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Leptin signalling in AgRP neurons modulates puberty onset and adult fertility in mice

Abbreviated title: Leptin acts via AgRP neurons to control fertility

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Abstract

The hormone leptin indirectly communicates metabolic information to brain neurons that control reproduction, using GABAergic circuitry. Agouti-related peptide (AgRP) neurons in the arcuate nucleus are GABAergic, express leptin receptors (LepR) and are known to influence reproduction. This study tested whether leptin actions on AgRP neurons are required and sufficient for puberty onset and subsequent fertility. Firstly, *Agrp*-Cre and *Lepr*-flox mice were used to target deletion of LepR to AgRP neurons. AgRP-LepR knockout female mice exhibited mild obesity and adiposity as previously described, as well as a significant delay in the pubertal onset of estrous cycles compared to control animals. No significant differences in male puberty onset or adult fecundity in either sex were observed. Next, mice with a floxed polyadenylation signal causing premature transcriptional termination of the *Lepr* gene were crossed with AgRP-Cre mice to generate mice with AgRP neuron-specific rescue of LepR. Lepr-null control males and females were morbidly obese, and exhibited delayed puberty onset, no evidence of estrous cycles and minimal fecundity. Remarkably, AgRP-LepR rescue partially or fully restored all of these reproductive attributes to levels similar to those of Lepr-intact controls despite minimal rescue of metabolic function. These results indicate that leptin signalling in AgRP neurons is sufficient for puberty onset and normal adult fecundity in both sexes when leptin signalling is absent in all other cells, and in females absence of AgRP neuron leptin signalling delays puberty. These actions appear to be independent of leptin's metabolic effects.

Significance statement

Sexual maturation and fertility are dispensable at the individual level but critical for species survival. Conditions such as nutritional imbalance may therefore suppress puberty onset and fertility in an individual. In societies characterised by widespread obesity, the sensitivity of reproduction to metabolic imbalance has significant public health implications. Deficient leptin signalling due to diet-induced leptin resistance is associated with infertility in humans and rodents, and treatments for human infertility show a decreased success rate with increasing body mass index. Here we show that the transmission of metabolic information to the hypothalamo-pituitary-gonadal axis is mediated by leptin receptors on AgRP neurons. These results provide conclusive new insights into the mechanisms that cause of infertility due to malnourishment.

Introduction

Leptin is an adipose-derived hormone that fluctuates in proportion to the nutritional status of the individual. This allows levels of oxidizable fuels to be effectively communicated to the CNS, where leptin acts to influence control of metabolic function and also fertility (Moschos et al., 2002, Quennell et al., 2009). Compromised leptin signalling due to mutations in the leptin or leptin receptor (*Lepr)* genes results in neuroendocrine dysfunction, including obesity and infertility. Leptin treatment is able to attenuate the effects of leptin deficiency, stimulating puberty onset, sexual maturation and gonadotropin secretion (Chehab et al., 1996).

Regulation of the reproductive system is initiated at the level of the hypothalamus by a neuronal network that converges on a small population of neurosecretory cells that synthesise and secrete gonadotropin-releasing hormone (GnRH). Many of the neurons that provide important input to the reproductive axis are also involved in appetite control and this allows the availability of metabolic fuels to be coordinated with fertility (Evans and Anderson, 2017). Leptin acts centrally via neurons to modulate the activity of the GnRH neuronal network (Quennell et al., 2009), but this occurs indirectly of GnRH neurons themselves as they do not contain LepR (Quennell et al., 2009). To identify the neurons required for leptin to communicate with GnRH neurons, Cre-LoxP technology has been used to delete LepR from specific neuronal populations but this has mostly proved to be unfruitful (e.g. Quennell et al., 2009, Shi et al., 2010, Donato et al., 2011b). Zuure et al. used a less focused approach to show that mice with glutamate neuron-specific LepR knockout have no reproductive deficits while GABA neuron-specific LepR knockout mice display delayed puberty and reduced fecundity. This indicates that leptin communicates with GnRH neurons through critical GABAergic neurons (Zuure et al., 2013), although the specific populations involved are yet to be identified.

Within the arcuate nucleus (ARC), LepR are colocalized with agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons (Donato et al., 2011a), among other cell types. AgRP/NPY neurons secrete two highly orexigenic peptides (Hahn et al., 1998) and are critically involved in the maintenance of energy homeostasis (Robertson et al., 2008). They are attractive candidates for metabolic control of reproduction due to their well-characterised role in metabolic regulation, the fact that they are also GABAergic (Horvath et al., 1997) and because they are known to influence the activity of GnRH neurons (Roa and Herbison, 2012, Sheffer-Babila et al.,

2013). Leptin signalling inhibits the activity of AgRP/NPY neurons, so it may be that these neuropeptides cause suppression of the HPG axis in conditions of leptin deficiency. Consistent with this, ablation of these neurons or knockout of the genes encoding either neuropeptide or the NPY Y4 receptor partially rescues the infertility phenotype of leptin-signalling-deficient mice (Sheffer-Babila et al., 2013, Wu et al., 2012, Erickson et al., 1996, Sainsbury et al., 2002).

The requirement or sufficiency of leptin signalling specifically in AgRP neurons for fertility has not previously been tested. Interestingly however, (van de Wall et al., 2008) reported normal fertility in mice with LepR knockout in both AgRP and pro-opiomelanocortin (POMC) neurons but did not investigate puberty onset. Since the reproductive effects of POMC and AgRP/NPY neuropeptides may oppose each other (Roa and Herbison, 2012), deleting leptin receptors from both cell types simultaneously could potentially cancel and mask the effects of deletion from the individual populations. We therefore utilised Cre-LoxP technology to specifically delete LepR from AgRP neurons to assess the requirement of this signalling pathway for reproductive function. We also assessed the sufficiency of leptin signalling through AgRP neurons by crossing mice with a floxed transcription blocker sequence in the *Lepr* gene with AgRP-Cre mice to generate mice with AgRP neuron-specific rescue of LepR. The effects of AgRP-LepR knockout and AgRP-LepR rescue on puberty onset and adult fecundity were evaluated in males and females to determine whether this pathway is required or sufficient for leptin's effects on the HPG axis.

Materials and Methods

Animals

To generate mice with deletion of LepR specifically from AgRP neurons, homozygous *Lepr* flox mice (*Lepr*$^{fl/fl}$; *loxP* sites flanking *Lepr* coding exon 17, a region that encodes a Janus kinase docking site required for STAT3 signaling) (McMinn et al., 2004) were bred to *Agrp*-IRES-Cre (Jax stock no 012899; IRES-Cre inserted in exon 3 of the *Agrp* gene) mice (Tong et al., 2008). The resulting *Lepr*$^{fl/+}$*,Agrp*-IRES-Cre mice were backcrossed to *Lepr*$^{fl/fl}$ mice to generate *Lepr*$^{fl/fl}$*,Agrp*-IRES-Cre conditional knockout mice (referred to as AgRP-LepR KO mice). To generate mice with specific rescue of LepR only in AgRP neurons, heterozygous Cre-dependent *Lepr* mice (*Lepr*$^{loxTB/+}$; Jax stock no 018989; *loxP* flanked transcription blocker sequence between exons 16 and 17 of the *Lepr* gene prevents transcription of the downstream exons) (Berglund et al., 2012) were bred to *Agrp*-IRES-Cre mice. The resulting *Lepr*$^{loxTB/+}$*,Agrp*-IRES-Cre mice were bred together to generate *Lepr*$^{loxTB/loxTB}$*,Agrp*-IRES-Cre conditional rescue mice (referred to as AgRP-LepR rescue mice). *Agrp*-Cre was visualized through *Agrp*-Cre-dependent green fluorescent protein (GFP) expression as a result of crossing *Agrp*-IRES-Cre mice with a *Tau*-GFP reporter line (Mayer et al., 2010, Wen et al., 2011) producing *Agrp*-GFP mice. All mouse lines were on a primarily C57BL/6J background strain. The AgRP-Cre mouse line has been previously validated (van de Wall et al., 2008). They report that 85 to 100% of AgRP immunoreactive neurons express Cre recombinase, and that all Cre-expressing neurons are AgRP immunoreactive. Furthermore, in AgRP-LepR KO mice, leptin treatment was unable to cause any increase in leptin signaling in AgRP neurons.

