BAC AgrpCre transgenesis:

AgrpCre transgenic mice were made on a mixed background (C57BL/6 x SJL)F2 as previously described (1). Briefly, a mouse *Agrp* BAC was modified using ET-recombination (Gene Bridges, Dresden, Germany) to insert a cDNA of codon-improved Cre (iCre) recombinase (Shimshek et al., 2002) at the initiator ATG of the *Agrp* coding sequence located in exon 2. The modified BAC was then linearized and injected into mouse zygotes at the University of Michigan Transgenic Core Facility. Eutopic expression of functional iCre was determined by immunostaining for Cre (1) and expression of Egfp (2) using the reporter strains Gt(ROSA)26Sor^{tm2Sho} (3).

Fluorescence in-situ hybridization (FISH)

Lymphocytes were isolated from two spleens of *AgrpCre* mice and cultured at 37 ^{o}C in RPMI 1640 medium supplemented with fetal calf serum, concanavalin A, lipopolysaccharide and mercaptoethanol. The mouse cells were harvested and metaphase chromosomal slides were made by conventional methods. The modified BAC was biotinylated and used as a DNA probe. The procedure for FISH detection was performed according to published methods (4, 5). FISH signals were observed under fluorescent microscopy and images were captured by a CCD camera.

Specific expression of cre recombinase in the arcuate nucleus

AgrpCre mice showed region-specific Cre expression restricted to Agrp/Npy neurons of the hypothalamic arcuate nucleus . Crossing *AgrpCre* mice with mice (129 x C57BL/6) in which exon 22 of the Stat3 gene was flanked by loxP sites (6) allowed for the deletion of functional Stat3 specifically from Agrp/Npy neurons. *AgrpCre/Stat3$^{flox/flox}$* mice were crossed with *Stat3$^{flox/flox}$* mice to produce equal numbers of mice with Stat3 deleted from Agrp/Npy neurons and littermate controls. Tail biopsies obtained at weaning were used to extract genomic DNA for genotyping. As reported previously (1), sporadic germline recombination in the *AgrpCre/Stat3$^{flox/flox}$* background also produced offspring carrying a single recombined allele of the *Stat3* gene (*Stat3$^{flox/-}$*). *Stat3$^{flox/flox}$* and *Stat3$^{flox/-}$* mice were combined as controls (*CON*) since no differences were detected between these genotypes (see supplemental Figure 1). Likewise, *AgrpCre/Stat3$^{flox/flox}$* and *AgrpCre/Stat3$^{flox/-}$* genotypes were combined to represent mice with complete deletion (*DEL*) of *Stat3* from Agrp/Npy neurons (Supplemental Figure 1).

Western blotting

Freshly dissected tissues were homogenized in ice-cold RIPA lysis buffer (50mM Tris-HCl, pH7.4, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA, 1mM PMSF, 1mM NaF and Roche's complete protease inhibitors). After a 60-min incubation on ice, samples were centrifuged at 13,200 rpm for 15 min at 4°Cand the protein concentration in supernatant measured by BCA Protein Assay Kit (Pierce, Rockford, IL). 50µg of total protein from each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was eliminated by incubating blots in 5% non fat dry milk in TBS (20mM Tris, 137mM NaCl,

pH7.6). Blots were then incubated overnight at 4°C with polyclonal anti-stat3 antibody (1:1000, #9132 Cell Signaling, Danvers, MA). Goat anti-rabbit HRP conjugated secondary antibody (1:2000, KPL, Gaithersburg, MD) were used to recognize primary antibody and the chemiluminescent signal was developed using a Pierce (Rockford, IL) SuperSignal West Dura kit according to the manufacturer's instruction. To determine the equivalence of protein amounts loaded among different samples, the developed membranes were washed and re-probed with monoclonal anti-GAPDH (1:2000, Abcam, Cambridge, MA) and an HRP-conjugated goat anti-mouse secondary antibody (1:2000, KPL, Gaithersburg, MD) was employed to detect GAPDH. Membranes were imaged on a Bio-Rad ChemiDoc (Hercules, CA).

Results

All results are from male mice unless otherwise indicated.

FISH analysis

Using the modified *AgrpCre* BAC as a hybridization probe, FISH analysis established that integration of the transgene into the genome of *AgrpCre*$^{+/-}$ mice was limited to a single integration site (see Supplemental Figure 2) which is important for stable transmission across generations.

Expression of cre recombinase is restricted to the arcuate nucleus of the hypothalamus.

Previous work has shown the virtually complete and cell-specific loss of Stat3 in Agrp/Npy neurons within the arcuate nucleus in *DEL* mice (1). To verify that cre recombinase expression in brain was restricted to the arcuate nucleus, *AgrpCre* mice were bred with cre reporter strains expressing Egfp (3). Immunostaining for Egfp showed expression of this reporter gene product in cell bodies to be restricted to the arcuate nucleus (Supplemental Figure 3). Western blotting of brain, whole hypothalamus, fat, liver and muscle showed no differences in the level of Stat3 protein between *AgrpCre/Stat3*$^{flox/flox}$ and *Stat3*$^{flox/flox}$ mice (data not shown).

References

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