Plxna1 and _Plxna3_ cooperate to pattern the nasal axons that guide gonadotropin-releasing hormone neurons

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Running title: PLXNAs in GnRH neuron migration

Summary statement: PLXNA1 and PLXNA3 convey the pathfinding of olfactory and vomeronasal axons as a prerequisite for neuroendocrine neurons to migrate into the hypothalamus and release gonadotropins into the circulation.

27 **Abstract**

28

29 The gonadotropin releasing hormone (GnRH) neurons regulate puberty onset and sexual

30 reproduction by secreting GnRH to activate and maintain the hypothalamic-pituitary-

31 gonadal axis. During embryonic development, GnRH neurons migrate along olfactory and

32 vomeronasal axons through the nose into the brain, where they project to the median

33 eminence to release GnRH. The secreted glycoprotein SEMA3A binds its receptors

34 neuropilin (NRP) 1 or NRP2 to position these axons for correct GnRH neuron migration,

35 with an additional role for the NRP co-receptor PLXNA1. Accordingly, mutations

36 in *SEMA3A*, *NRP1*, *NRP2* and *PLXNA1* have been linked to defective GnRH neuron

37 development in mice and inherited GnRH deficiency in humans. Here, we show that only

38 the combined loss of PLXNA1 and PLXNA3 phenocopied the full spectrum of nasal axon

39 and GnRH neuron defects of SEMA3A knockout mice. Together with *Plxna1,* the human

40 ortholog of *Plxna3* should therefore be investigated as a candidate gene for inherited

41 GnRH deficiency.

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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, whose cell bodies 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 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 KS patients (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.

77   Moreover, these findings illustrate that mouse models are powerful tools to uncover genes
78   that regulate GnRH neuron development and may be mutated in patients with inherited
79   GnRH neuron deficiency.

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

92

93   **Results and discussion:**

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95   ***Plxna1 and Plxna3 are co-expressed during GnRH neuron migration.***

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

106   To determine which cell types expressed PLXNA1 or PLXNA3 in the territories relevant to
107   GnRH neuron migration, we immunostained sections through wild type mouse embryo
108   heads. Double labelling with the TUJ1 antibody for neuronal-specific beta 3 tubulin
109   (nTUBB3) showed that PLXNA1 and PLXNA3 localised to the MM at E12.5 and to axons
110   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 (**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 wild type, single and double mutant embryos in litters 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 to 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

144 **_neuron, gonadal and olfactory system defects_**

145 To assess whether lack of GnRH neurons in the forebrain of E14.5 _Plxna1$^{-/-}$;Plxna3$^{-/-}$_

146 embryos results in an hypogonadal state in adulthood, we analysed postnatal _Plxna1$^{-/-}$_

147 _;Plxna3$^{-/-}$_ mice. The analysis of 5 litters from parents with combined _Plxna1_-null and

148 _Plxna3_-null alleles suggested that all genotypes were born at a normal Mendelian ratio

149 (**Table S2**), but there was a high rate of pre-weaning mortality. As the _Plxna3_ gene resides

150 on the X chromosome, males in these litters are either wild type or hemizygous for the

151 _Plxna3_-null mutation, i.e. _Plxna3$^{y/+}$_ or _Plxna3$^{y/-}$_, respectively. In contrast, females are

152 _Plxna3$^{+/+}$_, _Plxna3$^{+/-}$_ or _Plxna3$^{-/-}$_. Pooled male and female mice lacking _Plxna3_ are therefore

153 referred to as _Plxna3$^{-/-(y)}$_. We found that 2/5 juvenile _Plxna1$^{-/-}$;Plxna3$^{-/-(y)}$_ mutants were

154 small and appeared stressed when handled and had to be culled before weaning to

155 prevent suffering. To avoid the birth of further mutants with such severe adverse effects,

156 breeding was concluded, and all mutants obtained culled for analyses.

157 We next compared the GnRH neuron number in the MPOA of postnatal mutants and wild

158 type controls, because this is the final position these neurons should attain. Whereas the

159 GnRH neuron number in the _Plxna3$^{y/-}$_ MPOA was similar to that of wild type littermates,

160 3/5 _Plxna1$^{-/-}$_ mutants had slightly fewer and 4/4 _Plxna1$^{-/-}$; Plxna3$^{-/-(y)}$_ mutants contained

161 hardly any GnRH neurons in the MPOA (**Fig. 3D,E** and **Table S3**). Agreeing with a

162 severely reduced GnRH neuron number, GnRH staining of the ME was nearly absent in

163 _Plxna1$^{-/-}$;Plxna3$^{-/-(y)}$_ mutants (**Fig. 3F**), despite normal hypothalamic projections of

164 neuroendocrine neurons, such as those that secrete the corticotropin releasing hormone

165 CRH (**Fig. S3B**). Moreover, overall brain size was similar in all genotypes (**Table S4**). All

166 genotypes also had similar OB sizes (**Table S4**), but _Plxna1$^{-/-}$; Plxna3$^{-/-(y)}$_ mutants had a

167 smaller glomerular layer (GL) in the dorso-lateral OB compared to single mutants or wild

168 types (**Fig. S4**). Accordingly, the combined loss of PLXNA1 and PLXNA3 causes defects

169 in both the GnRH neuron and olfactory systems, the two co-existing hallmarks of KS.

170 Consistent with hypothalamic GnRH deficiency, 3/3 _Plxna1$^{-/-}$;Plxna3$^{-/-(y)}$_ mutants had

171 smaller gonads compared to single mutant littermates and wild types (2/2 males lacking

172 both PLXNA1 and PLXNA3 had smaller testes and seminiferous vesicles, and 1/1 female

173 lacking both PLXNA1 and PLXNA3 had smaller ovaries; **Fig. 3G-H**; **Fig. S5A,B**). Notably,

174 1/2 _Plxna1$^{-/-}$;Plxna3$^{y/-}$_ males examined only had one testis (**Fig. S5A**) and double mutant

175 testes appeared immature and contained hardly any spermatids (**Fig. S5C**). We also

176 detected PLXNA1 (Perälä et al., 2005) and PLXNA3 expression in the seminiferous

177 tubules and interstitial cells of the testes, but not in the ovary or pituitary (**Fig. S5D-F**).

178 Thus the severe testes phenotype of double mutants may result from the combined tissue-
179 specific loss of both plexins and hypothalamic GnRH deficiency.

180 A prior study reporting KS-like symptoms in adult *Plxna1*^{-/-} mice had focussed on the
181 analysis of males (Fig. 3 in Marcos et al., 2017). As we observed a genetic interaction of
182 *Plxna1* and *Plxna3*, it is conceivable that the mild and only partially penetrant defect
183 observed in *Plxna1*^{-/-} males might be explained, at least in part, by the hemizygous state of
184 *Plxna3* in males that impacts on GnRH neuron development. However, it is not known
185 whether *Plxna3* hemizygosity contributes to the increased incidence of KS in males
186 compared to females. Notably, 2/2 adult *Plxna1*^{-/-};*Pxna3*^{+/-} females had a severe reduction
187 of GnRH neurons in the MPOA, which exceeded that seen in 3/5 *Plxna1*^{-/-} male mutants
188 (**Table S3**). The intermediate phenotype severity in these females between *Plxna1*^{-/-} single
189 and *Plxna1*^{-/-};*Pxna3*[y/-] double mutants may be explained by random X chromosome
190 inactivation, as this has the potential to remove the functional copy of PLXNA3 in *Pxna3*^{+/-}
191 females and thereby decrease PLXNA3 dosage. Further work would be required to
192 investigate this hypothesis.

