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A critical period for the trophic actions of leptin on AgRP neurons in the arcuate nucleus of the hypothalamus

Anna Kamitakahara^{#1,2}, Karine Bouyer^{#1}, Chien-Hua Wang^{1,2}, and Richard Simerly^{1,3}

¹The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, California

²Program in Neuroscience, University of Southern California, Los Angeles, California

³Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee

[#] These authors contributed equally to this work.

Abstract

In the developing hypothalamus, the fat-derived hormone leptin stimulates the growth of axons from the arcuate nucleus of the hypothalamus (ARH) to other regions that control energy balance. These projections are significantly reduced in leptin deficient (*Lep^{ob/ob}*) mice and this phenotype is largely rescued by neonatal leptin treatments. However, treatment of mature *Lep^{ob/ob}* mice is ineffective, suggesting that the trophic action of leptin is limited to a developmental critical period. To temporally delineate closure of this critical period for leptin-stimulated growth, we treated *Lep^{ob/ob}* mice with exogenous leptin during a variety of discrete time periods, and measured the density of Agouti-Related Peptide (AgRP) containing projections from the ARH to the ventral part of the dorsomedial nucleus of the hypothalamus (DMHv), and to the medial parvocellular part of the paraventricular nucleus (PVHmp). The results indicate that leptin loses its neurotrophic potential at or near postnatal day 28. The duration of leptin exposure appears to be important, with 9- or 11- day treatments found to be more effective than shorter (5-day) treatments. Furthermore, leptin treatment for 9 days or more was sufficient to restore AgRP innervation to both the PVHmp and DMHv in *Lep^{ob/ob}* females, but only to the DMHv in *Lep^{ob/ob}* males. Together, these findings reveal that the trophic actions of leptin are contingent upon timing and duration of leptin exposure, display both target and sex specificity, and that modulation of leptin-dependent circuit formation by each of these factors may carry enduring consequences for feeding behavior, metabolism, and obesity risk.

Keywords

agouti-related peptide (AgRP); arcuate nucleus of the hypothalamus (ARH); Critical period; leptin; sexual dimorphism; RRID:IMSR_JAX:000632; RRID:AB_2313908; RRID:IMSR_JAX:007914; RRID:IMSR_JAX:006417; RRID:SCR_002668

1 INTRODUCTION

The arcuate nucleus of the hypothalamus (ARH) represents a critical interface between peripheral hormonal cues and neural circuits that control body weight (Hetherington & Ranson, 1940; Hewson, Tung, Connell, Tookman, & Dickson, 2002; Mirshamsi et al., 2004; Myers, Munzberg, Leininger, & Leshan, 2009; Saper, Chou, & Elmquist, 2002; Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Sternson, Atasoy, Betley, Henry, & Xu, 2016). Within the ARH, neurons that co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP) are activated or inhibited by signals that convey information about energy balance (Atasoy, Betley, Su, & Sternson, 2012; Betley, Cao, Ritola, & Sternson, 2013; Garfield et al., 2016; Gropp et al., 2005; Hahn, Breininger, Baskin, & Schwartz, 1998; Zarjevski, Cusin, Vettor, Rohner Jeanrenaud, & Jeanrenaud, 1993). The adipocyte-derived hormone, leptin, is required for central nervous system regulation of energy homeostasis and directly impacts the activity of AgRP neurons to rapidly alter feeding behavior and energy metabolism through connections to a distributed network of nuclei controlling downstream neuroendocrine and autonomic output (Atasoy et al., 2012; Betley et al., 2013, _S1_Reference72015; Bouyer & Simerly, 2013; Campfield, Smith, Guisez, Devos, & Burn, 1995; Carter, Soden, Zweifel, & Palmiter, 2013; Garfield et al., 2016; Grill et al., 2002; Halaas et al., 1995; Huo, Maeng, BjØrbæk, & Grill, 2007; Scott, Williams, Rossi, Lee, & Elmquist, 2011; Zhang et al., 1994).

The projections of ARH neurons are established primarily during the first two postnatal weeks, concomitant with a naturally occurring surge in leptin secretion (Ahima, Prabakaran, & Flier, 1998; Bouret, 2004). During this time, leptin signals through the b-form of its receptor (LepRb) at the level of the ARH to stimulate growth of axonal projections from AgRP neurons (Bouret, 2004; Bouret, Draper, & Simerly, 2004; Bouyer & Simerly, 2013). Thus, in the leptin deficient (Lep^{ob/ob}) mouse, AgRP projections to several key nuclei controlling energy balance are severely diminished, including projections to the paraventricular (PVH) and dorsomedial (DMH) nuclei of the hypothalamus (Bouret, 2004; Bouret et al., 2004; Bouyer & Simerly, 2013). This diminished innervation is largely rescued by treatment with exogenous leptin during the postnatal period (P4-P14) when the surge in leptin secretion normally occurs (Atasoy et al., 2012; Betley et al., 2013; Bouret et al., 2004; Bouyer & Simerly, 2013; Garfield et al., 2016; Gropp et al., 2005; Hahn et al., 1998; Zarjevski et al., 1993). Moreover, impairments in food intake, adiposity, and certain aspects of autonomic function are improved when Lep^{ob/ob} mice are treated with leptin only during this restricted postnatal period. However, leptin treatment in mature Lep^{ob/ob} animals is ineffective at restoring normal patterns of ARH innervation (Atasoy et al., 2012; Betley et al., 2013, 2015; Bouret et al., 2004; Bouyer & Simerly, 2013; Campfield et al., 1995; Carter et al., 2013; Garfield et al., 2016; Grill et al., 2002; Halaas et al., 1995; Huo et al., 2007; Scott et al., 2011; Zhang et al., 1994), suggesting that the trophic actions of leptin are restricted to a discrete developmental critical period.

