Defective gonadotropin-releasing hormone neuron migration in mice lacking SEMA3A signalling through NRP1 and NRP2: implications for the aetiology of hypogonadotropic hypogonadism

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Kallmann syndrome (KS) is a genetic disease characterized by hypogonadotropic hypogonadism and impaired sense of smell. The genetic causes underlying this syndrome are still largely unknown, but are thought to be due to a developmental defect in the migration of gonadotropin-releasing hormone (GnRH) neurons. Understanding the causes of the disease is hampered by lack of appropriate mouse models. GnRH neurons are hypothalamic cells that centrally control reproduction in mammals by secreting the GnRH decapeptide into the portal blood vessels of the pituitary to stimulate the production of gonadotropins. During development, these cells are born in the nasal placode outside the brain and migrate in association with olfactory/vomeronasal axons to reach the forebrain and position themselves in the hypothalamus. By combining the analysis of genetically altered mice with in vitro models, we demonstrate here that a secreted guidance cue of the class 3 semaphorin family, SEMA3A, is essential for the development of the GnRH neuron system: loss of SEMA3A signalling alters the targeting of vomeronasal nerves and the migration of GnRH neurons into the brain, resulting in reduced gonadal size. We found that SEMA3A signals redundantly through both its classical receptors neuropilin (NRP) 1 and, unconventionally, NRP2, while the usual NRP2 ligand SEMA3F is dispensable for this process. Strikingly, mice lacking SEMA3A or semaphorin signalling through both NRP1 and NRP2 recapitulate the anatomical features of a single case of KS analysed so far, and may therefore be used as genetic models to elucidate the pathogenesis of KS.

INTRODUCTION

Hypothalamic gonadotropin-releasing hormone (GnRH) neurons are neuroendocrine cells that centrally regulate the reproductive axis in mammals by projecting to the median eminence, where they secrete the decapetide into the pituitary portal vessels to induce the release of gonadotropins into the general circulation (1). They are unique among the other releasing factor neurons in that they are generated outside the brain and utilize an ‘entry’ mechanism to gain access to it. Specifically, GnRH neurons are born in the nasal placode, starting at embryonic day (E) 10.5 in mice, and migrate along olfactory/vomeronasal nerves towards the cribriform plate and into the forebrain. Once in the forebrain, they follow the caudal branch of the vomeronasal nerve to reach the hypothalamus by the time of birth (2). Loss of these neurons or their misdirected migration impairs sexual reproduction in mammals.

In humans, the deficiency of hypothalamic GnRH leads to hypogonadotropic hypogonadism (HH), causing absence or delay of puberty. When combined with anosmia, HH is referred to as Kallmann syndrome (KS), a genetic disorder...
that affects 1 in 10,000 males, with most cases being sporadic (3, 4). The aetiology of KS is still largely unknown, but is thought to be due to defective targeting of the olfactory/vomeronasal axons and therefore abnormal migration of GnRH neurons. Such a defect was shown in the single human foetus so far analysed, which carried a mutation in the gene responsible for the X-linked form of KS (KAL1); in this foetus, GnRH neurons failed to enter the brain and accumulated in a tangle with olfactory/vomeronasal nerves on the dorsal surface of the cribriform plate (5). To date, only a few mutated genes have been identified in patients with KS (6), implying that further genes crucial for GnRH neuron development still need to be discovered.

The molecular mechanisms underlying GnRH neuron development involve signals that directly regulate their migration or pattern the olfactory/vomeronasal nerves (reviewed in 7). Two recent studies have highlighted the importance of classical neuron/axon guidance cues, such as semaphorins, in this system (8, 9). The membrane-bound semaphorin (SEMA) 4D was shown to promote directional migration of GnRH neurons by acting as a long-range guidance cue (8), whereas we found that neuropilin (NRP) 2, which is best known as the receptor for the secreted SEMA3F, helps to organize the olfactory/vomeronasal axons that guide GnRH neurons along their migratory path (9). In the same study we suggested, based on in vitro experiments with an immortalized cell line, that the semaphorins SEMA3F and SEMA3A may also be involved in this process (9).

