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MOLECULAR METABOLISM

Leptin potentiates astrogenesis in the developing hypothalamus

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ABSTRACT

Background: The proper establishment of hypothalamic feeding circuits during early development has a profound influence on energy homeostasis, and perturbing this process could predispose individuals to obesity and its associated consequences later in life. The maturation of hypothalamic neuronal circuitry in rodents takes place during the initial postnatal weeks, and this coincides with a dramatic surge in the circulating level of leptin, which is known to regulate the outgrowth of key neuronal projections in the maturing hypothalamus. Coincidently, this early postnatal period also marks the rapid proliferation and expansion of astrocytes in the brain.

Methods: Here we examined the effects of leptin on the proliferative capacity of astrocytes in the developing hypothalamus by treating postnatal mice with leptin. Mutant mice were also generated to conditionally remove leptin receptors from glial fibrillary acidic protein (GFAP)-expressing cells in the postnatal period.

Results and conclusions: We show that GFAP-expressing cells in the periventricular zone of the 3rd ventricle were responsive to leptin during the initial postnatal week. Leptin enhanced the proliferation of astrocytes in the postnatal hypothalamus and conditional removal of leptin receptors from GFAP-expressing cells during early postnatal period limited astrocyte proliferation. While increasing evidence demonstrates a direct role of leptin in regulating astrocytes in the adult brain, and given the essential function of astrocytes in modulating neuronal function and connectivity, our study indicates that leptin may exert its metabolic effects, in part, by promoting hypothalamic astrogenesis during early postnatal development.

Keywords Astrocytes; Hypothalamus; Leptin

1. INTRODUCTION

Developmental programming of hypothalamic feeding circuits has profound effects on energy homeostasis throughout life, and dysregulation of this process could predispose individuals to obesity, type 2 diabetes and other metabolic disorders. In rodents, the hypothalamus continues to develop during the early postnatal weeks, a critical period when hypothalamic neuronal connections are laid down. The vast majority of hypothalamic neurons are born prenatally, but the outgrowth of their projections occurs after birth [1—3]. In contrast, glia comprise only about 6% of all cells in the rodent brain at birth, and this number increases markedly during the second and third postnatal weeks, stops abruptly at weaning, and eventually makes up about 50% of all brain cells in adulthood [4]. Astrocytes not only provide crucial metabolic support to neurons but also maintain ion homeostasis at synapses, participate in maintaining the blood—brain barrier, promote synaptogenesis, and trigger injury responses [5—7]. Recent evidence suggests that astrocytes may play an active role in metabolic regulation. For example, reactive astrogliosis occurs rapidly in the hypothalamus after short-term exposure to a high-fat diet, and is postulated to be a contributing factor for diet-induced obesity in adult animals [6,8,9]. While most studies on developmental programming of the hypothalamic feeding circuits have focused on understanding neuronal connectivity, the role of hypothalamic astrogenesis in this programming process is unknown.

Leptin, a white adipose tissue-derived hormone, is an essential regulator of diverse metabolic processes in adult mammals. Leptin acts on key hypothalamic neurons that are important for metabolic control, including those expressing the neuropeptides proopiomelanocortin (POMC), agouti-related protein (AgRP) and neuropeptide Y [10]. Interestingly, multiple lines of evidence indicate that, in addition to neurons, astrocytes in the adult hypothalamus also express functional leptin receptors [11—15]. Leptin regulates glutamate and glucose transporters in hypothalamic astrocytes [13] and disrupting leptin signaling in adult astrocytes leads to impaired metabolic regulation [14]. These studies suggest that in adults, leptin may exert its metabolic effects by coordinately regulating the function of both hypothalamic neurons and astrocytes.

In addition to exerting potent effects on adult hypothalamic neurons and glia, leptin also plays a role in the programming of hypothalamic

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feeding circuits during development. A dramatic surge in circulating leptin levels begins toward the end of the first postnatal week, peaks in the second postnatal week, and declines at weaning [16]. Unlike in adults, this postnatal leptin surge is independent of fat mass and food intake [16]. It is known that leptin-deficient *Lep*$^{ob/ob}$ mice have a lower brain weight and maintain an immature pattern of synaptic and glial protein expression, and these defects can be normalized by administering leptin [17]. In contrast to *Lep*$^{ob/ob}$ mice, lethal-yellow (A^{y}) mice, which are severely obese and hyperleptinemic, do not have smaller brains, suggesting that leptin deficiency, rather than obesity *per se*, exerts a limiting effect on brain growth and development [17]. Indeed, leptin promotes axonal outgrowth from POMC and AgRP neurons during the second and third postnatal weeks, supporting the notion that leptin is a trophic factor during postnatal development [18].

Intriguingly, both the postnatal leptin surge and the maturation of hypothalamic neuronal circuits coincide with a massive proliferation and expansion in the number of glia, which commences at the beginning of the second postnatal week in rodents [4]. Additionally, we observe that leptin-deficient *Lep*$^{ob/ob}$ mice have reduced number of arcuate astrocytes compared with the obese and hyperleptinemic *A*^{y} mice under normal chow-fed condition (unpublished results). This observation prompted us to investigate the possibility that leptin may modulate the generation of astrocytes, the functions of which are important for maturation and adult function of local hypothalamic neurons. We show here that leptin promotes astrogenesis in the developing hypothalamus. Our findings suggest that astrogenesis may be an integral feature of leptin's trophic functions as it acts to establish nascent hypothalamic feeding circuits during early postnatal development.

