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The Peripubertal Decline in *Makorin Ring Finger Protein 3* Expression is Independent of Leptin Action

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A critical body weight is necessary for pubertal development, an effect mediated in part by leptin. The potential regulation by leptin of *Makorin Ring Finger Protein 3 (MKRN3)*, in which loss-of-function mutations are the most common genetic cause of central precocious puberty, has not been previously explored. In mice, expression of *Mkrn3* in the hypothalamic arcuate nucleus is high early in life and declines before the onset of puberty. Therefore, we aimed to explore if leptin contributes to the decrease in hypothalamic *Mkrn3* mRNA levels observed in mice during pubertal development. We first used a leptin-deficient (*ob/ob*) mouse model. *Mkrn3* mRNA levels in the mediobasal hypothalamus (MBH), which includes the arcuate nucleus, and in the preoptic area (POA), both showed a significant decrease with age from postnatal day (PND) 12 to PND30 in *ob/ob* mice in both males and females, similar to that observed in wild-type mice. To further explore the effects of leptin on *Mkrn3* expression, we exposed prepubertal wild-type mice to high levels of leptin from age PND9-12, which did not result in any significant difference in *Mkrn3* expression levels in either the MBH or POA. In summary, regulation of *Mkrn3* expression by leptin was not observed in either the MBH or the POA, 2 hypothalamic sites important for pubertal maturation. These data suggest that the decline in *Mkrn3* at the onset of puberty may occur independently of leptin and support our hypothesis that MKRN3 is a bona fide controller of puberty initiation.

Key Words: MKRN3, puberty, GnRH, kisspeptin, leptin

The onset of puberty is influenced by a coordinated network of metabolic cues that converge at the hypothalamus [1, 2]. Leptin, a satiety hormone produced peripherally by adipocytes, acts at the level of the hypothalamus as a permissive metabolic signal to the reproductive axis [3]. Attainment of appropriate leptin levels is indispensable for the maturation of the hypothalamic-pituitary-gonadal (HPG) axis and normal pubertal progression. In healthy children, leptin levels increase before the onset of puberty [4]. In rodents, a neonatal leptin surge occurs at the end of the first week of life, with a peak around postnatal day (PND) 9 [5]. Blockade of the neonatal leptin surge results in delayed pubertal development in rats [6]. The importance of leptin for maturation of

Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; CPP, central precocious puberty; HH, hypogonadotropic hypogonadism; HPG, hypothalamic-pituitary-gonadal; MBH, mediobasal hypothalamus; *MKRN3*, *Makorin Ring Finger Protein 3*; PACAP, pituitary adenylate cyclase activating polypeptide; PMV, ventral premammillary nucleus; PND, postnatal day; POA, preoptic area.

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the reproductive axis is further highlighted by human and mouse models of leptin deficiency, because of mutations in leptin (*LEP/Lep)* or its receptor (*LEPR/Lepr)*, that fail to enter puberty and exhibit hypogonadotropic hypogonadism (HH) in addition to their metabolic phenotype of profound obesity, hyperphagia, and reduced energy expenditure [7]. The precise sites of action of leptin on the reproductive axis remain to be fully elucidated [8, 9], but leptin appears to act through intermediary gamma-aminobutyric acid (GABA)-ergic neurons, in which selective deletion of the leptin receptor in mice leads to delayed puberty in females [10, 11].

In contrast to the more than 30 other monogenic causes that have been described in individuals with delayed and/or absent puberty resulting from HH, which include loss-of-function mutations in *LEP* and *LEPR*, genetic causes of central precocious puberty (CPP) have not been widely described [12, 13]. *Makorin Ring Finger Protein 3* (*MKRN3)*, located within the maternally imprinted Prader-Willi syndrome region on chromosome 15q11.2, was reported as the first gene in which loss-of-function mutations are associated with CPP from premature activation of the HPG axis and is now the most known genetic cause of CPP [14, 15]. In mice, hypothalamic expression of *Mkrn3* in the arcuate nucleus (ARC) is high early in life and declines before the onset of puberty, remaining low into adulthood [14]. This expression pattern, together with the identification of loss-of-function mutations in children with CPP, supports a role for MKRN3 as an inhibitor of GnRH secretion [16]. The protein structure of MKRN3 predicts E3 ubiquitin ligase activity as well as RNA binding [16-18]. MKRN3 likely has multiple protein targets, supported by a protein–protein interaction study identifying 81 interacting proteins, including some involved in pubertal timing, insulin signaling, RNA metabolism, and cell-cell adhesion [17]. Additionally, murine Mkrn3 has been reported to bind and suppress neuronal pentraxin-1, a secreted protein important in neuronal development [18].