Transgenic mice were identified by PCR analysis of genomic DNA using the following primer sets, at an annealing temperature of 59^{o}C (*Lepr* flox), 61^{o}C (*Lepr*^{loxTB}) or 60^{o}C (*Agrp*-Cre). For *Lepr* flox identification: AAT GAA AAA GTT GTT TTG GGA CGA and CAG GCT TGA GAA CAT GAA CAC AAC AAC and CTG ATT TGA TAG ATG GTC TTG AG (200 b.p. product indicates the wild-type gene, 250 b.p. the floxed gene), for *Lepr*^{loxTB} identification: TGG CTT TTA AGC TCT GCA GTC and TAG GGC CAA ACC CAC ATT TA and CCC AAG GCC ATA CAA GTG TT (522 b.p. product indicates wild-type gene, 360 b.p. the floxed gene), for *Agrp*-Cre identification: GCT TCT TCA ATG CCT TTT GC and GTG TGT GGT TCC AGC ATG AC and GG AAC TGC TTC CTT CAC GA (199 b.p. product indicates the wild-type gene, 280 b.p. for the Cre gene). Animals were group housed or paired with an animal of the opposite

sex (for the fecundity experiments), except during food intake assessments. Mice were housed under conditions of controlled lighting (lights on from 0600–1800 h) and temperature (22 ± 1°C). They had free access from the date of weaning to standard rodent chow, except during overnight fasting as described. All mice were weighed every two weeks except for female mice when they were paired with male mice for the fecundity experiments. The University of Otago Animal Ethics Committee approved all animal experimental protocols.

Tissue collection and immunohistochemistry to identify leptin-responsive cells

At the end of the fecundity studies when mice were 5 months old, they were assessed for food intake over a 24 h period in individual cages. The animals were then fasted overnight to reduce the concentration of endogenous circulating leptin and then injected with recombinant leptin (1 mg/kg sc; National Hormone and Peptide Program). Two hours post-injection, they were anaesthetized with sodium pentobarbital (240 mg/kg ip) and transcardially perfused with 4% paraformaldehyde in 0.1M PBS, pH 7.4. Total abdominal fat mass was measured at this time. Coronal (30 µm thick) sections were cut throughout the ARC for each brain on a sliding microtome to be used for immunohistochemical staining. To visualize leptin-responsive cells in the ARC of AgRP-LepR KO and AgRP-LepR rescue mice and their respective control groups, immunohistochemical labeling of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) was performed. Antigen retrieval was performed by incubating for 15 min in 1 mM EDTA, pH 8.0 at 90^{o}C. To quench endogenous peroxidase activity the tissues were incubated in 1% H_2O_2 for 30 min. Sections were incubated for 24 hours in the primary antibody; monoclonal rabbit anti-pSTAT3 (Tyr705, D3A7 XP; 1:1000 dilution, Cell Signaling Technology, Danvers, MA). Tissue was then incubated for one hour in the secondary antibody, biotinylated goat anti-rabbit immunoglobulin (1:1000 dilution, Vector Laboratories Inc., Burlingame, CA). The signal was amplified by incubating in Vector Elite avidin-biotin peroxidase (Vector Laboratories) and then stained in diaminobenzidine solution to visualize pSTAT3 immunoreactivity. Omission of the primary antibody resulted in a complete absence of staining. Stained cells in the ventromedial ARC (vmARC) and ventromedial hypothalamic nucleus (VMH) (at least 3 sections per area from each animal) were counted.

Experiment 1: Are leptin actions on AgRP neurons required for normal puberty onset and fertility?

AgRP-LepR KO and $Lep^{fl/fl}$ littermate controls were used to evaluate the requirement of leptin signaling in AgRP neurons for puberty onset and subsequent fertility. In female mice, puberty onset was measured by assessing the age of vaginal opening and first estrus. From 21 days of age, all mice were checked daily for vaginal opening. Once this had occurred, vaginal cytology was used to detect occurrence of first estrus. Estrous cyclicity of adult females was then assessed for 14 consecutive days, starting at least 10 d after first estrus. Experimental and control female animals were paired with adult wild-type C57BL/6J males between 60 and 140 days of age to assess their fecundity (body weight measurements were not obtained from females over this time due to pregnancies). Male puberty progression was assessed visually based on the date of separation of the prepuce from the glans penis. Once this had occurred, male mice were paired with adult wild-type C57BL/6J females. The date of first successful mating was used as another measure of pubertal progression, calculated by subtracting the gestation period (21 days) from the date when their first litter was born. Assessment of male fecundity was carried out over a 100-day period. Cages were checked daily during male and female fecundity assessments for the presence of pups, and the date and size of the litter was recorded before pups were removed and culled.

Experiment 2: Are leptin actions on AgRP neurons sufficient for normal puberty onset and fertility?

In order to test the sufficiency of leptin signaling in AgRP neurons for puberty onset and subsequent fertility, we generated mice with specific rescue of LepR only in AgRP neurons (AgRP-LepR rescue mice). $Lepr^{loxTB/loxTB}$ and $Lepr^{wt/wt}$ littermates were used for the two control groups (referred to as Lepr-null and Lepr-intact controls, respectively). The Lepr-null control group has previously been shown to be essentially infertile (Cravo et al., 2013), and was included as a baseline reference against which any improvement of fertility could be compared in the AgRP-LepR rescue group. In male and female AgRP-LepR rescue, Lepr-null and Lepr-intact control mice, puberty onset, estrous cycles and adult fecundity were assessed as described for Experiment 1. At the completion of these studies, when the mice were 5 months old, we also assessed NPY fiber immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN), since it is known that leptin deficiency causes profound disruptions in the development of AgRP

feeding regulatory circuits to this region (Bouret et al., 2004). Hypothalamic tissue sections from Lepr-null, Lepr-intact control and AgRP-LepR rescue mice were quenched as described for pSTAT3 immunohistochemistry, incubated for 48 h in polyclonal rabbit anti neuropeptide Y (T-4070; 1:2000, Bachem, Torrance, CA) followed by biotinylated goat anti-rabbit immunoglobulin, avidin-biotin peroxidase and diaminobenzidine solutions as described for pSTAT3 immunohistochemistry. Omission of the primary antibody or overnight preabsorption of the primary antibody with 20 µg/ml human NPY (H6375; Bachem) resulted in a complete absence of staining. Fiber density within the PVN was quantified using ImageJ software after first thresholding the greyscale image to binary values.

The infertility anticipated in LepR-null (and possibly AgRP-LepR rescue) mice in Experiment 2 could reflect impaired estrogenic feedback on the hypothalamo-pituitary-gonadal axis. Therefore, negative feedback assessment was performed as described by Zuure et al. (2013). Briefly, female AgRP-LepR rescue, Lepr-null and Lepr-intact control mice were blood sampled (4 µl) from the tail tip (day 0; ovary intact). Ovariectomies were performed under isoflurane anesthesia, and 8 d later another blood sample was taken (OVX). Animals were subsequently implanted with a chronic slow-release 17β-estradiol subcutaneous implant (50 µg/ kg; 10–30 mm long depending on body weight); 8 d later, another blood sample was taken (OVX + estradiol implant). A sandwich ELISA (Evans et al., 2014) was used to measure LH concentrations in whole blood samples. The sensitivity of the assay was 0.2 ng/ml after correction for sample dilution, the intra-assay coefficient of variation was 6% and the inter-assay variation was 15%. All samples were analyzed in duplicate.

Statistical analysis

Values are presented as mean ±SEM. Differences were considered significant at $p<0.05$. In Experiment 1, unpaired Student's *t* tests were used to identify significant differences between control and AgRP-LepR KO animals when group sizes were greater than n=10. For smaller sample sizes the non-parametric Mann-Whitney U test was used. In Experiment 2, one-way ANOVA followed by the post-hoc Holm-Sidak test was used to identify significant differences between Lepr-null, AgRP-LepR rescue and Lepr-intact groups when group sizes were greater than n=10. For smaller sample sizes the non-parametric Kruskal Wallis H test was used followed

232 by Dunn's post hoc test. Body weight data was analyzed using a repeated measures two-way
233 ANOVA followed by the Holm-Sidak test.
234

Results

Experiment 1: Are leptin actions on AgRP neurons required for normal puberty onset and fertility?

