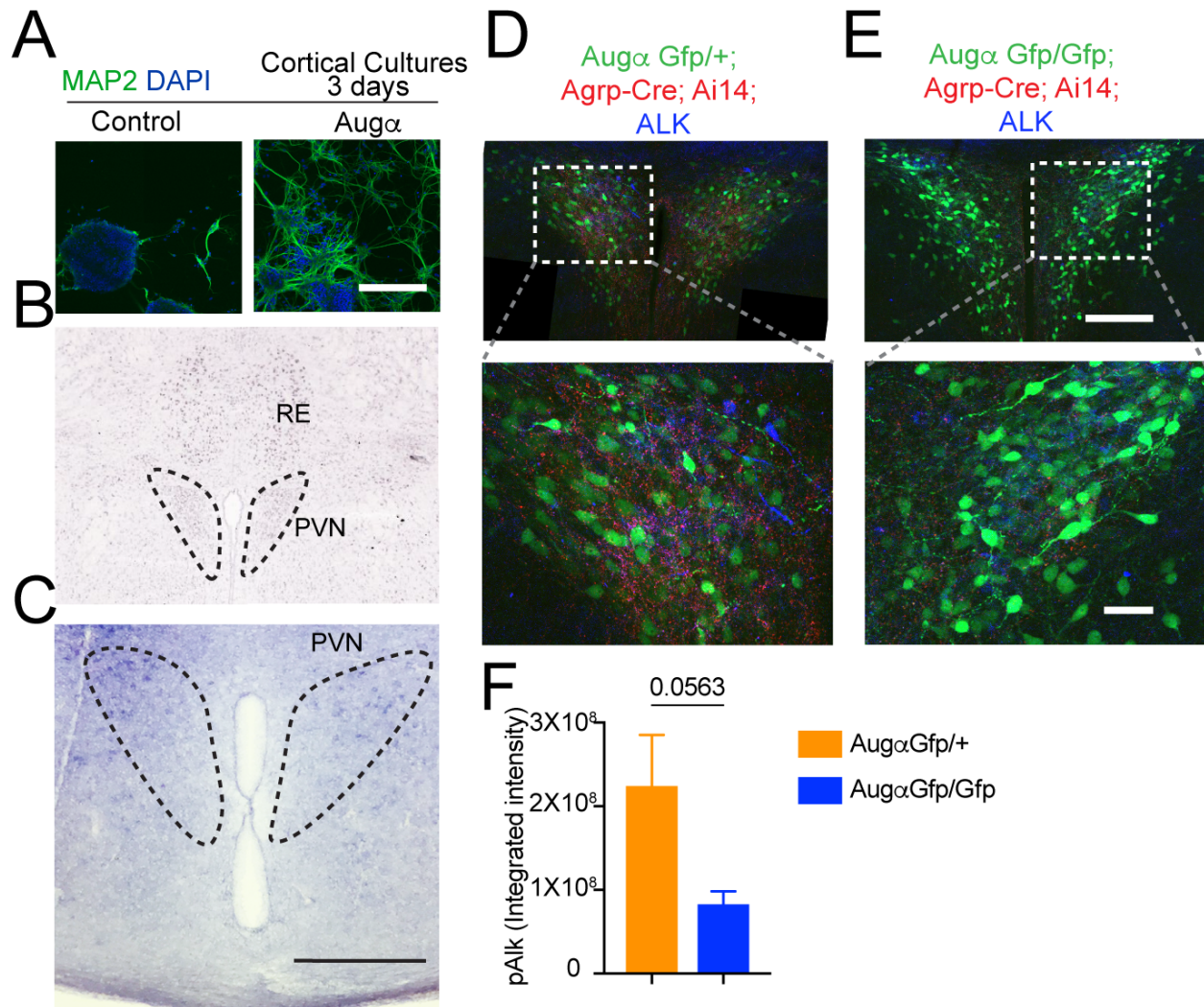


Fig. S6

Fig. S6. Augα induced neurodifferentiation, and Alk and Augα localization.

A. Image showing neurodifferentiation of neuronal stem cells isolated from E18 wild type brain (n = 3). Scale bar: 40 μm

B-C. In situ hybridizations from Allen brain atlas (B) and in-house (C) showing Alk expression within PVN (<https://portal.brain-map.org>). Scale bar: 500 μM

D-E. Image showing the Alk (Blue) immunostaining within the PVN neurons in Augα Gfp/+ and Augα Gfp/Gfp mice brains. Scale bar: 200 μM; 50 μM (zoom in)

F. pAlk quantification from Fig.5C-D. Mean integrated intensity of the pAlk stainings within PVN of Augα-Gfp/+ and Augα Gfp/Gfp were calculated (n = 4).