193

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

196 To better understand the underlying cause of abnormal GnRH neuron migration during
197 embryogenesis, we examined the patterning of their PRPH[+] axonal migratory scaffolds in
198 E14.5 embryos from parents carrying both *Plxna1*- and *Plxna3*-null alleles. This was also
199 important, because nasal axon defects were previously reported in 5/18 E14.5 *Plxna1*^{-/-}
200 mutants (Marcos et al., 2017). We found that 3/3 *Plxna1*^{-/-} and 3/3 *Plxna3*^{-/-} single mutants
201 had similar PRPH[+] axon organisation as wild type littermates, whereas 3/3 *Plxna1*^{-/-}
202 ;*Plxna3*^{-/-} double mutants contained PRPH[+] mistargeted axons between the OBs and axon
203 tangles at the CP (**Fig. 4A,B**). These axon defects therefore occur in areas in which GnRH
204 neurons accumulate (**Fig. 4B**; see also **Fig. 3B**). Even though cVN axons emerged from
205 the VNO in all genotypes analysed (**Fig. S6A**), double mutants lacked cVN axons in the
206 forebrain (**Fig. 4A,B**). Moreover, double, but not single null mutants, had defasciculated
207 and enlarged OLF axon bundles below the ventro-medial OBs (**Fig. S6B**) that may explain
208 the small size of the glomerular layer (GL) in the OB of double null mutants (see **Fig. S4**).

209 In summary, the combined loss of PLXNA1 and PLXNA3 severely disrupts the axons that
210 guide GnRH neurons through the nose and into the brain and additionally impairs olfactory
211 development. Notably, the axonal defects are similar to those reported for *Sema3a*-null

212 mutants (Cariboni et al., 2011; Hanchate et al., 2012), supporting the idea that PLXNA1

213 and PLXNA3 serve as co-receptors for SEMA3A during VN and OLF axon development.

214 Interestingly, partial PLXNA redundancy for SEMA3A-mediated axon targeting mirrors the

215 redundancy for SEMA3A's ligand binding receptors, as loss of semaphorin signalling

216 through both NRP1 and NRP2 is required to elicit the full spectrum of VN and OLF as well

217 as GnRH neuron migration defects that is observed in *Sema3a*-null mutants (Cariboni et

218 al., 2007; Cariboni et al., 2011).

219

220 **Conclusion**. Here we show that PLXNA1 and PLXNA3 cooperate to pattern the

221 SEMA3A/NRP-dependent axons that serve as migratory scaffolds for GnRH neurons *en*

222 *route* from the nasal placodes to the brain and also contribute to olfactory axon patterning.

223 Accordingly, the loss of both PLXNA1 and PLXNA3 from nasal axons impairs the

224 development of the GnRH neuron and olfactory systems to cause a KS-like phenotype in

225 adult mice (see working model, **Fig. 4C**). The human ortholog of *Plxna3,* like *PLXNA1,*

226 should thus be considered a candidate gene for mutation screening in patients with KS.

227 We further observed severe defects in testes formation in PLXNA1 and PLXNA3 mice that

228 exceed those seen in KS, suggesting that these genes might also be mutated in other

229 congenital diseases that affect gonad formation.

230

236

237 **Methods:**

238 **Mouse strains.** Mice lacking *Plxna1* or *Plxna3* (Cheng et al., 2001; Yoshida et al., 2006)

239 were used in a C57/Bl6 background for all embryonic studies or on a CD1 background to

240 increase postnatal survival of double mutants. As the *Plxna3* gene resides on the X

241 chromosome, we have indicated whether postnatal mice were male ($Plxna3^{y/-}$) or female

242 ($Plxna3^{-/-}$) and have referred to groups of both sexes as $Plxna3^{-/-(y)}$; the sex of mouse

243 embryos was not determined, and we therefore refer to all embryos lacking PLXNA3 as

244 $Plxna3^{-/-}$. To obtain mouse embryos of defined gestational ages, mice were mated in the

245 evening, and the morning of vaginal plug formation was counted as embryonic day (E) 0.5.

246 Due to the severe phenotype in 5/5 postnatal double mutants in the 5 litters obtained, we
247 abandoned further crosses to obtain additional adult mutants due to ethical considerations.
248 Genotyping protocols can be supplied on request. All animal procedures were performed
249 in accordance with Animal Welfare Ethical Review Body (AWERB) guidelines and under
250 UK Home Office licence and Italian Ministry of Health licences.

251 **Tissue preparation and cryosectioning:** E12.5 and E14.5 embryos were fixed for 3
252 hours in 4% formaldehyde, whereas post-natal tissues were dissected after perfusion in
253 4% formaldehyde. All samples were then cryoprotected overnight in 30% sucrose,
254 included in OCT and cryosectioned for immunohistochemistry or *in situ* hybridisation.
255 Schematic drawings showing the anatomical levels and orientation of the sections are
256 displayed in **Fig. S7**.

257 *In situ* **hybridisation.** Formaldehyde-fixed cryosections were incubated with digoxigenin
258 (DIG)-labelled anti-sense riboprobes for mouse *Plxna1, Plxna2, Plxna3 or Plxna4*
259 (Addgene plasmids no. 58237, 62353, 58238 and 58239, respectively; Schwarz et al.,
260 2008) or mouse *Gnrh* (Cariboni et al., 2015). For labelling, we used the DIG RNA labelling
261 kit (Roche). Hybridisation was performed in 50% formamide, 0.3 M sodium chloride, 20
262 mM Tris pH 7.5, 5 mM EDTA, 10% dextran sulphate and 1x Denhardt's solution overnight
263 at 65°C. Sections were washed in a saline sodium citrate buffer (50% formamide, 1x
264 saline sodium citrate buffer, 0.1% Tween20), incubated overnight with alkaline
265 phosphatase (AP)-conjugated anti-DIG IgG (1:1500; Roche) and developed overnight at
266 $37^{o}C$ with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate
267 disodium salt (Roche) dissolved in a buffer comprised of 100 mM Tris pH 9.5, 50 mM
268 $MgCl_2$, 100 mM NaCl and 1% Tween 20.

269 **Immunofluorescence labelling.** 25 µm cryostat sections of formaldehyde-fixed embryos
270 were incubated with serum-free protein block (DAKO) after permeabilisation of sections
271 with 0.1% TritonX-100. We used as primary antibodies rabbit anti–peripherin (1:100;
272 Merck Millipore, cat. no. AB1530), rabbit anti-GnRH, previously validated to recognise both
273 the pre-hormone and the processed hormone (Taroc et al., 2019) (1:400; Immunostar, cat.
274 no. 20075), rabbit anti-S100 (1:400, DAKO, cat. no. Z0311), mouse anti-TUBB3 (1:500,
275 clone Tuj1, Covance, cat. no. MMS- 435P), goat anti-OMP (1:200 WAKO, cat. no. 019-
276 22291), goat anti-PLXNA1 (1:200; R&D Systems, cat. no. AF4309) and goat anti-PLXNA3
277 (1:200; R&D Systems, cat. no. AF4075). Secondary antibodies used were Cy3-conjugated
278 donkey anti-goat and 488-conjugated donkey anti-rabbit Fab fragments (1:200; Jackson
279 Immunoresearch). Nuclei were counterstained with DAPI (1:10000; Sigma).

Immunoperoxidase labelling. 25 μm cryostat sections 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) and an appropriate species-specific biotinylated antibody (1:400; Vector Laboratories). Sections were developed with 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; Cariboni et al., 2015). 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 (H&E). 8 μm sections of formaldehyde-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 ± standard deviation (s.d.); error bars represent the standard deviation. We used a one-way ANOVA followed by a Dunnett's test to determine the statistical significance between values in multiple comparisons; a *P*-value of <0.05 was considered significant; P-values of <0.05, <0.01, <0.001 or <0.0001 were indicated with one, two, three or four asterisks, respectively. Statistical analysis was performed using Prism4 software (GraphPad Software, San Diego, CA, USA).