GFP expression indicative of cell bodies in the *Agrp*-GFP reporter mice was localised to the ventro-medial portion of the ARC (Fig. 1*D*), with virtually no soma seen elsewhere in the hypothalamus. This pattern of expression closely matches that previously reported for *Agrp* mRNA (Broberger et al., 1998) and AgRP immunohistochemistry using colchicine-treated rodents (Kloukina et al., 2012). This indicates that these animals were appropriate to use in experiments assessing the necessity and sufficiency of leptin signalling through AgRP neurons.

Phosphorylated STAT3 immunohistochemistry was used to detect the presence of any leptin-induced STAT3 signaling, which is a functional indicator of leptin-responsive cells, in all mice. Counting of pSTAT3-stained cells was restricted to the vmARC as this corresponded to the location of AgRP neurons in the reporter mice, and pSTAT3 responsive cells in the VMH were counted an internal control. Shown in Fig. 1 are representative images of the vmARC and VMH of control (*A*) and AgRP-LepR KO mice (*B*) used in Experiment 1. Consistent with a previous report based on these AgRP-LepR KO mice (van de Wall et al., 2008), a significant decrease in pSTAT3 cell numbers was observed in the vmARC of AgRP-LepR KO mice compared to controls ($U_{(12)} = 0$, $p < 0.01$) but no difference was seen for the VMH (*C*) or for the dorsolateral ARC (where few if any AgRP neurons are located; data not shown). Normal pSTAT3 staining was also evident in other hypothalamic regions in all mice, suggesting that the widespread 'ectopic' Cre expression previously reported in up to 5% of AgRP-Cre mice (Dietrich et al., 2015) did not occur in this experiment. (It should be noted that LepR excision-specific primers, were not used in this study to check for excision outside the ARC). The loss of leptin-responsiveness was previously shown to be specific to AgRP cells (van de Wall et al., 2008).

As reported previously (van de Wall et al., 2008), body weight of both male and female AgRP-LepR KO mice was slightly but significantly greater than control littermates ($F_{1,16} = 6.04$, $p < 0.05$ [males]; $F_{1,16} = 15.79$, $p < 0.05$ [females]), and this was confirmed by post-hoc tests at a number of time points as shown in Figure 2*A* and *B*. This effect was particularly apparent for female mice. Female AgRP-LepR KO mice also had significantly heavier abdominal fat pads compared to the control group ($U_{(10)} = 4$, $p < 0.05$) as shown in Figure 2*C*. Adiposity of male mice was not significantly different between the two groups suggesting that lack of leptin

signalling in AgRP neurons may have a greater impact on metabolism in female animals than in males. These observations align with the well-characterised role of AgRP in metabolism and validate the animal model used. Fig. 2*D* illustrates the amount of food consumed by control and AgRP-LepR KO animals over a 24-hour period. Consistent with a previous report (van de Wall et al., 2008), no significant difference was seen between the groups for either male or female mice suggesting that the slight obese phenotype of the KO mice was not caused by increased food intake.

Although no significant difference in age at vaginal opening was observed between control and AgRP-LepR KO females, the onset of first estrus was significantly delayed in AgRP-LepR KO mice by 3.4 ± 1.0 days compared to control littermates (Fig 3*B*, $U_{(16)} = 16$, $p < 0.05$), indicating that AgRP leptin signalling is required for normally timed female puberty. No significant difference was seen in the age of onset of male puberty (preputial separation or first fertile mating) between control and AgRP-LepR KO groups (Fig 3*A*).

Analysis of vaginal cytology revealed no significant difference in the time spent in each phase of the estrous cycle between control and AgRP-LepR KO animals (Fig. 4*A*). The average length of the estrous cycle was not significantly different between the two groups. The similarity between estrous cyclicity profiles is demonstrated in Fig. 4*B*. Fecundity of adult mice was assessed by litter frequency, number of pups per litter and inter-litter interval over 80 days (females) or 100 days (males). None of these measures differed between the groups in both males and females (Fig 4*C* and *D*). Reproductive function was also assessed by measuring the weight of reproductive organs. No significant difference was observed in the weight of the testes and seminal vesicles in males or of the uteri in females (data not shown).

Experiment 2: Are leptin actions on AgRP neurons sufficient for normal puberty onset and fertility?

Consistent with the vmARC-specific loss of leptin-responsive cells in AgRP LepR KO mice in Experiment 1, AgRP-LepR rescue restored responsiveness to leptin specifically in the vmARC. Fig. 5 shows representative images from Lepr-intact control (*A*), AgRP-LepR rescue (*B*) and Lepr-null control (*C*) animals used in Experiment 2. In all Lepr-null mice, no pSTAT3 positive cells were visible in any hypothalamic region. In all AgRP-LepR rescue animals, a group of leptin-responsive cells in the vmARC were visible but no staining was seen in any other

hypothalamic region (females only examined). The number of cells counted in the vmARC of AgRP-LepR rescue mice was not statistically different from Lepr-intact control animals but was significantly greater than Lepr-null animals (Fig. 5*D,* $F_{2,20} = 4.80, p < 0.001$), consistent with AgRP neuron-specific LepR rescue. Staining in the VMH of Lepr-intact control animals was significantly greater than both Lepr-null and AgRP-LepR rescue groups, in which pSTAT3 was essentially undetectable in this region (Fig. 5*A-D,* $F_{2,20} = 9.22, p < 0.001$).

As expected, Lepr-null male and female mice were significantly heavier than Lepr-intact control animals ($F_{2,28} = 126.2, p < 0.0001$ [males]; $F_{2,28} = 62.33, p < 0.0001$ [females]) and post-hoc tests showed this occurred from 34 days of age (Fig 6*A* and *B*). AgRP-LepR rescue mice were also metabolically compromised by lack of intact leptin signalling. This was demonstrated by their obesity compared to Lepr-intact control animals from 34 days of age. Post-hoc testing also showed that AgRP-LepR rescue mice of both sexes were significantly lighter than Lepr-null animals from 49 days of age ($p < 0.001$ [males]; $p < 0.01$ [females]). Abdominal adiposity of male and female Lepr-null and female AgRP-LepR rescue animals was also significantly increased compared to Lepr-intact controls (Fig. 6*C*, $F_{2,22} = 3.48, p < 0.05$ [males] $H_{2,23} = 14.98$, $p < 0.05$ [females]). Food intake results for male animals showed that both Lepr-null and AgRP-LepR rescue animals consumed significantly more food than Lepr-intact controls over a 24-hour period (Fig. 6*E*, $F_{2,28} = 12.23$, $p < 0.001$ and $p < 0.01$ respectively). There was no significant difference in food intake between Lepr-null and AgRP-LepR rescue mice. Food intake values for female animals are not shown due to the confounding effects of the different levels of parity across the treatment groups. Consistent with the minimal rescue from obesity by AgRP-LepR rescue, this group and the Lepr-null mice both exhibited impaired NPY fiber density in the PVN compared to Lepr-intact control animals (Fig. 6*G* and *H*, $F_{2,16} = 4.70, p < 0.05$), which has been previously shown to be a function of leptin-dependent outgrowth from ARC NPY/AgRP neurons during neonatal development (Bouret et al., 2004).

As expected, a significant delay in preputial separation of male mice was observed for Lepr-null mice when compared to Lepr-intact controls (Fig. 7*A*, $F_{2,28} = 17.56, p < 0.001$). While AgRP-LepR rescue mice were also significantly delayed compared to control animals ($p < 0.001$), preputial separation in this group happened significantly earlier than the Lepr-null group ($p < 0.05$) indicating that the presence of leptin signalling in AgRP neurons reduced the delay in this aspect of puberty onset experienced by Lepr-null mice. Age at first successful mating was

also significantly delayed for Lepr-null mice compared to Lepr-intact control mice (Fig. 7*B*, $F_{2,23} = 4.77$, $p < 0.05$), but remarkably no such delay occurred for AgRP-LepR rescue animals (Fig. 7*B*). This further indicates that the presence of LepR in AgRP neurons was sufficient to normalize puberty onset in male mice. For female mice as expected, vaginal opening in Lepr-null mice was significantly delayed when compared to Lepr-intact controls (Fig. 7*C*, $H_{2,23} = 12.1$, $p < 0.01$). As was the case with mating onset in males, AgRP-LepR rescue was sufficient to completely normalize this aspect of female puberty compared to the Lepr-intact control animals (Fig. 7*C*, $p < 0.05$ vs. Lepr-null mice). Lepr-null animals did not undergo first estrus during the monitoring period, while in marked contrast all AgRP-LepR rescue animals did albeit with a significant delay compared to Lepr-intact controls ($U_{(18)} = 0$, $p < 0.001$) (Fig. 7*D*). Collectively, these data suggest that the presence of leptin signalling in AgRP neurons is essentially sufficient to normalise puberty onset in these mice. In the month following puberty onset in Lepr-intact controls and AgRP-LepR rescue animals, Lepr-null mice showed no evidence of reproductive cycling (Fig. 8*A*, *B*); rather their vaginal cytological smears remained in a constant diestrus-like state. In marked contrast, AgRP-LepR rescue mice exhibited cycling patterns that were not significantly different from control animals in terms of cycle length or frequency of cycle stages (Fig. 8*A*, *B*).