2. MATERIALS AND METHODS

2.1. Animals

Lepr$^{flox/flox}$ mice [19] and GFAP-Cre ER^{T2} mice [20] were provided by Dr. Streamson Chua and Dr. Ken McCarthy, respectively. C57BL/6J (B6) and the Ai14 Cre-reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J) were purchased from the Jackson Laboratory. *Lep*$^{ob/+}$ mice (B6.Cg-*Lep*^{ob}/J) were purchased from the Jackson Laboratory, and *Lep*$^{ob/ob}$ mice were generated by crossing *Lep*$^{ob/+}$ male mice with *Lep*$^{ob/+}$ female mice. *Lep*$^{ob/+}$ or *Lep*$^{+/+}$ were used as controls. All mice were housed in a barrier facility with a 12/12 h light–dark cycle. Mice were fed standard mouse chow (Purina mouse diet #5058, 21.6% calories from fat; 23.2% from protein, and 55.2% from carbohydrates). All experiments were carried out under a protocol approved by the University of California at San Francisco Institutional Animal Care and Use Committee.

2.2. Induction of Cre-mediated recombination by tamoxifen

Tamoxifen (Sigma, T5648) was dissolved in corn oil (Sigma, C8267) at a concentration of 20 mg/mL. All mice were injected with tamoxifen at a dose 200 mg/kg of body weight. For mice injected at neonatal age (younger than P7) and subsequently kept alive for long periods of time (days to weeks), subcutaneous injection was used to minimize potential damage to internal organs by the injection needle due to the small sizes of the animals. For mice that were older than P8, intraperitoneal injection was used.

2.3. Leptin and BrdU administration

Leptin (National Hormone & Peptide Program) was dissolved in PBS, and all leptin administration was done by intraperitoneal injections. BrdU (Sigma, B5002) was dissolved in 0.9% saline at a concentration of 1 mg/mL. In the two gain-of-function experiments in which exogenous leptin was given to wild type postnatal pups, leptin was administered in the second postnatal week with the goal to prolong the duration of the postnatal leptin surge. In one of these experiments, mice were injected twice daily with leptin (2.5 mg/kg) for 5 days at P8–P12 and these mice were analyzed at 6 weeks of age. In the other experiment, mice were injected twice daily with leptin (5 mg/kg) for 3 days at an older age (P12–P14), and these mice were analyzed at P18. BrdU (50 mg/kg) was co-administered with either leptin or PBS. In the experiment to examine leptin responsiveness in periventricular GFAP cells, mice were injected with a single dose of leptin (5 mg/kg) at P2 before the onset of the endogenous leptin surge to avoid competition with the rising levels of endogenous leptin. These mice were killed 60 min later. In the LepR-removal experiment, control (*Lepr*$^{flox/flox}$) and mutant (*Gfap-Cre ER*^{T2}, *Lepr*$^{flox/flox}$) mice at P2 were injected with 10 mg/kg leptin and killed 30 min after the injection. A higher dose of leptin was injected to ensure sufficient transport of leptin into the brain in the hypothetical event that mutants show reduced leptin signaling.

2.4. Immunofluorescence analysis and confocal microscopy

P2 and P6 mice were killed by decapitation and the brains were placed in 4% PFA. The rest of the mice were transcardially perfused with 4% PFA after which the brains were removed, postfixed in 4% PFA, and transferred to 30% sucrose in PBS overnight at 4 °C. Brains were sectioned at 10 μm thickness using a cryostat. For GFAP and BrdU double staining, sections were boiled in 10 mM citrate solution, allowed to cool, washed with PBS and then placed in 60 °C HCl for 3 min. Section were then incubated in primary rabbit anti-GFAP (1:1000, Dako Z0334) and rat anti-BrdU (1:200, AbD Serotec OBT0030) overnight at 4 °C. For Ai14-Tdtomato and BrdU double labeling, images of Ai14-Tdtomato direct fluorescence were taken. Then the sections were processed for immunostaining with a BrdU antibody. Pictures of the BrdU staining were taken and then were merged with Ai14-Tdtomato pictures in Adobe Photoshop using anatomical landmarks. For pStat3 and GFAP double staining, sections were boiled in 10 mM citrate solution allowed to cool, washed with PBS and then were serially washed for 10 min in base solution (1% NaOH, 1% H_2O_2), PBS with 0.3% glycine, and PBS with 0.3% sodium dodecyl sulfate (SDS). Sections were then incubated in primary rabbit anti-pSTAT3 (1:100, Cell Signaling #9131) and mouse anti-GFAP (1:1000, Cell Signaling #3670) overnight at 4 °C. For AgRP and ACTH double immunofluorescence, brain sections were unmasked with 10 mM citrate, followed by 0.3% glycine and 0.3% SDS. Goat AgRP (1:500, Neuromics GT15023) and rabbit ACTH (1:500, National Hormone & Peptide Program) antibodies were used. Secondary antibodies were Alexa 488, and 594 of appropriate species (Life Technologies). Sections were mounted using Vectashield with 4′,6′-diamino-2-phenylindole (DAPI) (Vector Laboratories, H-1200). Fluorescence images were captured using an Olympus BX51WI microscope equipped with a Retiga 2000R digital camera (Qimaging). Due to high density of cells on the wall of the 3rd ventricle confocal images were taken to increase resolution between cells. Confocal images were captured using a Leica SP5 confocal microscope. Three 1 μm optical slices were captured and z-stacked to create a single image for analysis.

2.5. Cell counting

Images were blinded for analysis. Cells were counted utilizing the "Cell Counter" plugin in ImageJ software. Cells were counted as GFAP/BrdU or GFAP/pStat3 double positive, if the GFAP signal fully encircled either nuclear signal. Cells were counted as Ai14-tdTomato/BrdU double