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406

Figure legends:

408

409 **Figure 1. Expression of PLXNA1 and PLXNA3 on neuronal cell bodies and axons**.

410 (A,B) Expression of PLXNA1 and PLXNA3 on neurons and axons. Coronal sections of

411 E12.5 (A) and E14.5 (B) mouse heads were immunolabelled at the level of the VNO for

412 nTUBB3 together with PLXNA1 or PLXNA3. The corresponding single PLXNA1 or

413 PLXNA3 channels are shown below each image. White boxes indicate areas shown at

414 higher magnification on the right of the corresponding panel. Arrows and arrowheads

415 indicate examples of PLXNA1 and PLXNA3-positive neurons and axons, respectively.

416 (C,D) PLXNA1 and PLXNA3 are not expressed by S100-positive cells. Coronal sections of

417 E12.5 (C) and E14.5 (D) mouse heads at VNO level were immunolabelled for S100 to

418 detect OECs and PLXNA1 or PLXNA3. White boxes indicate areas shown at higher

419 magnification on the right of the corresponding panel. Clear arrowheads indicate lack of

420 PLXNA1 or PLXNA3 co-localisation with S100.

421 (E) Specificity of PLXNA1 and PLXNA3 antibodies. Coronal sections from E14.5 *Plxna1-*

422 and *Plxna3*-null mice at VNO level were immunostained for PLXNA1 or PLXNA3; lack of

423 staining indicates antibody specificity.

424 All sections were counterstained with DAPI.

425 Abbreviations: OE, olfactory epithelium; OB, olfactory bulb; NS, nasal septum; VNO,

426 vomeronasal organ; OEC, olfactory ensheathing cells.

427 Scale bars: 150 or 50 μm for lower and higher magnifications, respectively.

428

429 **Figure 2. PLXNA1 and PLXNA3 localise to nasal axons**.

430 (A-D) Coronal sections of E14.5 mouse heads were immunolabelled for PLXNA1 (A,B) or

431 PLXNA3 (C,D) together with PRPH (top panels) or GnRH (bottom panels). Sections are

432 shown at the level of the VNO (nose) or MPOA (forebrain). White boxes indicate areas

433 shown at higher magnification on the right of the corresponding panel, with single

434 channels shown also adjacent to the panel. Arrowheads in (A-C) indicate examples of

435 PRPH-positive axons with PLXNA1 and PLXNA3, respectively. Clear arrowheads in (D)

436 indicate examples of PRPH-positive axons that lack PLXNA3. Clear arrows in (A-D)

437 indicate examples of GnRH neurons that lack PLXNA1 and PLXNA3, respectively. All

438 sections were counterstained with DAPI.

Abbreviations: 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.

Figure 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 double mutant E14.5 embryo showing example of GnRH neurons accumulated cells at the CP and between OBs. (C) Quantification of GnRH neuron number in the E14.5 head; data are shown as 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 shown as 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.

Abbreviations: OB, olfactory bulb; CP, cribriform plate; MPOA, medial preoptic area; ME, median eminence; sem. ves., seminal vesicles.

Scale bars: 150 µm (A,E), 500 µm (C), 3 mm (F,G), 1.5 mm (H).

Figure 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
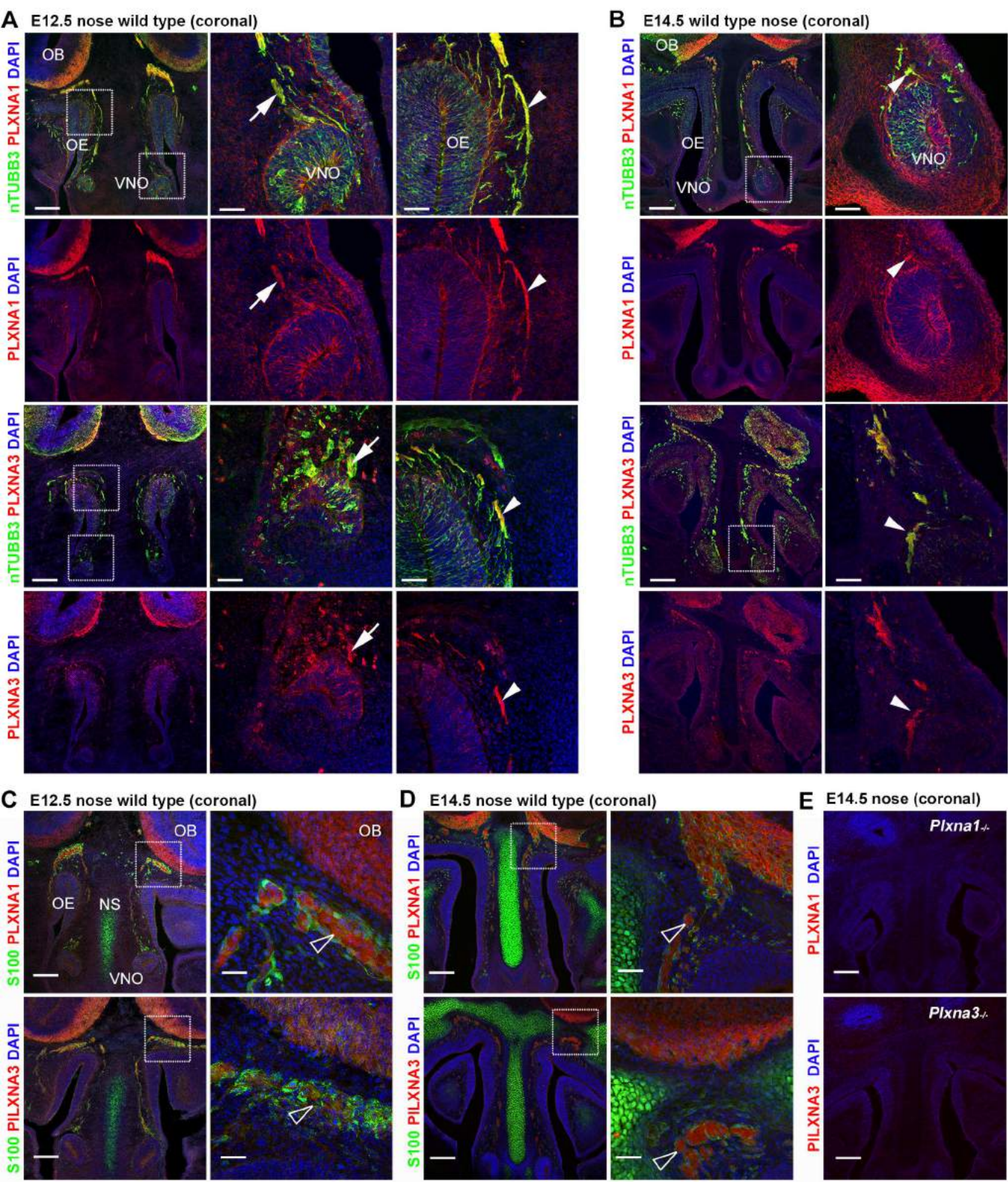
471   indicated with dotted lines in the wild type panel. Solid arrows and open arrowheads

472   indicate examples of ectopic axons and GnRH neurons between the OBs and at the CP,

473   respectively. Solid arrowheads indicate cVN axons in the MPOA. A lack of GnRH neurons

474   and cVN axons in the MPOA is indicated with Δ.