Here, we used a series of genetic mouse models as well as in vitro experiments to conduct a comprehensive analysis of the physiological roles of SEMA3A and SEMA3F and their neuropilin receptors (NRP1 and NRP2, respectively) in the establishment of the GnRH neuron system. We found that the development of this system relies on SEMA3A signalling through NRP1 and NRP2: mice lacking SEMA3A or mice lacking semaphorin signalling through both neuropilins show the phenotypical features reported for the X-linked KS foetus (5), with GnRH neurons and vomeronasal axons accumulating at the dorsal surface of the cribiform plate. We further explored the molecular and cellular mechanisms underlying class 3 semaphorin signalling in GnRH neuron development and found that SEMA3F is dispensable for this process, while SEMA3A unconventionally signals through both NRP1 and NRP2 to control vomeronasal axons (VNA) targeting into the brain and guide GnRH neurons towards the hypothalamus. These results demonstrate for the first time the essential role of SEMA3A signalling in the regulation of sexual reproduction and raise the possibility that genetic defects in components of the SEMA3A pathway may lead to KS.

**RESULTS**

**NRP1 and NRP2 are expressed in the axons that guide migrating GnRH neurons**

We had reported earlier that loss of NRP2 function impairs the fasciculation of the intermingled olfactory/vomeronasal axons that guide migrating GnRH neurons within the nose (9). Here, we first performed double-immunofluorescence staining for peripherin, a marker of these axons, and NRP2 on E14.5 mouse coronal sections to confirm the expression of NRP2 on the vomeronasal nerve (Fig. 1A). We then extended our analysis to NRP1 and found that this receptor, like NRP2, was expressed on vomeronasal axons and additionally on blood vessels within the nasal compartment (Fig. 1B). These observations raise the possibility that the two neuropilins cooperate to pattern the vomeronasal axons, the main substrate of the migrating GnRH neurons, on their journey from the nose into the forebrain and towards the hypothalamus.

**SEMA3F signalling is not essential for GnRH neuron migration**

Because NRP2 is essential for the fasciculation of the axons that guide migrating GnRH neurons, and the main NRP2 ligand SEMA3F repels immortalized GnRH neurons (GN11 cells) in vitro (9), we next asked whether SEMA3F was essential for GnRH neuron development. To answer this question, we compared the patterning of olfactory/vomeronasal axons and the position of GnRH neurons in Sema3f-null mutants and in their wild-type littermates (10), using immunohistochemistry for GnRH and the axon marker peripherin at E14.5, when migrating GnRH neurons are normally present in both the nose and forebrain (9). Unexpectedly, loss of SEMA3F did not compromise olfactory/vomeronasal axon-patterning (Fig. 2A and B) or GnRH neuron migration (Fig. 2D–F), and the number of GnRH neurons appeared similar in the medial preoptic area of postnatal SEMA3F-deficient mice and their wild-type littermates (Fig. 2G and H). These observations demonstrate that SEMA3F is dispensable for GnRH neuron migration in vivo, and suggest that NRP2 patterns their axonal substrate by serving as a receptor for a semaphorin other than SEMA3F.
SEMA3A is essential to pattern vomeronasal axons

We next investigated the role of SEMA3A in GnRH neuron development. This ligand is expressed in the developing olfactory system, and axons project aberrantly to the olfactory bulb in Sema3a-null mutants (11). In addition, SEMA3A repels in vitro (9). Using immunohistochemistry on sagittal sections of E14.5 mouse heads, we found that Sema3a-null mutants contained a normal number of GnRH neurons (compare Fig. 3A with B; Fig. 3D; Sema3a+/+ 1244 ± 39 versus Sema3a−/− 1197 ± 97; n = 3 each). Yet, few such neurons were present in the forebrain of mutants at this stage (Fig. 3B and E; Sema3a+/+ 496 ± 31 versus Sema3a−/− 53 ± 8; n = 3 each; P < 0.01). Instead of entering the forebrain, most GnRH neurons had accumulated in the nasal mesenchyme at the level of the cribriform plate (open arrowhead in Fig. 3B’ or in the tissue distal to the normal forebrain entry point (wavy arrow in Fig. 3B’; 326 ± 17 ectopic neurons in Sema3a−/− mutants; n = 3). The position of ectopic GnRH neurons coincided with the location of ectopic vomeronasal axons stained for peripherin (Fig. 3C).