The potential regulation of MKRN3 by leptin has not yet been explored. Thus, although we hypothesize that MKRN3 is a key controller of the timing of puberty onset, it must also be considered that MKRN3 may act downstream of other regulators of puberty such as leptin. Therefore, in this study, we aimed to explore if leptin contributes to the decrease in hypothalamic *Mkrn3* expression observed in mice during pubertal development. The purpose of this study was to test the hypothesis that leptin may act a negative regulator of hypothalamic *Mkrn3* in the ARC and/or anteroventral periventricular nucleus (AVPV). To this end, we first investigated whether leptin- or selective leptin receptor-deficient (*ob/ob* and *Vgat-Cre;Lepr*$^{lox/lox}$) mice have persistently high hypothalamic *Mkrn3* mRNA levels in adulthood, at a time when *Mkrn3* expression levels have previously shown to be low in wild-type mice, compared with prepubertal mice [14, 19, 20]. In an alternate model, we explored if leptin could induce a decline in hypothalamic *Mkrn3* mRNA expression by exposing wild-type prepubertal mice to high concentrations of leptin.

Materials and Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at Brigham and Women's Hospital and animals were housed at the Brigham and Women's Hospital Center for Comparative Medicine. Mice were maintained in a 12-hour light, 12-hour dark cycle and were fed a standard rodent diet. Leptin-deficient (*ob/ob*) mice were generated by crossing heterozygous B6.Cg-Lep^{Ob}/J mice from Jackson Laboratory (Bar Harbor, ME) and genotyped as described in the following section [21]. *Vgat-Cre;Lepr*$^{lox/lox}$ mice were generated and genotyped as described in the following section [11, 20]. Pups were maintained with the dam until weaning at PND21.

Genotyping

PCR was performed using DNA from digested tail samples. Briefly, for *ob/ob* mice, genotyping was performed by PCR using RFLP-F, RFLP-R, Lepob-R, and WtLep-F primers,

as previously described [21]. For *Vgat-Cre;Lepr*$^{lox/lox}$ mice, mice were genotyped by PCR to identify expression of the Cre amplicon, to recognize the lox P site, and to amplify ghrelin as an internal amplification control, as previously described [11, 20].

Exogenous leptin treatment

Wild-type male and female mice (n = 5 per group) were treated with recombinant leptin 2 mg/kg (National Hormone and Peptide Program, Torrance, CA) or vehicle (PBS) administered subcutaneously in the subscapular region every 12 hours from PND9 to PND12. Serum and tissue samples were collected 3 to 4 hours after the last dose. Dosing regimens were based on previously published protocols [22, 23]. Mice were weighed daily during the study period.

Hormone assays

Whole blood was collected by trunk decapitation from vehicle and leptin-treated mice on PND12 in a BD Serum Separator Tube (Franklin Lakes, NJ). Serum was extracted following centrifugation of whole blood at 7500 rpm for 5 minutes and stored at –80°C until assayed. Serum leptin levels were measured using the mouse leptin ELISA kit (RRID:AB_2722664, Crystal Chem USA, Downers Grove, IL) and measured on a Sunrise microplate absorbance reader (Tecan Trading TG, Switzerland). The intra- and inter-assay coefficients of variance for leptin were less than 10% and the minimum detectable concentration was 0.2 ng/mL.

Tissue collection

Tissues from the mediobasal hypothalamus (MBH) and preoptic area (POA) were collected from *ob/ob* male and female mice and from wild-type littermate controls at PND12 (n = 5 per group) and PND30 (n = 5 per group). These tissues were also collected from wild-type male and female mice at PND12 (n = 5 per group) treated with exogenous leptin or saline. Tissues from the ARC and POA were collected by microdissection from *Vgat*-Cre;*Lepr*$^{lox/lox}$ adult (6 to 8 weeks old) female mice (n = 4 per group).

RT-PCR

Total RNA was isolated from the collected tissue samples using TRIzol reagent (Invitrogen/Thermo Fisher Scientific, Inc., Waltham, MA) followed by chloroform/isopropanol extraction. One microgram of RNA was DNase-treated (RQ1 RNase-free DNase, Promega, Madison, WI) and reverse transcribed using Superscript III cDNA synthesis kit (Invitrogen/Thermo Fisher Scientific, Inc.). Quantitative RT-PCR assays were performed on an ABI Prism 7000 sequence detection system and software (Applied Biosystems). The cycling conditions were as follows: 2 minutes' incubation at 95°C (hot start), 45 amplification cycles (95°C for 30 seconds, 60°C for 30 seconds, and 45 seconds at 75°C), with fluorescence detection at the end of each cycle. *Mkrn3* (NM_011746) and *Socs3* (NM _007707) mRNA were detected using SYBR green mix (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions [14, 24]. Data were normalized using *Rpl19* (NM_009078) as an internal control [14].

