Characterization of the onset of leptin effects on the regulation of energy balance

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Abstract

Leptin is a hormone required for the regulation of body weight in adult animals. However, during the postnatal period, leptin is mostly involved in developmental processes. Because the precise moment at which leptin starts to exert its metabolic effects is not well characterized, our objective was to identify the approximate onset of leptin effects on the regulation of energy balance. We observed that male Lep⁰/₀ mice started to exhibit increased body fat mass from postnatal day 13 (P13), whereas in females, the increase in adiposity began on P20. Daily leptin injections from P10 to P22 did not reduce the weight gain of WT mice. However, an acute leptin injection induced an anorexigenic response in 10-day-old C57BL/6 mice but not in 7-day-old mice. An age-dependent increase in the number of leptin receptor-expressing neurons and leptin-induced pSTAT3 cells was observed in the hypothalamus of P7, P10 and P16 mice. Leptin deficiency started to modulate the hypothalamic expression of transcripts involved in the regulation of metabolism between P7 and P12. Additionally, fasting-induced hypothalamic responses were prevented by leptin replacement in 10-day-old mice. Finally, 12-day-old males and females showed similar developmental timing of axonal projections of arcuate nucleus neurons in both WT and Lep⁰/₀ mice. In summary, we provided a detailed characterization of the onset of leptin’s effects on the regulation of energy balance. These findings contribute to the understanding of leptin functions during development.

Introduction

The adipocyte-derived hormone leptin has fundamental importance on the regulation of body weight and feeding. In this respect, the brain uses circulating leptin levels as a major signal to indicate the balance between energy intake and expenditure (Ramos-Lobo & Donato 2017). Thus, genetic mutations that cause the absence of leptin or the leptin receptor (LepR) produce metabolic changes that resemble those observed during intense negative energy balance, including hunger and suppression of physiological processes that expend energy (Ahima et al. 1996). Consequently, mice deficient in leptin (Lep⁰/₀) or LepR (LepR⁰/₀) become morbidly obese in adulthood due to persistent hyperphagia and changes in energy metabolism. Leptin treatment in both Lep⁰/₀ mice and WT animals leads to reductions in food intake and body weight (Campfield et al. 1995, Halaas et al. 1995, Pelleymounter et al. 1995).
The powerful effects of leptin in energy homeostasis are mostly dependent on its central actions (de Luca et al. 2005, Ring & Zelser 2010). LepR expression was identified in several brain areas (Caron et al. 2010, Donato et al. 2010, da Silva et al. 2014), and numerous studies have investigated the importance of leptin signaling in different neural populations (Ramos-Lobo & Donato 2017). For example, leptin action on neurons that express proopiomelanocortin (POMC), agouti-related peptide (AgRP) or neuronal nitric oxide synthase regulates different aspects of metabolism (Ramos-Lobo & Donato 2017). These neural populations are distributed in several hypothalamic areas, including the arcuate nucleus (ARH) and ventral premammillary nucleus (PMv). Thus, leptin can influence a complex neural network to produce metabolic, behavioral and neuroendocrine changes.

Although the physiological effects of leptin have been widely investigated in adult animals, the precise role of leptin signaling during early life is less studied. Previous studies have shown that leptin action is required for the formation of the axonal projections that extend from ARH neurons to important second-order neurons, such as the paraventricular nucleus of the hypothalamus (PVH) (Bouret et al. 2004a,b). Besides this trophic effect, leptin signaling in early life also regulates the onset of puberty (Ahima et al. 1997, Chehab et al. 1997), brain mass and the expression of synaptic and glial proteins (Ahima et al. 1999, Ramos-Lobo et al. 2019). In accordance with the capacity of leptin to affect developmental processes and to induce the phosphorylation of the signal transducer and activator of transcription 3 (pSTAT3), brain Lepr mRNA and leptin-induced pSTAT3 expressions, respectively, are already present in suckling mice (Caron et al. 2010). Furthermore, leptin can induce electrochemical effects on ARH neurons in 2-week-old mice (Baquero et al. 2014). Taken together, these results show that leptin's action in the brain starts in early postnatal life. Nevertheless, daily leptin injections do not change the body weight gain level in 7- to 10-day-old mice. In addition, the food intake of 17-day-old WT or Lepob/ob pups is not affected by an acute intracerebroventricular leptin injection (Mistry et al. 1999). These findings suggest that leptin can regulate developmental processes in suckling animals, but the capacity of leptin to affect feeding and body weight may begin later in life. Notably, to our knowledge, a detailed temporal analysis of the onset of leptin effects on the regulation of energy balance has not yet been described. Therefore, the objective of the present study was to investigate at what point in postnatal life leptin begins its effects on feeding, body weight and in the regulation of classical neurocircuitry that regulate metabolism.

