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RESEARCH REPORT

PLXNA1 and PLXNA3 cooperate to pattern the nasal axons that guide gonadotropin-releasing hormone neurons

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) neurons regulate puberty onset and sexual reproduction by secreting GnRH to activate and maintain the hypothalamic-pituitary-gonadal axis. During embryonic development, GnRH neurons migrate along olfactory and vomeronasal axons through the nose into the brain, where they project to the median eminence to release GnRH. The secreted glycoprotein SEMA3A binds its receptors neuropilin (NRP) 1 or NRP2 to position these axons for correct GnRH neuron migration, with an additional role for the NRP co-receptor PLXNA1. Accordingly, mutations in *SEMA3A*, *NRP1*, *NRP2* and *PLXNA1* have been linked to defective GnRH neuron development in mice and inherited GnRH deficiency in humans. Here, we show that only the combined loss of PLXNA1 and PLXNA3 phenocopied the full spectrum of nasal axon and GnRH neuron defects of SEMA3A knockout mice. Together with *Plxna1*, the human orthologue of *Plxna3* should therefore be investigated as a candidate gene for inherited GnRH deficiency.

KEY WORDS: GnRH neuron, Olfactory bulb, Testes, Axon guidance, Plexin, Semaphorin, Hypogonadotropic hypogonadism, Kallmann syndrome

INTRODUCTION

GnRH-secreting neurons are hypothalamic neuroendocrine cells that regulate sexual reproduction in mammals by stimulating the pituitary secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Merchenthaler et al., 1984). GnRH deficiency is the common hallmark of two genetic reproductive disorders, hypogonadotropic hypogonadism (HH) and Kallmann syndrome (KS), which can be due to defective GnRH neuron development (Boehm et al., 2015; Stamou and Georgopoulos, 2018).

A key period in GnRH neuron development is their migration from the nasal placode, where they are born, to their final positions in the hypothalamus. In the nasal compartment, GnRH neurons migrate along the intermingled axons of olfactory (OLF) and vomeronasal (VN) neurons, the cell bodies of which are located in the nasal placode-derived olfactory epithelium (OE) and

vomeronasal organ (VNO), respectively (Fig. S1A). To enter the brain, GnRH neurons migrate along the caudal branch of the VN (cVN) nerve, also known as the cranial nerve 0 or terminal nerve (Taroc et al., 2017; Yoshida et al., 1995). The axons in this transient nerve turn caudo-ventrally into the brain at the level of the cribriform plate (CP), which separates the brain from the nasal compartment (Taroc et al., 2017) (Fig. S1A). Instead, other VN and OLF axons project to the main and the accessory olfactory bulb (OB), respectively (Fig. S1A). Finally, GnRH neurons settle in the medial preoptic area (MPOA) of the postnatal hypothalamus to project to the median eminence (ME), where they act as neuroendocrine cells to release GnRH into the hypophyseal portal circulation (Wierman et al., 2011) (Fig. S1B). Accordingly, GnRH neurons can be identified in several distinct compartments of the embryonic head that reflect their migratory route (Fig. S1C).

The importance of proper axon scaffolds for GnRH neuron migration is illustrated by the analysis of a human foetus with a KS mutation; in this foetus, GnRH neurons accumulated in neural tangles in the meninges at the level of CP (Schwanzel-Fukuda et al., 1989). Mice lacking the axon guidance cue SEMA3A similarly accumulate GnRH neurons within axon tangles at the CP and are therefore hypogonadal (Cariboni et al., 2011). Moreover, they have a defective olfactory system, with many aberrant OLF axons (Schwarting et al., 2004). Agreeing with combined GnRH neurons and olfactory defects in mice lacking SEMA3A, *SEMA3A* mutations were subsequently identified in a subset of individuals with KS (Hanchate et al., 2012; Young et al., 2012). These findings support the idea that proper axon guidance is essential to ensure GnRH neuron migration through the nose and into the brain. Moreover, these findings identify mouse models as powerful tools for uncovering genes that regulate GnRH neuron development and may be mutated in individuals with inherited GnRH neuron deficiency.

To exert its functions, SEMA3A usually binds to transmembrane receptors composed of a ligand binding subunit that is either neuropilin (NRP) 1 or NRP2, and a signal transducing subunit, typically a member of the A-type plexin (PLXNA) family (Alto and Terman, 2017). Accordingly, mice lacking SEMA3A signalling through NRP1 and NRP2 have similar axon and GnRH neuron defects to mice lacking SEMA3A (Cariboni et al., 2011). Mutations in *NRP1*, *NRP2* and *PLXNA1* have also been found in individuals with KS (Kotan et al., 2019; Marcos et al., 2017), although PLXNA1 loss affects the GnRH neuron and olfactory systems in mice only mildly (Marcos et al., 2017). These findings raise the possibility that PLXNA1 acts in partial redundancy with another A-type plexin. Here, we have compared the expression pattern of all four Plxna genes during GnRH neuron development in the mouse and examined whether *Plxna1* synergises with *Plxna3* during nasal axon guidance required for proper GnRH neuron migration.

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RESULTS AND DISCUSSION
PLXNA1 and PLXNA3 are co-expressed during GnRH neuron migration

To establish which of the four Plxna genes is expressed in a pattern consistent with a role in guiding the axons that ensure GnRH neuron migration, we performed *in situ* hybridisation of sections through the wild-type mouse embryo nose. Agreeing with prior reports (Marcos et al., 2017; Murakami et al., 2001; Suto et al., 2003), *Plxna1* was expressed at E12.5 and E14.5 in both the VNO and OE, and *Plxna3* had a similar expression pattern (Fig. S2A,B). Additionally, *Plxna1* and *Plxna3* transcripts were detected in the migratory mass (MM) (Fig. S2A), a mixed population of cells that includes neurons and olfactory ensheathing cells (OECs) (Miller et al., 2010). In contrast, *Plxna2* and *Plxna4* appeared only weakly expressed in the VNO, OE or MM cells (Fig. S2A). We therefore focussed subsequent work on *Plxna1* and *Plxna3*.