Data analysis

Data were analyzed using Prism statistics software (GraphPad, Inc., San Diego, CA). All data are presented as the mean ± SEM. Results were analyzed by unpaired *t* test for the comparison of mean differences between 2 groups. Multiple comparisons were analyzed by 1-way ANOVA followed by Tukey post hoc analysis. Differences were considered significant when $P < 0.05$.

Results

Mkrn3 *mRNA expression in leptin-deficient mouse models*

Our previous studies documented an age-related pattern of decline in *Mkrn3* mRNA levels in the ARC of both male and female wild-type mice, with decreases first documented at PND15 and continuing to decline and remaining low into adulthood [14]. The purpose of this experiment was to assess *Mkrn3* mRNA levels in *ob/ob* mice prepubertally (PND12), when hypothalamic *Mkrn3* expression is high in wild-type mice, compared with pubertal mice (PND30), when *Mkrn3* expression is low. We hypothesized that if leptin negatively regulates *Mkrn3* expression, then *Mkrn3* mRNA levels would remain elevated in pubertal mice in the absence of leptin.

In female mice, *Mkrn3* mRNA levels were analyzed by RT-PCR in the MBH in *ob/ob* mice, compared with wild-type littermate controls. In female wild-type mice, *Mkrn3* mRNA levels in the MBH showed a statistically significant decrease from PND12 to PND30 ($P < 0.0001$; Fig. 1A), concordant with prior studies [14]. In *ob/ob* mice, *Mkrn3* mRNA levels also decreased significantly from PND12 to PND30 in the MBH, concordant with wild-type mice ($P < 0.0001$; Fig. 1A). Similar to the age-related decline demonstrated in the MBH, there was also a decrease in *Mkrn3* mRNA expression in the POA from PND12 to PND30 in wild-type female mice ($P < 0.0001$; Fig. 1B). As in the MBH, *Mkrn3* mRNA expression in the POA of *ob/ob* mice also showed a significant

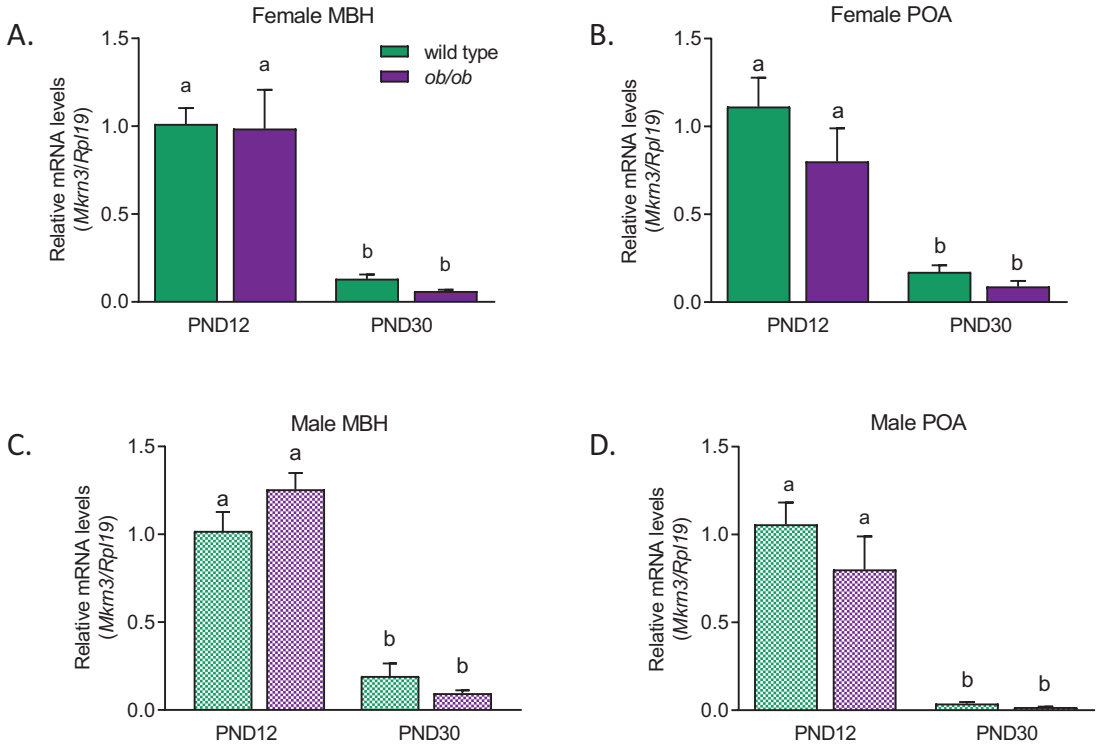

Figure 1. Peripubertal decrease in hypothalamic *Mkrn3* mRNA levels is not affected by leptin deficiency in mice. *Mkrn3* mRNA levels in the (A) MBH and (B) POA of female *ob/ob* mice (solid purple columns) compared with wild-type mice (solid green columns) at PND12 and PND30. *Mkrn3* mRNA levels in the (C) MBH and (D) POA of male *ob/ob* mice (dotted purple columns) compared with wild-type mice (dotted green columns) at PND12 and PND30. Data are shown as mean ± SEM, n = 5 per group. Different lowercase letters (a, b) above the bars indicate statistically significant differences between groups within each graph (1-way ANOVA, Tukey's post hoc analysis, $P < 0.0001$). For variables with the same letter, the difference between means was not statistically different. MBH, mediobasal hypothalamus; PND, postnatal day; POA, preoptic area.

