Rabconnectin-3α is required for the morphological maturation of GnRH neurons and kisspeptin responsiveness

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A few hundred hypothalamic neurons form a complex network that controls reproduction in mammals by secreting gonadotropin-releasing hormone (GnRH). Timely postnatal changes in GnRH secretion are essential for pubertal onset. During the juvenile period, GnRH neurons undergo morphological remodeling, concomitantly achieving an increased responsiveness to kisspeptin, the main secretagogue of GnRH. However, the link between GnRH neuron activity and their morphology remains unknown. Here, we show that brain expression levels of Dmxl2, which encodes the vesicular protein rabconnectin-3α, determine the capacity of GnRH neurons to be activated by kisspeptin and estradiol. We also demonstrate that Dmxl2 expression levels control the pruning of GnRH dendrites, highlighting an unexpected role for a vesicular protein in the maturation of GnRH neuronal network. This effect is mediated by rabconnectin-3α in neurons or glial cells afferent to GnRH neurons. The widespread expression of Dmxl2 in several brain areas raises the intriguing hypothesis that rabconnectin-3α could be involved in the maturation of other neuronal populations.

GnRH neurons are integral members of a complex neuronal network within the hypothalamus that controls puberty onset and fertility1. Puberty is due to the reactivation of the hypothalamic-pituitary-gonadal (HPG) axis, involving a robust increase in GnRH pulsatile release to elicit luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from the anterior pituitary2,3. LH and FSH in turn stimulate gonadal sex steroids secretion and gametogenesis. Since 2003, kisspeptins (Kp) have emerged as major hypothalamic peptides, which control GnRH release (for a review see ref. 4). In fact, Kp act through the activation of a G-protein coupled receptor (KISS1R (GPR54)), which is expressed on the surface of GnRH neurons. Loss of function of GPR54 has been initially shown to cause GnRH deficiency5,6. Thereafter, Kp neurons were revealed to mediate the negative feedback of sex steroid hormones, as well as the positive feedback of the estradiol on the gonadotropic axis. The reactivation of the gonadotropic axis at puberty is associated with an increase of Kp signaling in GnRH neurons. Failure of the GnRH system to develop or function properly has been largely described and may be associated with complex syndromes characterized by lack of pubertal onset and neurodevelopmental defects7,8. Although mutations have been identified in several genes linked to these syndromes, the mechanism of the neurodevelopmental defect remains obscure.

We recently described a new complex neuronal disorder in humans associated with pubertal and fertility defects due to low expression of DMXL29. Investigations in a Dmxl2 deficient mouse line revealed that haploinsufficiency of Dmxl2 in neurons (nes::cre;Dmxl2wt/loxp) causes infertility due to a partial GnRH deficiency9. Dmxl2 encodes the vesicular protein rabconnectin-3α (rbcn3-α), which was first identified as a scaffolding protein and a protein partner of the GTPase protein Rab3A10,11, specifically Rab3-GTPase activating protein (Rab3-GAP) and Rab-3 Exchange Protein (Rab3-GEPI). Rbcn3-α has also been shown important for cell-cell signaling and intracellular receptor trafficking12, as well as in the acidification of intracellular organelles by interacting with a subunit of the V-ATPase proton pump13,14.

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We previously found that rbcn-3α is expressed in the organum vasculosum of the lamina terminalis (OVLT) and in the median eminence (ME)9, where GnRH neurons undergo extensive dendritic morphogenesis during the juvenile period of rodent life15. Rbcn-3α-immunoreactive vesicles were previously found expressed inside GnRH nerve terminals in the ME9, suggesting a possible role of rbcn-3α in GnRH release. Interestingly, GnRH neuronal dendrites possess spines, receive extensive synaptic inputs along their entire length16 and exhibit a striking degree of structural and functional plasticity over postnatal rodent development15. However, the link between GnRH neuron activity and their morphology remains unknown.

We previously reported that nes::cre;Dmxl2wt/loxp mice display a 30% loss of GnRH-immunoreactive neurons9. As such defect cannot fully explain the reproductive deficit observed in these mice, we suspected that an additional functional alteration might affect the GnRH neuronal network of nes::cre;Dmxl2wt/loxp mice. As the use of the cre recombinase under the control of the nestin promoter results in the deletion of the critical exon in neuronal progenitors17, we thus sought to clarify whether low expression of rbcn3-α in neurons or glial cells may disturb the maturation and the activation of GnRH neurons, and what is the underlying mechanism leading to the reproductive deficit in these mice. As rbcn3-α is ubiquitously expressed in the brain and its function is vital after birth, we propose that the characterization of rbcn3-α function in the GnRH neuronal network will bring new insights on the understanding of neuronal maturation.

Results

Dmxl2 low expression impedes the morphological changes of the GnRH neuronal dendritic tree at puberty. Different studies have established that morphology, spine density, and topography of GnRH neurons are important for GnRH function15,18. In particular, GnRH neurons exhibit more complex/immature dendritic arborization during the juvenile period of rodent life15. Rbcn-3α-immunoreactive vesicles were previously found inside GnRH nerve terminals in the ME9, suggesting a possible role of rbcn-3α in GnRH release. Interestingly, GnRH neuronal dendrites possess spines, receive extensive synaptic inputs along their entire length16 and exhibit a striking degree of structural and functional plasticity over postnatal rodent development15. However, the link between GnRH neuron activity and their morphology remains unknown.