Fecundity of adult mice was measured by assessing litter frequency and average number of pups. In this experiment inter-litter interval was not a valid measurement for assessing fecundity as the majority of Lepr-null animals, and some of the AgRP-LepR rescue animals had only one litter. Males were left in breeding pairs for 100 days, while females were paired for 80 days. This was due to welfare considerations; Lepr-null and AgRP-LepR rescue females were prone to dystocia complications because of their obesity. Lepr-null male mice sired very few litters (6/11 males sired 1-3 litters each before becoming infertile) compared to both AgRP-LepR rescue and Lepr-intact control groups ($F_{2,28} = 27.21$, $p < 0.001$). AgRP-LepR rescue males had a litter frequency similar to that of Lepr-intact control males (Fig. 8*C*). There was no significant differences in the average number of pups per litter between any of the male groups (Fig. 8*D*). Leptin or LepR deficient female mice are usually infertile (e.g. Chehab et al., 1996, Quennell et al., 2009). Consistent with this, Lepr-null female mice produced almost no litters compared to both AgRP-LepR rescue and Lepr-intact control groups ($F_{2,32} = 16.2$, $p < 0.05$, $p < 0.01$ respectively; Fig. 8*C*). Surprisingly, given their apparent absence of reproductive cycles

immediately following vaginal opening, two out of the nine Lepr-null female mice produced a single litter. It is possible that pairing with a male provided an additional stimulus for reproductive cyclicity. These two females were not included in the analysis of litter size. Lepr-intact control animals had significantly larger litters when compared to AgRP-LepR rescue mice ($t_{(22)} = 2.41$, $p < 0.05$; Fig. 8*D*).

To test if the efficacy of estrogenic negative feedback is reduced in infertile LepR-null mice and if AgRP-LepR rescue overcomes this, measurements of LH concentration in whole blood samples were used to assess the effect of ovariectomy and subsequent estradiol replacement. As expected for female Lepr-intact control mice, there was a significant elevation of blood LH concentration in the ovariectomized state relative to both the ovary intact and ovariectomized + estradiol implant states ($F_{1.6,16} = 7.1$, $p < 0.01$), indicating their HPG axis response to estrogenic negative feedback. In contrast in Lepr-null mice, there was no statistically significant ovariectomy-induced rise in blood LH levels (ovary intact, $p = 0.13$; ovariectomized + estradiol implant, $p = 0.13$ vs. ovariectomized state). In the AgRP-LepR rescue groups the LH increase in response to ovariectomy was restored ($F_{1.2,5.9} = 30.13$, $p = 0.001$ when compared to the intact and ovariectomy + estradiol implant states; Fig. 8*E*). Indeed, when compared to the other two groups, AgRP-LepR rescue mice had significantly increased LH levels in the ovariectomized state ($F_{2,18} = 3.76$, $p < 0.05$).

Discussion

Under conditions of undernutrition, decreased circulating leptin levels are thought to lead to reproductive suppression, since exogenous leptin treatment is able to overcome this situation in female mice (Ahima et al., 1996) and women (Welt et al., 2004). Humans and mice with a congenital leptin or LepR deficiency are infertile despite being energy replete, and leptin treatment is sufficient to restore reproductive function in leptin-deficient individuals (e.g. Farooqi et al., 1999, Mounzih et al., 1997, Chehab et al., 1996). Mice exhibiting forebrain neuron-specific deletion of LepR are also infertile, highlighting the importance of leptin's central actions in regulating reproductive activity (Quennell et al., 2009). Recent experiments have narrowed down the pool of candidate neuronal populations that are required for control of reproduction by leptin, so that we now know that these neurons are likely to co-express GABA rather than glutamate (Zuure et al., 2013). In this study the importance of LepR signalling through GABAergic AgRP

neurons for the functioning of the hypothalamo-pituitary-gonadal axis was assessed to determine whether this pathway is required and/or sufficient for fertility. While the effects of deletion of LepR from AgRP neurons were limited to delayed female puberty, rescuing LepR expression solely in AgRP neurons revealed that leptin signalling through this population is almost entirely sufficient for normal puberty onset and fecundity in both sexes.

To assess specificity of Cre recombinase induced LepR knockout or rescue in AgRP neurons, leptin-induced pSTAT3 signalling was evaluated as a functional indicator of of leptin-responsive cells. In Experiment 1 when pSTAT3-stained cells in the vmARC were counted, significantly less pSTAT3 staining was observed in AgRP-LepR KO animals compared to Lepr-intact controls. This indicates that the animal model used was successful in preventing leptin signalling in this region, and presumably specifically in the AgRP neuronal population. In Experiment 2, Lepr-null animals were completely devoid of leptin signalling while AgRP-LepR rescue animals only expressed leptin signalling in the vmARC; presumably in the AgRP neurons.

Leptin signalling in AgRP neurons exerts minor body weight and food intake effects

AgRP acts as an antagonist to melanocortin receptors to promote feeding, while overexpression of NPY is associated with hyperphagia and obesity (Sheffer-Babila et al., 2013). Leptin inhibits the activity of AgRP neurons so deficient leptin signalling within these cells should lead to hyperphagia and increased body weight (van de Wall et al., 2008). In Experiment 1 both male and female mice that lacked leptin signalling in AgRP neurons showed significantly increased body weight compared to control littermates, although this difference was relatively minor and no difference in food intake was observed compared to controls. The body weight increase was greatest in female mice, in which a significant increase in abdominal adiposity also occurred. This mild and sex-specific metabolic phenotype and lack of difference in caloric intake of AgRP-LepR KO mice compared to controls has been confirmed by other researchers using this model (van de Wall et al., 2008). Consistent with the moderate bodyweight effect of AgRP-LepR KO, in Experiment 2, rescue of leptin signalling only in AgRP neurons led to a slight reduction in body weight in both sexes compared to the profoundly obese Lepr-null animals from 34 days of age. Abdominal fat mass and food intake were also unaffected or only minimally rescued in mice with restored leptin signalling in AgRP neurons, and the density of AgRP/NPY fibers in one of their primary target nuclei, the PVN, remained as defective as in the Lepr-null mice compared to

Lepr-intact controls. The latter results may indicate that leptin's trophic actions on AgRP neuronal wiring (Bouret et al., 2004) occurs indirectly to these cells, and is consistent with the idea that direct leptin signalling in AgRP neurons is not the key regulator of food intake circuitry. Previously it has been demonstrated by deletion of LepR from either AgRP or POMC neurons, or both, that the actions of leptin on these cell types are additive in regards to body weight and adiposity, but even collectively they do not account for the full extent of leptin's metabolic effects, particularly in regards to hyperphagia (van de Wall et al., 2008).

Leptin signalling in AgRP neurons is sufficient for puberty onset

In our study, female AgRP-LepR KO mice displayed a 3-day delay in onset of first estrus compared to control females. This indicates that lack of leptin signalling through AgRP neurons is a barrier for puberty onset in female mice, but that this can eventually be overcome so that reproductive function in adults is normal. It may be that redundant pathways such as ventral premammillary nucleus glutamatergic neurons (Donato et al., 2011b) or preoptic nitric oxide neurons (Bellefontaine et al., 2014) eventually compensate for lack of leptin signalling in AgRP neurons. In Experiment 2 the rescue of LepR in AgRP neurons was almost completely sufficient to allow normal puberty onset to occur although first estrus in AgRP LepR recue mice was delayed by 11 days compared to the Lepr-intact control group. Despite the delay, it is remarkable that the presence of leptin signalling solely in AgRP neurons is sufficient to restore onset of estrus cycles in these animals. This clearly indicates, for the first time, that leptin signalling in AgRP neurons is sufficient for puberty onset in mice. Presumably AgRP neurons are not the only leptin target population sufficient for puberty onset, since LepR re-expression in the ventral premammillary nucleus also rescued puberty onset (Donato et al., 2011b).