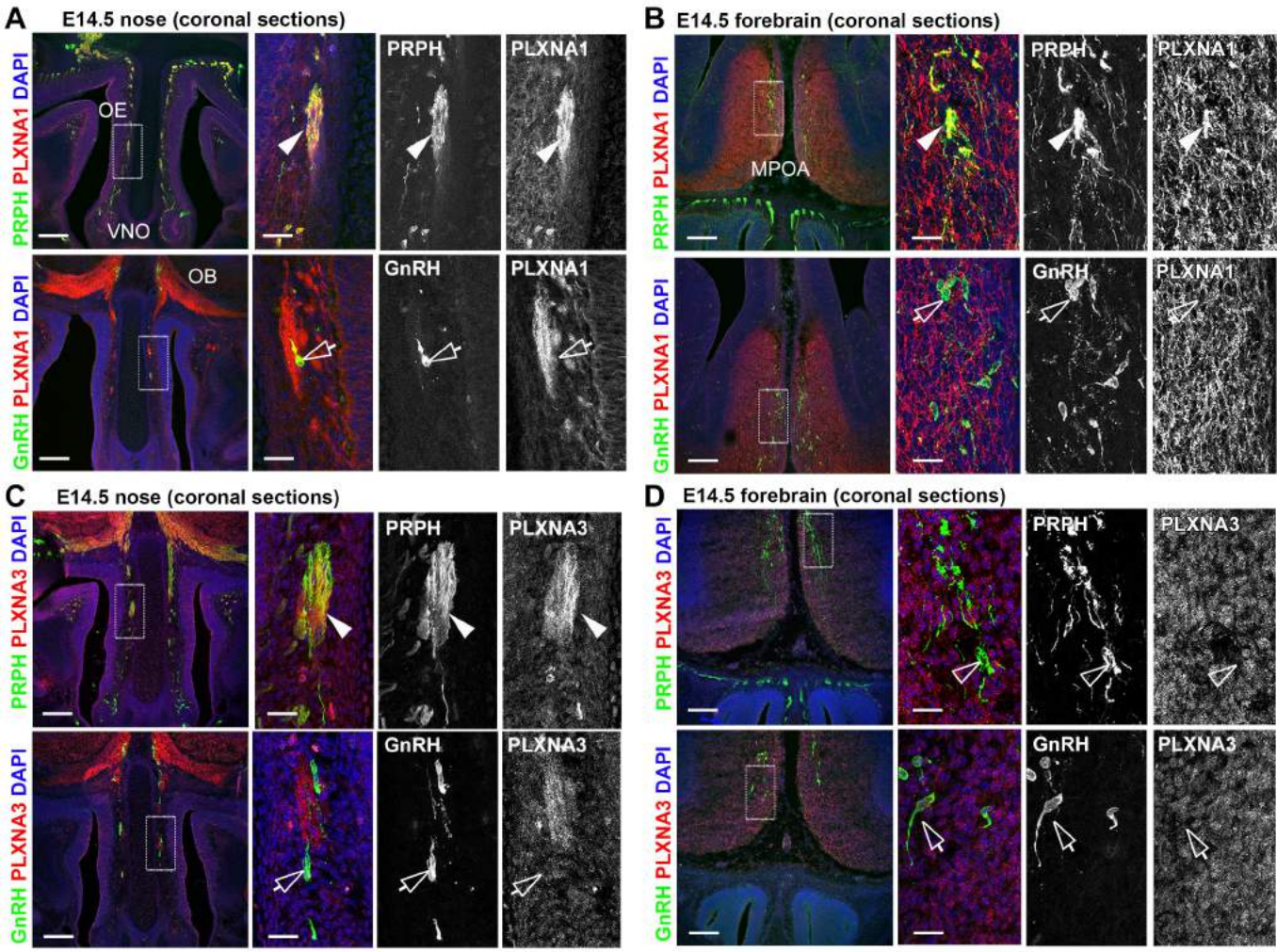475   Abbreviations: OB, olfactory bulb; CP, cribriform plate; MPOA, medial preoptic area.

476   Scale bar: 150 μm.

477   (C) Working model summarising the observed defects in GnRH neuron (green) migration

478   and axon organisation in wild type embryos vs. SEMA3A pathway mutants. Normal axon

479   projections are shown as continuous black lines, abnormal projections are shown as

480   continuous red line; the interrupted red line in mutants represents the missing cVN branch.

481   The corresponding, predicted signalling pathways are shown adjacent to each head

482   schematic.

A E12.5 nose wild type (coronal)

OB
nTUBB3 PLXNA1 DAPI
OE
VNO

PLXNA1 DAPI

nTUBB3 PLXNA3 DAPI

PLXNA3 DAPI

B E14.5 wild type nose (coronal)

OB
nTUBB3 PLXNA1 DAPI
OE
VNO

PLXNA1 DAPI

nTUBB3 PLXNA3 DAPI

PLXNA3 DAPI

C E12.5 nose wild type (coronal)

OB
S100 PLXNA1 DAPI
OE   NS
VNO

S100 PLXNA3 DAPI

D E14.5 nose wild type (coronal)

S100 PLXNA1 DAPI

S100 PlLXNA3 DAPI

E E14.5 nose (coronal)

PLXNA1 DAPI   *Plxna1-/-*

PlLXNA3 DAPI   *Plxna3-/-*

A — E14.5 nose (coronal sections)
PRPH PLXNA1 DAPI; OE; VNO
GnRH PLXNA1 DAPI; OB

B — E14.5 forebrain (coronal sections)
PRPH PLXNA1 DAPI; MPOA
GnRH PLXNA1 DAPI

C — E14.5 nose (coronal sections)
PRPH PLXNA3 DAPI
GnRH PLXNA3 DAPI

D — E14.5 forebrain (coronal sections)
PRPH PLXNA3 DAPI
GnRH PLXNA3 DAPI

A (columns: *wild type*, *Plxna1⁻/⁻*, *Plxna3⁻/⁻*, *Plxna1⁻/⁻;Plxna3⁻/⁻*)

Row 1 — GnRH / E14.5 CP: OB, OB, CP labels in wild type panel
B *Plxna1⁻/⁻;Plxna3⁻/⁻*

Row 2 — GnRH / E14.5 NOSE

Row 3 — GnRH / E14.5 MPOA

C GnRH neuron no. (E14.5)
nose, CP, forebrain, total
wild type, *Plxna1⁻/⁻*, *Plxna3⁻/⁻*, *Plxna1⁻/⁻;Plxna3⁻/⁻*
* ** ***

D P60 MPOA GnRH (columns: *wild type*, *Plxna1⁻/⁻*, *Plxna3^{y/-}*, *Plxna1⁻/⁻;Plxna3^{y/-}*)

E GnRH neuron no. (postnatal)
MPOA
wild type, *Plxna1⁻/⁻*, *Plxna3^{y/-}*, *Plxna1⁻/⁻;Plxna3^{-/-(y)}*
* ****

F ME / P60 (*wild type*, *Plxna1⁻/⁻;Plxna3^{y/-}*)

G P60 testes (*wild type*, *Plxna1⁻/⁻;a3^{y/-}*); P60 sem. ves. (*wild type*, *Plxna1⁻/⁻;a3^{y/-}*)

H P60 ovaries (*wild type*, *Plxna1⁻/⁻;a3⁻/⁻*)

A

wild type   *Plxna1⁻/⁻*   *Plxna3⁻/⁻*   *Plxna1⁻/⁻;Plxna3⁻/⁻*

E14.5 nose — PRPH (green)

E14.5 nose — GnRH (green)

B

E14.5 MPOA — PRPH (green)

E14.5 MPOA — GnRH (green)