Postnatal leptin also appears to influence targeting of ARH neurons to specific parts of the PVH. AgRP neurons project to two broad subgroups of neurons within the PVH: neuroendocrine neurons that send projections to the median eminence and posterior pituitary to mediate hormonal responses, and preautonomic neurons that project to the brainstem,

spinal cord, limbic system, and other hypothalamic sites to coordinate autonomic and goal-directed behaviors (Ahima et al., 1998; Biag et al., 2011; Bouret, 2004; Bouyer & Simerly, 2013; Cowley et al., 1999; Saper et al., 2002; Swanson, 2000). In *Lep^{ob/ob}* mice, deficits in innervation from the ARH to preautonomic regions of the PVH are rescued by treatment with exogenous leptin during the postnatal period (P4–14) when the surge in leptin secretion normally occurs (Bouret, 2004; Bouret et al., 2004; Bouyer & Simerly, 2013). However, leptin treatment from P4–14 is insufficient to restore AgRP projections to neuroendocrine regions of the PVH in males, suggesting that the growth of AgRP projections to individual target regions is not impacted uniformly by leptin (Bouret, 2004; Bouret et al., 2004; Bouyer & Simerly, 2013).

In order to understand how environmental stimuli presented at different times during development impact programming of CNS architecture, it is important to first define when circuits are capable of responding to leptin. To probe the site-specificity and temporal constraints for the neurotrophic action of leptin on development of ARH projections, we evaluated the density of AgRP projections to the PVH and DMH of *Lep^{ob/ob}* mice that received exogenous leptin treatment during several discrete postnatal time periods. The ability of leptin to rescue growth of AgRP projections was dependent on both duration and timing, with the period of effective leptin treatment closing around the 28th postnatal day. Furthermore, the neurotrophic actions of postnatal leptin appear to exhibit both target specificity and sexual dimorphism. Together, these findings reveal that the neurotrophic action of leptin does not extend past the fourth postnatal week, and exhibits anatomical and sex specific characteristics, that have long-term physiological consequences for metabolic function and obesity risk.

2 METHODS

2.1 Animals

All animal care and experimental procedures were performed in accordance with the Institutional Animal Care and Usage Committee of Children's Hospital Los Angeles. Mice were housed at 22°C on a 13:11-hr light:dark cycle (lights on at 0600h:lights off at 1900h) with ad libitum access to a standard chow diet (PicoLab Rodent Diet 20, #5053). Mice expressing *Cre* recombinase under the control of the leptin receptor promoter (*LRb^{cre}* mice) were provided by Dr. Martin Myers, University of Michigan (Leshan, Björnholm, Münzberg, & Myers, 2006). Mice expressing the *Cre* dependent reporter, tdTomato (stock 007914), mice expressing GFP under the control of the neuropeptide Y (NPY) promoter (*NPY^{GFP}* mice; stock 006417), and mice heterozygous for a mutation in the leptin gene (*Lep^{ob}* stock 000632) were obtained from The Jackson Laboratory.

Mice heterozygous for a mutation in the leptin gene were bred to generate homozygous, leptin deficient (*Lep^{ob/ob}*) mice, and control littermates (WT) with normal leptin expression. *Lep^{ob/ob}* mice were maintained on a C57/BL6J background. On postnatal day (P) 1, litter size was adjusted to 6–8 pups per litter to standardize nutrition during the lactation period. Male and female, *Lep^{ob/ob}* and WT littermates were treated daily (between 11:00 and 13:00 hours) by intraperitoneal (i.p.) injection of either leptin (10 mg/kg body weight; Peprotech Inc.) or vehicle (5 mM sodium citrate) during one of seven postnatal time

periods. Thus, genotyped male offspring were assigned to a treatment group (leptin or vehicle) corresponding to one of the following time periods: P4–8, P6–10, P8–12, P12–16, P4–14, P16–26, P28–38. In addition, groups of female mice were genotyped and treated with either leptin or vehicle during the following intervals: P4–14, P16–24, or P25–33. Mice from all experimental groups were weaned on postnatal day 22 onto an ad libitum standard chow diet. On P60–P70 mixed groups of leptin or vehicle treated mice were perfused and processed for immunohistochemistry together with Lep^{ob/ob} and WT littermates.

2.2 Immunohistochemistry

Mice were anesthetized with tribromoethanol and perfused transcardially with saline followed by fixative (4% paraformaldehyde in borate buffer, pH 9.5). Brains were postfixed in a solution of 20% sucrose in fixative, cryoprotected overnight in 20% sucrose in 0.2M potassium phosphate buffered saline (KPBS), and frozen in powdered dry ice. Four series of 20 µm-thick frozen sections were collected using either a sliding microtome or cryostat for immunohistochemical staining. Tissue sections were incubated overnight in blocking buffer (0.2M KPBS, 2% normal goat or donkey serum, 0.3% Triton X-100) at 4°C with constant agitation, followed by incubation for 72 hr at 4°C in blocking buffer containing combinations of antibodies directed against AgRP (Phoenix Pharmaceuticals Cat# H-003–53, RRID:AB_2313908), HuC/ D (Molecular Probes Cat# A21271, RRID:AB_221448), and/or estrogen receptor alpha (EMD Millipore Cat# 06–935, RRID:AB_310305). After several rinses in KPBS, sections were incubated for 2 hr at room temperature in blocking buffer containing a cocktail of species-specific Alexa Fluor conjugated secondary antibodies (Thermo Fisher Scientific Cat# A10042, RRID:AB_2534017; Thermo Fisher Scientific Cat# A-31571, RRID:AB_2536181; Thermo Fisher Scientific Cat# A-21206, RRID:AB_2535792). Sections were rinsed in KPBS, mounted onto gelatin-subbed slides and cover-slipped using Fluoromount G mounting medium (Southern Biotech, Birmingham, AL).