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Kp-10 fails to induce LH secretion and GnRH neuron activation in nes::cre;Dmxl2wt/loxp male mice. The abnormal GnRH dendritic morphologies in nes::cre;Dmxl2wt/loxp male mice suggested a defect in the functional maturation of GnRH neurons. To address this, we analyzed if Kp-10 intraperitoneal (i.p) injections were able to induce LH secretion in nes::cre;Dmxl2wt/loxp male mice compared to Dmxl2wt/loxp littermates. Indeed, Kp-10 induced a large increase in LH concentration in Dmxl2wt/loxp male mice, whereas no rise in LH concentration was seen after nes::cre;Dmxl2wt/loxp mice were subjected to Kp-10 (Fig. 2a). We next investigated if the absence of this response could be due to a failure of Kp-10 to induce GnRH neuron activation. To address this, analysis of cFos positive labeled rPOA GnRH neuronal nuclei was used as a read-out of Kp-10-induced GnRH neuron activation. Injections of Kp-10 (i.p.) in Dmxl2wt/loxp male mice induced an increase in the percentage of rPOA cFos-GnRH positive nuclei as compared to PBS-treated Dmxl2wt/loxp male mice (Fig. 2b). By contrast, the percentage of dually labeled cFos-GnRH neurons in Kp-10-treated nes::cre;Dmxl2wt/loxp mice was not different to that of PBS-treated nes::cre;Dmxl2wt/loxp male nor PBS-treated Dmxl2wt/loxp mice (Fig. 2b).

GnRH neurons in nes::cre;Dmxl2wt/loxp female mice do not respond to the E2 positive feedback paradigm. We previously reported that nes::cre;Dmxl2wt/loxp female mice exhibit a normal development of antral follicles but lower number of corporal lutea, which was associated with infertility. The infertility in female nes::cre;Dmxl2wt/loxp mice could therefore be related to the inability of GnRH neurons to respond to the Estradiol (E2)-induced positive feedback. In rodents, high E2 leads to positive feedback, which is mediated by increased Kp-10 signaling in GnRH neurons, leading to a GnRH/LH surge at the time of lights out on the day of proestrus. Ovariectomized (OVX)-Dmxl2wt/loxp female mice implanted with low E2 capsule for 1-week exhibited higher LH values at 18:00 h (LH surge) when given a bolus of E2 as compared to vehicle (Veh)-treated OVX-Dmxl2wt/loxp females (Fig. 3a). However, nes::cre;Dmxl2wt/loxp mice could not elicit a LH surge at the time of lights out despite the hormonal regimen (Fig. 3a). Indeed, LH levels in tail-tip sampling over two hours between 18:00–20:00 h remained very low, showing no sign of an LH peak or surge, whereas OVX+E2-treated Dmxl2wt/loxp exhibited a LH surge (Fig. 3b). Unexpectedly, nes::cre;Dmxl2wt/loxp mice exhibited significantly higher LH values at 10:00 h as compared to 18:00 h when given a bolus of E2, and the same results were found when they were given vehicle treatment (Fig. 3a,b).

To assess proper GnRH neuron activation at the time of the LH surge, the percentage of cFos-GnRH neurons was analyzed at 18:00 h in OVX+E2 female mice. A significant increase in cFos-GnRH positively labeled GnRH neurons was observed in E2-treated Dmxl2wt/loxp female mice as compared to Veh-treated Dmxl2wt/loxp mice (Fig. 3c). By contrast, E2-treated nes::cre;Dmxl2wt/loxp female mice exhibited the same percentage of cFos-GnRH positively labeled GnRH neurons as Veh-treated nes::cre;Dmxl2wt/loxp females (Fig. 3c). To note, Veh-treated nes::cre;Dmxl2wt/loxp female mice exhibited a significant higher cFos-GnRH positive staining at 18:00 h when compared to Veh-treated Dmxl2wt/loxp females. Collectively, these data show that nes::cre;Dmxl2wt/loxp female mice do...
not display a proper positive feedback response to E₂, which is compounded by an abnormal timing of the LH surge in the morning. In addition, a constitutive neuronal activation was observed at 18:00 h in both Veh- and E₂-treated nes::cre;Dmxl2wt:loxp animals.

We next assessed whether the defect in GnRH-neuronal activation in nes::cre;Dmxl2wt/loxp mice was potentially associated to their defect in GnRH morphological maturation. To test this, cFos-GnRH expression was quantified per group of GnRH neurons based on their morphology in Kp-10 treated and OVX + E₂ treated Dmxl2wt/loxp mice. Solid line: nes::cre;Dmxl2wt/loxp mice. Dashed lines; Dmxl2wt/loxp mice. Black bar: nes::cre;Dmxl2wt/loxp mice. White bar: nes::cre;Dmxl2wt/loxp mice.