To determine which cell types expressed PLXNA1 or PLXNA3 in the territories relevant to GnRH neuron migration, we immunostained sections through wild-type mouse embryo heads. Double labelling with the TUJ1 antibody for neuronal-specific β3 tubulin (nTUBB3) showed that PLXNA1 and PLXNA3 localised to the MM at E12.5 and to axons emerging from the OE and VNO at E12.5 and E14.5 (Fig. 1A,B). Double labelling for the OEC marker S100 showed that OECs lacked PLXNA1 and PLXNA3, but that they surrounded PLXNA1/PLXNA3 double-positive axons (Fig. 1C,D). Immunostaining of *Plxna1*$^{−/−}$ and *Plxna3*$^{−/−}$ mouse tissues validated antibody specificity (Fig. 1E). Double labelling for peripherin (PRPH), a marker of OLF and VN, including cVN axons (Fueshko and Wray, 1994; Taroc et al., 2017), confirmed that both PLXNs localised to E14.5 nasal axons; PLXNA1 was also prominent on cVN axons in the forebrain, whereas PLXNA3 staining of cVN axons was undetectable (Fig. 2). A prior study reported PLXNA1 immunostaining of E12.5 GnRH neurons (Marcos et al., 2017); in agreement, we observed low *Plxna1* transcript levels in FACS-isolated E13.5 GnRH neurons (Cariboni et al., 2007). Nevertheless, GnRH neurons in the E14.5 nose and ventral forebrain lacked obvious PLXNA1 or PLXNA3 protein (Fig. 2).

Together, these findings raise the possibility that PLXNA1 and PLXNA3 pattern the axons that guide GnRH neurons, either directly or by regulating the behaviour of pioneer cells in the MM that act as guide-post cells for the first nasal axons (Miller et al., 2010). Additionally, GnRH neurons may themselves express PLXNA1 for some time during their development.

Reduced GnRH neuron migration into the forebrain of *Plxna1*/*Plxna3*-null embryos

We next investigated whether combined loss of PLXNA1 and PLXNA3 impairs GnRH neuron migration more severely than loss of PLXNA1 alone. Thus, we analysed the number and position of GnRH neurons in embryos from parents carrying *Plxna1*-null and *Plxna3*-null alleles (Cheng et al., 2001; Yoshida et al., 2006). GnRH immunostaining showed that *Plxna1*$^{−/−}$ and *Plxna3*$^{−/−}$ single as well as *Plxna1*$^{−/−}$; *Plxna3*$^{−/−}$ double mutants had an overall similar number of GnRH neurons compared with wild-type littermates at E14.5 (Fig. 3A,C and Table S1). Whereas *Plxna1*$^{−/−}$ and *Plxna3*$^{−/−}$ single mutants had a similar number of neurons as wild types in the forebrain, *Plxna1*$^{−/−}$; *Plxna3*$^{−/−}$ double mutants contained significantly fewer GnRH neurons in the forebrain (Fig. 3A,C and Table S1). The loss of GnRH neurons from the brain of *Plxna1*$^{−/−}$; *Plxna3*$^{−/−}$ double mutants was explained by the statistically significant retention of GnRH neurons in the nose, including at the CP (Fig. 3B,C and Table S1). Similar results were obtained by *Gnrh in situ* hybridisation (Fig. S3A). As the overall number of GnRH neurons was similar in all these genotypes at this stage, the primary GnRH neuron defect in double mutants is likely the impaired forebrain entry.

Combined PLXNA1 and PLXNA3 loss increases morbidity and causes GnRH neuron, gonadal and olfactory system defects

To assess whether lack of GnRH neurons in the forebrain of E14.5 *Plxna1*$^{−/−}$; *Plxna3*$^{−/−}$ embryos results in an hypogonadal state in adulthood, we analysed postnatal *Plxna1*$^{−/−}$; *Plxna3*$^{−/−}$ mice. The analysis of five litters from parents with combined *Plxna1*-null and *Plxna3*-null alleles suggested that all genotypes were born at a normal Mendelian ratio (Table S2), but there was a high rate of pre-weaning mortality. As the *Plxna3* gene resides on the X chromosome, males in these litters are either wild type or hemizygous for the *Plxna3*-null mutation, i.e. *Plxna3*$^{y/+}$ or *Plxna3*$^{y/−}$, respectively. In contrast, females are *Plxna3*$^{+/+}$, *Plxna3*$^{+/−}$ or *Plxna3*$^{−/−}$. Pooled male and female mice lacking *Plxna3* are therefore referred to as *Plxna3*$^{−/−(y)}$. We found that two out of five juvenile *Plxna1*$^{−/−}$; *Plxna3*$^{−/−(y)}$ mutants were small and appeared stressed when handled and had to be culled before weaning to prevent suffering. To avoid the birth of further mutants with such severe adverse effects, breeding was concluded and all mutants obtained were culled for analyses.