Immunohistochemical labeling of androgen receptor (EMD Millipore 06–080, AB_310214) or pSTAT3 (Cell Signaling Technology Cat# 9131L, RRID:AB_331587) was performed as described above, with slight modification, as described previously (Levin, Dunn- Meynell, & Banks, 2004). Briefly, sections were treated with 0.5% NaOH, 0.5% H₂O₂, in KPBS for 20 min, rinsed several times in KPBS, then incubated in 0.3% glycine in KPBS for 10 min. Following several rinses in KPBS, sections were then treated with 0.03% sodium dodecyl sulfide, and rinsed again several times in KPBS before blocking for 20 min in 4% normal goat serum (NGS), 0.4% triton X-100, 1% bovine serum albumin (BSA, fraction V) in KPBS. Sections were incubated in anti-pSTAT3 or androgen receptor antibody diluted to 1:1,000 in 1% NGS, 0.4% triton X-100, 1% BSA in KPBS for 48 hr at 4°C. Sections were rinsed several times in KPBS before incubating in secondary goat anti-rabbit Alexa 488 (Molecular Probes Cat# A-11008, RRID:AB_143165) (1:500) in 1% NGS, 0.3% triton X-100 in KPBS for 2 hr at room temperature. Sections were rinsed several times in KPBS, and a coverslip applied using Fluoromount G mounting medium (Southern Biotech).

2.3 Antibody characterization

Antibodies used in this study are summarized in Table 1, and described in greater detail by corresponding number below.

1. The polyclonal rabbit anti-AgRP antibody (AB_91683) was generated against synthetic peptide sequence N'-TRSCP⁺NMATGRCYCF-ANFFRCACPDCCPVQQGSEHLRVCRSS-C'. Specificity of this antibody has been demonstrated by immunolabeling in mouse hypothalamic tissue sections, which was blocked completely by preadsorption with AgRP peptide (83–132) (Nilsson, Lindfors, Fetissof, Hökfelt, & Johansen, 2007). A single band between 14–21 kDa was recognized following SDS-PAGE in duck total brain and hypothalamic tissue samples (Mirabella, Esposito, Squillacioti, De Luca, & Paino, 2004). Furthermore, patterns of immunoreactivity in histological staining with AB_91683 are consistent with in situ hybridization data from mouse ARH (Hahn et al., 1998), as well as our own staining for AgRP in the hypothalamus in developing and adult mice.
2. Polyclonal anti-AR antibody used in this study was generated using KLH-conjugated linear peptide corresponding to the N-terminus of rat androgen receptor as the immunogen according to the manufacturer. Specificity of this antibody was confirmed by SDS-PAGE in African Cichlid fish demonstrating that it recognizes two bands between 70 and 100 kDa corresponding to alpha and beta forms of the receptor (Munchrath & Hofmann, 2010). In addition, preadsorption with the immunogen followed by immunofluorescence staining in tissue from rat brain sections completely abolished all signal (Munchrath & Hofmann, 2010).
3. According to the information provided by the manufacturer, polyclonal rabbit anti-estrogen receptor alpha antisera (AB_310305) recognizes the C-terminus amino acids of human, rat, and mouse. Specificity of this antibody, then referred to as C1355, has been demonstrated by western blot, which revealed that the antibody recognizes several bands of molecular weight 64–66 kDa, 50–55 kDa, 40–45 kDa, and 20–24 kDa, corresponding to different ER α forms (Friend, Resnick, Ang, & Shupnik, 1997). Immunodetection of all bands was eliminated by preadsorption with excess antigen (Friend et al., 1997). Furthermore, immunostaining in sensory and autonomic ganglia using AB_310305 is consistent with other antiestrogen receptor alpha antisera, and signal is not diminished by preabsorption with estrogen receptor beta protein (Papka et al., 2001).
4. Antibodies directed against HuC/D in this study have been widely cited in published literature used in human, mouse, rat, pig, reptile, non-human primate, sheep, zebrafish, chicken, horse, xenopus, and guinea pig according to the manufacturers information. The mouse monoclonal antibody was described by Marusich, et al. as binding several Hu/Elav neuronal proteins visualized by western blot around 40 kDa in human, avian, and mouse neural tissue (Marusich, Fur-neaux, Henion, & Weston, 1994). Preadsorption with HuD protein abolished

antibody labeling in western blot (Marusich et al., 1994). The antibodies bind exclusively to neuronal antigens and are used in this study to identify the cellular boundaries of the PVH and DMH.

5. Polyclonal pSTAT3 antibody recognizes both alpha and beta forms revealed as 79 and 86 kDa molecular weight bands following western blot, according to the manufacturer information. Specificity of the antibody has been demonstrated in tissue from transgenic animals in which Tyr 1183 of the leptin receptor was mutated disrupting STAT3 signaling, referred to as 's/s' mice (Bates et al., 2003). Robust pSTAT3 signal is observed in wild-type mice following leptin administration, but no immunoreactive signal was detected in tissue from s/s mice by either western blot or immunohistochemistry (Bates et al., 2003).

2.4 Image acquisition and analysis

The density of immunolabeled neuronal fibers was measured in discrete regions of interest (ROI) in the medial parvocellular region of the PVH (PVHmp) or ventral DMH (DMHv). Preautonomic regions of the PVH were not assessed due to inherent difficulties in visualization without the use of retrograde tracer injections to label specific populations, a technique that is not feasible given the size of cohorts necessary for this study. Anatomical features were identified cytoarchitecturally, using the neuronal marker HuC/D and nuclear regions classified as defined in Dong, 2008 (DongThe Allen Institute for Brain Science, 2008). All images were obtained using a laser scanning confocal microscope (Zeiss LSM 710) equipped with high N.A. objectives. Confocal image stacks were collected through the z-axis of each ROI at a frequency of 0.4 μm using a high-magnification, 63X oil-corrected objective (NA 1.4). Using Volocity software (Perkin Elmer), axonal fibers containing AgRP or tdTomato fluorescence were visualized in each image stack by using a thresholded intensity value. Identified fibers were then skeletonized to one-pixel thickness and the total length of each line measured and summed. The resulting values represent fiber density in a defined volume, which closely correspond to other methods used to measure axonal density such as use of the lipophilic tracer, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Bouret et al., 2004, 2008). While expression of AgRP peptide is, itself, leptin dependent (Morrison, 2005; Ollmann, 1997), statistical evaluation of AgRP fiber density following leptin treatment was made through comparisons between leptin-deficient *Lep^{ob/ob}* mice weeks after leptin treatment cessation, such that hormonal effects on gene expression would be comparable between groups.