C

wild type

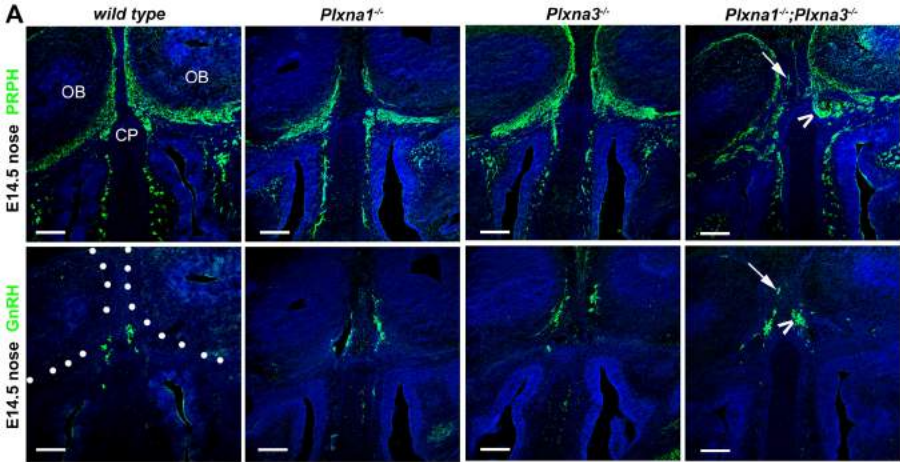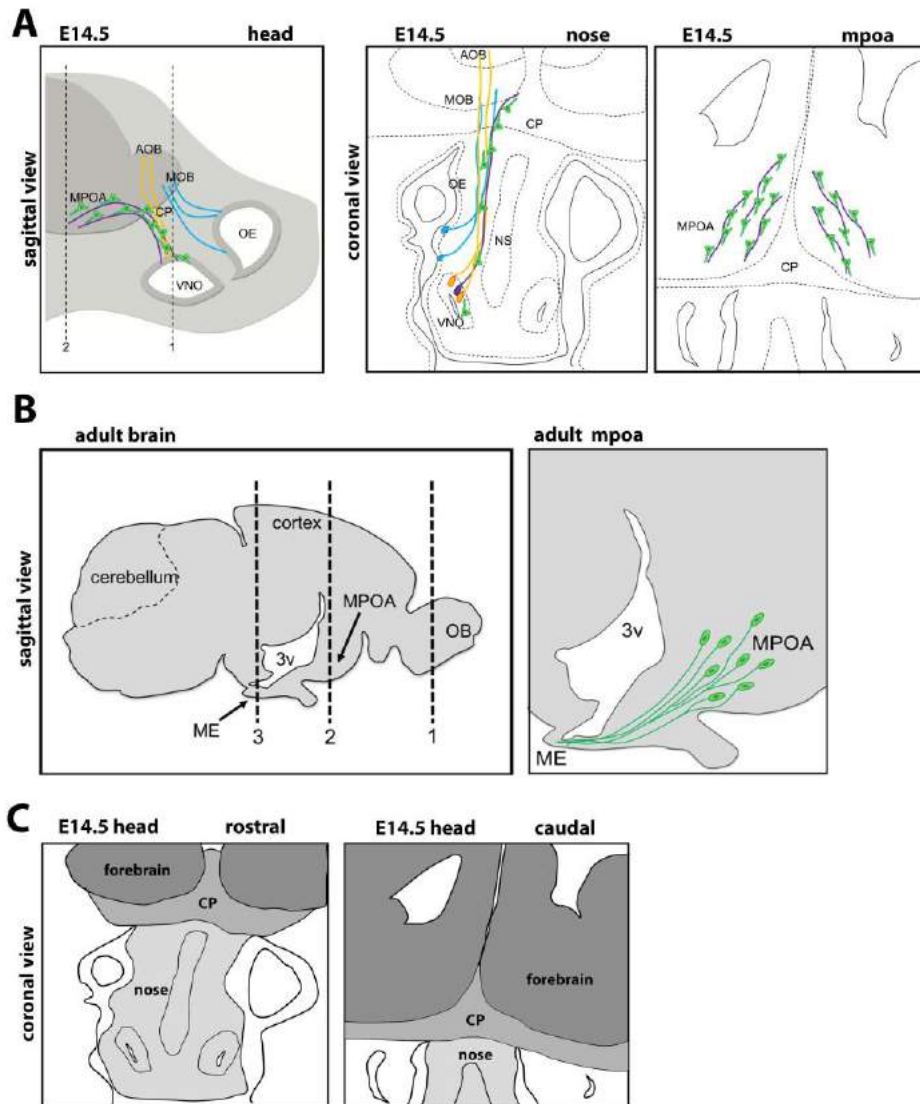*Sema3a⁻/⁻*
Nrp1^sema/sema ;Nrp2⁻/⁻
Plxna1⁻/⁻;Plxna3⁻/⁻

SUPPLEMENTARY MATERIAL

6 supplementary figures with legends

4 supplementary tables

Fig. S1

**Fig. S1. Head structures relevant to GnRH neuron migration and position.**

(**A**) Sagittal view of an E14.5 mouse head; dashed lines (1, 2) indicate the anatomical levels at which coronal sections were obtained for immunostaining in this study. The adjacent schematics show coronal views of the structures present in these sections. Nasal axons and their cell bodies are differently colored: OLF axons in blue, VN in orange and cVN/TN in purple; GnRH neurons are shown in green.

(**B**) Sagittal view of an adult brain; dashed lines (1-3) indicate the anatomical levels at which coronal sections were obtained for immunostaining in this study. The adjacent schematic shows that GnRH neurons in the adult MPOA project their axons towards the ME.

(**C**) Coronal view of an E14.5 mouse head at nasal (left panel) and MPOA (right panel) level. The anatomical compartments referred to in this study and used as reference points for for GnRH neuron number quantifications are distinguished by different shades of grey.

Abbreviations: OB, olfactory bulb; MPOA, medial preoptic area; VNO, vomeronasal organ; NS, nasal septum; CP, cribriform plate; ME, median eminence, 3v, third ventricle.

Fig. S2
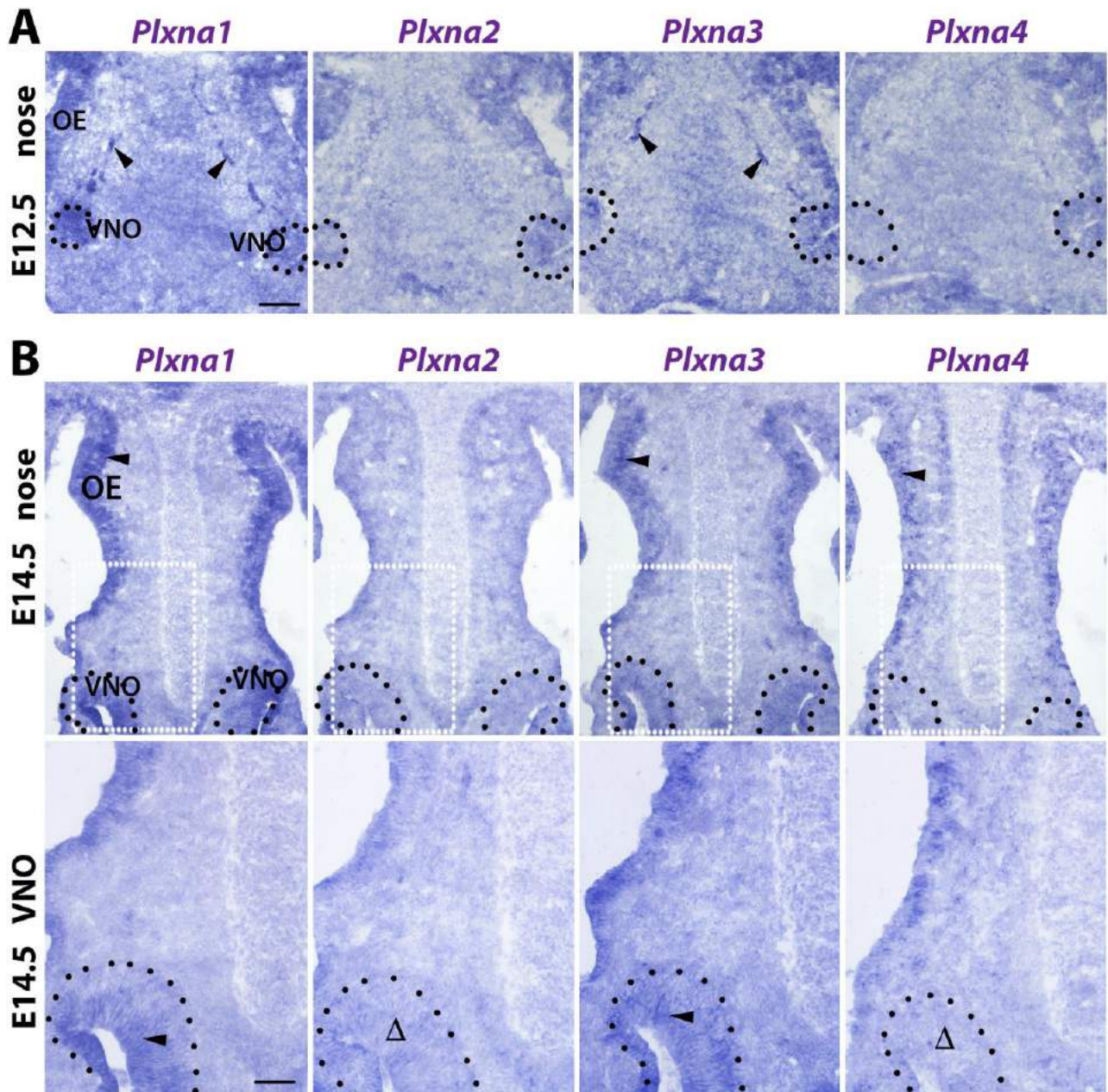
Fig. S1. *Plxna* expression in the mouse embryo nose.