We next compared the GnRH neuron number in the MPOA of postnatal mutants and wild-type controls, because this is the final position most neurons should attain. Whereas the GnRH neuron number in the *Plxna3*$^{y/−}$ MPOA was similar to that of wild-type littermates, three out of five *Plxna1*$^{−/−}$ mutants had slightly fewer and four out of four *Plxna1*$^{−/−}$; *Plxna3*$^{−/−(y)}$ mutants contained hardly any GnRH neurons in the MPOA (Fig. 3D,E and Table S3). Agreeing with a severely reduced GnRH neuron number, GnRH staining of the ME was nearly absent in *Plxna1*$^{−/−}$; *Plxna3*$^{−/−(y)}$ mutants (Fig. 3F), despite normal hypothalamic projections of other neuroendocrine neurons, such as those that secrete the corticotropin-releasing hormone CRH (Fig. S3B). Moreover, overall brain size was similar in all genotypes (Table S4). All genotypes also had similar OB sizes (Table S4), but *Plxna1*$^{−/−}$; *Plxna3*$^{−/−(y)}$ mutants had a smaller glomerular layer (GL) in the dorso-lateral OB compared with single mutants or wild types (Fig. S4). Accordingly, the combined loss of PLXNA1 and PLXNA3 causes defects in both the GnRH neuron and olfactory systems: the two co-existing hallmarks of KS.

Consistent with hypothalamic GnRH deficiency, three out of three *Plxna1*$^{−/−}$; *Plxna3*$^{−/−(y)}$ mutants had smaller gonads compared with single mutant littermates and wild types (two out of two males lacking both PLXNA1 and PLXNA3 had smaller testes and seminiferous vesicles, and one out of one female lacking both PLXNA1 and PLXNA3 had smaller ovaries; Fig. 3G-H; Fig. S5A,B). Notably, one out of two *Plxna1*$^{−/−}$; *Plxna3*$^{y/−}$ males examined had only one testis (Fig. S5A) and double mutant testes appeared immature and contained hardly any spermatids (Fig. S5C). We also detected PLXNA1 (Perälä et al., 2005) and PLXNA3 expression in the seminiferous tubules and interstitial cells of the testes, but not in the ovary or pituitary (Fig. S5D-F). Thus, the severe testes phenotype of double mutants may result from the combined tissue-specific loss of both plexins and hypothalamic GnRH deficiency.
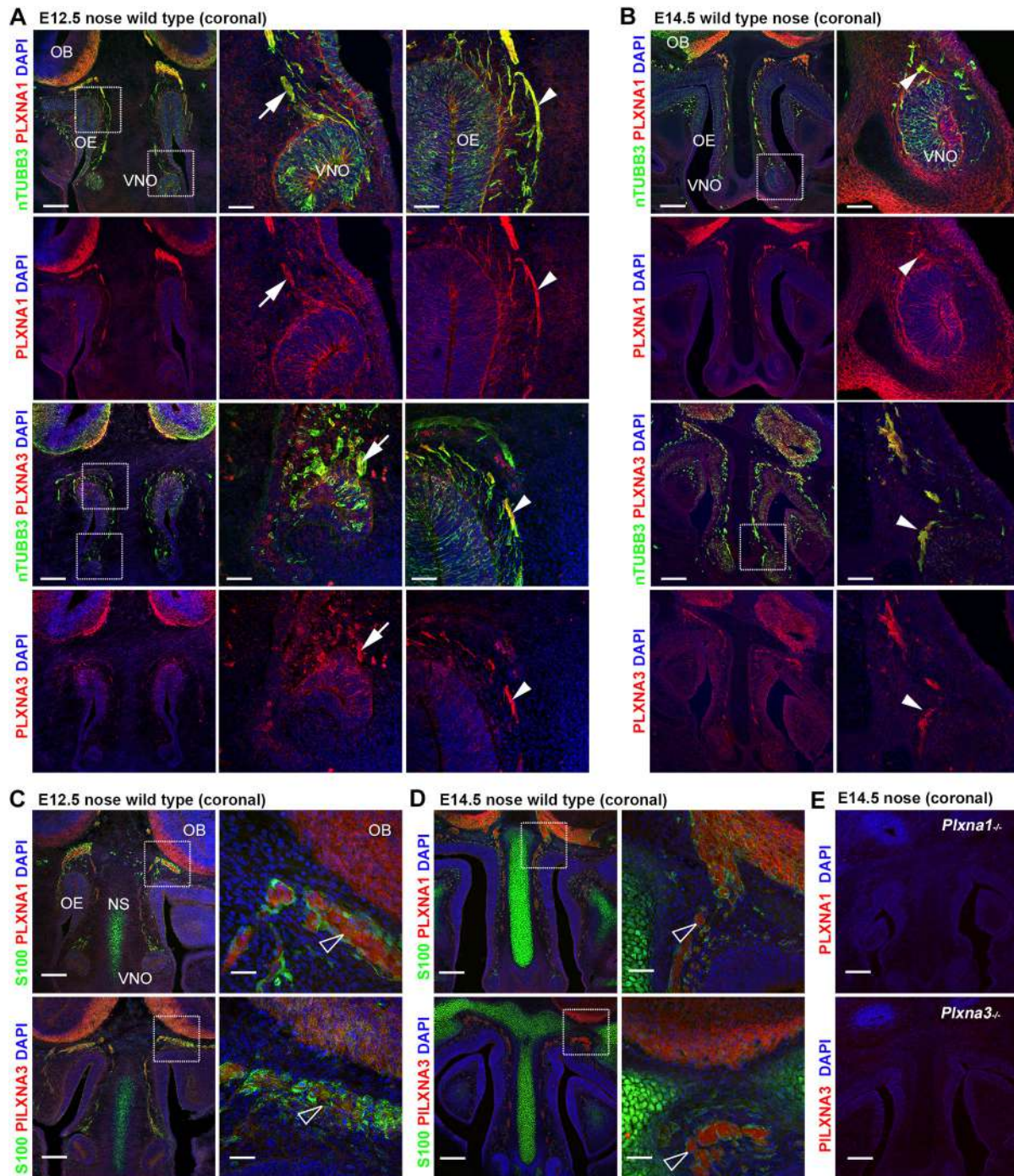
Fig. 1. Expression of PLXNA1 and PLXNA3 on neuronal cell bodies and axons. (A,B) Expression of PLXNA1 and PLXNA3 on neurons and axons. Coronal sections of E12.5 (A) and E14.5 (B) mouse heads were immunolabelled at the level of the VNO for nTUBB3 together with PLXNA1 or PLXNA3. The corresponding single PLXNA1 or PLXNA3 channels are shown below each image. White boxes indicate areas shown at higher magnification on the right. Arrows and arrowheads indicate examples of PLXNA1- and PLXNA3-positive neurons and axons, respectively. (C,D) PLXNA1 and PLXNA3 are not expressed by S100-positive cells. Coronal sections of E12.5 (C) and E14.5 (D) mouse heads at VNO level were immunolabelled for S100 to detect OECs and PLXNA1 or PLXNA3. White boxes indicate areas shown at higher magnification on the right of the corresponding panel. Open arrowheads indicate lack of PLXNA1 or PLXNA3 colocalisation with S100. (E) Specificity of PLXNA1 and PLXNA3 antibodies. Coronal sections from E14.5 *Plxna1*- and *Plxna3*-null mice at VNO level were immunostained for PLXNA1 or PLXNA3; lack of staining indicates antibody specificity. All sections were counterstained with DAPI. OE, olfactory epithelium; OB, olfactory bulb; NS, nasal septum; VNO, vomeronasal organ. Scale bars: 150 or 50 µm for lower and higher magnifications, respectively.