Materials and methods

Mice

All experiments were carried out in accordance with and approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo (Protocol number: 79/2015). Mice were bred and maintained in standard conditions of light (12 h light: 12 h darkness cycle) and received a regular rodent chow (2.99 kcal/g; 9.4% calories from fat). In the experiments, we used C57BL/6 WT mice, Lepob/ob mice (Stock No: 000632; The Jackson Laboratory, Bar Harbor, ME) and LepR-reporter mice, which was produced by breeding the LepR-Cre mouse (Stock No: 008320; The Jackson Laboratory) with the Cre-inducible tdTomato-reporter mouse (Stock No: 007909, The Jackson Laboratory).

Evaluation of body composition changes during development

To determine the precise moment when the absence of leptin starts to affect energy homeostasis, body weight, fat mass and lean body mass were monitored three times per week from birth until 6 weeks of life in male and female Lepob/ob mice and their lean littermates. Body composition was measured by time-domain NMR using the LF50 body composition mice analyzer (Bruker, Mannheim, Germany).

Evaluation of the acute and chronic effects of exogenous leptin on food intake, body weight and vaginal opening

To determine the acute effects of exogenous leptin on the food intake of suckling mice, we used a protocol published previously (Mistry et al. 1999, Buonfiglio et al. 2015, 2016, Ramos-Lobo et al. 2018). Briefly, 7- and 10-day-old C57BL/6 mice were initially separated from their mothers for 4 h. After that, the pups were weighed and received a s.c. injection of leptin (10 µg/g of body weight, from Dr AF Parlow, National Hormone and Peptide Program, Torrance, CA) or an equal volume of PBS. Then, the pups were allowed to lactate and their body weights were recorded 2 and 5 h later. To evaluate the chronic effects of leptin, 10- and 45-day-old C57BL/6 mice received daily injections of either PBS or leptin (1 µg/g) for 12 days. Their body weights were recorded daily. In females, we also determined the age of vaginal opening, as previously described (Ahima et al. 1997, Chehab et al. 1997, Donato et al. 2011, Bohlen et al. 2016).
Detection of leptin-responsive cells in the brain during development

A LepR-reporter mouse model (da Silva et al. 2014, Nagaishi et al. 2014) was used to allow the visualization of LepR-expressing neurons in different hypothalamic nuclei in mice at 7 (n = 5), 10 (n = 3) and 16 (n = 2) days of life. To identify leptin-responsive neurons, 7- (n = 3), 10- (n = 2) and 16-day-old (n = 2) C57BL/6 WT mice fed ad libitum received an acute s.c. injection of mouse recombinant leptin (10 µg/g body weight). After 90 min, mice were perfused and their brains processed to detect the immunoreactivity to pSTAT3 as a marker of leptin-responsive cells (Scott et al. 2009, Caron et al. 2010), using a protocol previously described (Campos et al. 2020). For the whole-cell patch-clamp recordings, 8- to 16-day-old LepR-reporter male mice (n = 7) were anesthetized, decapitated and their brains were immediately processed, as previously described (Silveira et al. 2017). In current-clamp mode, neurons were recorded under zero current injection (I = 0). The resting membrane potential (RMP) of LepR-expressing neurons in the ARH and PMv was monitored for at least 5 min (basal), followed by the addition of 100 nM mouse recombinant leptin to the bath for approximately 5 min. The RMP values were compensated to account for the junction potential (~8 mV).

Hypothalamic gene expression analysis in Lep<sup>ob/ob</sup> and fasted WT mice

In this study, 7- or 12-day-old male and female Lep<sup>ob/ob</sup> mice and lean littermates fed ad libitum were decapitated, and the entire hypothalamus was collected. To evaluate the effects of fasting and leptin replacement, 10-day-old C57BL/6 mice were subjected to an overnight fast (16 h). At the moment of separation from the dams, the pups received a s.c. injection of either PBS or leptin (10 µg/g of body weight). The following morning, the pups received a second injection of PBS or leptin and were sacrificed 3 h later. A group of 10-day-old C57BL/6 mice fed ad libitum also received PBS injections at the same time and were used as controls. The hypothalamus was collected to determine the gene expression. Total RNA was extracted with TRIzol (Invitrogen). Real-time PCR was performed using the 7500TM Real-Time PCR System (Applied Biosystems), Power SYBR Green Gene Expression PCR Master Mix (Applied Biosystems) and specific primers for target genes (Table 1). Data were normalized to the geometric average of Actb, Gapdh and Ppia.