Immunofluorescence analysis of contiguous coronal sections at E14.5 with antibodies for peripherin and GnRH further corroborated that axon guidance errors were the likely cause of the ectopic positions of GnRH neurons in Sema3a-null mutants (Fig. 4). Thus, GnRH neurons were seen to accumulate at the cribriform plate and between the olfactory bulbs, in a location distal to their normal forebrain entry point (Fig. 4C), and significantly fewer neurons were present in the forebrain (Fig. 4G). Moreover, this abnormal GnRH neuron distribution correlated with the presence of ectopic axons at the cribriform plate (compare Fig. 4C with...
lack of GnRH neurons and VNAs in the basal FB of
GnRH neurons at the level of the cribriform plate (CP), respectively. The
olfactory bulbs (OB); open arrowheads indicate axon tangles and excess
GnRH neurons at the level of the cribiform plate (CP), respectively. The
lack of GnRH neurons and VNAs in the basal FB of Sema3a−/− mutants is
indicated with Δ in (G) and (H). Scale bars: 100 μm in A–H.

Figure 4. Aberrant olfactory/vomeronasal axon patterning and GnRH neuron
migration in Sema3a null mice. (A–H) Contiguous coronal sections of E14.5
mouse heads were immunolabelled to reveal GnRH neurons (green) and olfac-
tory/vomeronasal axons (red) in the nasal compartment/olfactory bulb and the
basal forebrain (FB) of wild-type and Sema3a−/− littersmates. Wavy arrows
indicate mistargeted axons and associated ectopic GnRH neurons between
the olfactory bulbs (OB); open arrowheads indicate axon tangles and excess
GnRH neurons at the level of the cribiform plate (CP), respectively. The
Sema3a−/− mutants were smaller than those of wild-type littermates
(P = 0.01; Fig. 3F; compare Fig. 3G with H). Because of the reduced entry of GnRH
neurons in the forebrain, there were very few GnRH-positive fibres in the hypothalamus at this age (Fig. 3I and J). Consist-
ent with these findings in foetal stages, GnRH neuron projections
to the median eminence were markedly reduced in adult
Sema3a−/− null males compared with wild-type littermates.

Figure 5. GnRH neurons migrate abnormally in single Nrp1Sema−/− and
Nrp2−/− mutants, and are affected more severely in compound
Nrp1Sema−/− Nrp2−/− mice. (A–E) Sagittal sections of E14.5 mouse heads
were immunolabelled for GnRH to reveal migrating neurons in the nasal com-
partmen and forebrain (FB) of the genotypes shown. Examples of migrating
neurons are indicated with arrowheads; the boundary of each FB is marked
with a black dotted line. In Nrp1Sema−/− mutants, a few GnRH neurons
migrated towards the meninges (wavy arrow in B). In Nrp2−/− mutants,
small aggregates of GnRH neurons were seen in the nasal compartment
(clear arrow in C). (E) In double-mutants, most GnRH neurons failed to
enter the FB (Δ) but, instead, accumulated at the level of the cribiform
plate (open arrowhead) or took an ectopic path towards the meninges (wavy
arrow). (F) Quantitation of GnRH neuron number in the FB at E14.5. Scale
bar: 100 μm in A–E.

D) and the absence of the vomeronasal axons in the forebrain
(compare Fig. 4G with H). Because of the great severity of the
axon defect in the Sema3a-null mutants, we could not ascen-
tain whether Sema3A played an additional and direct role
in guiding GnRH neurons.