A prior study reporting KS-like symptoms in adult *Plxna1*$^{-/-}$ mice had focussed on the analysis of males (Fig. 3 in Marcos et al., 2017). As we observed a genetic interaction of *Plxna1* and *Plxna3*, it is conceivable that the mild and only partially penetrant defect observed in *Plxna1*$^{-/-}$ males might be explained, at least in part, by the hemizygous state of *Plxna3* in males that impacts on GnRH neuron development. However, it is not known whether *Plxna3* hemizygosity contributes to the increased incidence of KS in males
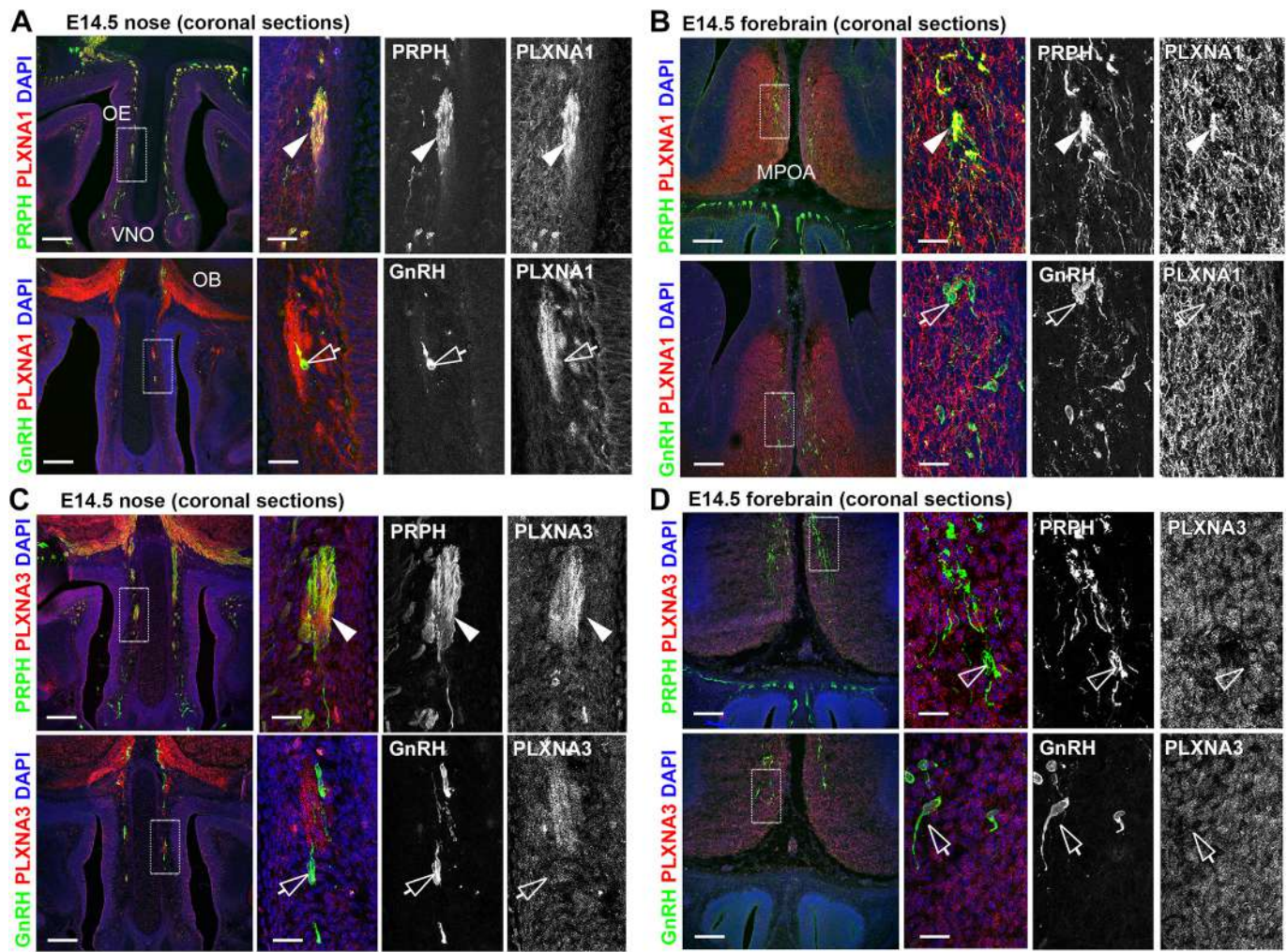
Fig. 2. PLXNA1 and PLXNA3 localise to nasal axons. (A-D) Coronal sections of E14.5 mouse heads were immunolabelled for PLXNA1 (A,B) or PLXNA3 (C,D) together with PRPH (top panels) or GnRH (bottom panels). Sections are shown at the level of the VNO (nose) or MPOA (forebrain). White boxes indicate areas shown at higher magnification on the right of the corresponding panel, with single channels shown also adjacent to the panel. Arrowheads in A-C indicate examples of PRPH-positive axons with PLXNA1 and PLXNA3. Open arrowheads in D indicate examples of PRPH-positive axons that lack PLXNA3. Open arrows in A-D indicate examples of GnRH neurons that lack PLXNA1 and PLXNA3. All sections were counterstained with DAPI. OE, olfactory epithelium; OB, olfactory bulb; VNO, vomeronasal organ; MPOA, medial preoptic area. Scale bars: 150 or 50 μm for lower and higher magnifications, respectively.