Analysis of innervation of ARH neurons

To determine possible sexually dimorphic differences in the developmental timing of the innervation of ARH neurons, 12-day-old male (n = 5) and female (n = 7) C57BL/6 mice and male (n = 5) and female (n = 5)Lep<sup>ob/ob</sup> mice were transcardially perfused with saline, followed by 4% formaldehyde fixative solution. Subsequently, brains were cut in 40-µm thick sections using a freezing microtome, and the brain sections were subjected to immunofluorescence staining in order to evaluate the integrated optical density (IOD) of POMC and AgRP fibers in the PVH, as previously described (Ramos-Lobo et al. 2018, 2019). A Zeiss Axiomager A1 microscope (Zeiss, Munich, Germany) was used to obtain the epifluorescence photomicrographs. Then, the ImageJ software (http://rsb.info.nih.gov/ij) was used to determine the IOD in the PVH that was subtracted by the IOD assessed in adjacent areas

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences.</th>
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Statistical analysis

Changes in body weight and composition along time and the effects of leptin on food intake were analyzed by repeated-measures two-way ANOVA and the contrasts were identified by Sidak’s multiple comparisons test. Comparisons between two groups were performed using Student’s t-test, and for analysis between more than two groups, we used one-way ANOVA followed by Newman–Keuls multiple comparison post-hoc test. Possible outliers were checked by the ROUT method and removed from the analysis. All results were expressed as mean ± S.E.M. Statistical analyses were performed using GraphPad Prism software (GraphPad), considering \( P < 0.05 \) as statistically significant.

Results

Absence of leptin induces obesity in mice between the second and third week of life

To determine the onset of leptin effects on the regulation of energy balance, the body weight, fat mass and lean body mass were determined from birth to 6 weeks of age in male and female \( \text{Lep}^{ob/ob} \) mice and their lean littermates (Fig. 1). Interestingly, we did not find significant changes in body weight between WT and \( \text{Lep}^{ob/ob} \) mice before the sixth week of life in males (Fig. 1A) or females (Fig. 1B). After this period, \( \text{Lep}^{ob/ob} \) mice exhibited a well-established increase in body weight (data not shown). However, the body composition analysis revealed that male \( \text{Lep}^{ob/ob} \) mice exhibited increased fat mass from P13 (interaction (F\(_{17, 442} = 11.03, P < 0.0001\)); Fig. 1C), whereas female \( \text{Lep}^{ob/ob} \) mice showed higher fat mass from P20 in comparison with WT littermates (interaction (F\(_{17, 559} = 22.53, P < 0.0001\)); Fig. 1D). Conversely, \( \text{Lep}^{ob/ob} \) mice presented reduced lean body mass from P20, compared to WT littermates, in both males (interaction (F\(_{16, 394} = 15.33, P < 0.0001\)); Fig. 1E) and females (interaction (F\(_{17, 636} = 5.781, P < 0.0001\)); Fig. 1F). Thus, the absence of endogenous leptin induces obesity in mice from the second week of life.

Chronic leptin injections in suckling mice do not reduce weight gain

To determine whether bodyweight of suckling mice is affected by exogenous leptin treatment, 10-day-old CS7BL/6 mice received daily injections of PBS or leptin for 12 consecutive days. We observed that chronic leptin treatment did not affect the weight gain in males (F\(_{1, 18} = 0.0046, P = 0.9466\)) or females (F\(_{1, 18} = 0.0098, P = 0.922\)), compared to PBS-treated mice (Fig. 2A and B). For comparison, a group of young adult (45-days old) mice also received leptin treatment for the same period of time, and in this case, leptin caused a sustained suppression in weight gain (F\(_{1, 12} = 10.42, P = 0.0072\)), compared to PBS-injected animals (Fig. 2C). Thus, exogenous leptin treatment is unable to affect the bodyweight of mice before the fourth week of life. Notably, although leptin treatment between P10 and P22 did not affect body weight, leptin-treated females presented earlier vaginal opening, compared to PBS-injected mice (t\(_{18} = 1.834, P = 0.0416\); Fig. 2D).