To confirm effective leptin signaling in *LRb^{cre}*-expressing neurons, the number of pSTAT3 immunoreactive cells colocalized with *LRb^{cre}*tdTomato endogenous fluorescence was measured in sections containing the ARH. Confocal images were collected in a defined ROI through a 1:4 series of sections through the ARH (unilaterally). The number of cells labeled for pSTAT3 immunoreactivity, tdTomato, or both were counted manually, aided by Volocity software. The total number of labeled cells in the ARH was estimated by multiplying the number of counted cells by 2, to account for bilateral ARH cell numbers, and then multiplied by 4, to account for the use of a single series of tissue sections (of 4 series collected) for quantification. This calculation approximates the total number of neurons in the ARH positive for pSTAT3, tdTomato, and colocalized populations.

For analysis of AgRP projections derived from neurons that express LRb, the density of fibers doubly labeled for AgRP and tdTomato was measured in LRb^{cre}tdTomato mice using Volocity software. The density of fibers containing both AgRP and tdTomato signals was measured and expressed as a percentage of singly labeled AgRP containing fibers in order to estimate the relative penetrance of LRb expression in AgRP projections to each target.

2.5 Statistical analyses

Statistical significance was analyzed using GraphPad Prism software, for which data are expressed as mean values $\pm$ SEM. Normality of the data was assessed using the D'Agostino Pearson test. For multiple comparisons with one independent variable, a one-way ANOVA followed by Holm-Sidak post hoc test was used. Comparisons included were between leptin treatments of equal duration. *p* values less than .05 were considered significant.

3 RESULTS

3.1 The trophic effect of leptin on AgRP axonal growth is temporally restricted to a discrete developmental period

To evaluate temporal constraints of leptin's trophic action on ARH outgrowth, the density of AgRP projections to the DMHv was measured on P60 in male Lep^{ob/ob} mice and WT littermates treated daily with either vehicle or leptin during several discrete time periods designed to test the timing and duration of effective leptin exposure. Consistent with previous findings (Bouret et al., 2004), the density of AgRP innervation in the DMHv was significantly decreased in vehicle injected Lep^{ob/ob} animals (Figure 1b,e) compared to vehicle injected WT animals, which displayed robust AgRP innervation of the DMHv (Figure 1a,d). Treatment of Lep^{ob/ob} mice with leptin from P4–14 or P16–26 significantly increased the density of AgRP labeled fibers in the DMHv compared to vehicle injected Lep^{ob/ob} mice. However, leptin treatment from P28–38 was not effective in increasing the density of AgRP projections to the DMHv (Figure 1c,f,g), suggesting that the neurotrophic actions of leptin on the development of ARH projections does not extend beyond the 4th week of postnatal life in mice.

In addition to these 11-day treatment groups, several shorter (5-day) treatment groups were included in the study, including daily leptin injections from P4–8, P6–10, P8–12, and P12–16. None of the Lep^{ob/ob} mice in the 5-day leptin treatment groups exhibited a statistically significant increase in the density of AgRP containing projections to the DMHv compared to that of Lep^{ob/ob} animals injected with vehicle (Figure 1g).

3.2 Neurotrophic action of leptin treatment displays target specificity

In contrast to the effects of leptin on AgRP projections to the DMHv, previous studies have demonstrated that leptin treatment from P4–14 does not rescue AgRP innervation to the medial parvocellular compartment of the PVH (PVHmp), in male Lep^{ob/ob} mice (Bouyer & Simerly, 2013). Here, we confirm and extend this observation demonstrating that leptin treatment from P4–8, P6–10, P8–12, P12–16, P4–14, P16–26, or P28–38 was insufficient to rescue AgRP innervation of the PVHmp in male Lep^{ob/ob} mice (Figure 2). Innervation of the PHVmp was examined in the same cohort of animals used to study rescue of ARH

projections to the DMHv in $Lep^{ob/ob}$ mice, thus confirming that the inability to rescue AgRP innervation in the PVHmp is not due to technical issues, such as poor leptin lot efficacy, because leptin treatments were sufficient to stimulate growth of AgRP projections to the DMHv in the same animals.

AgRP neurons do not appear to project equally to all nuclei innervated by AgRP neurons. Subpopulations of AgRP neurons provide inputs to distinct subsets of ARH targets and $LepRb$ does not appear to be expressed in AgRP neurons that project to hypothalamic targets in adult mice (Betley et al., 2013). Therefore, it is possible that $LepRb$ may be expressed transiently by subsets of ARH cells that project to either the PVHmp or DMHv. To test whether AgRP neurons that project to the DMHv or PVHmp have the capacity to respond to leptin during development, $LepRb^{cre}tdTomato$ mice were used to visualize neurons that express $LepRb$ in neonatal mice (P10). Following leptin injection, most (89%) tdTomato labeled ARH neurons displayed pSTAT3 immunoreactivity, indicating that the tdTomato reporter faithfully labels neurons expressing leptin receptors in neonatal mice, and that these neurons are capable of initiating downstream signaling (Figure 3a). To determine whether the AgRP neurons that project to the PVHmp and DMHv are differentially sensitive to leptin during development, the degree of colocalization between tdTomato and AgRP was measured in axonal projections labeled by immunofluorescence staining in P10 mice. Furthermore, this analysis was done in both males and females to determine whether a sexual dimorphism exists in the expression of leptin receptor by neurons projecting to either the PVHmp or DMHv. The data indicate that approximately 30% of AgRP immunoreactive fibers in the PVHmp contained the tdTomato label (i.e., are derived from leptin receptor expressing neurons) in both male and female mice on P10 (Figure 3b, d, f). In the DMHv, more than 20% of AgRP immunoreactive fibers colocalized tdTomato labeling in both males and females (Figure 3c, e, f). Therefore, functional $LepRb$ signaling appears to be intact in AgRP neurons of male and female neonates that project to either the PVHmp or DMHv.