compared with females. Notably, two out of two adult $Plxna1^{-/-}$; $Pxna3^{+/-}$ females had a severe reduction of GnRH neurons in the MPOA, which exceeded that seen in three out of five $Plxna1^{-/-}$ male mutants (Table S3). The intermediate phenotype severity in these females between $Plxna1^{-/-}$ single and $Plxna1^{-/-}$; $Pxna3^{y/-}$ double mutants may be explained by random X-chromosome inactivation, as this has the potential to remove the functional copy of PLXNA3 in $Pxna3^{+/-}$ females and thereby decrease PLXNA3 dose. Further work would be required to investigate this hypothesis.

Mispatterned OLF/VN axons in *Plxna1*/*Plxna3*-null embryos form axon tangles at the cribriform place that retain GnRH neurons

To better understand the underlying cause of abnormal GnRH neuron migration during embryogenesis, we examined the patterning of their PRPH+ axonal migratory scaffolds in E14.5 embryos from parents carrying both *Plxna1*- and *Plxna3*-null alleles. This was also important, because nasal axon defects were previously reported in five out of 18 E14.5 $Plxna1^{-/-}$ mutants (Marcos et al., 2017). We found that three out of three $Plxna1^{-/-}$ and three out of three $Plxna3^{-/-}$ single mutants had

similar PRPH+ axon organisation as wild-type littermates, whereas three out of three $Plxna1^{-/-}$; $Plxna3^{-/-}$ double mutants contained PRPH+ mistargeted axons between the OBs and axon tangles at the CP (Fig. 4A,B). These axon defects therefore occur in areas in which GnRH neurons accumulate (Fig. 4B; see also Fig. 3B). Even though cVN axons emerged from the VNO in all genotypes analysed (Fig. S6A), double mutants lacked cVN axons in the forebrain (Fig. 4A,B). Moreover, double, but not single null mutants, had defasciculated and enlarged OLF axon bundles below the ventro-medial OBs (Fig. S6B) that may explain the small size of the glomerular layer (GL) in the OB of double-null mutants (see Fig. S4).