We next extended our analysis of Sema3a-null mice to
E18.5, when the migration of GnRH neurons is largely com-
plete, and confirmed that the number of these cells in the fore-
brain remained severely reduced (Sema3a+/+ 631 ± 55 versus
Sema3a−/− 101 ± 46; n = 3; P < 0.01; Fig. 3F; compare
Fig. 3G with H). Because of the reduced entry of GnRH
neurons in the forebrain, there were very few GnRH-positive
fibres in the hypothalamus at this age (Fig. 3I and J). Consist-
ent with these findings in foetal stages, GnRH neuron projections
to the median eminence were markedly reduced in adult
Sema3a-null males compared with wild-type littersmates
(compare Fig. 3K with L). Accordingly, the testes of adult
mutants were smaller than those of wild-type littersmates
(Fig. 3M; n = 3 each). We conclude that Sema3A is essential
to organize the axons that are used by GnRH neurons to enter
the forebrain and is therefore required for the formation of the
GnRH neuron system.

Mice lacking semaphorin signalling through NRP1 only
partially phenocopy the GnRH neuron defect of
Sema3a-null mutants

Because NRP1 is considered to be the obligatory SEMA3A
receptor [e.g. refs. (12–15)], we anticipated that loss of semi-
aphorin signalling through NRP1 would phenocopy the vomer-
onasal axon and, therefore, the GnRH neuron defect of
Sema3a-null mutants. Thus, we analysed GnRH neuron develop-
ment in a Nrp1 mutant mouse strain that is deficient in semaphorin signalling through NRP1, because it carries
point mutations that prevent the interaction with the SEMA
domain [Nrp1Sema−/− mice; (16)]. The previously reported
similarity of axon-patterning defects between Nrp1Sema−/−
and Sema3a−/− mice in the cranial and spinal nerves had
already established that this Nrp1 allele is effective in disrupt-
ing SEMA3A signalling through NRP1, but that it leaves
the vascular NRP1 functions intact [e.g. (16–18)]. Therefore,
using Nrp1Sema−/− mice circumvents the mid-gestation embryonic
lethality of full Nrp1-null mutants that is caused by cardiovas-
cular defects (16).

Immunohistochemical analysis of sagittal sections of E14.5
Nrp1Sema−/− mutants demonstrated a mild defect in the pat-
terning of peripherin-positive axons, with most vomeronasal
axons projecting into the forebrain and only a few ectopic
axons projecting into the meningeal tissue surrounding the
forebrain (data not shown). This mild axonal defect correlated
with the presence of a few ectopic GnRH-positive neurons
in the areas containing the abnormal axons (compare Fig. 5A
with B) and with a small, but significant reduction of
GnRH-positive cells in the forebrain (Fig. 5F; Nrp1Sema+/+
509 ± 44 versus Nrp1Sema−/− 349 ± 26; n = 4 each; P <
0.05). The overall number of GnRH neurons in the head of the
Nrp1Sema−/− mutants was normal (Nrp1Sema+/+ 1265 ± 75
versus Nrp1Sema−/− 1211 ± 48; n = 3 each). Because the severity of the vomeronasal axon defect of Sema3a-null mice was not recapitulated in Nrp1Sema−/− mutants, which
contrasts the similarity of both mutants in cranial and spinal
nerve axon patterning, we hypothesized that SEMA3A
signals redundantly through NRP1 and a second receptor in
the axons that guide GnRH neurons. A likely candidate was
NRP2, as it contributes to GnRH neuron migration in vivo
(9) independently of its main ligand SEMA3F (Fig. 2).
SEMA3A signals through both NRP1 and NRP2 to organize the axons that guide GnRH neurons into the forebrain