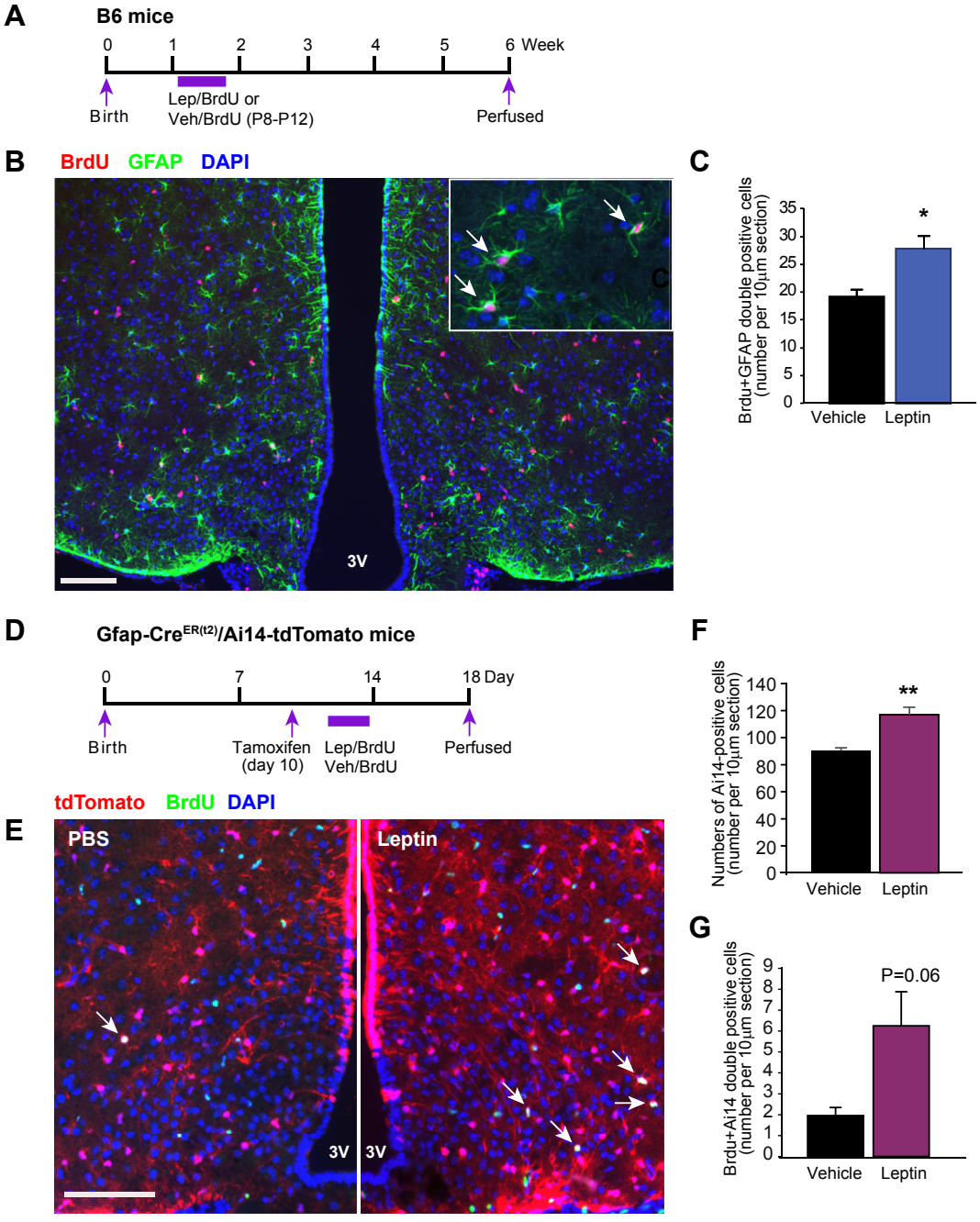

Figure 1: Administration of leptin during early postnatal life stimulates astrocyte proliferation in the hypothalamus. (**A**–**C**) Leptin (2.5 mg/kg) or vehicle (PBS), together with BrdU (50 mg/kg) were injected twice daily between P8 and P12 in B6 male mice. GFAP and BrdU double immunofluorescence analysis was performed when mice reached 6 weeks of age. (**A**) Experimental scheme. (**B**) A representative image from a vehicle-treated mouse showing GFAP immunoreactivity (green) and BrdU-positive cells (red) in the hypothalamus. Arrows show examples of astrocytes that incorporated BrdU. (**C**) Postnatal (P8–P12) leptin treatment increased number of GFAP and BrdU double-positive cells in the arcuate nucleus of 6-week-old mice. n = 4 mice per group. (**D**–**G**) Lineage tracing to examine effects of leptin administration on astrocyte proliferation. GFAP-Cre ER^{t2}/Ai14-tdTomato mice were injected intraperitoneally with tamoxifen (200 mg/kg) at P10 to induce expression of Ai14-tdTomato in GFAP-expressing cells. At P12–P14 mice were intraperitoneally injected twice daily with either vehicle or leptin (5 mg/kg), both groups were co-injected with BrdU (50 mg/kg). Mice were killed at P18 and analyzed for Ai14-tdTomato direct fluorescence and BrdU immunofluorescence. (**D**) Experimental scheme. (**E**) Representative images showing hypothalamus of P18 male mice that were treated with PBS (left panel) or leptin (right panel). Arrows show examples of Ai14-tdTomato cells (red) that incorporated BrdU (green). (**F**) Leptin treatment increased the number of Ai14-tdTomato expressing cells in the arcuate nucleus of the hypothalamus. n = 4–5 mice per group, 3 sections/mouse. (**G**) Leptin treatment increased the number of Ai14-tdTomato and BrdU double-positive cells in the arcuate nucleus of the hypothalamus. n = 4–5 mice per group, 3 sections/mouse. Data represent mean ± SEM. *P < 0.05, **P < 0.01 by 2-tailed student's *t*-test. 3V = 3rd ventricle. Scale bars in (B) and (E): 100 µm.

positive if the two signals were colocalized (BrdU showing nuclear localization whereas tdTomato signal is both nuclear and cytoplasmic). Presence of DAPI signal was required for any signal to be counted as a cell.

2.6. Statistics

Mean values in the text and figures are expressed as mean ± SEM (standard errors of the mean). Two-tailed Student *t* tests were used to compare difference between two independent groups.