decline from PND12 to PND30 ($P < 0.001$; Fig. 1B), similar to that in wild-type mice. Levels of *Mkrn3* mRNA expression did not differ between the wild-type and *ob/ob* mice at PND12 or at PND30 in either the MBH (PND12, $P = 0.97$; PND30, $P = 0.10$; Fig. 1A) or the POA (PND12 $P = 0.48$; PND30 $P = 0.99$; Fig. 1B).

In wild-type male mice, the *Mkrn3* mRNA expression pattern in the MBH recapitulated the findings in female mice, showing a decrease in *Mkrn3* mRNA levels from PND12 to PND30 ($P < 0.0001$; Fig. 1C), consistent with previous findings [14]. *Mkrn3* mRNA expression in the MBH of male *ob/ob* mice also significantly decreased from the PND12 to PND30 ($P < 0.0001$; Fig. 1C). In the POA of wild-type male mice, *Mkrn3* mRNA levels significantly decreased in wild-type mice from PND12 to PND30, similar to the POA of wild-type female mice and MBH of wild-type male mice ($P < 0.0001$; Fig. 1D). The *Mkrn3* expression in *ob/ob* mice mimicked the pattern in wild-type mice with a significant decline in the POA from PND12 to PND30, similar to that in the MBH ($P < 0.0001$; Fig. 1D). There was no significant difference in *Mkrn3* mRNA expression levels between wild-type and *ob/ob* male mice at PND12 or PND30 in either the MBH (PND12, $P = 0.90$; PND 30, $P = 0.39$; Fig. 1C) or POA (PND12, $P = 0.45$; PND30, $P = 0.99$; Fig. 1D).

In addition to assessing the change in expression with age in both sexes, *Mkrn3* mRNA levels in the MBH were compared between female and male wild-type mice directly at PND12 and at PND30. No difference between females and males was found at either age (PND12, $P = 0.35$; PND30, $P = 0.99$; Fig. 2A). Similarly, in the POA, *Mkrn3* mRNA expression levels did not statistically differ between female and male wild-type mice at either PND12 or PND30 (PND12, $P = 0.44$; PND30, $P = 0.99$; Fig. 2B). These data suggest a lack of sexual dimorphism in expression of *Mkrn3* in the hypothalamus, in contrast to the sexual dimorphism of *Kiss1* expression, where Kiss1 neurons are nearly absent in the AVPV of males, in contrast to their presence in females [25].

To corroborate our findings in another model, we examined a more selective leptin receptor-deficient model, using *Vgat-Cre;Lepr*$^{lox/lox}$ adult female mice in which the leptin receptor has been selectively deleted from GABAergic neurons [10, 11, 20]. Similar to *ob/ob* mice, *Vgat-Cre;Lepr*$^{lox/lox}$ female mice were obese and failed to undergo pubertal maturation and had delayed or absent vaginal opening [10, 11]. There was no significant difference in *Mkrn3* mRNA expression in the ARC or AVPV between adult *Vgat-Cre;Lepr*$^{lox/lox}$ and wild-type mice (ARC, $P = 0.86$; AVPV, $P = 0.78$; Fig. 3).