3.3 Neurotrophic action of leptin treatment on AgRP projections to the PVHmp is sexually dimorphic

The density of AgRP projections to the PVHmp was significantly reduced in both male and female $Lep^{ob/ob}$ mice treated with vehicle, and remained low in male $Lep^{ob/ob}$ mice treated with leptin from P4-P14, confirming previous results (Bouyer & Simerly, 2013). In contrast, treatment of female $Lep^{ob/ob}$ mice with leptin during the same period was sufficient to restore the density of AgRP inputs to the PVHmp (Figure 4) indicating that this action of leptin is sexually dimorphic.

The timing of the critical period for AgRP outgrowth was also examined in female $Lep^{ob/ob}$ mice and WT littermates treated with either vehicle or leptin from P4–14, P16–24, and P25–33. Despite a shorter (9-day) treatment period, treatment of $Lep^{ob/ob}$ females from P16–24 was sufficient to significantly increase the density of AgRP containing fibers in both the PVHmp and DMHv compared to $Lep^{ob/ob}$ females treated with vehicle. However, treatment of $Lep^{ob/ob}$ females from P25–33 was not sufficient to restore AgRP innervation density in either the PVHmp or DMHv, suggesting that the capacity for leptin to promote growth of

ARH projections to hypothalamic targets is lost around P25-P28 in both males and females (Figure 4).

To determine whether the ability of leptin to rescue ARH projections to the PVHmp is due to a sexual dimorphism in the timing of the growth of AgRP projections, we quantified the density of AgRP containing fibers in the PVHmp of male and female WT or *Lep^{ob/ob}* mice during postnatal development. On P6, the density of AgRP containing axonal projections in the PVHmp was not different between sexes or genotypes, and no significant differences in the density of AgRP projections were found between male and female genotype-matched animals at any time point. However, by P24, the density of AgRP fibers was significantly lower in male *Lep^{ob/ob}* mice, when compared with that of WT males (Figure 3g). No statistically significant effect was found in the density of AgRP projections between male and female age- and genotype- matched samples, suggesting that there is not a sexual dimorphism in the timing of the growth of AgRP projections.

The sexually dimorphic response in the ability of leptin treatment to stimulate growth of AgRP projections to the PVHmp could also result from differential sensitivity to gonadal hormones. To determine whether males and females differentially express receptors for gonadal steroids in AgRP neurons, we used immunohistochemistry to examine the expression of the androgen receptor (AR) and estrogen receptor alpha (ER α) in the ARH on P10. Mice expressing GFP in NPY neurons were labeled immunohistochemically for AR and ER α to compare cellular patterns of hormone receptor expression in AgRP/NPY neurons of the ARH. NPY and AgRP have been demonstrated to be co-produced in 94% of NPY neurons in the ARH (Hahn et al., 1998), therefore, GFP was used as a proxy for AgRP neurons in these experiments. While a significant number of ARH neurons express AR or ER α on P10, only a very small percentage of NPY neurons appear to express either gonadal hormone receptor at this age (Figure 5).

4 DISCUSSION

The capacity for synaptic plasticity and the impact of environmental influences on brain architecture is not constant throughout life. The periods when the greatest changes occur are often limited to prenatal or early postnatal development. The ability of leptin to promote innervation of hypothalamic nuclei by AgRP neurons in the ARH appears to display such a restricted developmental sensitive period, because the severe deficits in AgRP projections apparent in *Lep^{ob/ob}* mice can be rescued with exogenous leptin treatment only in neonatal mice (Bouret et al., 2004; Bouyer & Simerly, 2013).

In the current study, we defined the closure of this critical neurodevelopmental period. The neurotrophic effect of leptin on growth of AgRP projections is quite robust in early postnatal life, extending into the fourth postnatal week, but does not extend beyond P28, after which exogenous leptin is no longer able to rescue innervation of the DMHv in leptin deficient mice. In addition to its temporal specificity, leptin does not appear to affect all ARH projections equally, nor does it function during development of AgRP projections identically in males and females. In females, exogenous leptin treatment is sufficient to rescue the density of AgRP projections to both the PVHmp and DMHv. However, in males, postnatal

leptin treatment rescues AgRP projections to the DMHv, but not to the PVHmp, suggesting that leptin facilitates growth of these projections in conjunction with another sex specific mechanism. Together, the data identify a developmental window of environmental sensitivity, in which ARH circuits are structurally specified, with lasting consequences for metabolic status that may be distinct for males and females (for summary, see Figure 6).

4.1 Timing and duration of a critical period for leptin-stimulated ARH outgrowth

Defining the timing of cellular responsiveness to signals that control formation of circuit architecture is essential in the context of obesity and metabolic disease, as it specifies windows of potential vulnerability, or opportunity for circuit manipulation. In the current study, we reveal a critical period of neural development in which leptin (and possibly other stimuli such as nutrition, or other hormones and environmental stimuli) can shape ARH connectivity and function. The use of the leptin deficient mouse allows us to model this manipulation and provides a foundation for elucidation of the mechanisms responsible for developmental plasticity of ARH projections, with implications for circuit programming relevant to childhood metabolic disease.