3. RESULTS

3.1. Leptin administration early in postnatal life stimulates astrocyte proliferation in the hypothalamus

To investigate whether leptin influences the proliferation of astrocytes that occurs in the developing hypothalamus, leptin (2.5 mg/kg body weight) and mitotic marker BrdU (50 mg/kg body weight) were injected twice daily in pups from postnatal day 8—12 (P8—P12), and subsequent analyses were performed when the mice reached 6 weeks of age. Pups injected with BrdU alone served as controls (Figure 1A). Hypothalamic sections from the mice were processed for double immunofluorescence analysis using antibodies against BrdU and glial fibrillary acidic protein (GFAP), an intermediate filament protein and well-established marker for astrocytes. GFAP-expressing cells were found in the hypothalamic parenchyma, and their morphology was characteristic of mature parenchymal astrocytes (e.g. small nuclei, stellate cell bodies, ramified processes). In addition, GFAP-expressing cells were also found in the ventricular and subventricular zones of the 3rd ventricle, and these cells had a morphology reminiscent of radial glia [21] (Figure 1B). Transient postnatal leptin treatment (P8—P12) did not alter body weight throughout the course of the experiment (data not shown), but resulted in significant increase in the number of BrdU/GFAP double-positive cells in the hypothalamic parenchyma of 6-week-old mice (Figure 1C).

Although GFAP is a specific marker for parenchymal astrocytes, GFAP expression levels are known to be heterogeneous among different astrocyte subsets. Therefore, it is possible that GFAP expression in certain subsets of astrocytes could fall below the detection limit of immunofluorescence method we used. It is also possible that leptin treatment might increase GFAP protein expression [12,13], leading to an erroneous detection of more astrocytes. To exclude these possibilities, we performed lineage tracing experiments using mice carrying both a tamoxifen-inducible GFAP-Cre ER^{T2} [20,22,23] and a Cre-activatable-tdTomato (Ai14) reporter. tdTomato expression, once turned on by tamoxifen, is placed under the control of the strong and constitutive CAG promoter specifically in *Gfap*-expressing cells and any daughter cells emerging from them. Importantly, tdTomato expression in this context is independent of *Gfap* transcriptional activity and hence unlikely to be influenced by leptin. We therefore injected mice expressing both GFAP-Cre ER^{T2} and the Ai14-tdTomato reporter with tamoxifen at postnatal day 10 (P10), injected them twice daily with BrdU in the presence or absence of leptin from P12 to P14, and then sacrificed the mice at P18 (Figure 1D). Consistent with the results we obtained by measuring GFAP immunofluorescence, postnatal leptin treatment increased the numbers of both tdTomato-positive cells (Figure 1F) and tdTomato/BrdU double-positive cells (Figure 1G) in the arcuate nucleus of the hypothalamus. Since bioavailability of BrdU is less than 2 h [24], BrdU-positive cells mark proliferating GFAP-expressing cells during the BrdU injection period and any daughter cells emerging from them. Together, these findings indicate that leptin stimulates hypothalamic astrocyte proliferation during the early postnatal period.

3.2. Removal of leptin receptors from GFAP-expressing cells during early postnatal life reduces the normal proliferation of hypothalamic astrocytes

Multiple studies with different experimental approaches have shown that GFAP-expressing cells express functional leptin receptors [11—15]. We thus sought to evaluate whether removing the leptin receptor from GFAP-expressing cells affects the proliferative capacity of astrocytes in the developing hypothalamus. Mice carrying the GFAP-Cre ER^{T2} and Ai14-tdTomato alleles were crossed with mice carrying a floxed allele of the leptin receptor, $Lepr^{flox/flox}$ [19]. Control (*Gfap-Cre* ER^{T2}, *Ai14*, $Lepr^{flox/+}$) and mutant mice (*Gfap-Cre* ER^{T2}, *Ai14*, $Lepr^{flox/flox}$) were injected with tamoxifen and BrdU at postnatal day 4 and 5 (P4—P5) to delete leptin receptors from GFAP-expressing cells. Mice were subsequently killed at P21 (Figure 2A), and their brains were analyzed for immunofluorescence. Consistent with our initial studies, the number of BrdU/Ai14-tdTomato double positive cells in the arcuate nucleus was reduced in the mutant animals (Figure 2B—C). These results further solidify the concept that leptin signaling is required for hypothalamic astrogenesis during postnatal development.

3.3. Removing leptin receptors from GFAP-expressing cells during early postnatal life does not affect the number of hypothalamic neurons

Beyond marking astrocytes, GFAP is also expressed by a subset of neural progenitor cells that give rise to neurons [21]. Upon close examination of the morphology of tdTomato-positive cells in the hypothalamic parenchyma at P21, the overwhelming majority of these cells exhibited astrocyte morphology, suggesting that they are mature astrocytes. However, a small number of tdTomato-positive cells also expressed the neuronal marker HuC/D and exhibited neuronal morphology, consistent with the notion that some GFAP-expressing cells may serve as neural progenitor cells [21]. Nevertheless, the number of these tdTomato and HuC/D double-positive cells in the arcuate nucleus were similar between control and mutant mice. The numbers of POMC and AgRP neurons, two important neuronal subtypes in the arcuate nucleus with opposing functions, were comparable between control and mutant animals (Figure 3). These findings suggest that leptin signaling in GFAP-progenitors during the postnatal period is not required for postnatal neurogenesis that emanates from these GFAP-expressing neural precursor cells.