In summary, the combined loss of PLXNA1 and PLXNA3 severely disrupts the axons that guide GnRH neurons through the nose and into the brain, and additionally impairs olfactory development. Notably, the axonal defects are similar to those reported for *Sema3a*-null mutants (Cariboni et al., 2011; Hanchate et al., 2012), supporting the idea that PLXNA1 and PLXNA3 serve as co-receptors for SEMA3A during VN and OLF axon development. Interestingly, partial PLXNA redundancy for SEMA3A-mediated axon targeting mirrors the redundancy for SEMA3A ligand-binding
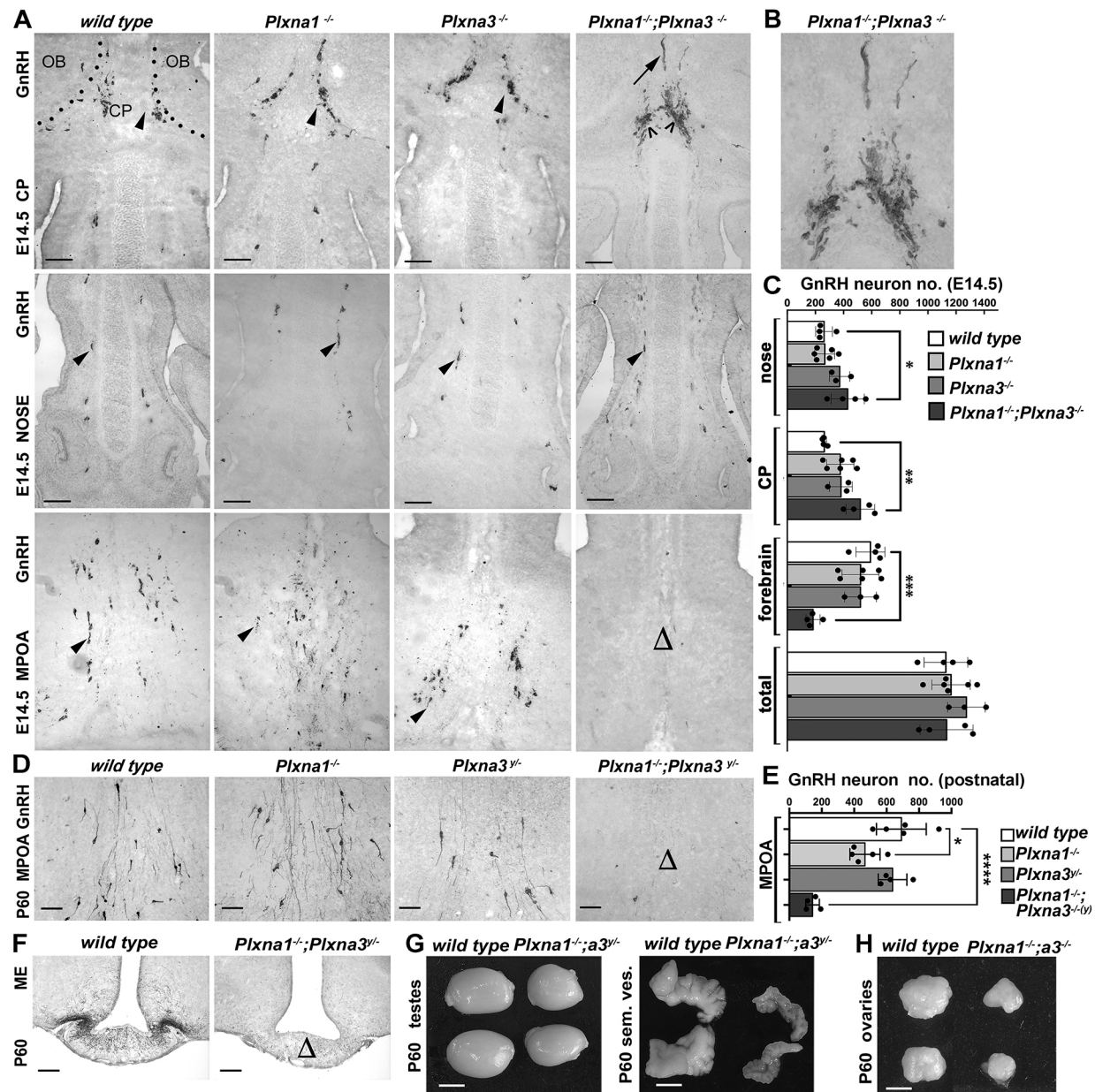
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Fig. 3. Combined PLXNA1 and PLXNA3 loss decreases GnRH neuron number, ME innervation and testes size in adult mice. (A-C) Embryonic GnRH neuron analysis. (A) Coronal sections of E14.5 mouse heads with the indicated genotypes were immunolabelled for GnRH. The OB boundaries are indicated with black dotted lines in the wild-type panel. Arrowheads indicate examples of GnRH neurons at the CP (top panels), in the nasal parenchyma (middle panels) and in the MPOA (bottom panels). The black arrow and open arrowheads indicate GnRH neuron clumps between the OBs and at the CP, respectively. Δ indicates a lack of GnRH neurons in the MPOA. (B) High-magnification image of a double mutant E14.5 embryo showing an example of GnRH neurons accumulating at the CP and between OBs. (C) Quantification of GnRH neuron number in the E14.5 head; data are mean±s.d.; ***$P<0.001$, **$P<0.01$, *$P<0.05$ (one-way ANOVA with Dunnett's test). (D-F) Adult GnRH neuron analysis. Coronal sections of P60 brains with the indicated genotypes at the level of the MPOA (D) and ME (F) were immunolabelled for GnRH. Δ indicates a lack of GnRH staining. (E) Quantification of GnRH neuron number in the P60 MPOA; data are mean±s.d.; *$P<0.05$, ****$P<0.0001$ (one-way ANOVA with Dunnett's test). (G,H) Adult gonad size. Micrographs show paired testes (G, left panel), seminal vesicles (G, right panel) and ovaries (H) of P60 littermate mice. OB, olfactory bulb; CP, cribriform plate; MPOA, medial preoptic area; ME, median eminence; sem. ves., seminal vesicles. Scale bars: 150 μm in A,F; 100 μm in B; 250 μm in D; 3 mm in G; 1.5 mm in H.

receptors, as loss of semaphorin signalling through both NRP1 and NRP2 is required to elicit the full spectrum of VN and OLF, as well as the GnRH neuron migration defects that are observed in *Sema3a*-null mutants (Cariboni et al., 2007, 2011).