In the current study, we find that treatment of Lep^{ob/ob} mice with exogenous leptin for an 11-day period, as late as P16–26, restored the growth of AgRP projections to the DMHv. By contrast, leptin treatment from P28–38 was not sufficient to rescue the deficits in projections observed in Lep^{ob/ob} mice, suggesting that leptin loses its trophic action on AgRP neurons at approximately the fourth postnatal week. There was no discernable difference between early leptin treatments (P4–P14) and treatments during the third postnatal week (P16–P26). This finding implies that leptin sensitivity during development of AgRP projections to the DMHv remains relatively constant during the first four weeks of life. This observation is interesting in view of the rapid fall in leptin levels that occurs near the second to third postnatal week (Ahima et al., 1998), because it suggests that the brain remains responsive to the trophic action of leptin long after leptin levels fall from their peak, which occurs at approximately P8–12. Nevertheless, the ability of AgRP neurons to respond to the developmental actions of leptin extends for at least 1 week beyond the end of the naturally occurring postnatal surge in leptin secretion.

While leptin administration beyond the critical period is not sufficient to rescue large-scale axonal elaboration, synaptic inputs to ARH neurons can be influenced by leptin in adulthood. Acute leptin treatment in adult Lep^{ob/ob} mice reduces the ratio of excitatory to inhibitory synaptic inputs onto NPY neurons within 6 hr of treatment (Pinto et al., 2004). It remains to be determined whether these rapid plastic effects are under the control of activity dependent mechanisms, or leptin receptor-driven signaling cascades such as pSTAT3 or pERK, which have been shown to be responsible for the large-scale axonal outgrowth examined in the current study (Bouret, Bates, Chen, Myers, & Simerly, 2012). Nevertheless, it is clear that leptin functions not only to modulate synaptic density and neuronal excitability acutely, but also impacts the organization of ARH projections, which may

permanently alter how regulation of these neurons by leptin in adulthood affects other components of metabolic circuitry.

The postnatal critical period for the developmental effects of sex steroid hormones on several populations of hypothalamic neurons displays a temporal organization that is quite different from that of leptin. In rats, a single injection of testosterone during the first few days of life effects a dramatic masculinization of both brain structure and physiology, and the critical period for estrogen's action appears to be restricted largely to the first ten days of life (Arnold & Gorski, 1984; Dohler et al., 1984). However, steroid sensitivity and timing vary for different cell types. For example, treatment of female rats with sex steroid hormones for 24 hr induces caspase-dependent cell death in the anteroventral periventricular nucleus, but not if the hormone treatment is delayed until after the sixth postnatal day (Waters & Simerly, 2009), whereas full masculinization of serotonergic inputs to the medial preoptic nucleus requires prenatal exposure to testosterone (Simerly, Swanson, & Gorski, 1984). The postnatal surge in testosterone in males, known to be responsible for most aspects of sexual differentiation, occurs on the first day of life, so even in the case of sex steroids the capacity of neurons to respond to exogenous hormone treatment extends well beyond the time when hormone exposure normally occurs in vivo.

In contrast to the rapid actions of sex steroids on brain development, we did not detect acute effects of leptin on the growth of AgRP projections to the DMHv in male mice. There also appears to be a minimum duration of leptin exposure required to achieve robust target innervation. While leptin treatment for 11 days, from P4–14 or P16–26, was sufficient to cause significant increases in growth of AgRP projections, leptin treatment for 5 days was ineffective. However, fiber densities in the DMHv following 5 days of leptin treatment tended to be intermediate between those observed for *Lep^{ob/ob}* mice treated with leptin for 11 days, and vehicle treated controls. Thus, the length of leptin exposure appears to be positively correlated with the density of AgRP projections. This finding is consistent with the temporal dynamics of signaling cascades mediating leptin-induced growth of ARH projections. In the mature brain, the leptin receptor signals through three known signal transduction cascades including the STAT3, MAP kinase/ERK, and AKT/PI3 pathways (Bates et al., 2003; Bjorbaek et al., 2001; Björnholm et al., 2007; Goetze et al., 2002; Hill et al., 2008). During development, pSTAT3 and pERK mediated signaling is required for normal growth of AgRP projections (Bouret, et al. 2012). The kinetics of STAT3 and MEK/ERK activation occur on rapid timescales, with signals peaking and returning to basal levels within a few hours (El-Haschimi, Pierroz, Hileman, BjØrbæk, & Flier, 2000; Mirshamsi et al., 2004). Thus, our results are consistent with the notion that these signaling pathways need continued activation in order to couple effectively with growth promoting pathways affecting axon targeting of AgRP neurons.

The timing of the closure of the critical period for leptin dependent stimulation of axonal outgrowth from AgRP neurons is coincident with other indicators of their maturation, including establishment of mature electrophysiological properties of AgRP neurons, and the ability of leptin to inhibit food intake. In mature AgRP neurons, leptin signaling induces membrane hyperpolarization (Spanswick, Smith, Groppi, Logan, & Ashford, 1997; Takahashi & Cone, 2005; van den Top, Lee, Whyment, Blanks, & Spanswick, 2004). By

contrast, during hypothalamic development, leptin appears to activate AgRP neurons, inducing membrane depolarization (Baquero et al., 2014). The transition from leptin induced depolarization to hyperpolarization as AgRP neurons mature is complete by P30, commensurate with an increase in the expression of K_{ATP} channels, which is known to facilitate membrane hyperpolarization and suppression of activity (Baquero et al., 2014). In addition, leptin itself has no impact on food intake until P28 (Mistry, Swick, & Romsos, 1999), despite the fact that the PVH, DMH, and LHA receive ample innervation from the ARH well before this time (Bouret, 2004). Taken together, these findings suggest that AgRP projections reach functional maturity at approximately 4 weeks of age, when they become refractive to the developmental actions of leptin and achieve adult electrophysiological properties.