An acute leptin injection induces an anorexigenic response in 10-day-old mice but not in 7-day-old mice

Next, the acute anorexigenic effects of leptin were determined in 7- (P7) and 10-day-old (P10) CS7BL/6 mice (Fig. 3). Since mice are still suckling at this age, the effects of leptin on feeding were indirectly evaluated through the weight gain in pups that had been separated from...
their mothers for 4 h before the test. Compared to PBS-injected pups, an acute leptin injection did not affect the weight gain of P7 mice ($F(1, 9) = 0.0001, P = 0.9975$; Fig. 3A). However, leptin reduced the weight gain in P10 mice ($F(1, 12) = 4.783, P = 0.0493$; Fig. 3B), suggesting that the acute anorexigenic effect of leptin is already present in P10 mice but not earlier.

Temporal characterization of the LepR expression and leptin-induced pSTAT3 in the hypothalamus of young mice

Given the critical role played by hypothalamic neurons in mediating the effects of leptin on energy homeostasis (de Luca et al. 2005, Ring & Zeltser 2010), we now performed a temporal characterization of the LepR expression and leptin-induced pSTAT3 in the hypothalamus of mice at 7 (P7), 10 (P10) and 16 (P16) days of life. Using a LepR-reporter mouse, we observed that a significant number of LepR-expressing neurons are already present in the medial preoptic area (MPA), ARH and PMv of P7 mice, whereas few cells could be observed in the dorsomedial nucleus of the hypothalamus (DMH) and lateral hypothalamic area (LHA; Fig. 4). In P10 mice, a modest increase in the number of LepR-expressing neurons was observed in the MPA and DMH (Fig. 4 and Table 2). The number of LepR-expressing neurons further increased in the ARH, ventromedial nucleus of the hypothalamus (VMH), DMH and LHA of P16 mice. To demonstrate that the activation of LepR is able to recruit the STAT3 transcription factor, C57BL/6 mice received a leptin injection before perfusion. Leptin induced pSTAT3 only in the MPA, ARH and PMv of P7 mice (Fig. 5). By P10, increased responsiveness to leptin was observed in the MPA and even more in the DMH and LHA (Fig. 5 and Table 2). Furthermore, increased numbers of leptin-induced pSTAT3 cells were observed in all hypothalamic nuclei of P16 mice. Remarkably, the VMH had not shown responsiveness to leptin at younger ages (Fig. 5 and Table 2). To further demonstrate that LepR is functional in sucking mice, electrophysiological experiments were performed in brain slices of 8- to 16-day-old LepR-reporter mice. LepR-expressing neurons in the ARH and PMv were recorded before and during leptin administration (Fig. 5M).
We observed that three out of eight cells in the ARH (38%) and six out of eight cells in the PMv (75%) exhibited significant changes in the RMP after leptin administration (Fig. 5N).

**Temporal characterization in the hypothalamic expression of several transcripts involved in the regulation of energy balance in postnatal Lep$^{ob/ob}$ mice**

To determine whether endogenous leptin is able to regulate the hypothalamic expression of transcripts involved in the regulation of energy balance in young mice, we analyzed the hypothalamic gene expression of 7- and 12-day-old male and female Lep$^{ob/ob}$ mice (Fig. 6). In P7 Lep$^{ob/ob}$ male mice, only a reduction in hypothalamic Crh ($t_{(7)}=2.592$, $P=0.358$) mRNA levels was observed, as compared to WT littermates (Fig. 6A). No significant changes were observed in the hypothalamic mRNA levels of Agrp, Npy, Pomc, Cartpt, Hcrt, Pmch, Trh, Oxt, Kiss1, Nos1, Lepr or Socs3 (Fig. 6A). In P7 female mice, we observed an increase in Agrp ($t_{(5)}=2.652$, $P=0.0453$) mRNA levels in the hypothalamus of Lep$^{ob/ob}$ mice, whereas Hcrt ($t_{(4)}=3.312$, $P=0.0212$) and Crh ($t_{(4)}=2.94$, $P=0.0424$) mRNA levels decreased, as compared to WT littermates (Fig. 6A). In P12 male mice, the absence of leptin action induced an upregulation in the expression of Agrp ($t_{(13)}=3.457$, $P=0.0043$), Npy ($t_{(13)}=4.798$, $P=0.0003$), Pomc ($t_{(11)}=2.204$, $P=0.0497$), Kiss1 ($t_{(13)}=2.059$, $P=0.0289$) and Lepr ($t_{(13)}=4.767$, $P=0.0004$) mRNA levels in the hypothalamus, whereas Cartpt ($t_{(13)}=2.174$, $P=0.0487$), Hcrt ($t_{(13)}=5.128$, $P=0.0002$), Pmch ($t_{(13)}=4.013$, $P=0.0015$), Nos1 ($t_{(13)}=2.675$, $P=0.0191$) and Socs3 ($t_{(13)}=2.326$, $P=0.0368$) expression reduced in Lep$^{ob/ob}$ male mice.
Table 2  Temporal characterization of the leptin receptor (LepR) expression and leptin-induced pSTAT3 in the hypothalamus of mice at 7 (P7), 10 (P10) and 16 (P16) days of life.