Leptin signalling in AgRP neurons is sufficient, but not required, for adult fertility

While no requirement of AgRP leptin actions for estrous cyclicity and was evident in Experiment 1, results of Experiment 2 clearly showed that leptin signalling through AgRP neurons is completely sufficient for normal reproductive cycles and to maintain males and female fecundity in the absence of all other leptin signalling pathways. This role is consistent both with previous reports that ablation of these neurons or knockout of the genes encoding AgRP, NPY or the NPY Y4 receptor partially rescues the infertility phenotype of leptin-signalling-deficient mice

(Sheffer-Babila et al., 2013, Wu et al., 2012, Erickson et al., 1996, Sainsbury et al., 2002) and the idea that leptin's inhibition of AgRP neurons reduces their suppression of GnRH neuronal activity.

Surprisingly, a few Lepr-null male and female mice were able to sire or give birth to litters. This suggests that the dogma of leptin requirement for fertility in not absolute in all cases. In fact, the degree of infertility in leptin-signalling-deficient mice has been reported to be dependent of the genetic background and sex of the mice (Ewart-Toland et al., 1999).

In female mice, removal of the ovaries disrupts negative feedback resulting in a gradual increase in circulating LH levels on the days following ovariectomy. The expected increase in LH levels after OVX was observed in Lepr-intact control mice, but in, Lepr-null animals this effect appeared to be blunted, suggesting mild hypogonadotropic hypogonadism in the absence of gonadal steroids. In contrast in AgRP-LepR rescue mice, a marked increase in circulating LH levels was seen following ovariectomy. As we reported previously for GABA-specific LepR knockout females (Zuure et al., 2013), the negative feedback actions of estradiol remained intact in both Lepr-null and AgRP-LepR rescue mice, suggesting that impairments in this neuroendocrine action are not to blame for the infertility of leptin-signalling-deficient females.

The transgenic models used in this study relied on the removal of LepR early in development. This may permit other types of leptin-responsive neurons to develop sufficient roles to compensate where previously they may have been only minor players. The reciprocal approaches of Experiments 1 and 2 helps to reveal roles that might otherwise be masked by a network of compensatory mechanisms. A role that was compensated for in a knockout experiment would be expected to be apparent or even exacerbated in a 'rescue' experiment, where all other leptin-GnRH pathways are absent. While it is possible obesity may contribute to reduced fertility, it has been shown that fertility can be maintained in morbidly obese mice (Bates and Myers, 2003, Singireddy et al., 2013). The AgRP-LepR rescue mice in Experiment 2 provide another example of a mouse model that is essentially fully fertile while being morbidly obese, and suggest that it is lack of leptin signaling rather than obesity per se that is primarily responsible for infertility in leptin signalling deficient mouse lines.

These results demonstrate that AgRP neurons are involved in the transmission of information from leptin receptors to the hypothalamo-pituitary-gonadal axis. It is likely this action occurs via modulation of GnRH neuronal activity. AgRP and NPY inhibit pulsatile LH

483 release (Catzeflis et al., 1993, Vulliémoz et al., 2005), which directly reflects GnRH release. It
484 seems that one mechanism by which leptin signaling deficiency leads to infertility is through
485 overexpression of AgRP and NPY which in turn leads to the suppression of GnRH release.
486 Consistent with AgRP neurons exerting direct actions on GnRH neurons (Roa and Herbison,
487 2012), AgRP is also a potent antagonist of the stimulatory effects of αMSH on the melanocortin-
488 4 receptor (MC4R) (Butler and Cone, 2002). Approximately half of murine GnRH neurons
489 express *Mc4r*, and MC4R activation can increase c-Fos coexpression and firing rate in GnRH
490 neurons (Israel et al., 2012). AgRP may also influence GnRH neurons indirectly, since AgRP
491 deficiency upregulates *Tac2* (coexpressed by a subpopulation of arcuate kisspeptin neurons) gene
492 expression in LepR-deficient mice (Sheffer-Babila et al., 2013). It is likely that a complex
493 network exists between AgRP neurons and other neurons of the GnRH neuronal network to
494 coordinate reproduction.

495 In summary, we have demonstrated here that leptin signaling in arcuate AgRP neurons is
496 sufficient to permit all aspects of puberty onset and fertility in male and female mice, and this
497 action appears to be independent of leptin's metabolic affects. The requirement of leptin actions
498 in these neurons for fertility is relatively minimal, however. These findings are consistent with
499 the existence of multiple redundant leptin-responsive inputs to the GnRH neurons that govern the
500 reproductive axis.

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Figure legends

Figure 1. Leptin-induced pSTAT3 signaling in the ventromedial ARC (vmARC) and ventromedial hypothalamus (VMH). Representative examples of pSTAT3 immunoreactivity in the ARC and VMH of control (***A***) and AgRP-LepR KO (***B***) animals. ***C,*** no difference in leptin-induced pSTAT3 immunoreactivity between control and KO groups was observed in the VMH but a significant decrease (p = 0.004) was seen in the vmARC of AgRP-LepR KO animals compared to controls. ***D,*** representative coronal section showing GFP immunofluorescence in AgRP Cre-positive neurons of the vmARC. Controls, n=7-8; AgRP-LepR KO, n=6-8 per group. **p < 0.01. 3V, third ventricle. Scale bar represents 200 μm.

Figure 2. Effects of AgRP-LepR KO on body weight, adiposity and food intake in male and female mice. ***A,*** AgRP-LepR KO male mice were significantly heavier than control males at the marked time points (n=9 per group). ***B,*** AgRP-LepR KO female mice were significantly heavier than control females from 49 days of age (controls, n=8; AgRP-LepR KO, n=10 per group). ***C,*** there was no significant difference in adiposity of male AgRP-LepR KO and control animals (n=9 per group), but AgRP-LepR KO females had significantly increased (p = 0.030) abdominal adiposity compared to control females (controls, n=5; AgRP-LepR KO, n=7 per group). ***D,*** daily food intake for male or female AgRP-LepR KO animals was not significantly different from male or female control mice (males, n=9 per group; female controls, n=8; female AgRP-LepR KO, n=10 per group). *p < 0.05, **p <0.01, ***p < 0.001.

Figure 3. Age at puberty onset in male and female AgRP-LepR KO mice compared to control animals. ***A,*** no significant difference in puberty onset was observed between male AgRP-LepR KO and control animals (n=9 per group). ***B,*** no significant difference in vaginal opening was observed between female AgRP-LepR KO and control animals, but a significant delay (p = 0.030) in age at first estrus was observed in the AgRP-LepR KO group (controls, n=8; AgRP-LepR KO, n=10 per group). *p < 0.05.