3.4. GFAP-expressing cells in the periventricular zone of the 3rd ventricle are responsive to leptin during the initial postnatal week

During early postnatal days (P2), GFAP-expressing cells were sparse in the parenchyma of the hypothalamus. Instead, abundant GFAP-expressing cells were found in the ventricular and subventricular zone of the 3rd ventricle (Figure 4A). The morphology of many of these periventricular GFAP-expressing cells resembles that of radial glia, which are postulated to give rise to astrocytes [21,25]. Interestingly, leptin receptors are expressed along the periventricular zone of the hypothalamic 3rd ventricle at early postnatal time points such as P4 [26]. Leptin injection at this age strongly increases mRNA expression of suppressor of cytokine signaling 3 (*Socs3*), a direct transcriptional target of STAT3, in periventricular cells along the 3rd ventricular wall [26]. This observation suggests that functional leptin receptors are expressed along the periventricular cells of the 3rd ventricle in the early postnatal hypothalamus. With this in mind, we examined phosphorylation of STAT3 (pSTAT3), a major leptin-induced signaling step, in GFAP-expressing cells located along the periventricular zone of the 3rd ventricle. At P2, no basal pSTAT3 signal was detected in these GFAP-expressing cells. However, at P6, a time that approximates the onset of

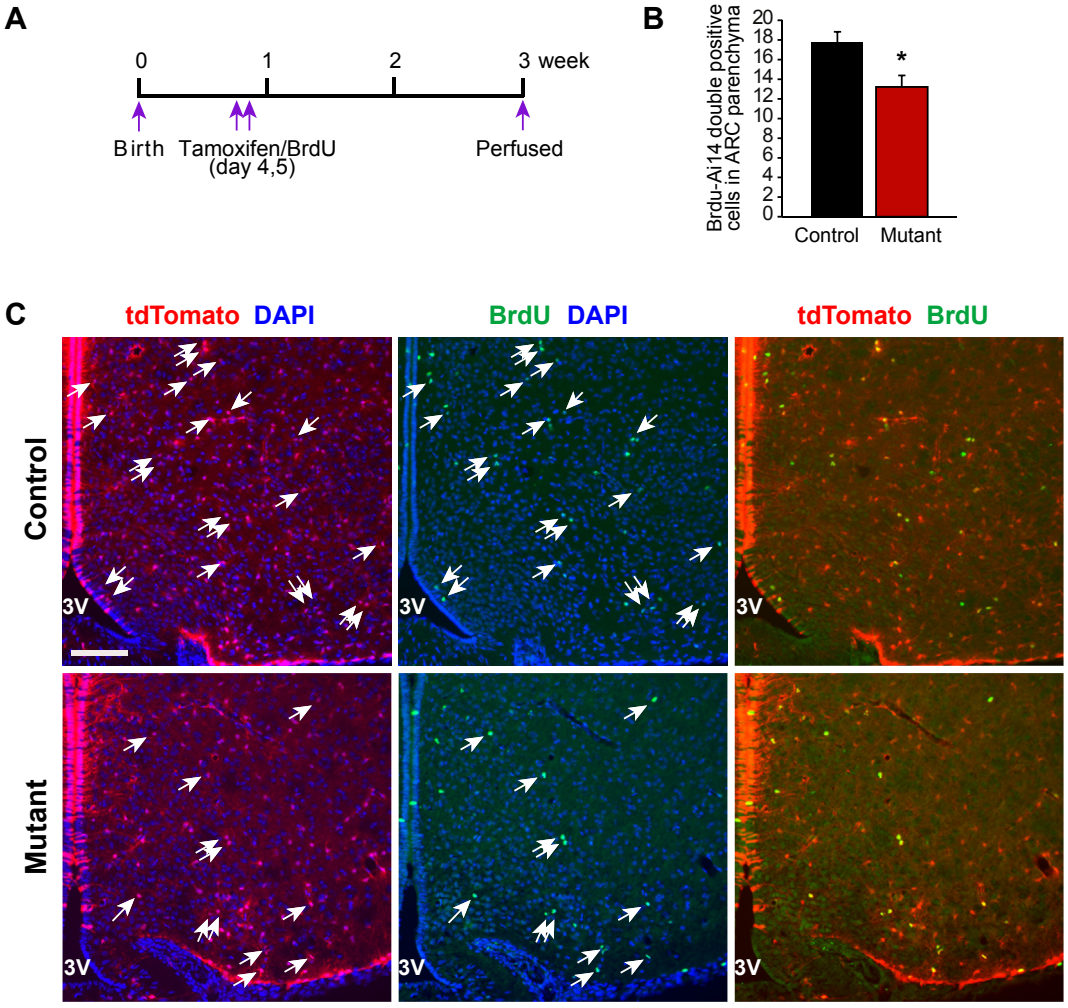

Figure 2: Removal of leptin receptors from GFAP-expressing cells during early postnatal life reduces the normal proliferation of hypothalamic astrocytes. (**A**) Experimental scheme. Control (*Gfap-Cre ER^{T2}*, *Ai14*, *Lepr$^{flox/+}$*) and mutant (*Gfap-Cre ER^{T2}*, *Ai14*, *Lepr$^{flox/flox}$*) female mice were injected subcutaneously once a day with tamoxifen (200 mg/kg) at P4—P5 to delete leptin receptors from GFAP-expressing cells and induce expression of Ai14-tdTomato in the same cells. On the same days mice were subcutaneously injected with BrdU (100 mg/kg). Mice were analyzed at 3-weeks of age. (**B**) Deletion of leptin receptors from GFAP-expressing cells decreased the number of Ai14-tdTomato and BrdU double-positive cells in the arcuate nucleus of the hypothalamus. n = 3—5 per group, 3 sections/mouse. (**C**) Representative images showing hypothalami of 3-week-old control and mutant mice that had leptin receptors deleted from GFAP-expressing cells from P4—P5. Arrows show Ai14-tdTomato cells (red) that incorporated BrdU (green). Data represent mean ± SEM. *P < 0.05 as determined by 2-tailed student's *t*-test. 3V = 3rd ventricle. Scale bar: 100 μm.

the postnatal leptin surge [16], basal pSTAT3 signal was readily detected in these cells by confocal microscopy (Figure 4A—B, **left panels**). Basal pSTAT3 in GFAP-expressing periventricular cells is restricted to the postnatal period as no such signal was detected in these cells in adulthood (data not shown). This result suggests that pSTAT3 expression is stimulated transiently by an endogenous signal in GFAP-expressing periventricular cells in the developing hypothalamus.