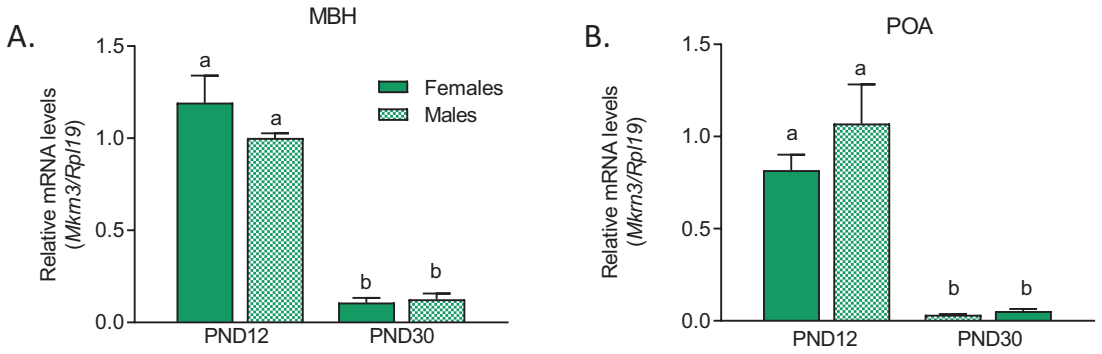

Figure 2. Lack of sexual dimorphism of hypothalamic *Mkrn3* mRNA expression in the hypothalamus. *Mkrn3* mRNA levels in the (A) MBH and (B) POA of wild-type female mice (solid green columns) compared with male mice (dotted green columns) at PND12 and PND30. Data are shown as mean ± SEM (n = 5 per group). Different lowercase letters (a, b) above the bars indicate statistically significant differences between groups (1-way ANOVA, Tukey's post hoc analysis, $P < 0.0001$). For variables with the same letter, the difference between means was not statistically different. MBH, mediobasal hypothalamus; PND, postnatal day; POA, preoptic area.

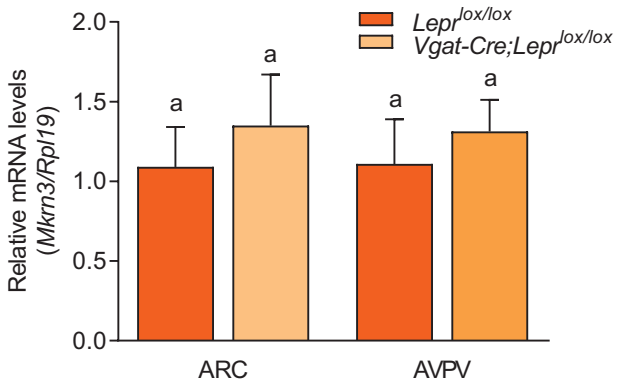

Figure 3. Hypothalamic *Mkrn3* expression is not affected in selective leptin receptor deficient female mice. *Mkrn3* mRNA levels in the ARC and AVPV of adult female *Vgat-Cre; Lepr*$^{lox/lox}$ mice (light orange columns) compared with *Lepr*$^{lox/lox}$ control mice (dark orange columns). Data are shown as mean ± SEM (n = 4 per group). The same lowercase letter (a) above all bars indicates the lack of statistically significant differences among groups (1-way ANOVA, Tukey's post hoc analysis, ARC, $P = 0.86$; AVPV, $P = 0.78$). ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus.

Effect of exogenous leptin administration on Mkrn3 expression in wild-type juvenile mice

In a contrasting model, wild-type juvenile mice were given exogenous leptin because there may be differences in how the HPG axis responds to a leptin-deficient compared with a leptin-sufficient state. If leptin negatively regulates MKRN3, then we would expect to observe inhibition of *Mkrn3* mRNA expression in prepubertal mice following leptin administration. Wild-type mice were treated from PND9 to PND12 with supraphysiologic doses of leptin (2 mg/kg, subcutaneously) based on prior dosing protocols [22, 23]. *Mkrn3* mRNA levels in the MBH and POA of leptin-treated mice were compared with those in vehicle-treated control mice 3 to 4 hours after the last injection on PND12.

In female mice, *Mkrn3* mRNA levels in the MBH did not differ in leptin-treated mice at PND12 compared with vehicle-treated controls ($P = 0.67$; Fig. 4A). Similarly, in the POA, *Mkrn3* mRNA levels did not differ significantly from leptin-treated mice at PND12 compared with vehicle-treated controls ($P = 0.98$; Fig. 4B). In male mice, there was also no significant difference in *Mkrn3* mRNA expression in either the MBH or the POA in leptin-treated compared with vehicle-treated mice at PND12 (MBH, $P = 0.21$; POA, $P = 0.34$; Fig. 4C, D).

To confirm that exogenous leptin administration was effective at the doses used, leptin levels were measured in serum collected 3 to 4 hours after the final dose of leptin was administered. In female mice, serum leptin levels were significantly higher in leptin-treated mice compared with vehicle-treated controls (36.0 ± 5.8 vs 4.2 ± 0.6 ng/mL, respectively; $P < 0.01$; Fig. 5A). In male mice, serum leptin levels were also significantly higher in leptin-treated mice compared with vehicle-treated controls (57.6 ± 7.8 vs 11.6 ± 0.9 ng/mL, respectively; $P < 0.01$; Fig. 5B). To confirm that peripheral leptin administration was reaching and activating leptin receptors centrally, *Socs3* mRNA expression was measured in the collected MBH tissue samples. *Soc3* expression is induced by leptin after activation of the Jak-STAT3 pathway [26]. In female mice at PND12, *Socs3* expression in the MBH was significantly higher in leptin-treated mice compared with controls ($P < 0.05$; Fig. 5C). In male mice at PND12, *Soc3* expression in the MBH was also significantly higher in leptin-treated mice compared with controls ($P < 0.05$; Fig. 5D). Body weight was also measured from PND9 through PND12, whereas leptin was being administered