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Classification: –, very low expression or virtually absent; +, low density of cells covering only parts of the nucleus; ++, moderate density of cells covering only parts of the nucleus; ++++, high density of cells covering most of the nucleus; +++, high density of cells covering the whole extension of the nucleus.

ARH, arcuate nucleus; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; MPA, medial preoptic nucleus; PMv, ventral premammillary nucleus; VMH, ventromedial nucleus of the hypothalamus.

compared to WT littermates (Fig. 6B). The hypothalamic gene expression was also analyzed in P12 females. As seen in males, the absence of leptin increased the hypothalamic expression of Agrp (t_{10}=4.109, P=0.0026), Npy (t_{10}=2.856, P=0.0189) and Kiss1 (t_{10}=2.784, P=0.0213) mRNA levels. However, in contrast to the results found in males, P12 Lep^{ob/ob} females exhibited increased hypothalamic Cartpt (t_{10}=3.751, P=0.0045), Trh (t_{10}=5.369, P=0.0005) and Nos1 (t_{10}=5.27, P=0.0005) mRNA levels, compared to WT mice (Fig. 6B).

Leptin treatment prevents fasting-induced changes in the expression of hypothalamic transcripts involved in energy-balance regulation

To determine whether suckling mice are responsive to fasting-induced hypothalamic changes and if leptin replacement can prevent these adaptations, the hypothalamic gene expression was analyzed in fed, fasted or leptin-treated fasted 10-day-old C57BL/6 mice (Fig. 7). Fasted 10-day-old mice exhibited increased expression of Agrp (F_{2, 28}=5.205, P=0.012), Npy (F_{2, 30}=3.973, P=0.0295) and Hcrt (F_{2, 30}=8.857, P=0.0005) mRNA, compared to fed mice, whereas Kiss1 (F_{2, 27}=11.29, P=0.0003) and Nos1 (F_{2, 19}=4.177, P=0.0314) mRNA levels suppressed by fasting (Fig. 7). Notably, leptin replacement prevented the fasting-induced increase in Agrp, Npy and Hcrt expression (Fig. 7). In contrast, leptin treatment in fasted mice did not restore Kiss1 and Nos1 mRNA levels (Fig. 7). We also observed increased expression of Crh (F_{2, 30}=11.9, P=0.0002) and Socs3 (F_{2, 14}=14.02, P=0.0005) only in fasted+leptin mice (Fig. 7). Neither fasting nor leptin treatment affected the hypothalamic expression of Pomc, Cartpt, Pmch, Trh, Oxt, and Lepr mRNA in 10-day-old mice (Fig. 7).

Male and female show similar developmental timing of ARH neuronal projections

To determine whether possible differences in the developmental timing of the innervation of ARH neurons could explain the sexually dimorphic differences in the timing of obesity in Lep^{ob/ob} mice, we compared the innervation of ARH neurons to the PVH between 12-day-old male and female WT or Lep^{ob/ob} mice (Fig. 8). We found that 12-day-old WT males and females showed similar density of AgRP fibers in the PVH (t_{10}=0.94, P=0.3717) (Fig. 8A, B and C). Regarding POMC innervation, we also observed an equivalent density of POMC fibers in the PVH between male and female WT mice (t_{10}=1.876, P=0.0902) (Fig. 8D, E and F). Likewise, 12-day-old Lep^{ob/ob} male and female mice showed similar density of AgRP (t_{10}=0.8373, P=0.4267) (Fig. 8G, H and I) and POMC (t_{10}=1.865, P=0.1044) (Fig. 8J, K and L) fibers in the PVH.