In situ hybridisation to examine the expression of the indicated *Plxna* genes, performed with coronal sections from E12.5 (**A**) and E14.5 (**B**) mouse heads at the level of the VNO. Higher magnifications of the boxed areas are shown below each image. The black dots delineate the VNO. Arrowheads in (**A**) indicate examples of *Plxna1*- or *Plxna3*-positive cells that appear to be migrating from the VNO. Arrowheads in (**B**) indicate expression of *Plxna1, Plxna3* and *Plxna4* in the olfactory epithelium (OE, top row) or expression of *Plxna1* and *Plxna3* in the VNO (bottom row). Δ indicates lack of *Plxna2* and *Plxna4* expression in the VNO in (**B**).

Scale bars: 150 μm (**A,** top row in **B**), 100 μm (bottom row in **B**).

Abbreviations: VNO, vomeronasal organ; OE, olfactory epithelium.

Fig. S3
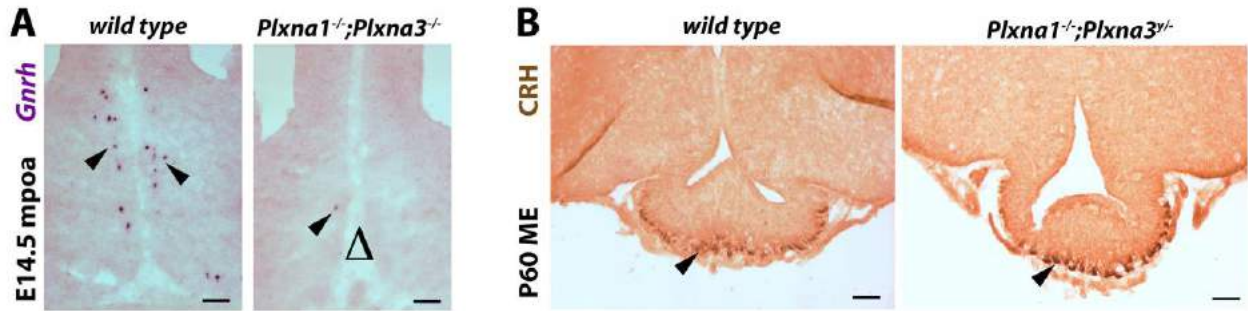
**Fig. S3. GnRH deficiency is not due to loss of GnRH peptide synthesis or an absent ME.**
(**A**) *In situ* hybridisation for the *Gnrh* transcript on coronal sections of E14.5 mouse heads at the level of the medial preoptic area (MPOA) in the indicated genotypes. Arrowheads indicate *Gnrh-*expressing neurons. Lack of *Gnrh+* cells in the *Plxna1-/-;Plxna3-/-* MPOA is indicated with Δ.
(**B**) Median eminence (ME) innervation by corticotropin-releasing hormone (CRH)+ fibers in coronal sections of a P60 *Plxna1-/-;Plxna3y/-* mutant and littermate wildtype control.
Scale bars: 150 μm.
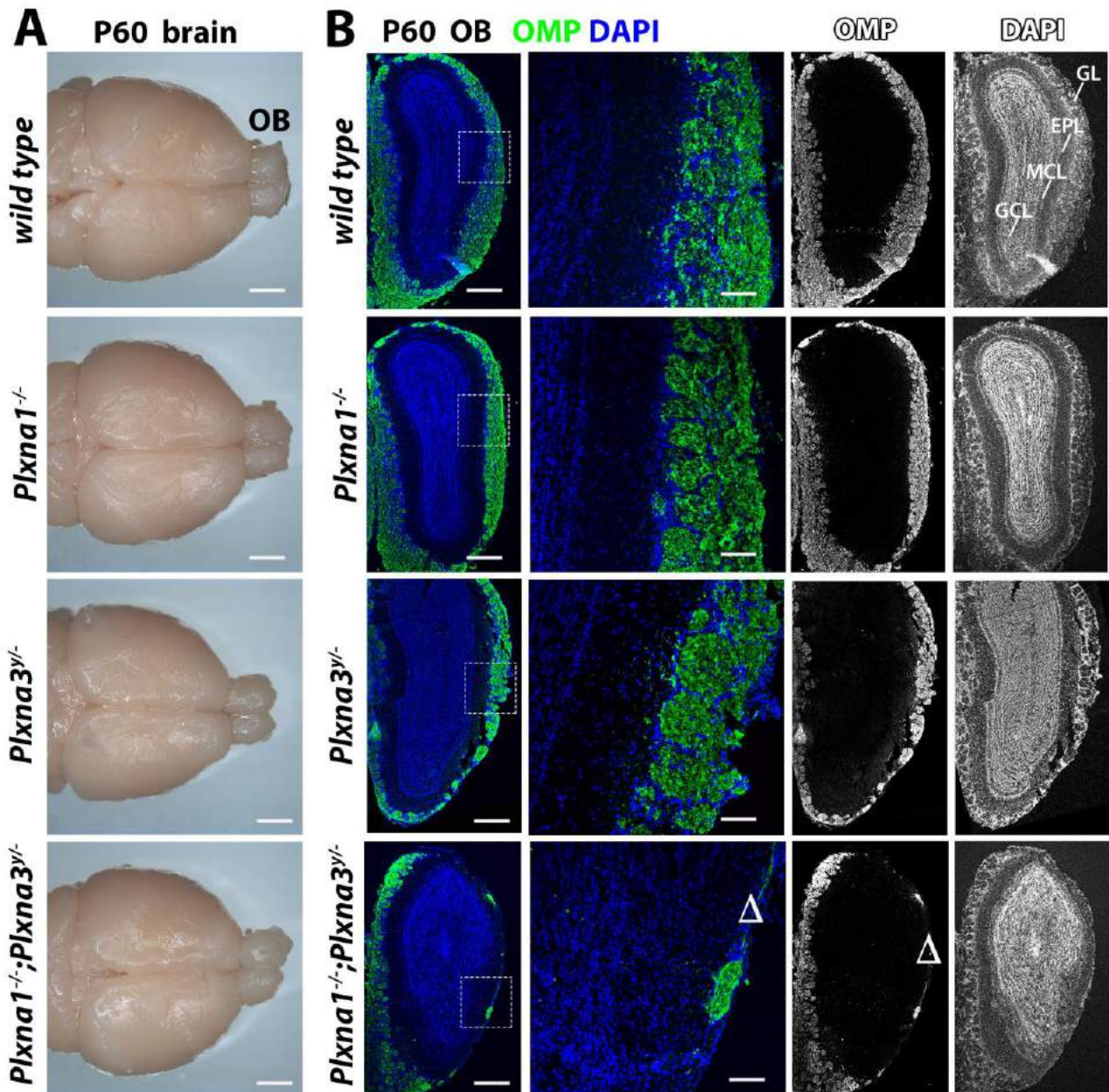
**Fig. S4. Impaired OB development in mice lacking PLXNA1 and PLXNA3.**

(**A**) Micrographs of P60 brains from mice of the indicated genotypes. The brain and olfactory bulbs (OBs) appear to be of similar size in all genotypes (see Supplemtal Table 4 for quantification).