**Figure 3.** GnRH neurons in OVX nes::cre;Dmxl2wt/loxp female mice are unresponsive to E₂-positive feedback. (a) Plasma LH levels measured at 10:00 h and 18:00 h in both E₂- and Veh-treated nes::cre;Dmxl2wt/loxp and Dmxl2wt/loxp ovariectomized (OVX) female mice (n = 5 for each group and time point, mean ± SEM, **p < 0.01, ****p < 0.0001). (b) Top graph depicts the LH levels in a pulsatile manner during 10-min intervals for 2-hours in OVX + E₂ Dmxl2wt/loxp (n = 4) and OVX + E₂ nes::cre;Dmxl2wt/loxp mice (n = 4) mice between 10:00 h and 12:00 h. Bottom graph depicts LH concentrations over 30-min intervals for 2-hours between 18:00 h and 20:00 h in OVX + E₂ Dmxl2wt/loxp (n = 5) and OVX + E₂ nes::cre;Dmxl2wt/loxp mice (n = 4). Dashed lines; Dmxl2wt/loxp mice. Solid line; nes::cre;Dmxl2wt/loxp mice. (c) cFos positive rPOA GnRH-ir neurons in OVX + E₂- and OVX + Veh-treated nes::cre;Dmxl2wt/loxp and Dmxl2wt/loxp adult female mice. Percentage of the total number of GnRH neurons (d) cFos positive GnRH neurons related to the GnRH neuron morphology in Kp-10-treated male (n = 4) and E₂-treated female mice at 18 h (n = 4) (mean ± SEM, **p < 0.001, ****p < 0.0001). Percentage of cFos-GnRH-ir neurons to the total number of counted GnRH neuron in each group. The numbers of unipolar, bipolar and complex cFos-positive GnRH neurons counted in each subgroup were 74, 60, 8 in Dmxl2wt/loxp mice and 16, 12, 2 in nes::cre;Dmxl2wt/loxp mice, respectively. White bar: nes::cre;Dmxl2wt/loxp mice. Black bar: nes::cre;Dmxl2wt/loxp mice.
$Dmxl2^{wt/loxp}$ and $nes::cre;Dmxl2^{wt/loxp}$ male and female mice. Unipolar GnRH neurons displayed a higher cFos staining than bipolar neurons in wild type mice (Fig. 3d). Despite the fact that complex/immature GnRH neurons responded to Kp-10 and E2, in $Dmxl2^{wt/loxp}$ mice, it should be noted that there were extremely few immature GnRH neurons in $Dmxl2^{wt/loxp}$ mice. By contrast, $nes::cre;Dmxl2^{wt/loxp}$ mice exhibited significantly more complex/immature GnRH neurons with almost no cFos-GnRH positive labeling (Fig. 3d). With this, $nes::cre;Dmxl2^{wt/loxp}$ mice displayed significantly less cFos-GnRH positive labeling in unipolar and complex GnRH neurons, whereas no change was observed in bipolar neurons (Fig. 3d). In summary, the number of complex GnRH neurons was higher in $nes::cre;Dmxl2^{wt/loxp}$ mice and their capacity to be activated was almost completely abolished. In contrast to unipolar or complex neurons, bipolar neurons appeared to be insensitive to low expression of $Dmxl2$ in neuronal progenitor-derived cells.

Kisspeptin expression in the antero-ventral periventricular nucleus (AVPV/PeN) is disturbed in $nes::cre;Dmxl2^{wt/loxp}$ mice. To analyze the consequences of $Dmxl2$ neuronal haploinsufficiency on kisspeptin neurons, we first quantified Kiss1 mRNA by quantitative PCR (qRT-PCR) from total RNA extracted from hypothalamus of $nes::cre;Dmxl2^{wt/loxp}$ mice. This analysis was performed in males and in diestrus females at PND 60. In both sexes, we did not observe any difference of the relative levels of hypothalamic Kiss1 mRNA to GAPDH between $Dmxl2^{wt/loxp}$ and $nes::cre;Dmxl2^{wt/loxp}$ mice (Fig. 4a). This first result indicated that the gonadotropic deficiency observed in $nes::cre;Dmxl2^{wt/loxp}$ mice was not due to a dramatic change of Kiss1 expression in the hypothalamus. However, our analysis did not take into account the fact that Kp neurons in the anteroventral periventricular (AVPV/PeN) nucleus are positively controlled by estradiol in female mice, whereas in the arcuate nucleus, estradiol acts as a negative regulator of Kiss1 expression in both sexes. It was therefore necessary to assess Kp expression by another approach which differentiated Kp expression in the AVPV/PeN from the arcuate nucleus. To do this, we quantified the total number of Kp-ir neurons in the AVPV/PeN. In $nes::cre;Dmxl2^{wt/loxp}$ males, we observed significantly higher number of AVPV/PeN Kp-ir neurons as compared to control males (Fig. 4b). In female mice, results were more complex. As expected, O VX + E2 treated $Dmxl2^{wt/loxp}$ females displayed higher number of Kp-ir neurons in the AVPV/PeN at 18:00 h when compared to 10:00 h (Fig. 4c). By contrast, there were significantly more AVPV/PeN Kp-ir neurons in O VX + E2 treated $nes::cre;Dmxl2^{wt/loxp}$ mice at 10:00 h as compared to 18:00 h (Fig. 4c). This staining corresponded to the inverted LH levels (See Fig. 3a,b). To note, Kp-ir staining in the arcuate nucleus was similar between wild type and $nes::cre;Dmxl2^{wt/loxp}$ female mice in diestrus (data not shown). The consequences of low expression of rbcn3α in the brain thus differs between sexes. In $nes::cre;Dmxl2^{wt/loxp}$ male mice, GnRH neurons exhibit a resistance to Kp associated with an increase of Kp expression in the AVPV/PeN. In $nes::cre;Dmxl2^{wt/loxp}$ female mice, Kp expression analysis suggests a defect in the control of its diurnal expression in AVPV/PeN.