Discussion

The well-established importance of leptin on the regulation of body weight is based on studies in adult animals, whereas, in early life, leptin is mostly involved in developmental processes (Ahima et al. 1999, Bouret et al. 2004a,b, Ramos-Lobo et al. 2019). Remarkably, a detailed temporal characterization of the onset of leptin effects on the regulation of energy balance is not available. Data in humans indicate that the absence of leptin action does not affect birth weight but leads to severe obesity that starts at an average age of 4.1 months (Huang et al. 2015). In mice, some studies have shown that 17- or 18-day-old Lep^{ob/ob} mice already exhibited changes in bodyweight, characterizing the onset of obesity, although these studies did not evaluate males and females separately (Dubuc 1976, Mistry et al. 1999). However, another study did not find statistically significant changes in bodyweight of 3.5-week-old Lep^{ob/ob} mice, although the analysis of body fat indicated higher adiposity (Traquair & Fuller 1980). This study and our findings highlight the importance of using body adiposity, instead of bodyweight, as the main readout to detect metabolic changes in Lep^{ob/ob} mice during development. In this regard, changes in body weight were detected in Lep^{ob/ob} mice only after 6 weeks of age, whereas
statistically significant changes in body adiposity were observed between the second and third week of age. The greater adiposity of *Lep*<sup>ob/ob</sup> mice did not lead to significant changes in bodyweight because they were offset by a lower gain in lean mass during that period. The causes of the lower gain in lean mass in *Lep*<sup>ob/ob</sup> mice are unknown but may be related to a blunted or delayed onset of the somatotropic axis. Accordingly, while lean littersmates exhibit the normal peak of growth hormone (GH) secretion around puberty, *Lep*<sup>ob/ob</sup> mice maintain reduced serum GH levels over development and at adulthood (Larson et al. 1976). The GH/insulin-like growth factor-1 axis becomes functional between the third and fourth week of age in mice (Donahue & Beamer 1993), which represents the

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**Figure 5**
Temporal characterization in the capacity of leptin to induce pSTAT3 in the hypothalamus of young mice. (A, B, C, D, E, F, G, H, I, J, K and L) Representative photomicrographs showing the pSTAT3 induced by a s.c. leptin injection in the hypothalamus of C57BL/6 mice at 7 (P7), 10 (P10) and 16 (P16) days of life. Abbreviations: 3V, third ventricle; OVLT, organum vasculosum lamina terminalis. Scale bar = 200 µm. (M) Example of a current-clamp recording of a LepR-expressing neuron that exhibited a hyperpolarization after application of leptin. Dashed line indicates the resting membrane potential (RMP) before leptin treatment. (N) Changes in the RMP induced by leptin in LepR-expressing neurons of the ARH and PMv in 8 to 16-day-old LepR-reporter male mice.
moment when the difference in lean mass between WT and \textit{Lep}^{ob/ob} mice starts. It is worth mentioning that the absence of leptin causes an interruption in sexual maturation (Ahima et al. 1997, Chehab et al. 1997), and the onsets of puberty and the somatotropic axis are generally interconnected (Veldhuis et al. 2006). Accordingly, gonadal steroids act directly in growth hormone-releasing hormone neurons to modulate the somatotropic axis during the pubertal transition (Garcia-Galiano et al. 2020).

Not only body adiposity is altered early in life in \textit{Lep}^{ob/ob} mice but several metabolic parameters as well. In this sense, 15-day-old \textit{Lep}^{ob/ob} mice exhibited increased carcass lipogenesis (Godbole et al. 1980). Furthermore, 15-day-old \textit{Lep}^{ob/ob} mice already exhibited decreased thermogenesis in brown adipose tissue and body temperature, as well as increased serum insulin levels, compared to lean animals (Dubuc 1976, Godbole et al. 1980, Goodbody & Trayhurn 1982, Hull & Vinter 1984). In addition, insulin-stimulated glycogen synthesis is already impaired in the adipose tissue of 3-week-old \textit{Lep}^{ob/ob} mice (Kaplan & Leveille 1981). These findings and the present results indicate that the absence of leptin action starts to induce significant metabolic consequences in mice around the end of the second week of age.

Our findings are in accordance with a previous study that showed the lack of effect of exogenous leptin injections on the bodyweight of suckling mice (Mistry et al. 1999). However, while we observed a significant acute reduction in food intake in leptin-injected 10-day-old mice, Mistry (1999) did not observe significant changes in food intake after leptin injection in 17-day-old WT mice.
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Methodological differences may explain these divergent findings since we assessed food intake indirectly through changes in body weight after suckling, whereas Mistry (1999) evaluated the intake of a powdered stock diet. Despite this divergence, it seems that exogenous leptin treatment in suckling mice produces metabolic changes that are more difficult to detect in comparison with adult animals. Nevertheless, we observed that leptin treatment between P10 and P22 induced an earlier vaginal opening, which is in accordance with previous studies that treated 3-week-old mice with leptin and observed puberty advancement (Ahima et al. 1997, Chehab et al. 1997). However, given the fact that we did not determine additional reproductive parameters, like first estrous or sex hormone levels, further experiments are necessary to confirm whether the earlier vaginal opening observed in our study is indeed associated with earlier onset of puberty.