While leptin is a plausible endogenous signal that could activate pSTAT3 in GFAP-expressing ventricular cells in the developing hypothalamus, other factors that signal through STAT3 are known to be developmentally regulated [27,28]. To this end, we sought to examine whether leptin could induce pSTAT3 in GFAP-expressing cells at P2, an age prior to the onset of postnatal leptin surge. Wild type mice at P2 were injected with leptin or vehicle (PBS) and killed 60 min later. While very few cells expressed detectable levels of pSTAT3 in vehicle-treated mice, the number of GFAP-pSTAT3 double positive cells was markedly increased by leptin treatment (Figure 4A). This experiment indicates that leptin acts on GFAP-expressing cells at the periventricular zone of the early postnatal hypothalamus.

Multiple lines of evidence suggest that mature astrocytes in the adult hypothalamic parenchyma express functional leptin receptors [11—15], and that removing them from adult astrocytes disrupts normal energy homeostasis [14]. However, we were not able to detect leptin-induced pSTAT3 signal in adult astrocytes despite that robust pSTAT3 was detected in surrounding neurons (data not shown). Since activation of the functional leptin receptors induces multiple cellular signaling pathways, including STAT3, PI3K, SHP2, ERK and SH2B1 [10], these observations imply that leptin may regulate astrocyte functions via a STAT3-independent mechanism, although it is possible that such activation is below the detection limit of current methods. The lack of observing an induction of pSTAT3 by leptin in adult hypothalamic astrocytes also prompts us to wonder whether the endogenous pSTAT3 signal we observed in periventricular GFAP cells at P6 was induced by endogenous leptin. To this end, we examined

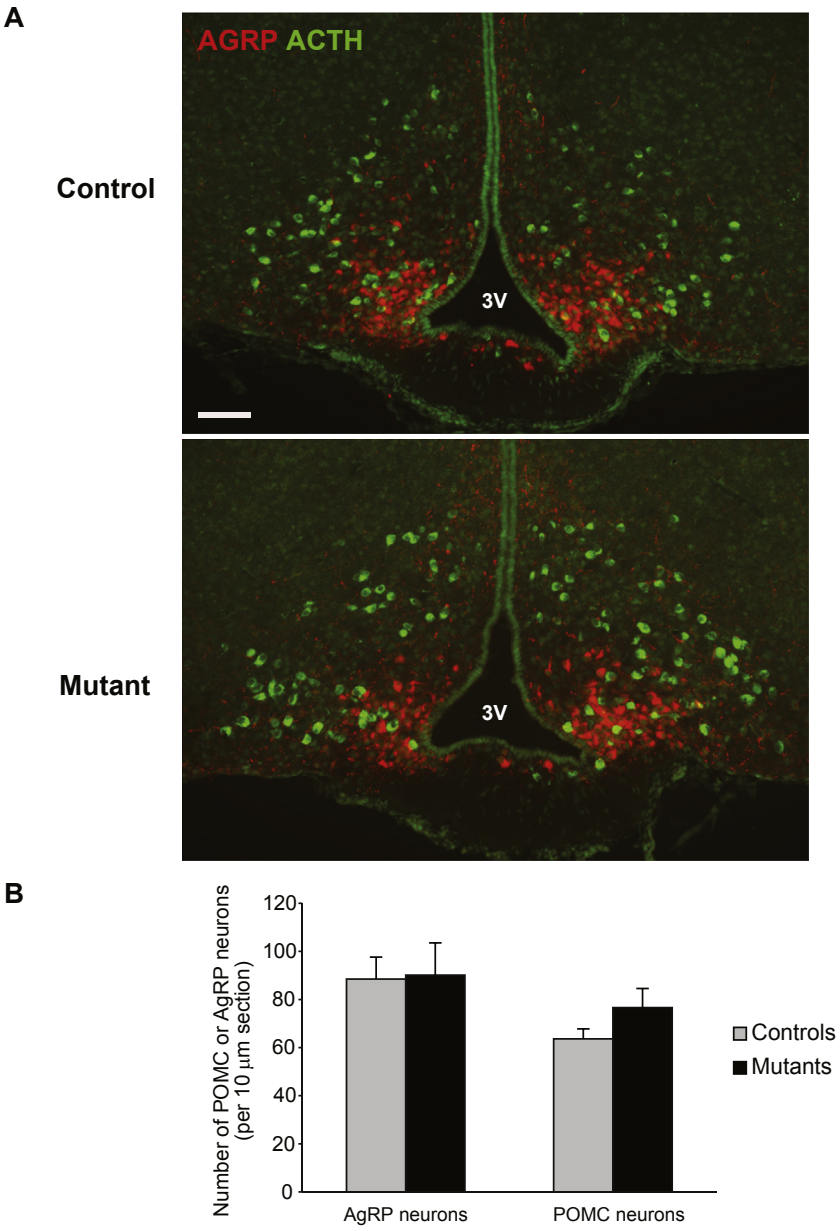

Figure 3: Removing leptin receptors from GFAP-expressing cells during early postnatal life does not affect the number of POMC or AgRP neurons. Control (*Gfap-Cre ER^{T2}*, *Ai14*, *Lepr$^{flox/+}$*) and mutant (*Gfap-Cre ER^{T2}*, *Ai14*, *Lepr$^{flox/flox}$*) female mice were injected subcutaneously once a day with tamoxifen (200 mg/kg) at P4–P5 to delete leptin receptors from GFAP-expressing cells. POMC and AgRP immunoreactivity were analyzed in 3-week-old mice by double immunofluorescence analysis using an ACTH and an AgRP antibody. (**A**) Representative images showing AgRP (red) and POMC (green) expressing neurons in the arcuate nucleus of control and mutant mice. (**B**) Deletion of leptin receptors from GFAP-expressing cells at P4–P5 did not affect the number of AgRP or POMC neurons when analyzed at 3 weeks of age. n = 4–7 per group. Data represent mean ± SEM. 3V = 3rd ventricle. Scale bar: 100 µm.