Lack of $Dmxl2$ expression in GnRH neurons does not affect their functional maturation. As rbcn3-α is expressed in GnRH neurons, the functional defect observed in $nes::cre;Dmxl2^{wt/loxp}$ could be attributable to deletion of $Dmxl2$ in GnRH neurons alone, or cumulative defects in both afferent and GnRH neurons as well as in afferent neurons or glial cells only. To test this hypothesis, we created and analyzed another mouse model with ablated $Dmxl2$ in GnRH neurons. $GnRH::cre;Dmxl2^{wt/loxp}$ mice exhibited a slight delay in pubertal onset and $GnRH::cre;Dmxl2^{wt/loxp}$ mice showed a mild reproductive phenotype with a delayed first ovulation but a normal ovarian cyclicity in adult female mice, and a normal ano-genital distance (AGD) in male mice (Fig. 5a–e). To assess if there was a defect in the number of GnRH neurons, we analyzed the number and the distribution of GnRH neurons in the three genotypes. $GnRH::cre;Dmxl2^{wt/loxp}$ and $GnRH::cre;Dmxl2^{wt/loxp}$ had significantly less GnRH-ir neurons as compared to $Dmxl2^{wt/loxp}$ mice (Fig. 6a). This loss of GnRH-ir neurons was most significant in the OVLT in both $GnRH::cre;Dmxl2^{wt/loxp}$ and $GnRH::cre;Dmxl2^{wt/loxp}$ mice as compared to wild type mice (Fig. 6b,c). Because $nes::cre;Dmxl2^{wt/loxp}$ mice harbored a more severe reproductive phenotype to that of $GnRH::cre;Dmxl2^{wt/loxp}$ mice, yet a similar GnRH neuronal loss to that of $GnRH::cre;Dmxl2^{wt/loxp}$ mice and $GnRH::cre;Dmxl2^{wt/loxp}$, we questioned what separated the phenotype in $GnRH::cre;Dmxl2^{wt/loxp}$ knock out mice from that of $nes::cre;Dmxl2^{wt/loxp}$ mice. To test this, we subjected $Dmxl2^{wt/loxp}$, $GnRH::cre;Dmxl2^{wt/loxp}$ and $GnRH::cre;Dmxl2^{wt/loxp}$ mice to Kp-10 stimulation. A Kp-10-induced LH increase was observed in mice of all genotypes, (Fig. 6d). Confirming proper GnRH neuron activation, we found that the number of cFos positive GnRH neurons in Kp-10-treated $GnRH::cre;Dmxl2^{wt/loxp}$ mice did not differ from that of Kp-10-treated $Dmxl2^{wt/loxp}$ mice (Fig. 6e). The cumulative reproductive deficit in $nes::cre;Dmxl2^{wt/loxp}$ mice thus extends beyond the GnRH neuron deficit alone.

Discussion

Herein, we found that $Dmxl2$ deficiency in mouse brain reduces the physiological transition of immature GnRH neurons toward mature neurons along with a reduced GnRH immunoreactivity within the OVLT and an absence of GnRH neuronal responsiveness to Kp-10 in males. This resistance to Kp-10 was associated to an increase of Kp expression in the AVPV/PeN. By contrast to male mice, $nes::cre;Dmxl2^{wt/loxp}$ female mice did not develop a kisspeptin resistance but rather an abnormal control of Kp expression during the day. Although we observed a decrease of GnRH-ir neurons in mice in which $Dmxl2$ was deleted solely in GnRH neurons, the reproductive phenotype observed was mild and did not yield the functional defect as to which we observed in $nes::cre;Dmxl2^{wt/loxp}$ mice. These results reveal the critical role of the expression level of rbcn3α in the brain for the post-natal homeostasis of the GnRH neuronal network.

The analysis of $GnRH::cre;Dmxl2^{wt/loxp}$ knock-out mice highlights that the cumulative functional defect observed in $nes::cre;Dmxl2^{wt/loxp}$ mice is most likely due to the extrinsic role of rbcn3-α in afferent neurons or in glial cells, with a less important intrinsic role in GnRH neurons. Indeed, cre-recombinase under the control of the nestin promoter leads to a deletion of the critical exon at the stage of neuronal progenitors which can be further
differentiated in neurons or glial cells. In addition to the crucial role of several hypothalamic neurons, the contribution of glial cells in the control of GnRH neurons is well known. Glial cells secrete small molecules that directly activate GnRH neurons. Glial cells also participate in the control of the GnRH release at the neurohemal junction in the ME upon the control of nitric oxide produced by endothelial cells. As rbcn3-α is expressed in tanycytes in the ME, a dysfunction in the structural plasticity of tanycytes could perturb the control of GnRH release and thus could contribute to the phenotype in nes::cre;Dmxl2wt/loxp female mice. However, we suspect that this effect likely plays a minor role in the phenotype of these mice when compared to the abnormal maturation of GnRH neuronal dendrites with the disorganized control of Kp expression.