To investigate whether NRP2 cooperates with NRP1 to convey SEMA3A signals in the axons that guide GnRH neurons, we compared single Nrp1<sup>Sema</sup>−/− and Nrp2<sup>−/−</sup> mutants with compound Nrp1<sup>Sema</sup>−/− Nrp2<sup>−/−</sup> mice, which lack all semaphorin signaling through neuropilins and survive to birth (16). As previously shown (9), we found that Nrp2<sup>−/−</sup> mutants contained small aggregates of GnRH neurons within the nasal compartment at E14.5 (clear arrow in Fig. 5C). Accordingly, the number of aggregates of GnRH neurons within the nasal compartment at E14.5 (Fig. 5D–F; double immunofluorescence labelling was not possible with the antibodies available). GnRH neurons had reached the forebrain at E14.5, while GnRH neurons had completely lacked GnRH neurons in the basal forebrain at E14.5 (Fig. 5D–F; normal through both NRP1 and NRP2 to guide vomeronasal axons. We therefore asked next whether SEMA3A was able to bind NRP2 <em>in situ</em>. To answer this question, we compared the binding of previously tested alkaline phosphatase (AP)-tagged SEMA3A and SEMA3F proteins to tissue sections [e.g. (18,19)]. We first confirmed that SEMA3F and SEMA3A bound to wild-type tissue in regions that express NRP1 and NRP2, and that binding could be competed with the non-tagged ligands (Supplementary Material, Fig. S1). We then confirmed that SEMA3F bound to head tissue from wild-type mice, which express NRP2, but not to head tissue from NRP2 knockout mice. As expected, loss of NRP1 or the Nrp1<sup>Sema</sup>−/− mutation did not affect SEMA3F binding, because it depends on NRP2 rather than NRP1 (Supplementary Material, Fig. S2A and B, Fig. 7A–D).

We subsequently found that SEMA3A bound to some areas in head tissue from single Nrp1<sup>Sema</sup>−/− or Nrp2<sup>−/−</sup> mutants almost as well as to tissue from their wild-type littermates (Fig. 7E–G). We therefore asked if SEMA3A also bound to head tissue lacking NRP1 expression completely i.e. NRP1 knockout mice. For this experiment, we reacted the SEMA3A-AP protein with sections from littermate embryos at E12.5, the latest time point at which homozygous mutants in the outbred CD1 background are still viable (20). We found that SEMA3A binding to wild-type tissue at this stage was less intense than at E14.5, but still clearly detectable (Supplementary Material, Fig. S2C). Thus, SEMA3A bound to the olfactory bulb (OB) of wild-types, but binding to the OB was abolished in mice lacking NRP1 (Supplementary Material, Fig. S2C and D), consistent with the idea that SEMA3A/NRP1 signalling patterns olfactory axons (11). However, SEMA3A still bound to nasal tissue lacking NRP1 (Supplementary Material, Fig. S2D), and was completely abolished only in compound Nrp1<sup>Sema</sup>−/− or Nrp2<sup>−/−</sup> mutants (Fig. 7H). Together, these findings confirm that the Nrp1<sup>Sema</sup> allele is not hypomorphic with respect to SEMA3A binding,

**SEMA3A binds to tissue from Nrp1<sup>Sema</sup>−/− or Nrp2<sup>−/−</sup> mutants, but not to tissue from compound neuropilin mutants**