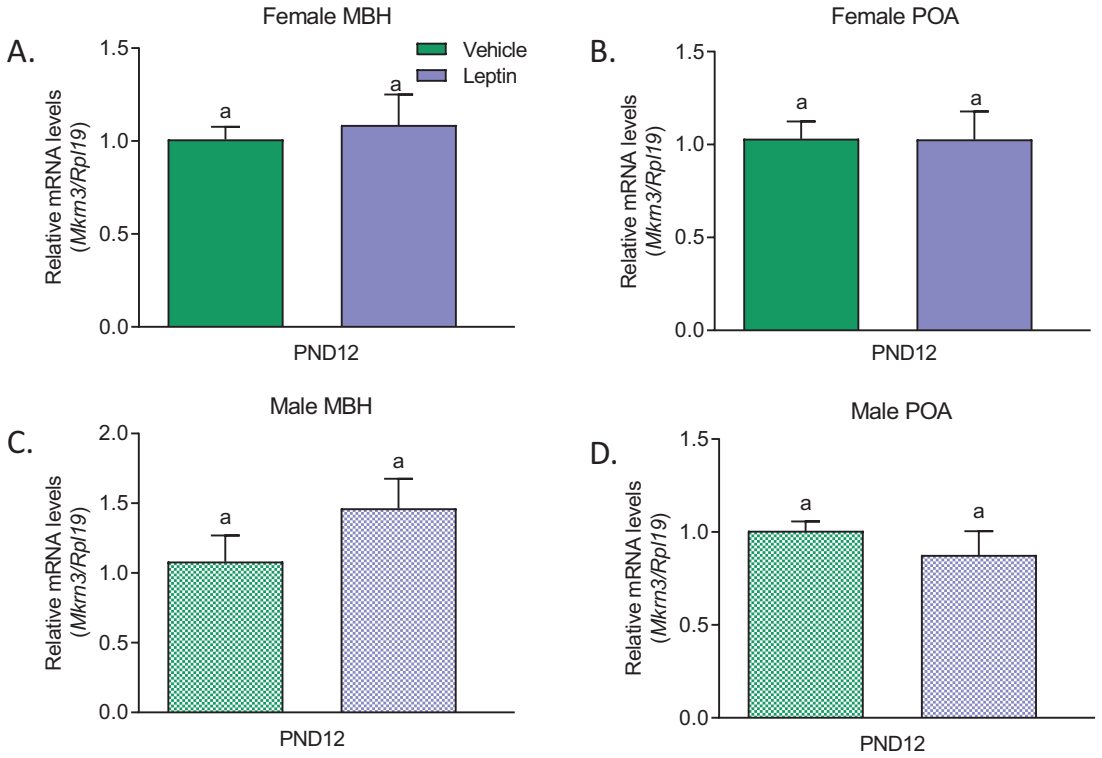

Figure 4. *Mkrn3* mRNA expression is not altered by exogenous leptin administration in wild type mice at PND12. *Mkrn3* mRNA expression in the (A) MBH and (B) POA of leptin-treated female (solid blue columns) compared with vehicle-treated mice (solid green columns). *Mkrn3* mRNA expression in the (C) MBH and (D) POA of leptin-treated male (dotted blue columns) compared with vehicle-treated mice (dotted green columns). Data are shown as mean ± SEM (n = 5 per group). The same lowercase letter (a) above all bars indicates the lack of statistically significant differences among groups (unpaired *t* test, female: MBH, $P = 0.67$; POA, $P = 0.98$, male: MBH, $P = 0.21$; POA, $P = 0.34$). MBH, mediobasal hypothalamus; PND, postnatal day; POA, preoptic area.

until animals were sacrificed, and levels did not differ significantly compared to vehicle-treated controls (Fig. 5E).

Discussion

In this study, the potential relationship between MKRN3 and leptin was explored using leptin-deficient and leptin-excess models. The relationship between reproduction and metabolism is well established and leptin is an important cue for energy balance, with important permissive effects for the onset and maintenance of puberty and reproduction [2]. Models of leptin deficiency in both humans and mice exhibit central HH [3]. Conversely, short-term administration of leptin to wild-type mice has been suggested in some studies to advance pubertal onset [22, 23] and exogenous leptin administration to leptin deficient *ob/ob* mice corrects their hypogonadism and infertility [27]. Additionally, transgenic "skinny" mice overexpressing leptin exhibit advanced pubertal onset, even in the absence of any apparent adipose tissue [28]. Nonetheless, in some studies, leptin has not conclusively accelerated pubertal onset, and is generally considered a "permissive" factor for pubertal onset [29].