GnRH neurons respond to endogenous Kp concomitantly with their morphological remodeling, however, the functional link between these two events has not been previously explored. To date, complex GnRH dendritic morphology was reported in only one mouse model exhibiting delayed puberty and fertility defects, yet the functional relevance of the immature GnRH morphology was not explored. In our study, we found an increase of complex/immature GnRH dendritic morphology in the OVLT in nes::cre;Dmxl2wt/loxp mice, associated with a decrease of unipolar morphology, and no changes in the bipolar GnRH dendritic morphology. These data indicate that the balance between the stabilized-to-destabilized GnRH dendritic morphology is disturbed when the expression of Dmxl2 is low in the brain. Complex/immature GnRH dendritic morphology in nes::cre;Dmxl2wt/loxp

Figure 4. Kisspeptin expression is altered in nes::cre;Dmxl2wt/loxp mice. (a) Relative expression of hypothalamic Kiss1 mRNA in Dmxl2wt/loxp (males: n = 7; females: n = 5) and nes::cre;Dmxl2wt/loxp adult mice (males: n = 10; females: n = 8) (p > 0.05). (b) Left: Photomicrograph depicting Kp-ir neurons in the AVPV/PeN in one adult Dmxl2wt/loxp (top) and nes::cre;Dmxl2wt/loxp (bottom) male mice. Scale bars 300-μM. Quantification of AVPV/PeN Kp-ir neurons in Dmxl2wt/loxp (n = 5) and nes::cre;Dmxl2wt/loxp male mice (n = 5; Mean ± SEM, ****p < 0.0001). (c) Photomicrograph depicts Kp-ir staining in the AVPV/PeN in OVX + E2 treated Dmxl2wt/loxp (left) and nes::cre;Dmxl2wt/loxp (right) female mice at 10:00 h and 18:00 h. Scale Bar 10-μM. (d) Quantification of AVPV/PeN Kp-ir neurons in OVX + E2 treated Dmxl2wt/loxp (n = 4–5) and nes::cre;Dmxl2wt/loxp (n = 4–5) female mice at 10:00 h and 18:00 h (Mean ± SEM, ***p < 0.001). White bar: nes::cre;Dmxl2wt/loxp mice. Black bar: nes::cre;Dmxl2wt/loxp mice.
mice failed to exhibit cFos activation after Kp-10 and/or E2 treatment, whereas mature unipolar GnRH neurons, albeit at a lesser level, still exhibited significantly higher cFos activation than complex neurons. Unipolar and bipolar GnRH neurons are thus more prone to be stimulated by Kp-10 and E2 than complex neurons in nes::cre;Dmxl2wt/loxp mice. These results pinpoint that the dendritic pruning of GnRH neurons during the juvenile period could participate to the pubertal increase of GnRH neuron responsiveness to Kp and E2. It could also be a concomitant event without any functional relationship to the increase Kp effect on GnRH neurons at puberty. Surprisingly, LH blood concentrations were increased at 10:00 h in E2-treated nes::cre;Dmxl2wt/loxp mice. The correlation between LH concentrations with Kp expression in the AVPV/PeN in OVX + E2 nes::cre;Dmxl2wt/loxp mice at different times of the day, implies that GnRH neurons likely responded to endogenous Kp in the morning (10:00 h) instead at night of proestrus at lights out (18:00 h). In nes::cre;Dmxl2wt/loxp adult males, the relatively mild effect that Dmxl2 haploinsufficiency had on testis weight contrasts with the complete resistance of GnRH neurons to Kp-10. In fact, Kiss1R knockout mice exhibit very small testes in adulthood5. Further studies are necessary to delineate the age at which GnRH neurons become resistant to Kp-10 in nes::cre;Dmxl2wt/loxp male mice. We suspect the major difference of Kp signaling between sexes in nes::cre;Dmxl2wt/loxp mice is related to the sexual dimorphism of the GnRH neuronal network24. High LH levels were also observed in Veh-treated nes::cre;Dmxl2wt/loxp mice indicating that this increase in LH is likely E2 independent. The abnormal high expression of Kp in the AVPV/PeN at 10:00 h nes::cre;Dmxl2wt/loxp female mice revealed an advanced phase of Kp expression. The circadian timing of the GnRH neuronal network is under the control of neurons of the suprachiasmatic nucleus (SCN)25. As rbcn3-α is expressed in the ventral part of the SCN9, an abnormal control of the GnRH neuronal network by the SCN may be one explanation for the advanced phase of the LH release. Arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP) are both expressed by SCN neurons. AVP and VIP neurons were shown to contact Kp neurons and GnRH neurons, respectively26. AVP exerts daily signals onto Kp neurons, of which is highly dependent on circulating levels of mice.
estradiol, but not on the time of the day. VIP neurons has also been shown to participate to the GnRH/LH surge but it is probably less critical than AVP for the timing of the surge during the late afternoon of proestrus. In addition, Kp neurons were shown to express clock genes and to display an intrinsic circadian oscillator upon the control of estradiol. Further studies will delineate whether the abnormal daily expression of Kp in the AVPV/PeN in nes:cre;Dm xl2lox/lox mice is related to an abnormal control of the GnRH neuronal network by AVP/VIP neurons and/or to an alteration of the intrinsic circadian oscillator of Kp neurons.

Changes in synaptic inputs and cell-cell signaling onto GnRH neurons contribute to the GnRH neuronal plasticity. These synaptic inputs can change afferent neuronal activity as well as retraction or apposition.