Figure 4. Estrous cyclicity and fecundity of AgRP-LepR KO male and female mice compared to control animals. ***A,*** frequency of occurrence of cycles stages in females. Stage of the estrous cycle was determined by the predominant presence of leukocytes (proestrus), cornified epithelial

651 cells (estrus) or nucleated epithelial cells (metestrus or diestrus). There were no significant
652 differences between AgRP-LepR KO and control animals. ***B,*** representative examples of the
653 cyclicity of AgRP-LepR KO and control female mice. ***C,*** there were no significant differences in
654 the number of litters produced over 100 days between male or female AgRP-LepR KO and
655 control animals. ***D,*** there were no significant differences in the average number of pups produced
656 per litter between male or female AgRP-LepR KO and control animals. Male controls, n=9; male
657 AgRP-LepR KO, n=8 per group; female controls, n=8; female AgRP-LepR KO, n=10 per group.
658 P; proestrus, E; estrus, M/D; metestrus/diestrus.
659
660 Figure 5. Leptin-induced pSTAT3 signaling in the ARC and VMH of Lepr-null control, AgRP-
661 LepR rescue and Lepr-intact control animals. ***A,*** Representative Lepr-intact control section
662 showed staining throughout both the vmARC and VMH. ***B,*** Representative AgRP-LepR rescue
663 section showing staining in the ventromedial ARC (vmARC). ***C,*** no pSTAT3 staining was
664 observed in any region in Lepr-null animals. ***D,*** quantification of leptin-induced pSTAT3
665 immunoreactivity, showing that leptin responsiveness was rescued in the vmARC of AgRP-LepR
666 rescue mice ($p = 0.0003$ vs Lepr-null mice). In the VMH, the response to leptin remained
667 undetectable in both AgRP-LepR rescue and Lepr-null animals (Lepr-intact controls, n=7; AgRP-
668 LepR rescue and Lepr-null, n=8 per group). ***$p < 0.001$. Scale bar represents 200 μm.
669
670 Figure 6. The effect of AgRP-LepR rescue on body weight, adiposity, food intake and NPY fiber
671 density. AgRP-LepR rescue and Lepr-null male (***A***) and female (***B***) mice were significantly
672 heavier than Lepr-intact control animals from 5 weeks of age (#), while AgRP-LepR rescue
673 animals were significantly lighter than Lepr-null animals (**, ***) from 6-7 weeks of age (n=10-
674 11 per group). Lepr-null male (***C***) and female (***D***) animals had significantly increased abdominal
675 fat mass compared to Lepr-intact controls, and AgRP-LepR rescue females also had significantly
676 increased ($p = 0.0003$) adiposity compared to Lepr-intact control females (male Lepr-intact
677 controls, n=9; male AgRP-LepR rescue and Lepr-null, n=8 per group; female Lepr-intact
678 controls, n=9; female AgRP-LepR rescue, n=11; Lepr-null, n=5 per group). ***E,*** daily food intake
679 of both Lepr-null ($p = 0.0001$) and AgRP-LepR rescue ($p = 0.008$) males was significantly
680 increased compared to Lepr-intact controls (Lepr-intact controls and AgRP-LepR rescue, n=10;
681 Lepr-null, n=11 per group). ***F,*** NPY fiber density in the paraventricular nucleus was significantly

682 reduced in both Lepr-null (p = 0.046) and AgRP-LepR rescue (p = 0.012) animals compared to
683 Lepr-intact controls (Lepr-intact controls and AgRP-LepR rescue, n=6; Lepr-null, n=7 per
684 group). Representative examples are shown in ***G***. *p < 0.05, **p < 0.01, ***p < 0.001. 3V, third
685 ventricle. Scale bar represents 100 µm.
686
687 Figure 7. Puberty onset in male and female AgRP-LepR rescue compared to Lepr-null and Lepr-
688 intact control animals. Age at preputial separation (***A***) and the first fertile mating (***B***) was
689 significantly delayed in Lepr-null compared to Lepr-intact control males and this was partially
690 overcome by AgRP-LepR rescue (preputial separation: p = 0.046 vs Lepr-null mice; first fertile
691 mating: not significant vs either Lepr-intact or Lepr-null mice). Note that only 6/11 of Lepr-null
692 males were able to sire a litter. Lepr-intact controls and AgRP-LepR rescue, n=10; Lepr-null,
693 n=11 per group. ***C,*** age at vaginal opening in Lepr-null females was significantly delayed
694 compared to both Lepr-intact control (p = 0.002) and AgRP-LepR rescue (p = 0.012) females. ***D,***
695 first estrus did not occur in any Lepr-null animals during the monitoring time, whereas first estrus
696 occurred in all AgRP-LepR rescue females albeit delayed (p = 0.0001) compared to Lepr-intact
697 control animals (Lepr-intact controls and AgRP-LepR rescue, n=10; Lepr-null, n=5 per group).
698 *p < 0.05, ** p < 0.01, ***p < 0.001.
699
700 Figure 8. Estrous cyclicity and fecundity of AgRP-LepR rescue males and females compared to
701 Lepr-null and Lepr-intact control animals, and plasma LH concentration in female mice. ***A,***
702 frequency of occurrence of estrous cycle stages. Lepr-null mice showed lack of cycling,
703 remaining in a constant diestrus-like state, whereas no statistically significant differences
704 between AgRP-LepR rescue and control females. ***B,*** representative examples of the cycling
705 pattern seen in AgRP-LepR rescue and Lepr-intact control animals and the lack of cycling in
706 Lepr-null animals. ***C,*** a significant reduction in litter frequency was observed when Lepr-null
707 male and female animals were compared with AgRP-LepR rescue (males: p = 0.0001; females: p
708 = 0.008) and Lepr-intact controls (males: p = 0.0001; females: p = 0.0003), whereas no
709 significant difference was observed between LepR rescue mice and Lepr-intact controls. Male
710 Lepr-intact controls and male AgRP-LepR rescue, n=10; male Lepr-null, n=11 per group; female
711 Lepr-intact controls, n=14; female AgRP-LepR rescue, n=11; female Lepr-null, n=9 per group. ***D,***
712 there was no significant difference in litter size between any of the male groups, but AgRP-LepR