(**B**) Coronal sections of P60 OBs from mice of the indicated genotypes were immunolabelled for OMP to reveal OB innervation and counterstained with DAPI. Higher magnifications of the boxed areas are shown adjacent to each image. Δ indicates a near absent glomerular layer (GL) in the dorso-lateral region of double mutants.

Scale bars: 3 mm (A), 500 and 100 µm (lower and higher magnifications in B, respectively).

Abbreviations: OB, olfactory bulb; GL, glomerular layer; EPL, external plexiform layer; MCL mitral cell layer; IPL, inner plexiform layer.

Fig. S5
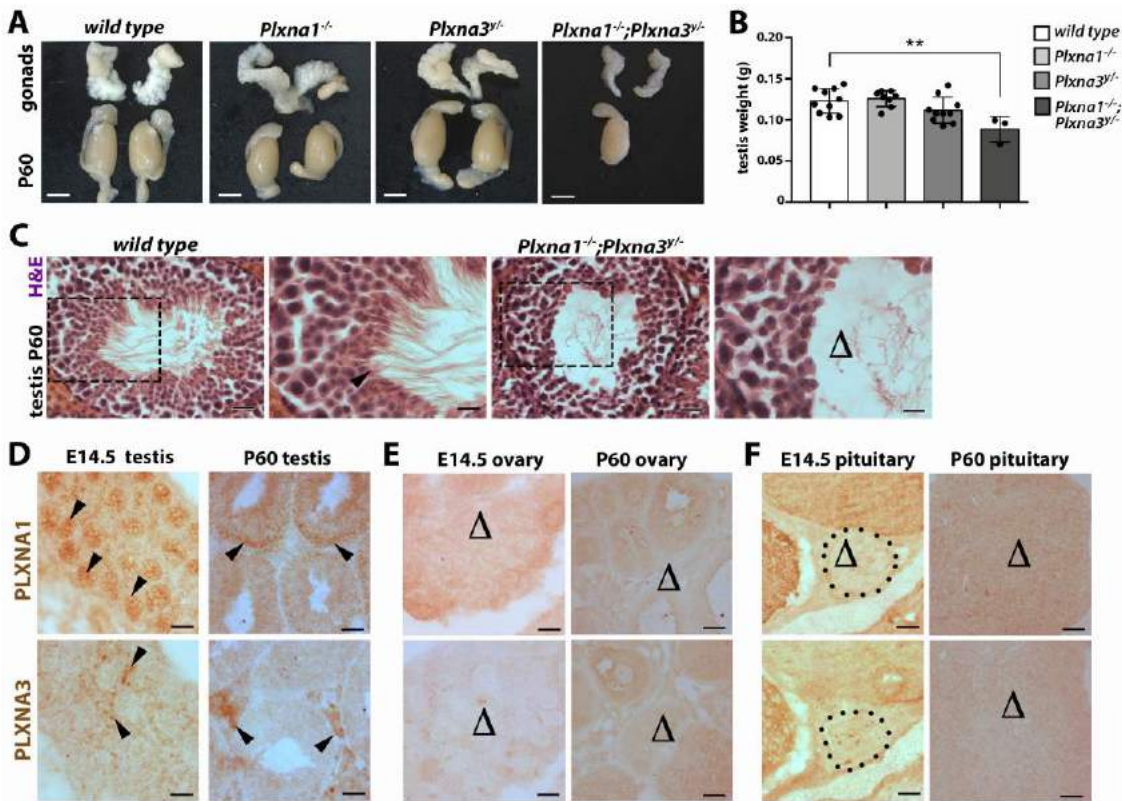
Fig. S5. PLXNA1 and PLXNA3 cooperate for testis formation but are not expressed in the ovaries or pituitary gland.

(**A**) Micrographs show pairs of seminal vesicles (top) and testes (bottom) in P60 mice of the indicated genotypes. The double mutant shown had only one testis. Scale bar: 3 mm.

(**B**) Individual testis weights in the indicated genotypes at P60. Data are shown as mean ± s.d,, and P-values compared to wild type were calculated by one-way ANOVA followed by a Dunnett's test; wildtype 0.123 ± 0.015 g (n = 10 testes); $Plxna1_{-/-}$ 0.126 ± 0.010 g (n = 8 testes), P > 0.05 (not significant); $Plxna3_{y/-}$ 0.112 ± 0.016 g (n = 10 testes), P > 0.05 (not significant); $Plxna1_{-/-};Plxna3_{y/-}$ 0.0849 ± 0.016 g (n = 3 testes), **P < 0.01.

(**C**) Haematoxylin and eosin (H & E) staining of P60 testes illustrate seminiferous tubules in wild type and double mutant mice; black boxes indicate areas shown at higher magnification adjacent to each image. The arrowhead indicates spermatozoa within the lumen of seminiferous tubules; Δ indicates near absence of spermatozoa. Scale bars: 50 μm or 25 μm for lower and higher magnifications, respectively.