The site of action of leptin on the reproductive axis remains to be fully understood because leptin signaling in Kiss1 neurons appears to arise only after sexual maturation is completed [30]. Additionally, selective deletion of leptin receptors on Kiss1 neurons does not alter rodent pubertal timing or fertility and leptin receptors are not expressed on GnRH

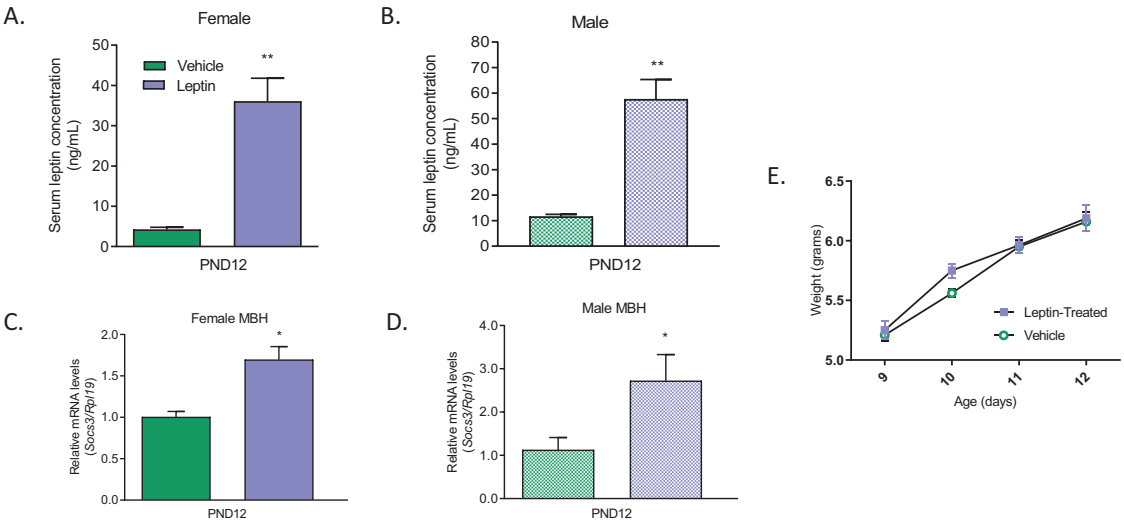

Figure 5. Serum leptin levels and hypothalamic *Socs3* mRNA levels are increased in wild-type mice at PND12 after treatment with exogenous leptin. Serum leptin levels in (A) female and (B) male mice treated with exogeneous leptin (blue columns) compared with vehicle-treated controls (green columns). *Socs3* mRNA expression in the MBH of (C) female and (D) male mice in leptin-treated (blue columns) compared with vehicle-treated controls (green columns). Data are shown as mean ± SEM (n = 5 per group; unpaired *t* test: $*P < 0.05$; $**P < 0.01$). (E) Mean body weight of male and female mice treated from PND9 to PND12 with leptin or vehicle (n = 10 per group). (Blue squares = leptin-treated; green open circles = vehicle-treated.)

neurons [8, 9]. The actions of leptin on puberty and reproduction appears to be through upstream GABA neurons as selective deletion of the leptin receptor in GABAergic, but not glutamatergic, neurons in mice resulted in absent or delayed pubertal onset [10, 11]. The ventral premammillary nucleus (PMV) has been suggested as a site of action of leptin because ablation of this area in rodents in *ob/ob* mice attenuates the ability of exogenous leptin to rescue the phenotype of delayed sexual maturation and re-expression of the leptin receptor in PMV neurons rescues the phenotype of delayed puberty in leptin receptor null mice [9]. The effects in the PMV may be mediated by the neuromodulator pituitary adenylate cyclase activating polypeptide (PACAP) because deletion of PACAP from leptin-responsive neurons in the PMV led to delayed puberty and impaired reproduction in female mice [31]. Additionally, AgRP neurons in the ventromedial nucleus, which are GABAergic, have been suggested as the site of action for leptin's influence on reproduction [32, 33]. However, whether leptin's actions on puberty may be influenced by regulation of MKRN3 has not been previously explored.

In this study, given that an increase in leptin levels occurs in mice before pubertal onset and is indispensable for reproductive maturation, and given that *Mkrn3* expression decreases before pubertal onset, we hypothesized that this decline in *Mkrn3* expression might be due to inhibitory actions of leptin. The failure of leptin-deficient mice to undergo pubertal maturation could be because loss of leptin prevents the decline of *Mkrn3* expression in the hypothalamus, such that Mkrn3 continues to prevent activation of the HPG axis, potentially through inhibition of kisspeptin and/or GnRH neurons. Using the *ob/ob* mouse, a model of leptin deficiency, we found that *Mkrn3* expression in the MBH and POA of the hypothalamus declined with age from PND12 to PND30 in a manner indistinguishable to that of wild-type mice in both males and females, despite the fact that *ob/ob* mice exhibit delayed or absent pubertal onset.