Figure 6. GnRH::cre;Dm xl2 knock-out mice exhibit GnRH neuronal loss yet a normal GnRH neuron response to Kp-10. (a–c) Quantification and distribution of GnRH-ir neurons in Dm xl2lox/lox, GnRH::cre;Dm xl2lox/lox, and GnRH::cre;Dm xl2lox/lox mice (n = 5 in all genotypes). (a) Total number of GnRH-ir neurons in the hypothalamus and (b) Rostral to caudal distribution of GnRH-ir neurons, where OVLT is marked numerically as 0, with rostral GnRH-ir neurons in negative numerical values and caudal GnRH-ir neurons in positive numerical values. Dm xl2lox/lox (blue), GnRH::cre;Dm xl2lox/lox (green), GnRH::cre;Dm xl2lox/lox (red) (c) Total number of GnRH-ir neurons in the OVLT. (d) Plasma LH concentration in Kp-10 and PBS-treated Dm xl2lox/lox, GnRH::cre;Dm xl2lox/lox, and GnRH::cre;Dm xl2lox/lox mice (n = 5 in all groups and genotypes). (e) cFos-GnRH neurons in both Kp-10 and PBS-treated Dm xl2lox/lox and GnRH::cre;Dm xl2lox/lox male mice (n = 5 in all groups and genotypes) (mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001). White bar: Dm xl2lox/lox. Gray bar: GnRH::cre;Dm xl2lox/lox. Black bar: GnRH::cre;Dm xl2lox/lox.
of synapses on GnRH neuron perykaria. A defect in the synaptic plasticity in GnRH neurons in \textit{nes::cre}\textsubscript{Dmxl2}\textsuperscript{lox/lox} mice could explain in part the altered response to Kp-10 and E\textsubscript{2} stimulation. The biochemical function of rbcn3-\alpha supports the hypothesis of a synaptic defect when \textit{Dmxl2} expression is low in neurons. Indeed, rbcn-3 subparticipates in the acidification of intracellular vesicles through the control of the V-ATPase activity, where acidification is necessary for an optimal synaptic function. Many other cellular processes such as protein-processing and receptor-mediated endocytosis may also be disturbed. GnRH neurons receive inputs from GABAergic and glutamatergic neurons, of which both express estrogen receptor 1 (ESR1). The homeostasis of the GnRH neuronal network is highly disturbed in ESR1-deleted glutamatergic neuron mice. In some way, the phenotype observed in \textit{nes::cre}\textsubscript{Dmxl2}\textsuperscript{lox/lox} female mice is similar to the disrupted homeostasis of the GnRH neuronal network observed in ESR1-deleted glutamatergic neuron mice. The increase of the basal cFos staining in GnRH neurons in \textit{nes::cre}\textsubscript{Dmxl2}\textsuperscript{lox/lox} female mice as opposed to wild type females implies there could be an imbalance between excitatory and inhibitory synaptic inputs on GnRH neurons. This could be also due to the fact that there are less mature GnRH neurons and thus a compensatory mechanism is trying to override the poorly functional immature GnRH neurons. These results suggest a functional link between estrogen receptor signaling pathways, glutamatergic inputs on the GnRH neuronal network and the control of the V-ATPase activity.

Altogether, these results revealed the crucial role of the expression level of \textit{Dmxl2} in the brain for the functional and morphological maturation of GnRH neurons by afferent neurons or glial cells in mice. We also revealed the role of a vesicular protein on the timing of the LH surge upon the control of estradiol. This is a new mechanism of gonadotropic deficiency in mice, which is likely similar in \textit{DMXL2} mutated patients. This study opens new avenues for a better understanding of the mechanisms leading to GnRH neuron dysfunction.

**Methods**

**Animals.** \textit{nes::cre}\textsubscript{Dmxl2} and \textit{GnRH::cre} mice have been previously characterized. To obtain \textit{GnRH::cre}\textsubscript{Dmxl2}\textsuperscript{lox/lox} mice, we crossed \textit{Dmxl2}\textsuperscript{lox/lox} mice with \textit{GnRH::cre} transgenic mice. Both \textit{nes::cre}\textsubscript{Dmxl2} and \textit{GnRH::cre}\textsubscript{Dmxl2} lines were weaned at post-natal day (PND) 21 and tail biopsies were harvested for genotyping. \textit{GnRH::cre}\textsubscript{Dmxl2} female mice were checked for Vaginal Opening (VO) starting at PND 21. Upon VO, females were cycled for estrous cyclicality for 21 days to obtain 5 cycles. Anogenital Distance (AGD) was measured in male \textit{GnRH::cre}\textsubscript{Dmxl2} mice starting at PND 21 until PND 60. Animals were housed 4–5 per cage on a 12 h light: 12 h dark cycle (lights on 6am, lights off 18h).

Animal use was in compliance with Inserm guidelines for the care and use of laboratory animals in accordance with Paris Diderot University and Inserm. Mammalian research was approved by the Institutional Ethics Committees of Care and Use of Experimental Animals by Inserm and Paris Diderot University with the ethical approval number 2012-15-676-0099.