Conclusions

Here, we show that PLXNA1 and PLXNA3 cooperate to pattern the SEMA3A/NRP-dependent axons that serve as migratory scaffolds for GnRH neurons *en route* from the nasal placodes to the brain, and also contribute to olfactory axon patterning. Accordingly, the loss of both PLXNA1 and PLXNA3 from nasal axons impairs the development of the GnRH neuron and olfactory systems to cause a KS-like phenotype in adult mice (see working model, Fig. 4C). The human orthologue of *Plxna3*, like *PLXNA1*, should thus be considered a candidate gene for mutation screening in individuals with KS. We further observed
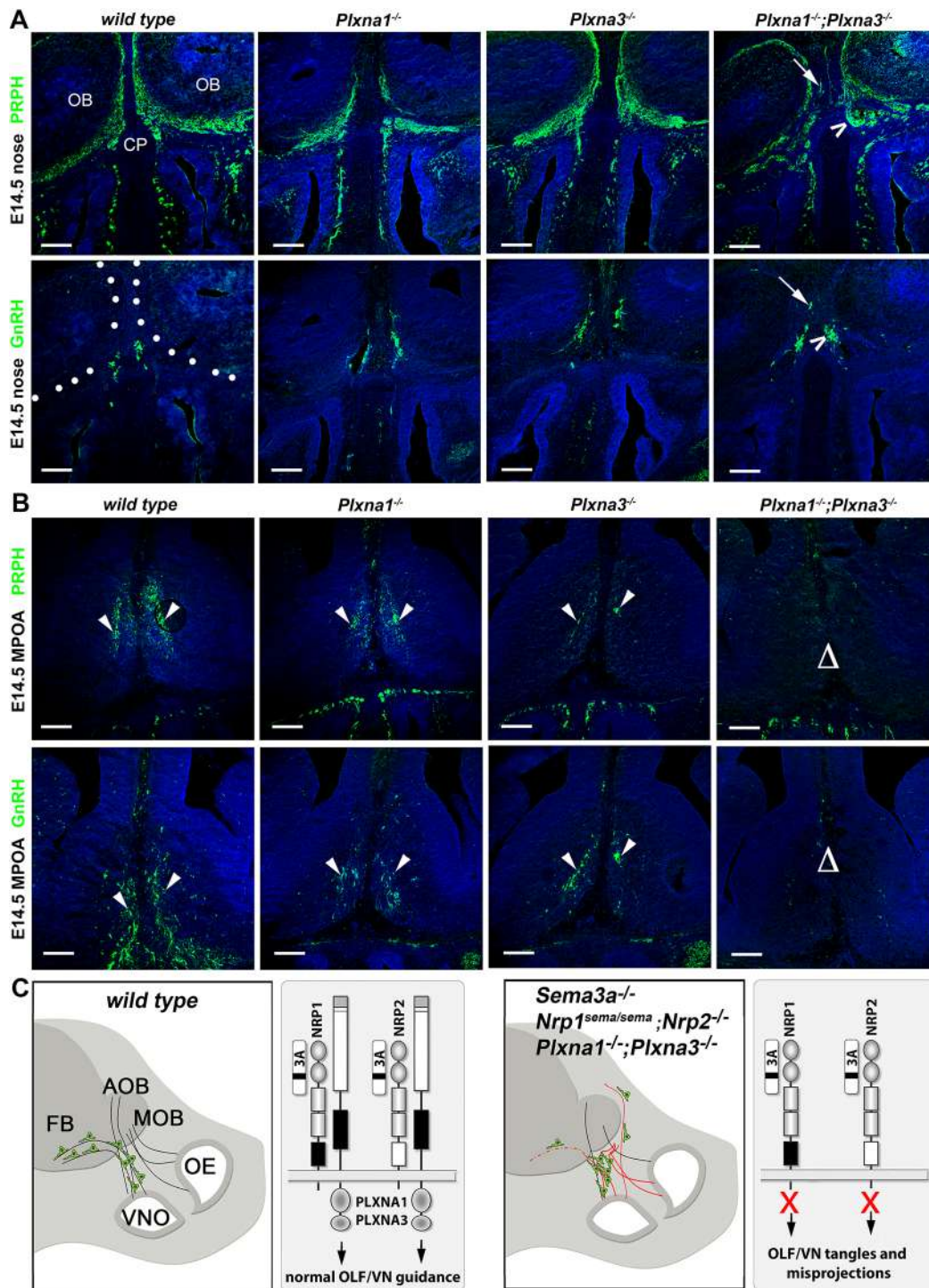
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Fig. 4. Combined PLXNA1 and PLXNA3 loss impairs nasal axon and GnRH neuron distribution. (A,B) Adjacent coronal sections of E14.5 mouse heads of the indicated genotypes at the level of the CP (A) and MPOA (B) were immunolabelled for PRPH to reveal OLF, VN and cVN axons (top panels) and GnRH neurons (bottom panels). The OB boundaries are indicated with dotted lines in the wild-type panel. Solid arrows and open arrowheads indicate examples of ectopic axons and GnRH neurons between the OBs and at the CP, respectively. Solid arrowheads indicate cVN axons in the MPOA. A lack of GnRH neurons and cVN axons in the MPOA is indicated with Δ. OB, olfactory bulb; CP, cribriform plate; MPOA, medial preoptic area. Scale bars: 150 μm. (C) Working model summarising the observed defects in GnRH neuron (green) migration and axon organisation in wild-type embryos versus SEMA3A pathway mutants. Normal axon projections are shown as continuous black lines, abnormal projections are shown as continuous red lines; the interrupted red line in mutants represents the missing cVN branch. The corresponding predicted signalling pathways are shown adjacent to each head schematic.

severe defects in testes formation in PLXNA1 and PLXNA3 mice that exceed those seen in KS, suggesting that these genes might also be mutated in other congenital diseases that affect gonad formation.