4.2 Sexually dimorphic target dependence of leptin action

In our experiments, leptin administration is limited to discrete postnatal periods, but it should be noted that following the postnatal treatments, $Lep^{ob/ob}$ mice are completely leptin deficient. Despite the absence of circulating leptin, adult $Lep^{ob/ob}$ mice that were treated with leptin postnatally display dense AgRP projections to the DMHv, indicating that sustained exposure to leptin is not required for maintenance of this pathway. Although leptin is required for normal development of AgRP projections to the PVHmp in male $Lep^{ob/ob}$ mice, these projections are not rescued by postnatal leptin treatments alone, raising the possibility that these projections may require sustained circulating leptin in order to be maintained. The apparent transient expression of LRb in AgRP projections to the PVH argues against this hypothesis, but may represent a possible mechanism for limiting the developmental actions of leptin to the perinatal period (Betley et al., 2013). In contrast to males, AgRP projections to the PVHmp in female $Lep^{ob/ob}$ mice are rescued by postnatal leptin treatment alone. Thus, the developmental actions of leptin during the postnatal critical period display not only target specificity, but there also appear to be sex-dependent mechanisms that impact the trophic actions of leptin to induce ARH growth and connectivity.

Interestingly, no sexual differences were detected in expression of receptors for either leptin or gonadal steroid hormones in AgRP neurons. Furthermore, the growth of AgRP neurons to the PVH of WT males and females display similar developmental timelines. Although, the mechanisms underlying the sex specific effects of leptin on development of AgRP projections remain unknown, they may reflect changes in responses of ARH neurons caused by prior exposure to steroid hormones. The ARH exhibits a sexually dimorphic glial morphology as early as P1 (Mong & McCarthy, 2002), and the number of neuropeptide Y expressing neurons in the caudal ARH is determined by postnatal testosterone exposure (Urban, Bauer-Dantoin, & Levine, 1993). Furthermore, ARH neurons exhibit sexually dimorphic patterns of synaptic inputs (Matsumoto & Arai, 1980). Perhaps these reported sex differences in ARH structure impact the ability of leptin to rescue AgRP projections to the PVHmp. The possibility that sex differences in synaptic and neuronal-glial architecture within the ARH may impact the neurotrophic action of leptin should be explored.

In summary, the results presented here demonstrate that the ability of leptin to stimulate growth of ARH axons to their targets is restricted to a discrete time period that extends until postnatal day 28 in the mouse. Furthermore, the data indicate that the density of AgRP containing projections to the PVHmp is permanently specified by postnatal leptin in females, but requires an additional sex-specific factor in males. While age correlations between mice and humans are only general approximations, these data suggest that the corresponding sensitive period for leptin-mediated ARH growth in humans may extend into early postnatal life, underscoring the importance of proper nutrition and hormonal balance during this important period of human hypothalamic development.

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FIGURE 1.

The trophic effect of leptin on AgRP axonal growth is restricted to the first 4 postnatal weeks. On P60, the density of AgRP projections to the DMHv was measured in male WT and Lep^{ob/ob} mice treated with either vehicle or leptin during one of several discrete time periods in early postnatal life. Representative images of AgRP innervation of the DMH in (a) WT vehicle injected, (b) Lep^{ob/ob} vehicle injected, and (c) Lep^{ob/ob} leptin treated mice in the treatment group spanning from P4–14. Dashed lines indicate the anatomical borders of the DMH. d, dorsal; c, central; v, ventral subregion of the DMH. 3V denotes the third ventricle. Scale bar, 70 μm. Square indicates the ROI in the DMHv used for quantification further illustrated in d-f using a high-magnification 63X oil corrected lens. Scale bar, 13 μm. (g) Quantification of AgRP axonal fiber density in the DMHv for all treatment groups revealed that after postnatal day 28, leptin no longer stimulates axonal growth from AgRP neurons. OB, Lep^{ob/ob}; Veh, Vehicle; Lep, Leptin. (WT Veh, $n = 32$, OB Veh, $n = 22$, OB Lep P4–8, $n = 5$, OB Lep P6–10, $n = 4$, OB Lep P8–12, $n = 3$, OB Lep P12–16, $n = 4$, OB Lep P4–14, $n = 4$, OB Lep P16–26, $n = 5$, OB Lep P28–38, $n = 4$) Data are expressed as mean $\pm$ SEM of the density of AgRP-containing axonal fibers within the DMHv in a set volume. Significance between groups was determined by one-way ANOVA and Holm-Sidak's multiple comparisons test; * indicates $p < .0001$ compared to WT vehicle, ^ indicates $p < .05$ compared to Lep^{ob/ob} vehicle

FIGURE 2.

Leptin treatment is not sufficient to restore AgRP innervation to the PVHmp in leptin-deficient males. Representative images of AgRP innervation of the PVH in (a) WT mice injected with vehicle, (b) Lep^{ob/ob} mice injected with vehicle, and (c) Lep^{ob/ob} mice treated with leptin from P4–14. Dashed lines indicate the anatomical borders of the PVH. PVHmp, medial parvicellular part of the PVH. 3V, third ventricle. Scale bar, 40 μm. Square indicates the ROI in the PVHmp used for quantification, further illustrated in (d–f). Scale bar, 13 μm. (g) Quantification of AgRP axonal fiber density in the PVHmp for all treatment groups. OB, Lep^{ob/ob}; Veh, Vehicle; Lep, Leptin. (WT Veh, $n = 32$, OB Veh, $n = 31$, Lep P4–8, $n = 5$, OB Lep P6–10, $n = 4$, OB Lep P8–12, $n = 3$, OB Lep P12–16, $n = 4$, OB Lep P4–14, $n = 4$, OB Lep P16–26, $n = 5$, OB Lep P28–38, $n = 4$) Data are expressed as mean $\pm$ SEM of the density of AgRP-containing axonal fibers within each target region in a set volume. Significance between groups was determined by one-way ANOVA and Holm-Sidak multiple comparisons test; * indicates $p < .0001$ compared to WT vehicle

FIGURE 3.