713 rescue females had smaller litters ($p = 0.024$) when compared to Lepr-intact control females. Not
714 enough litters were born to Lepr-null females to enable comparison. ***E,*** plasma LH concentration
715 in female mice in the intact state, ovariectomized state (OVX) and OVX + estradiol implanted
716 state. A significant increase in LH levels in response to ovariectomy and a subsequent decrease
717 following estradiol replacement was seen in both Lepr-intact control ($p = 0.001$) and AgRP-LepR
718 rescue ($p = 0.0001$) mice, but not Lepr-null mice (Lepr-intact controls, n=11; female AgRP-LepR
719 rescue, n=6; Lepr-null, n=5 per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
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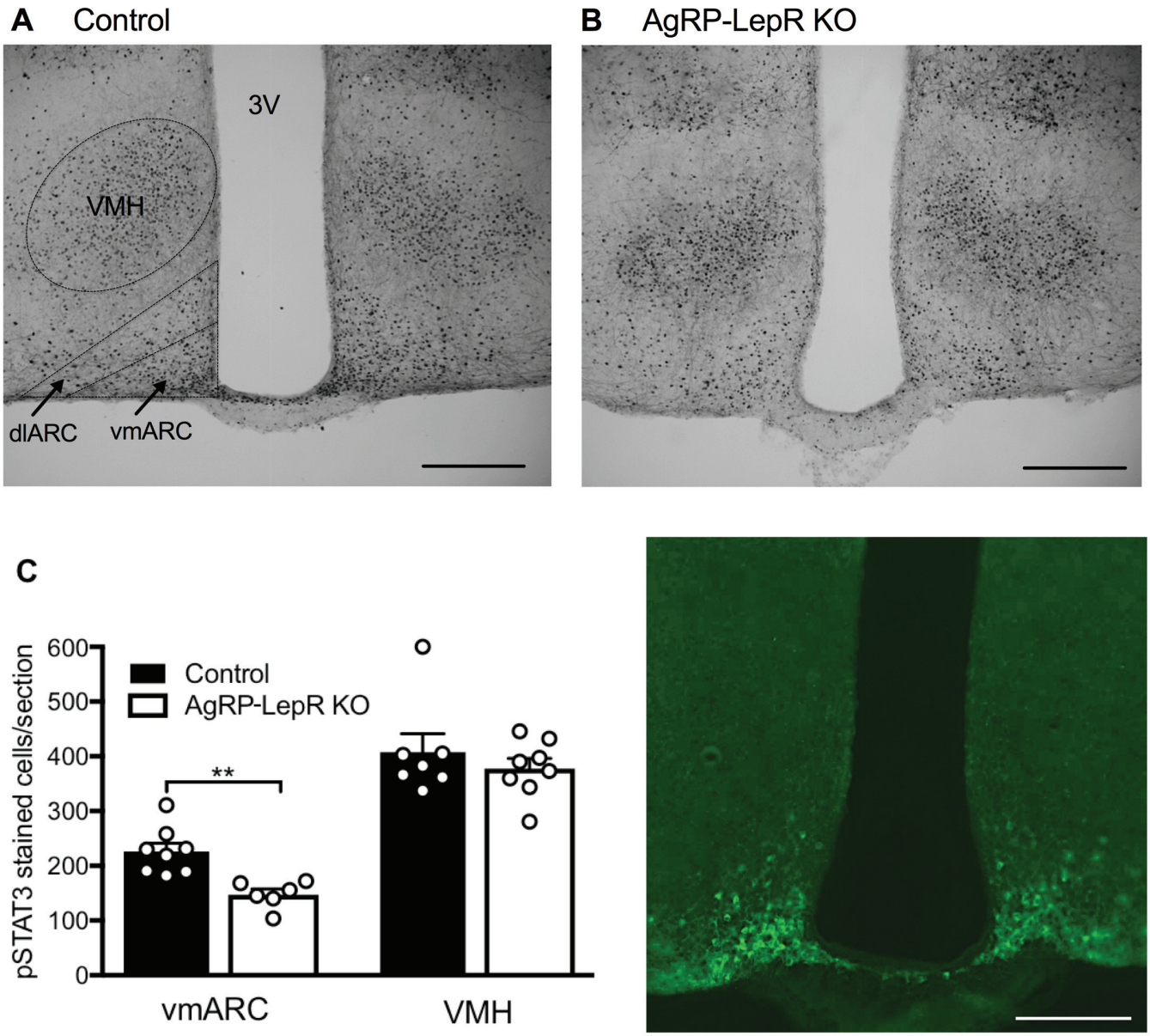
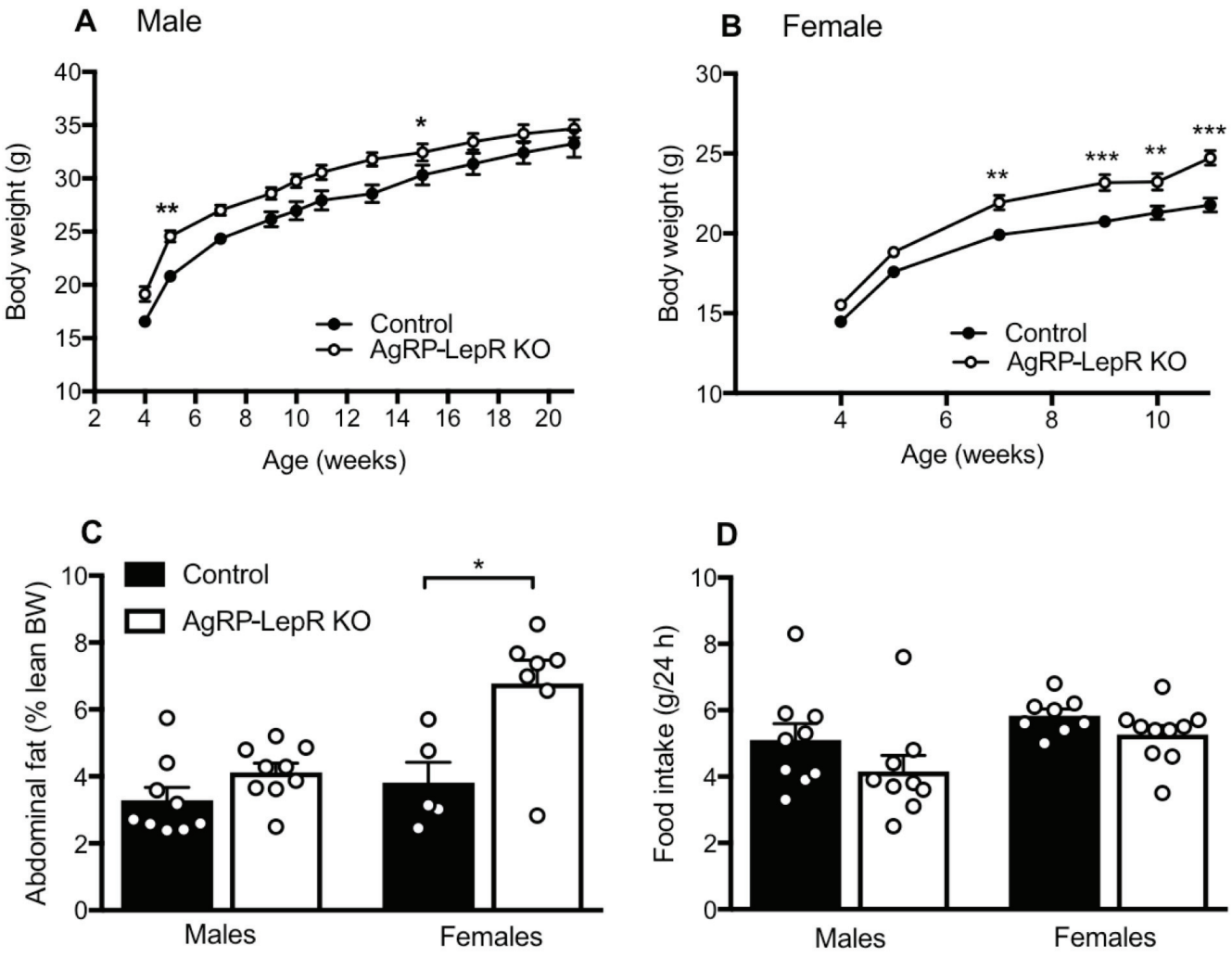
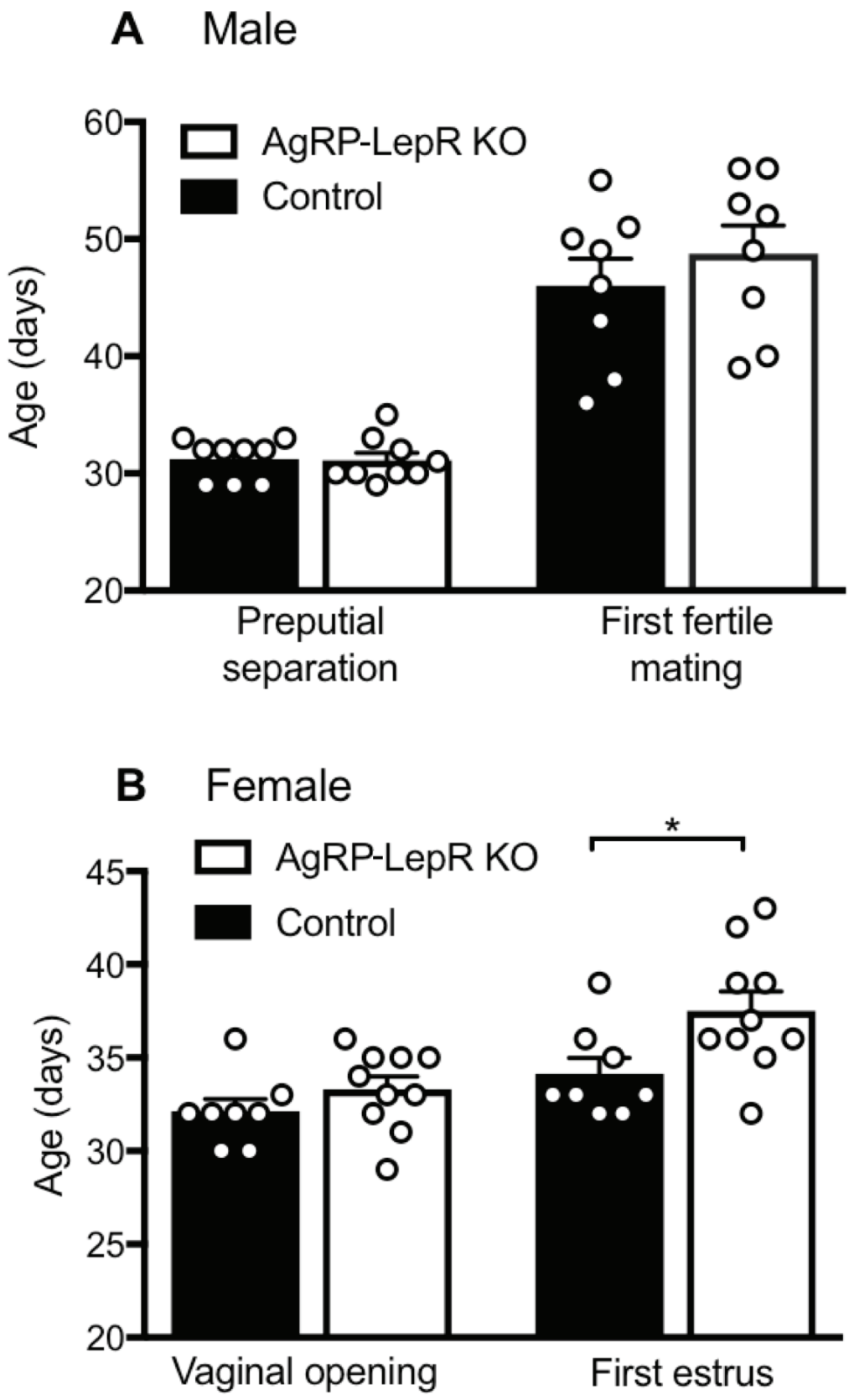
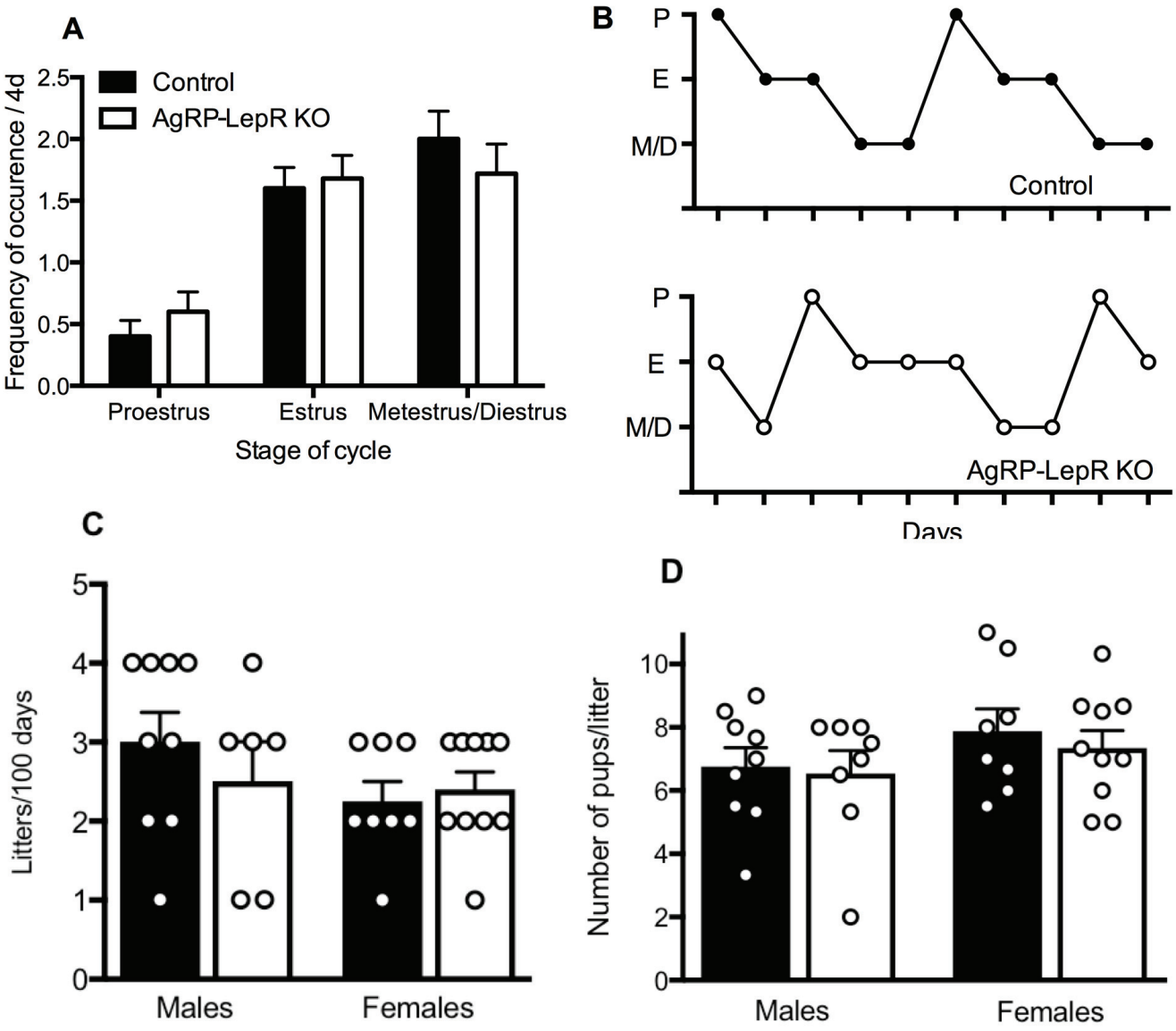
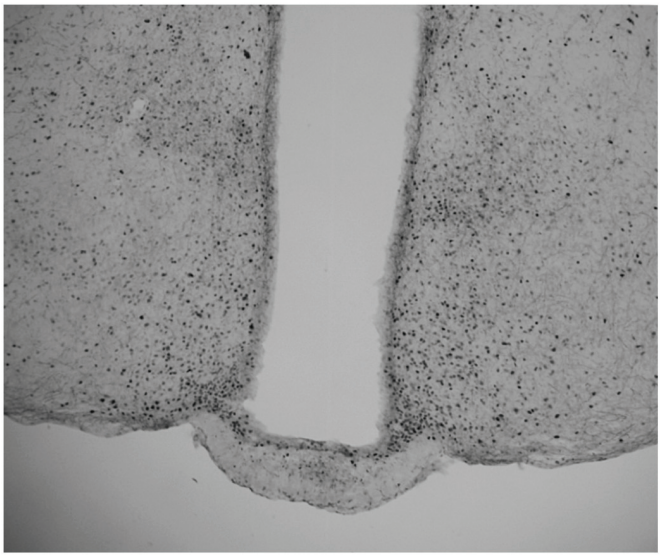