pSTAT3 signals in GFAP cells in leptin-deficient *Lep$^{ob/ob}$* mice at P6, an age when pSTAT3 was readily detectable in control mice. No difference in the numbers of pSTAT3/GFAP double positive cells was found between *Lep$^{ob/ob}$* mice and their control littermates (Figure 4B). Separately, control (*Lepr$^{flox/flox}$*) and mutant mice (*Gfap-Cre ER^{T2}*, *Lepr$^{flox/flox}$*) were injected with tamoxifen at P0 to delete leptin receptors from GFAP-expressing cells. These mice were then injected with leptin at P2, and their brains were processed 30 min later to examine pSTAT3 and GFAP immunoreactivity by confocal microscopy. Again, no difference in leptin-induced pSTAT3 signal was observed between control and mutant mice (Figure 4C). Together, these results suggest that GFAP-expressing cells in the periventricular zone of the 3rd ventricle are responsive to leptin during the initial postnatal week. However, our results also suggest that leptin is unlikely to be the major endogenous signal that induces pSTAT3 in these cells.

4. DISCUSSION

Developmental programming of hypothalamic feeding circuits plays an important role in metabolic regulation, and abnormalities in this programming are associated with a predisposition to obesity and other metabolic abnormalities [29]. Leptin, a crucial regulator of metabolic function, has been shown to regulate the outgrowth of neuronal projections in the postnatal hypothalamus. Intriguingly, the postnatal leptin