AgRP neurons in the ARH that project to the PVHmp and DMHv are leptin sensitive during early postnatal life. (a) Representative image of leptin-induced pSTAT3 (green) colocalized to tdTomato expressing neurons (magenta) in the ARH of a LRb^{cre}tdTomato animal on P10, 1 hr following leptin injection. 3V, third ventricle. Dotted lines illustrate the boundaries of the nucleus. Scale bar, 40 μm. (b, c) Immunohistochemical labeling of AgRP projections (green) targeting the PVH (b), and DMH (c) in LRb^{cre}tdTomato mice. Squares indicate the ROI in the PVHmp and DMHv quantified and illustrated in (d) and (e). Representative high-magnification images of AgRP immunoreactivity (green) in the PVHmpd (d) and DMHv (e) colocalized to axonal projections from leptin receptor expressing neurons (magenta). Scale bar, 13 μm. (f) Quantification of the percentage of AgRP immunoreactivity (ir) colocalized to tdTomato labeled fibers in male and female LRb^{cre}tdTomato mice on P10 (male, $n = 3$; female, $n = 3$). (g) The density of AgRP projections to the PVHmp measured in male and female, WT and Lep^{ob/ob} mice on P6, P10, P16, and P24 are graphed. Data are expressed as mean $\pm$ SEM of the density of AgRP-containing axonal fibers within the PVHmp in a defined volume. Significance between groups was determined by one-way ANOVA and Holm-Sidek multiple comparisons test performed at each age; * indicates $p < .01$ compared to WT Male. Data are expressed as mean $\pm$ SEM

FIGURE 4.

Postnatal leptin treatment is sufficient to restore AgRP innervation to the PVHmp in leptin-deficient females. Representative images of the PVH in (a) WT vehicle treated, (b) *Lep^{ob/ob}* vehicle treated, and (c) *Lep^{ob/ob}* leptin treated female mice treated from P4–14. Dashed lines indicate the anatomical borders of the PVH. 3V denotes the third ventricle. Scale bar, 40 μm . Square indicates the ROI in the PVHmpd used for quantification, further illustrated in (d–f). Scale bar, 13 μm . (g) Quantification of AgRP axonal fiber density in the PVHmpd and DMHv in WT and *Lep^{ob/ob}* females treated with vehicle or leptin. OB, *Lep^{ob/ob}*; Veh, Vehicle; Lep, Leptin. (WT Veh, $n = 18$, OB Veh, $n = 19$, OB Lep P4–14, $n = 4$, OB Lep Leptin P16–24, $n = 4$, OB Lep P25–33, $n = 5$) Data are expressed as mean SEM of the density of AgRP-containing axonal fibers within each target region in a set volume. Significance between groups was determined by one-way ANOVA and Holm-Sidak multiple comparisons test; * indicates $p < .0001$ compared to WT vehicle, ^ indicates $p < .05$ compared to *Lep^{ob/ob}* vehicle

FIGURE 5.

AgRP neurons do not express androgen receptor or estrogen receptor alpha during early postnatal development.

Representative images of estrogen receptor alpha (a and c; ERα; magenta) or androgen receptor (b and d; AR; magenta) immunoreactivity in the ARH of male (a and b) and female (c and d) *NPY^{GFP}* animals on P10. Square in (a) denotes the location of insets. 3V, third ventricle

FIGURE 6.

Schematic model of the early postnatal trophic effects of leptin on development of AgRP neuronal projections. (a) The timing of the naturally occurring early postnatal surge in serum leptin, known to stimulate the growth of ARH projections, is graphed. Treatment of *Lep^{ob/ob}* mice with exogenous leptin during several discrete time periods enabled identification of the closure of a critical period for growth of ARH AgRP projections (Bars below graph indicate each leptin treatment period. Green bars indicate treatment periods that rescued innervation and red bars indicate treatment periods that were not effective). Short duration, 5-day, leptin treatment periods from P4–8, P6–10, P8–12, or P12–16 were not sufficient to rescue AgRP innervation density to the DMHv in *Lep^{ob/ob}* mice. By contrast, longer duration, 11-day, leptin treatment periods from P4–14 and P16–26 were able to rescue innervation deficits in the DMHv of *Lep^{ob/ob}* mice. However, leptin treatment of *Lep^{ob/ob}* mice from P28–38 was no longer effective, suggesting that the critical period for the trophic actions of leptin is closed at this time. (b) Early postnatal leptin treatment also revealed striking sexually dimorphic, target-dependent effects on innervation of the PVHmp. While treatment of *Lep^{ob/ob}* mice with leptin (for 9 or 11 days during the critical period) was sufficient to rescue deficits in projections to the DMHv in both males and females, deficits in innervation to the PVHmp were only rescued in female *Lep^{ob/ob}* mice treated with leptin.

Table 1

Primary antibodies

Antibody	Immunogen	Manufacturer, Catalog #, RRID, Host Species, Monoclonal/Polyclonal	Concentration used
1. Agouti Related Peptide (AgRP)	Synthetic peptide sequence N'-TRSCP _N MATGRCYCFANFFR-CACPDCCPVQQGSEHLRVCRSS-C'	Phoenix Pharmaceuticals, H-003-53, AB_2313908, Rabbit Polyclonal	1:2,500
2. Androgen Receptor (AR)	KLH-conjugated linear peptide corresponding to the N-terminus of rat Androgen Receptor (MEVQLGLGRVYPRPPSKTYRGC)	EMD Millipore, 06-680, AB_310214, Rabbit Polyclonal	1:1,000
3. Estrogen Receptor alpha (ER α)	KLH-conjugated linear peptide corresponding to the C-terminus of rat Estrogen Receptor alpha (TYYIPPEAEGFPNTI)	EMD Millipore, 06-935, AB_310305, Rabbit Polyclonal	1:10,000
4. Human HuC/HuD neuronal protein (HuC/D)	Human HuC/HuD neuronal protein	Molecular Probes, A21271, AB_221448, Mouse Monoclonal	1:500
5. phospho-Signal Transducer and Activator of Transcription 3 (pSTAT3)	Synthetic phosphopeptide corresponding to residues surrounding Tyr705 of mouse Stat3 (ADPGSAAPyLKTKFIC)	Cell Signaling Technology, 9131L, AB_331587, Rabbit Polyclonal	1:1,000