Previous studies have already shown that suckling mice exhibit Lepr mRNA expression in several hypothalamic nuclei, and leptin is able to induce pSTAT3 in 10-day-old mice (Caron et al. 2010). In the current study, we observed an age-dependent increase in the number of LepR-expressing neurons and leptin-induced pSTAT3 cells in the hypothalamus of mice between days 7 and 16 of life. Of note, the distribution of LepR-expressing neurons and leptin-induced pSTAT3 in the hypothalamus of 16-day-old mice resembles the pattern observed in adult mice (Scott et al. 2009, Caron et al. 2010, da Silva et al. 2014, Nagaishi et al. 2014). The pattern of ARH projections to different hypothalamic nuclei is similar to that found in adult mice only around 18 days of life (Bouret et al. 2004a).

Differences in the timing when responsiveness to leptin begins in different nuclei are interesting. In this regard, MPA, ARH and PMv neurons exhibit an earlier expression of LepR and/or leptin-induced pSTAT3, compared to other hypothalamic nuclei. Whether this difference has biological meaning is unknown, but all these three hypothalamic areas are associated with the regulation of onset of puberty and reproduction (Donato et al. 2011, 2013 Wu et al. 2012, Bellefontaine et al. 2014, Egan et al. 2017). Thus, during development, leptin may first modulate hypothalamic neuronal populations involved in the

Figure 8
Male and female C57BL/6 or Lepob/ob mice show similar developmental timing of neural projections of arcuate nucleus neurons. (A, B and C) Quantification of the AgRP innervation density in the paraventricular nucleus of the hypothalamus (PVH) of 12-day-old male (n = 5) and female (n = 6) C57BL/6 WT mice. (D, E and F) Quantification of the POMC innervation density in the PVH of 12-day-old male (n = 5) and female (n = 7) C57BL/6 WT mice. (G, H and I) Quantification of the AgRP innervation density in the PVH of 12-day-old male (n = 5) and female (n = 5) Lepob/ob mice. (J, K and L) Quantification of the POMC innervation density in the PVH of 12-day-old male (n = 4) and female (n = 5) Lepob/ob mice. Scale bar = 200 µm.
control of neuroendocrine axes, while the appearance of neurons that regulate the energy balance may occur later.

The absence of leptin causes limited effects in the hypothalamic gene expression of 7-day-old mice. The exceptions were an increase in Agrp expression and a decrease in Crh mRNA level, both observed in male and female Lep<sup>ob/ob</sup> mice. In contrast, 12-day-old Lep<sup>ob/ob</sup> mice exhibit significant changes in the hypothalamic expression of several transcripts that regulate energy balance and are influenced by leptin action. Both males and females showed increased Agrp, Npy and Kiss1 mRNA levels in the hypothalamus. Since leptin exerts an inhibitory effect on AgRP/NPY neurons (Ramos-Lobo & Donato 2017), an upregulation in the hypothalamic Agrp and Npy expression is expected and observed in both adult Lep<sup>ob/ob</sup> and Lep<sup>rb/rd</sup> mice (Mizuno & Mobbs 1999, de Luca et al. 2005, Ramos-Lobo et al. 2019). Although adult Lep<sup>ob/ob</sup> mice show reduced hypothalamic Kiss1 mRNA levels (Smith et al. 2006), we observed the opposite effect in pre-pubertal Lep<sup>ob/ob</sup> mice. This contrasting result may be explained by the fact that leptin signaling in Kiss1-expressing neurons only arises after pubertal development (Cravo et al. 2013). Thus, indirect factors possibly led to the increased Kiss1 expression in the hypothalamus of 12-day-old Lep<sup>ob/ob</sup> mice. Intriguingly, Pmc mRNA levels are suppressed in the hypothalamus of adult mice lacking leptin or its receptor (Schwartz et al. 1997, de Luca et al. 2005, Ramos-Lobo et al. 2019), whereas this transcript was either unchanged or increased in 12-day-old Lep<sup>ob/ob</sup> mice. Therefore, POMC neurons seem to require a longer developmental time to respond to the lack of leptin than AgRP/NPY neurons. Accordingly, in 7- and 10-day-old mice, leptin-induced pSTAT3 cells are mostly observed in the ventromedial ARH, where AgRP/NPY neurons are found (Ramos-Lobo & Donato 2017), while leptin-responsive neurons in the lateral ARH, where POMC neurons are mainly distributed, are only observed in 16-day-old mice. Additionally, some evidence of sexual dimorphism was observed in the hypothalamic expression of neuropeptides during development. While 12-day-old male Lep<sup>ob/ob</sup> mice exhibited decreased hypothalamic expression of Cartpt, Hcrt, Pnch, Nos1 and Socs3, compared to lean animals, females showed increased expression of Cartpt, Trh and Nos1. Overall, our findings indicate that leptin deficiency begins to modulate the hypothalamic expression of transcripts involved in the regulation of metabolism between the seventh and twelfth days of life.