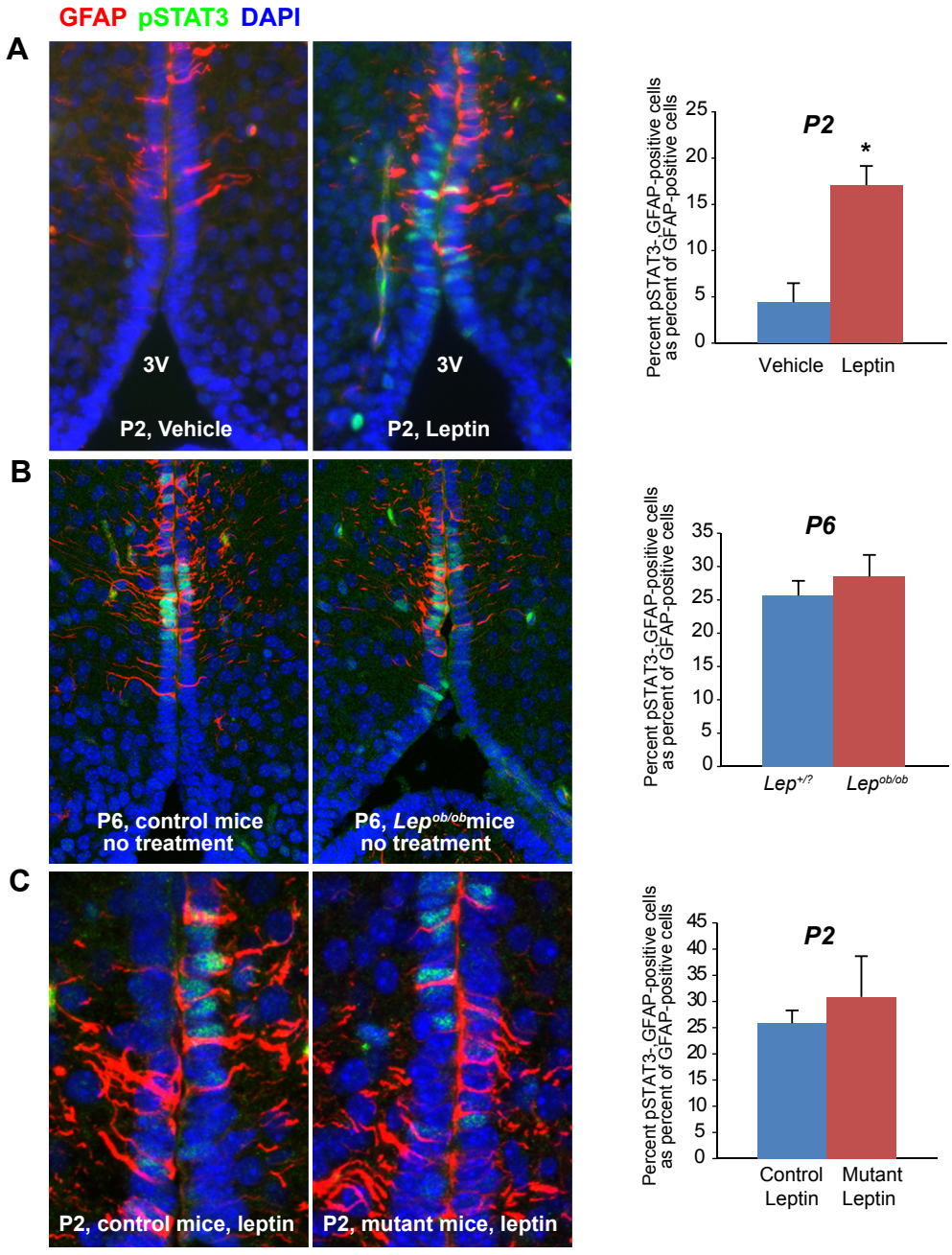

Figure 4: GFAP-expressing cells in the periventricular zone of the 3rd ventricle are responsive to leptin during the initial postnatal week. (**A**) P2 wild-type male mice were injected with vehicle (PBS) or leptin (5 mg/kg) and killed 60 min later. GFAP and pSTAT3 immunoreactivity in the hypothalamus was examined by confocal microscopy. A collapsed Z-stack of three 1 μm optical slices was used to assess pStat3 (green, nuclear signal) expression in GFAP (red)-expressing cells. Leptin treatment significantly increased pStat3 in GFAP-expressing cells in the periventricular zone of the 3rd ventricle. n = 4–6 per group, 4 sections per mouse. (**B**) P6 *Lep*$^{+/?}$ *or Lep*$^{ob/ob}$ male mice were killed without any treatment and immunofluorescence analysis was performed. A collapsed Z-stack of three 1 μm immunofluorescent optical slices was used to assess the pStat3 (green) expression in GFAP (red)-expressing cells in the periventricular zone of the 3rd ventricle. There was no significant difference in number of pStat3 and GFAP double-positive cells between the genotypes. n = 3–4 mice per group, 2 sections per mouse. (**C**) Control (*wt*, *Lepr*^{floxflox}) and mutant (*Gfap-Cre ER*^{T2}, *Lepr*$^{flox/flox}$) male mice were subcutaneously injected with tamoxifen (200 mg/kg) at P0. Mice were then intraperitoneally injected with leptin (10 mg/kg) and killed 30 min later. n = 3–4 per group, 4 sections per mouse. Deletion of leptin receptor did not alter pStat3 expression in GFAP-expressing cells in the periventricular zone of the 3rd ventricle. Data represent mean ± SEM. *P < 0.05 as determined by 2-tailed student's *t*-test. 3V = 3rd ventricle.

surge and maturation of the hypothalamic neuronal circuits also coincide with the marked proliferation and expansion of astrocytes. In this study, we show that leptin also modulates the proliferation of astrocytes in the developing hypothalamus. Leptin administration during postnatal period increases proliferation of astrocytes while deleting leptin receptors from astrocytes reduces their proliferation. Given the importance of astrocytes in neuronal connectivity and function, our study indicates that leptin may exert its metabolic effects, in part, by promoting hypothalamic astrogenesis during early postnatal development.

To date, the signaling mechanism by which leptin regulates the proliferation or function of astrocytes is not well understood. Our

result suggests that leptin can enhance pSTAT3 expression in GFAP-expressing cells in the periventricular zone of the developing hypothalamus. This result is consistent with previous findings showing that both leptin receptor expression and the ability of leptin to stimulate SOCS3 expression is manifest in periventricular cells along the 3rd ventricle only during early postnatal period and then dissipates prior to adulthood [26]. Despite this, our results indicate that leptin itself is not required for pSTAT3 activation in these cells, even though this activation is concordant with the onset of the leptin surge. STAT3 is a well-known transcriptional regulator of astrocyte gene expression and astrogenesis during development. STAT3 is activated by leukemia inhibitory factor (LIF), and forms a complex with Smad1 to synergistically induce astrocytes from neuroepithelial cells [27,28]. Thus, leptin may enhance astrogenesis by further potentiating STAT3 signaling in astrocyte progenitors. Future work will be required to test this hypothesis, as the relative insensitivity of the current immunofluorescence techniques precludes us from precisely comparing pSTAT3 intensity in control mice with that in mice lacking leptin receptors in GFAP-expressing cells (Figure 4).

Astrocytes are known to be highly heterogeneous not only in different brain regions but also within the same brain region. Moreover, different subpopulations of astrocytes may arise from distinct progenitor cell pools [25,30]. Astrocytes have been shown to be spatially restricted to specific locations in the CNS that are close to the sites of origins where their progenitor cells are located. Positional identity, an organizing principle governing the generation of neuronal subtype diversity, has been shown to govern astrocyte subtype diversification [31]. Thus, it is possible that parenchymal astrocytes in the hypothalamus consist of distinct subpopulations, and that leptin function may be required for the development of one or more subsets of astrocytes but not others. This notion is consistent with the modest impairment of astrogenesis caused by disrupting leptin signaling in GFAP-expressing cells, although we cannot exclude that incomplete Cre-mediated recombination may also play a role. It is also possible that leptin function can be partially compensated by other gliogenic factors. In addition, our study does not rule out the possibility that leptin may affect the survival of a specific subset of hypothalamic astrocytes. The future identification of distinct subpopulations of hypothalamic astrocytes will help us understand the physiological role of these cells in metabolic regulation under normal and pathologic conditions.

Astrocytes are vital for neuronal function. They provide crucial metabolic support to neurons, promote synaptogenesis, modulate glucose sensing and neural transmitter homeostasis at synapses, mediate transport across the blood—brain barrier, exert neural protective effects and help mount injury responses [5—7]. The recent demonstration that disrupting leptin function in adult astrocytes distorts neuronal function and energy homeostasis further strengthens the notion that leptin coordinately orchestrates the function of both neurons and glia [13,14]. Our study suggests that leptin plays an important developmental role in promoting astrogenesis during a critical time of neuronal maturation. Thus, altering the timing or abundance of leptin during early postnatal development, as occurs in maternal and postnatal malnutrition [32], could influence hypothalamic neuronal function or their connectivity in an astrocyte-dependent manner. For example, premature onset of the leptin surge in the neonatal period does not alter body weight or fat mass in adulthood when mice are fed with regular chow but predisposes animals to high-fat-diet-induced obesity later in life [32]. Moreover, neonatal overnutrition causes increased GFAP expression and an increase in both astrocyte numbers and extensions in the absence of inflammation [13]. It is worth noting that the rodent brain between postnatal days P1 and P10 corresponds approximately to the third trimester of human gestation [33]. In the developing human brain, astrogenesis continues through the second half of intrauterine life as well as after birth [34]. Leptin concentration in the arterial cord blood of human fetuses increases dramatically after 34 weeks of gestation, and newborns with intrauterine growth retardation have significantly lower serum leptin values than those with normal growth [35]. With the increased prevalence of maternal diabetes and childhood obesity in recent years, it has now recognized that human hypothalamic development is influenced by both maternal and early postnatal environments [29]. Our study raises the possibility that altered regulation of astrogenesis by leptin during early development may affect the programming of the hypothalamic feeding circuits, which could predispose these individuals to metabolic dysfunction late in life.

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CONFLICT OF INTERESTS

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.08.005.

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