MATERIALS AND METHODS
Mouse strains

Mice lacking *Plxna1* or *Plxna3* (Cheng et al., 2001; Yoshida et al., 2006) were used in a C57/Bl6 background for all embryonic studies or on a CD1

background to increase postnatal survival of double mutants. As the *Plxna3* gene resides on the X chromosome, we have indicated whether postnatal mice were male (*Plxna3*$^{y/-}$) or female (*Plxna3*$^{-/-}$), and have referred to groups of both sexes as *Plxna3*$^{-/-(y)}$; the sex of mouse embryos was not determined, and we therefore refer to all embryos lacking PLXNA3 as *Plxna3*$^{-/-}$. To obtain mouse embryos of defined gestational ages, mice were mated in the evening, and the morning of vaginal plug formation was counted as embryonic day (E) 0.5. Owing to the severe phenotype in five out of five postnatal double mutants in the five litters obtained, we abandoned further crosses to obtain additional adult mutants due to ethical considerations. All animal procedures were performed in accordance with Animal Welfare Ethical Review Body (AWERB) guidelines and under UK Home Office licence and Italian Ministry of Health licences.

Tissue preparation and cryosectioning
E12.5 and E14.5 embryos were fixed for 3 h in 4% formaldehyde, whereas postnatal tissues were dissected after perfusion in 4% formaldehyde. All samples were then cryoprotected overnight in 30% sucrose, embedded in OCT and cryosectioned for immunohistochemistry or *in situ* hybridisation. Schematic drawings showing the anatomical levels and orientation of the sections are displayed in Fig. S1A,B.

In situ hybridisation
Formaldehyde-fixed cryosections were incubated with digoxigenin (DIG)-labelled antisense riboprobes for mouse *Plxna1*, *Plxna2*, *Plxna3* or *Plxna4* (Addgene plasmids 58237, 62353, 58238 and 58239, respectively; Schwarz et al., 2008) or mouse *Gnrh* (Cariboni et al., 2015). For labelling, we used the DIG RNA labelling kit (Roche). Hybridisation was performed in 50% formamide, 0.3 M sodium chloride, 20 mM Tris (pH 7.5), 5 mM EDTA, 10% dextran sulphate and 1× Denhardt's solution overnight at 65°C. Sections were washed in a saline sodium citrate buffer (50% formamide, 1× saline sodium citrate buffer, 0.1% Tween20), incubated overnight with alkaline phosphatase (AP)-conjugated anti-DIG IgG (1:1500; Roche) and developed overnight at 37°C with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (Roche) dissolved in a buffer comprising 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl and 1% Tween 20.

Immunofluorescence labelling
Cryostat sections (25 µm) of formaldehyde-fixed embryos were incubated with serum-free protein block (DAKO) after permeabilisation of sections with 0.1% TritonX-100. We used as primary antibodies rabbit anti-peripherin (1:100; Merck Millipore, AB1530), rabbit anti-GnRH, previously validated to recognise both the pre-hormone and the processed hormone (Taroc et al., 2019) (1:400; Immunostar, 20075), rabbit anti-S100 (1:400, DAKO, Z0311), mouse anti-TUBB3 (1:500, clone Tuj1, Covance, MMS- 435P), goat anti-OMP (1:200 WAKO, 019-22291), goat anti-PLXNA1 (1:200; R&D Systems, AF4309) and goat anti-PLXNA3 (1:200; R&D Systems, AF4075). Secondary antibodies used were Cy3-conjugated donkey anti-goat and 488-conjugated donkey anti-rabbit Fab fragments (1:200; Jackson Immunoresearch). Nuclei were counterstained with DAPI (1:10,000; Sigma).

Immunoperoxidase labelling
Cryostat sections (25 µm) of formaldehyde-fixed samples were incubated with hydrogen peroxide to quench endogenous peroxidase activity, and sequentially incubated with 10% heat-inactivated normal goat serum in PBS or serum-free blocking solution (DAKO) and then immunostained with the above antibodies to GnRH (1:1000), PLXNA1 (1:500) and PLXNA3 (1:500) or antibodies to CRH (1:400, Proteintech, cat. no. 10944-1-AP) followed by an appropriate species-specific biotinylated antibody (1:400; Vector Laboratories). Sections were developed using the ABC kit (Vector Laboratories) and 3,3-diaminobenzidine (DAB; Sigma). To determine the total number of GnRH neurons at E14.5, 25-µm coronal sections through each entire head were immunolabelled for GnRH, and all GnRH-positive cells in the nose, CP area and forebrain were counted, as previously reported (Cariboni et al., 2011, 2015) (Fig. S1C). To help distinguish individual

GnRH neurons found in cell clumps at the CP of double mutants, high-magnification images were analysed. To determine the number of GnRH neurons in the MPOA of postnatal adult male brains, 25-µm coronal sections through the MPOA from a position around 200 µm after the end of ME to the area in which the two hemispheres separate (60 sections/brain) were immunolabelled and all GnRH-positive cells counted in all sections.

Haematoxylin and Eosin staining
Sections (8 µm) of formalin-fixed testes from P60 mice were stained as previously described (Macchi et al., 2017).

Statistical analysis
Sample sizes for expression and mouse phenotyping analyses were estimated based on prior experience and those in the existent literature. Typically, embryo samples were taken from at least three different litters for each group. Randomization was not used to assign samples to experimental groups or to process data, but samples were allocated to groups based on genotypes. The researcher analysing the data was blind to the genotypes during analysis. Loss of sections during cryosectioning of embryo heads, damaged tissue and unspecific immunostaining were pre-established criteria for sample exclusion, otherwise all samples were included in the analyses. All data are expressed as mean±s.d. We used a one-way ANOVA followed by a Dunnett's test to determine the statistical significance between values in multiple comparisons; $P<0.05$ was considered significant; $P<0.05$, $P<0.01$, $P<0.001$ or $P<0.0001$ are indicated with one, two, three or four asterisks, respectively. Statistical analysis was performed using Prism4 software (GraphPad Software).

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.176461.supplemental

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