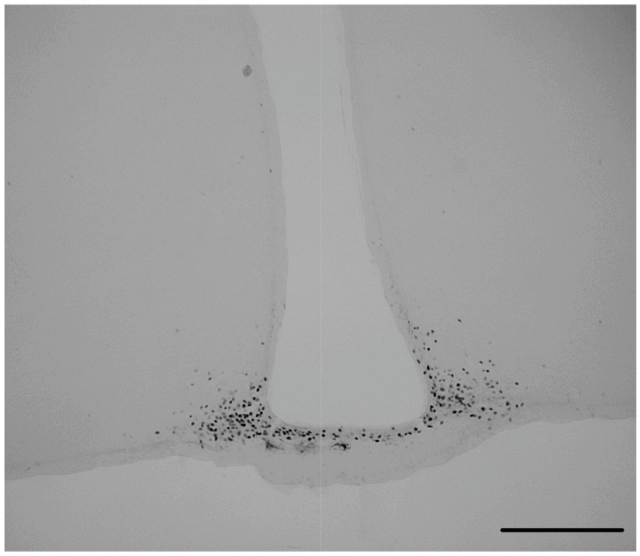




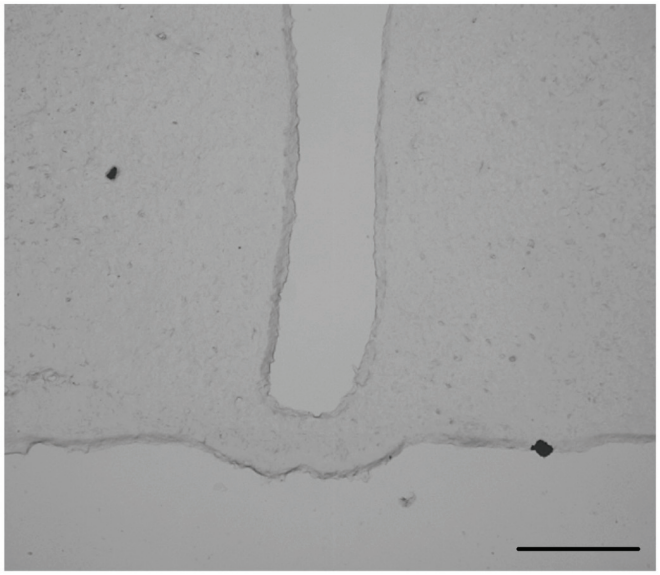
A LepR-intact control



B AgRP-LepR rescue



C LepR-null



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