**Perfusion.** Animals were given an overdose of pentobarbital (3 mg/100-\mu l) and intracardially perfused with 4% paraformaldehyde (PFA) in 0.1 M Phosphate Buffer (PB) (pH 7.6). Brains were post-fixed overnight at 4°C, placed in 30% Sucrose/TBS (pH 7.6), frozen in 99% isopentane and kept at –80°C until sectioning.

**Immunohistochemistry.** For each experiment, five adult males (>PND 60) from each genotype, tissues were sectioned (Leica) in 40-\mu m serial coronal sections from the the Medial Septum (MS) to the rostral preoptic Area (rPOA).

**Chromagen labeling.** Endogenous peroxidase activity was inhibited with 40% methanol, 1% H\textsubscript{2}O\textsubscript{2} and 0.05 M TBS (pH 7.6). Sections were rinsed with TBS1x and blocked in an incubation solution (ICS) containing 10% normal goat serum, 0.25% BSA in 0.05 M TBS and 0.3% Triton 100-X, pH 7.6 for 1-hour at RT. Sections were incubated in ICS containing 10% NGS in 1/5000 guinea-pig polyclonal GnRH antibody (generous gift from Dr. Greg Anderson, Table S1) for 72-hrs at 4°C for GnRH neuron immunoreactivity, or in 1/5000 rabbit polyclonal anti-Kisspeptin-10 antibody (AC564, generous gift from Dr. Alain Caraty, Table S1). After rinses, sections were incubated in biotinylated goat anti-guinea pig antibody or biotinylated donkey anti-rabbit antibody (1/200; Vector Laboratories) for 90-mins at RT, washed and incubated with avidin-peroxidase (1/200) in ICS for 90 mins at RT (Vectastain ABC Kit, Vector Laboratories). GnRH and Kp-10 immunoreactivity was revealed using 0.05% diaminobenzadine (DAB) (Sigma Aldrich) with 0.01% H\textsubscript{2}O\textsubscript{2} in TBS 0.05 M (pH 7.6) for 15 mins at RT, then rinsed and coverslipped for analysis.

**Dual chromagen labeling** was carried out using the same method above with an addition step of using the glucose-oxidase/NiDAB method. Briefly, sections were incubated in rabbit polyclonal cFos primary antibody (1/5000; SC-52, Santa Cruz Biotechnology, Santa Cruz, CA), and in 1/15000 guinea-pig polyclonal GnRH antibody (GA04; a generous gift from Dr. Greg Anderson, Table S1) in ICS solution at 4°C for 48-hrs. After rinses, sections were incubated in biotinylated anti-rabbit (1/200; Vector Laboratories) for 90-min at RT, rinsed, and reacted with glucose oxidase and NiDAB for 5-mins at RT. Sections were then incubated in 10% NGS with polyclonal goat anti-guinea, (1/200; Vector Laboratories) for 90-mins at RT and reacted with 0.05% DAB (Sigma-Aldrich) in 0.01% H\textsubscript{2}O\textsubscript{2} and 0.05 M TBS1x for 15-mins, rinsed and coverslipped for analysis.

**Quantification of GnRH neurons in \textit{GnRH::Cre}\textsubscript{Dmxl2} mice.** Five adult males and females (>PN day 60) from each genotype were perfused and brains were sectioned coronally (45-\mu m) on a sliding microtome. Sections were processed for GnRH chromagen labeling immunohistochemistry (see above). Sections were mounted in rostral to caudal order from the Medial Septum (MS) to the Arcuate Nucleus (ARN). To obtain the global distribution of GnRH neurons, sections before the OVLT (rostral) were labeled with negative numbers and sections after the OVLT (caudal) were considered positive numbers. The OVLT was labeled as zero. GnRH
neurons were quantified in order and plotted in overall distribution from the diagonal band of broca to the median eminence, as previously reported.

Quantification of dually labeled GnRH-cfos positive neurons in the OVLT-rPOA.  To quantify cFos positive GnRH neurons, GnRH neurons were separated into two populations (OVLT and rPOA) where the distribution and number of dually labeled cFos-GnRH neuronal nuclei were counted in the regions of the Franklin and Paxinos brain atlas plates 22–24, 25–27, and 28–31, respectively (Franklin and Paxinos, 1997).

Quantification of GnRH dendritic morphologies.  Three coronal sections containing GnRH-ir neurons from the MS to the rPOA were viewed on a Nikon brightfield microscope and morphologies were scored, as previously described. Briefly, GnRH-ir neuronal morphologies were quantified based on three criteria: unipolar (mature; one dendrite directly off the GnRH soma), bipolar (mature; two dendrites directly off of the GnRH soma), or complex (immature; three or more dendritic processes directly off of the GnRH soma). Values of quantified GnRH dendritic morphologies were expressed as the percentage of the total GnRH neuron population analyzed.

In vivo studies.  Kisspeptin-10 (Kp-10) Injections.  Five male adult nes::cre;Dmxl2<sup>wt/loxp</sup> and GnRH::cre;Dmxl2<sup>2loxp/loxp</sup> mice were given intraperitoneal (i.p.) injections of 100-μl of 1-nmol Kp-10 (Sigma Aldrich) or PBS (control), 10-μl after injections, 4-μl of tail-tip blood was collected in duplicates. Mean LH levels were pooled through tail-tip sampling 10-mins after Kp-10 injections where tail tip blood was harvested for 2-hrs. LH levels were analyzed using an ELISA Luteinizing Hormone (LH) Sandwich Assay, as previously described. For quantification of cFos labeling, 2-hours after i.p. injection of Kp-10 or PBS (control), male mice were perfused, as described above, and brains were prepared for immunolabeling (see Immunohistochemistry).