(**D-F**) Expression analysis for PLXNA1 and PLXNA3. Cryosections of wild type E14.5 and P60 testis (**D**), ovary (**E**) and pituitary (circled in **F**) were immunolabelled for PLXNA1 (upper panels) or PLXNA3 (lower panels). Arrowheads in (**D**) indicate PLXNA1 localisation to seminiferous tubule cells and PLXNA3 localisation to interstitial cells. Lack of PLXNA1 or PLXNA3 expression in (**E,F**) is indicated with Δ. Scale bars: 150 μm (E14.5) and 50 μm (P60).
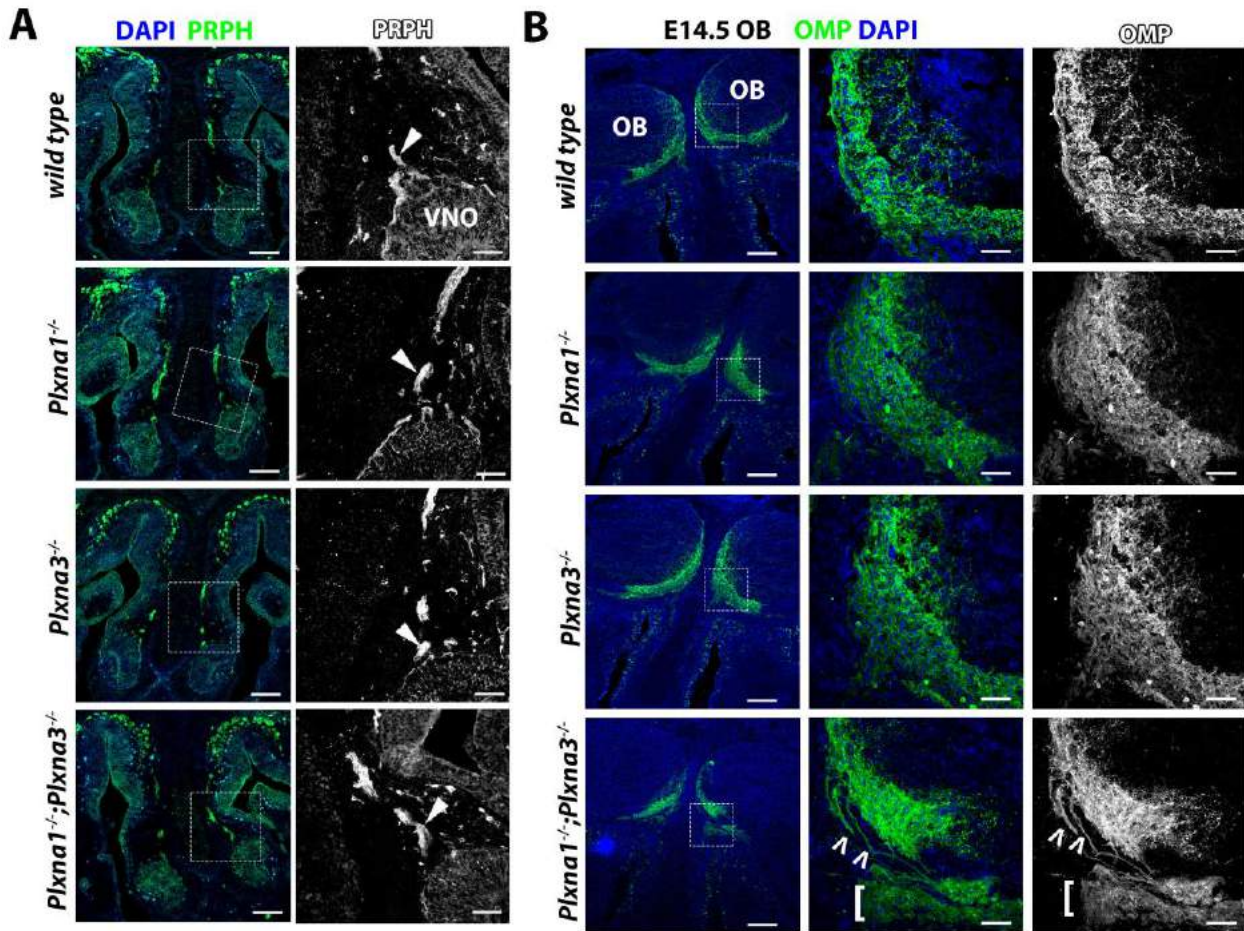
Fig. S6

Fig. S6. Combined PLXNA1 and PLXNA3 loss does not preclude axon projection out of the VNO, but compromises olfactory axon projection.

(**A**) Coronal sections of E14.5 mouse heads of the indicated genotypes at the level of VNO were immunolabelled for PRPH to reveal VN and cVN axons and counterstained with DAPI. White boxes indicate areas shown at higher magnification on the right of the corresponding panel as the single channel for PRPH in grey scale. Solid arrowheads indicate PPRH+ axons that emerge from the VNO and therefore represent intermingled VN and cVN axons. Scale bars: 125 and 40 μm for lower and higher magnifications, respectively.

(**B**) Coronal sections of E14.5 mouse heads of the indicated genotypes were immunolabelled for OMP to reveal OLF axons and were counterstained with DAPI. White boxes indicate areas shown at higher magnification adjacent to the corresponding image, including single channels for OMP in grey scale. Open arrowheads indicate examples of defasciculated axons, whereas the brackets indicate an area with abnormal OMP+ axons below the ventro-medial OB.

Scale bars: 150 and 50 μm (higher and lower magnifications, respectively).

Abbreviations: VNO, vomeronasal organ; OB, olfactory bulb.

Table S1.

GnRH+ cell number in E14.5 mouse heads of the indicated genotypes. CP, cribriform plate. Data are shown as mean ± s.d. and P-values were calculated with a one-way ANOVA followed by a Dunnett's test for mutants relative to wild type; ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant (P > 0.05).

Genotype	Nose	CP	Forebrain	Total
Wild type (n = 4 mice)	261.5 ± 59.04	263.5 ± 16.18	591.8 ± 104.0	1228 ± 154.9
Plxna1-/- (n = 6 mice)	266.3 ± 71.16 ns	377.0 ± 96.71 ns	521.0 ± 131.3 ns	1164 ± 136.6 ns
Plxna3-/- (n = 3 mice)	372.0 ± 71.63 ns	381.7 ± 81.38 ns	520.3 ± 112.5 ns	1274 ± 133.1 ns
Plxna1-/-;Plxna3-/- (n = 4 mice)	429.5 ± 118.5 *	519.5 ± 101.6 **	183.5 ± 50.13 ***	1133 ± 188.2 ns

Table S2.

Mendelian ratio of postnatal mice in 5 different litters obtained from matings of *Plxna1+/-;Plxna3+/-* females with *Plxna1+/-;Plxna3y/-* males.

Genotype	Number of mice in litters	% expected			% observed		
		total	male	female	total	male	female
Plxna1+/+;Plxna3y/+, Plxna1+/+;Plxna3+/-	6	12.50	6.25	6.25	10.79	8.97	1.82
Plxna1+/-;Plxna3y/+	8	12.50	12.50	0	14.58	14.27	0
Plxna1-/-;Plxna3y/+, Plxna1-/-;Plxna3+/-	8	12.50	6.25	6.25	14.75	9.45	5.30
Plxna1+/+;Plxna3y/-, Plxna1+/+;Plxna3-/-	9	12.50	6.25	6.25	16.09	16.09	0
Plxna1+/-;Plxna3+/-	4	12.50	0	12.5	6.97	0	6.97
Plxna1+/-;Plxna3y/-, Plxna1+/-;Plxna3-/-	15	25.00	12.50	12.5	27.33	6.67	18.33
Plxna1-/-;Plxna3y/-, Plxna1-/-;Plxna3-/-	5	12.50	6.25	6.25	9.48	10.00	2.50

Table S3.

Total GnRH neuron number in postnatal MPOAs from mice of the indicated genotypes; data are shown as mean ± s.d.; P-values were calculated with a one-way ANOVA followed by a Dunnett's test for mutants relative to wild type; *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$; ns, not significant ($P > 0.05$).

Genotype	MPOA GnRH neuron number
Wild type (n = 5 males)	691.0 ± 153.4
Plxna1$_{-/-}$ (n = 5 males)	466.4 ± 93.0 (*)
Plxna3$_{y/-}$ (n = 4 males)	637.0 ± 88.04 (ns)
Plxna1$_{-/-}$;Plxna3$_{-/-(y)}$ (n = 4; 3 males, 1 female)	143.3 ± 41.91 (****)
Plxna1$_{-/-}$;Plxna3$_{+/-}$ (n = 2 females)	231 ± 79.2 (***)

Table S4.

Hemisphere and OB area in images of brains dissected from P60 mice of the indicated genotypes, indicated as mean ± s.d.; P-values were calculated with a one-way ANOVA followed by a Dunnett's test for mutants relative to wild type; ns, not significant ($P > 0.05$).

Genotype	Hemisphere area (mm$_2$)	OB area (mm$_2$)
Wild type (n = 3 mice; 2 males, 1 female)	222.0 ± 6.21	26.53 ± 4.12
Plxna1$_{-/-}$ (n = 2 males)	225.8 ± 14.89 (ns)	33.15 ± 1.29 (ns)
Plxna3$_{y/-}$ (n = 3 males)	224.1 ± 10.26 (ns)	28.98 ± 1.75 (ns)
Plxna1$_{-/-}$;Plxna3$_{-/-(y)}$ (n = 3; 2 males, 1 female)	215.4 ± 8.09 (ns)	29.31 ± 2.06 (ns)