Noteworthy, the ability of exogenous leptin to prevent fasting-induced changes in the hypothalamic expression of Agrp, Npy and Hcrt is already present in 10-day-old C57BL/6 mice. This finding suggests that the cellular machinery necessary for the response to leptin emerges early in life, even before Lep<sup>ob/ob</sup> mice start to exhibit significant changes in body adiposity. Interestingly, fasting-induced changes in Nos1 and Kiss1 mRNA levels were not reversed by leptin treatment. However, in adult animals, fasting also suppresses hypothalamic Nos1 and Kiss1 expression via leptin-independent mechanisms (Donato et al. 2010, True et al. 2011). Although fasting did not affect the hypothalamic expression of Crh or Socs3, leptin-treated mice exhibited increased levels of these transcripts, compared to the other groups. Increased Socs3 mRNA levels are expected in mice treated with leptin since Socs3 expression is induced by LepR signaling (Andreoli et al. 2019, Pedroso et al. 2019). The causes of the increased hypothalamic expression of Crh in leptin-treated mice are unknown.

A remarkable observation of the present study is that male Lep<sup>ob/ob</sup> mice exhibit an earlier onset of obesity, compared to female Lep<sup>ob/ob</sup> mice. Although the onset of leptin’s effects is not necessarily linked to puberty timing, it is interesting that, on average, puberty begins earlier in girls than in boys (Partsch et al. 2002). Less information is available about the comparison in the timing of puberty between male and female rodents. However, the first ovulation of female rats and mice usually occurs between P35 and P45 (Nelson et al. 1990, Neill 2006), whereas spermatzoa only begin to be observed in seminiferous tubules in 45-day-old rats (Clermont & Perey 1957, Neill 2006). The onset of leptin’s effects may also be linked with changes in the development of the neural circuits that connect ARH neurons to second-order neurons. Of note, ARH neuronal projections develop during the first weeks of postnatal life (Bouret et al. 2004a). Thus, based on our results, one could expect that males would present an earlier development of ARH neuronal projections than females, given the long time it takes for them to respond to the absence of leptin. However, we observed a similar density of AgRP and POMC fibers in the PVH of 12-day-old males and females. At this age, male Lep<sup>rb/rd</sup> mice are almost exhibiting differences in fat mass, whereas female Lep<sup>ob/ob</sup> mice need one additional week to show increased body adiposity, compared to lean littersmates. Although the development of ARH projections was not studied separately in males and females, previous studies also reported that the developmental timing of ARH projections was similar between the sexes (Bouret et al. 2004a). Therefore, the earlier onset of obesity in male Lep<sup>ob/ob</sup> mice, as compared to females, cannot be explained by the timing in which ARH neuronal projections are established.

In the present study, we provided a detailed characterization of the onset of leptin’s effects on the
regulation of energy balance, indicating that leptin begins to influence the metabolism in mice around the end of the second week of postnatal life. Nevertheless, the ability of exogenous leptin administration to induce changes in body weight seems to be reduced in suckling mice. We provide evidence of sexual dimorphic differences in the timing of obesity caused by the absence of leptin, in which Lep^{ob/ob} males exhibit an earlier onset of obesity compared to females. However, this sexual dimorphism cannot be explained by differences in the developmental timing of ARH neuronal projections or the hypothalamic gene expression of transcripts that control metabolism. Thus, our findings contribute to the understating of leptin functions during development.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**

P D S T, A M R L, M R T and F W performed the in vivo, molecular and histological experiments. R F performed the electrophysiological experiments. J D J designed the study. P D S T, R F and J D J analyzed the data. J D J wrote the paper. All authors revised and approved the final manuscript.

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