Positive Feedback Paradigm and Evaluation of E<sub>2</sub>-mediated positive feedback effects on LH levels.  Adult nes::cre;Dmxl2<sup>wt/loxp</sup> and Dmxl2<sup>2loxp/loxp</sup> female mice, housed 4–5 per cage on a 12 h light (6am): 12 h dark (6 pm) were bilaterally ovariectomized (OVX) (Day 0) and implanted with 1-cm of low-dose E<sub>2</sub> silastic capsules containing 17-3-estradiol (1 μg/20 g) (Sigma Aldrich; inner diameter 0.212 cm; Dow Corning, MI), and Silastic medical-grade adhesive (0.1 mg/ml; Dow Corning), as previously reported. Six days after low-E<sub>2</sub> treatment, OVX female mice were subjected to subcutaneous injections of either 1 μg/20 estradiol benzoate (E<sub>2</sub>) mixed in sesame oil (100 μl) or sesame oil alone (Veh) as a control at (9:00 h). To measure LH pulsatility and to obtain LH pulsatility and the LH surge at the time of lights out (18:00), tail-tip blood samples were harvested every 10 mins between 10:00 h and 12:00 or between 18:00–20:00 h the day after E<sub>2</sub> and Veh treatment (See LH Elisa). To analyze cFos activation of GnRH neurons as well as AVPV Kp-10 immunoreactivity at different time points, we generated another group of adult OVX + E2 Dmxl2<sup>2loxp/loxp</sup> and nes::cre; Dmxl2<sup>2loxp/loxp</sup> females. After one week under hormonal regimen and 36-hrs after Estradiol Benzoate injection or Vehicle (Veh) treatment, animals were perfused for cFos analysis and for Kp immunoreactivity in AVPV/PeN (See Immunohistochemistry). To quantify Kp-ir neurons, three AVPV/PeN sections from each animal and treatment were quantified for the average number of Kp-ir neurons.

Image Analysis.  Light microscopy image acquisition was performed using an Zeiss LSM710 Apoime. For Chromagen labeling for GnRH, cFos, and kisspeptin neurons, images were acquired using Axiophot software using both 10x and 20x Plan Neofluor objectives for imaging (Numerical Aperature 0.3 and 0.5, respectively). Images were transferred to ImageJ software was used to quantify the number of cFos positive GnRH neurons and KP-positive neurons within defined regions. For cFos positive GnRH neurons were separated into two populations (OVLT and rPOA) where the distribution and number of dually labeled cFos-GnRH neuronal nuclei were counted in the regions of the Franklin and Paxinos brain atlas plates 22–24, 25–27, and 28–31, respectively (Franklin and Paxinos, 1997).

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LH ELISA assay.  LH levels were determined by a sandwich ELISA as described previously using the mouse LH–RP reference provided by A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). For all experiments, animals were habituated for 3-weeks prior to experiments, and on the day of experiments, blood was harvested in 10-min intervals (4 ul/sample) over a 2-hr period for each sex and genotype in PBS-Tween (0.05%).

qRT-PCR.  Adult nes::cre;Dmxl2<sup>wt/loxp</sup> and Dmxl2<sup>2loxp/loxp</sup> mice (n = 5–10 of each genotype and sex) were anesthetized with isoflurane vapor and immediately decapitated. After hypothalamic dissection, mRNA was extracted using Trizol (Invitrogen, Carlsbad CA, USA), as previously described. 1 μg of total RNA was used for the synthesis of OligoDT cdNA from the Superscript III First-Strand cdNA Synthesis kit (Invitrogen), following the manufacturer’s instructions. 2 μl of 1/50 diluted hypothalamic cdNA in duplicates was used for real time quantitative PCR (RT-qPCR) using SyberGreen MasterMix (Bio-Rad, Hercules, CA). Samples were processed in an iCycler qRT-PCR machine (Bio-Rad). Primer sequences for Kiss1 were 5′-TAACGAGTTCTCTCGGCTCCG-3′; 5′-CTCCTCCTTCTCTGCTTG1T-3′. GAPDH was used as an internal control 5′-GATGCTCTTGGCCACCACTCTTCT-3′; 5′-AATTGTGGTCCGTTGGATCGA-3′. All primers were used at a concentration of 0.15 μM. qRT-PCR conditions leading to an efficiency between 95–110% were selected. Relative differences in the cdNA concentration between baseline and experimental conditions were calculated using the comparative threshold cycle (Ct) method.

Statistical Analysis.  Statistical analyses were carried out using Prism software (GraphPad, La Jolla, CA). Comparisons between two groups were analyzed with a non-parametric Mann Whitney unpaired t-test. For multiple treatments and comparisons, one-way Anova and a post-hoc Newman-Keuls test were performed. Differences were considered significant when p < 0.05. All data are expressed as mean ± SEM for each group, genotype, and experiment.
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Author Contributions
B.K.T and N.D.R designed research. B.K.T C.H., Z.C, S.P and S.J performed research. B.K.T., Z.C, S.P and N.D.R analyzed data. B.K.T and N.D.R wrote the paper.

Additional Information
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