In a second experimental model, we administered leptin to wild-type mice before initiation of puberty onset, when *Mkrn3* expression is high in the hypothalamus compared with adult mice. If MKRN3 is inhibited by leptin, then we would anticipate that4*Mkrn3*

expression would be suppressed following exogenous administration of leptin. However, we found that *Mkrn3* mRNA levels were no different in the leptin-treated mice compared with wild-type control mice. Taken together, the findings from these 2 models suggest that leptin does not exert a direct or indirect inhibitory effect on *Mkrn3* expression, and further suggests that leptin is important for pubertal onset through pathways independent or downstream of MKRN3 action.

To support this conclusion, it was necessary to confirm the adequacy of our leptin administration paradigm. Serum leptin levels were demonstrated to be increased in the leptin-treated juvenile mice compared with control mice, confirming that the doses and routes of leptin administration were sufficient to increase circulating leptin levels. To further ensure that the leptin was biologically active and activated leptin receptors centrally, we measured *Socs3* expression in the MBH. *Socs3* expression is induced by leptin-mediated activation of cellular Jak-STAT3 signaling pathways [24]. We confirmed that *Socs3* mRNA levels were indeed increased in the MBH in leptin-treated mice compared with vehicle-treated control mice. The effect of leptin administration on body weight at this early age has not been examined in prior studies, and we documented that exogenous leptin administration from PND9 to PND12 did not affect body weight. There may be a critical window, or duration of exposure, during which body weight is affected by leptin because, conversely, leptin-deficient mice do not increase body weight compared with control animals until after 4 weeks of age [34].

A potential limitation of this study is that the dose of leptin administered may be have been inadequate to exert an effect on *Mkrn3* mRNA expression. This possibility was in large part addressed by measuring serum leptin levels and *Socs3* mRNA expression in the MBH, although we cannot exclude the possibility that the levels of leptin needed to affect *Mkrn3* expression may be higher than those needed to increase *Socs3* mRNA in the MBH and POA. Alternatively, because this study examined potential regulation of Mkrn3 by leptin in the MBH and POA, another possibility is that leptin could regulate Mkrn3 in different regions of the brain. Additionally, it is possible that leptin could exert an effect on Mkrn3 in an age-dependent manner not captured by the ages of mice examined in this study; however, the aim of this study was to explore the potential impact of leptin on Mkrn3 during a developmental period when Mkrn3 levels are high maximize the ability to observe a potential regulatory effect [14].

Taken together, these data support that although both leptin and MKRN3 are vital in pubertal onset, they appear to act through independent pathways. Because *Mkrn3* mRNA expression in the hypothalamus was not affected by either the absence nor by supplementation of leptin, MKRN3 may be acting in a parallel pathway or, alternatively, leptin may be acting downstream of MKRN3. Our results show high levels of *Mkrn3* mRNA expression at PND12, with lower levels at PND30, recapitulating the expression pattern in wild-type mice previously reported in the ARC and demonstrating a similar expression pattern in the POA [14]. As *Mkrn3* mRNA expression levels decline in these hypothalamic areas before pubertal onset, these findings indirectly support the hypothesis that MKRN3 may be exerting an inhibitory effect on kisspeptin and/or GnRH neurons. Thus, when MKRN3 declines with age, this contributes to a lessening of inhibitory input and promotion of stimulatory factors on GnRH secretion, allowing puberty to begin.

Interestingly, our results do not demonstrate sexual dimorphism in *Mkrn3* mRNA expression in the POA at PND12 or PND30, in contrast to the sexually dimorphic Kiss1 neuronal populations in the AVPV [25]. Female mice have Kiss1 neurons in the AVPV, whereas this population is nearly absent in male mice [25]. The exploration of sex-specific *MKRN3* expression in the hypothalamus has not been previously reported but is noteworthy because girls with loss-of-function mutations in *MKRN3* leading to CPP exhibit a significantly earlier age of pubertal onset compared with boys [15]. The mechanisms by which MKRN3 exerts differential sex-specific effects on the timing of puberty remain to be determined, but do not appear to be related to differences in MKRN3 expression. The possibility of sex-specific differences in the accurate diagnosis of CPP also needs to be considered [35].

In summary, regulation of *Mkrn3* expression by leptin was not observed in either the MBH or the POA, 2 hypothalamic regions important for pubertal maturation. These data suggest that the decrease in *Mkrn3* expression just before the onset of puberty may occur independently of leptin and that other regulators of MKRN3 remain to be elucidated. Further investigation is needed to understand the mechanism of action of MKRN3 and the regulatory factors leading to the decline in *Mkrn3* expression before pubertal onset.

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Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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