Our <em>in vivo</em> analysis implied that SEMA3A could signal through both NRP1 and NRP2 to guide vomeronasal axons.
Figure 7. NRP2 serves as a SEMA3A receptor in vivo and in vitro. (A–I) Alkaline phosphatase (AP)-ligand binding assay on tissues from neuropilin mutants. Sagittal sections of E14.5 mouse heads of the indicated genotypes were incubated with SEMA3F-AP or SEMA3A-AP ligand. As expected, SEMA3F-AP bound olfactory axons in wild-type mice and Nrp1Sema2−/− mutants (arrowheads in A and B), but not in Nrp2-null mutants or compound neuropilin mutants (Δ in C and D). As expected, SEMA3A-AP-bound wild-type tissues (arrowhead in E), and binding was not obviously affected in Nrp2-null mutants (arrowhead in G). Binding of SEMA3A to Nrp1Sema2−/− tissue was reduced, but not abolished (F). Moreover, in both wild-types and single mutants, SEMA3A-AP bound to an area in which the caudal branch of the vomeronasal nerve is located (arrows in E–G). SEMA3A-AP binding was abolished in compound neuropilin mutants (Δ in H). The FB boundary is indicated with a black dotted line. (I–M) Nrp2 shRNA reduces the responsiveness of GN11 cells to SEMA3A and SEMA3A in the Boyden chamber chemotactic assay. The immunofluorescent staining of GN11 cells for NRP2 and NRP1 (red in K and L) demonstrated that the transfection of a bicistronic plasmid encoding GFP and a Nrp2 shRNA knocked-down NRP2 expression (transfected cells are green in L and outlined with white dots in J). In contrast, the Nrp2 shRNA did not affect the expression of NRP1 (indicated by yellow cells in K; arrowheads indicate transfected NRP1-expressing cells in L). Note that some cells naturally express low levels of NRP1. (M) Quantitation of the number of GN11 cells that migrated in response to COS cell-conditioned medium (control), or COS cell-conditioned medium containing SEMA3F or SEMA3A. The number of cells that had migrated after transfection of the scrambled Nrp2 shRNA (control) is shown in the grey columns, while the number of cells that had migrated after transfection of the inhibitory Nrp2 shRNA is shown in white columns. Abbreviations: OB, olfactory bulb. Scale bars: 100 μm in A–H, 25 μm in I–L.

but that NRP2 compensates for NRP1 in at least some SEMA3A-binding tissues.

Reducing the level of NRP2 impairs SEMA3A signalling in vitro

Previous experiments in the non-neuronal COS cell line, which was transfected with neuropilin expression vectors, had suggested that NRP1, but not NRP2, serves as the SEMA3A receptor [e.g. (12–15,19,21)]. However, our results on axon-patterning and ligand-binding in vitro supported the idea that NRP2 serves as a SEMA3A receptor to help organize vomeronasal axons. These findings are consistent with a recent report that showed SEMA3A binding to both neuropilins in cancer cell lines (22). We, therefore, wanted to re-examine the possibility that NRP2 contributes to SEMA3A signalling in a neuronal tissue culture model. For these studies, we took advantage of the fact that GN11 cells, like vomeronasal axons, express both NRP1 and NRP2 and can be used to measure migratory responses to secreted semaphorins (9).

To study the contribution of NRP2 to SEMA3A signalling, we transfected GN11 cells with a bicistronic plasmid encoding green fluorescent protein (GFP) and an inhibitory short hairpin RNA (shRNA) that targets Nrp2 (23). Even though neuropilin expression was somewhat heterogeneous between individual GN11 cells, with low- and high-level expressing cells, immunofluorescence labelling confirmed that NRP2 expression was low in most cells expressing GFP (Fig. 7I and J). The Nrp2 shRNA, therefore, knocked down NRP2 effectively, in a proportion of cells. In contrast, NRP1 was present at normal levels in most GFP-expressing cells, establishing that the shRNA was specific for NRP2 (Fig. 7K and L).

The transfection of the inhibitory Nrp2 shRNA or a control vector expressing scrambled shRNA did not affect GN11 chemotaxis in the Boyden chamber assay in the absence of SEMA3A or SEMA3F, confirming that they did not impair cell migration (control, Fig. 7M). As expected, the inhibitory Nrp2 shRNA, but not the control shRNA, significantly reduced the responsiveness of GN11 cells to SEMA3F [P < 0.05; Fig. 7M; see also (9)]. Consistent with our in vivo finding suggesting a role for NRP2 in conveying SEMA3A signals, the inhibitory Nrp2 shRNA also significantly reduced the responsiveness of GN11 cells to SEMA3A (P < 0.05; Fig. 7M). These observations confirm that both NRP1 and NRP2 are capable of conferring SEMA3A signals in neuronal cells that normally co-express these neuropilins.

DISCUSSION

Gonadotropin-releasing hormone neurons are a small group of neuroendocrine cells in the hypothalamus that play a central role in the initiation of puberty and in the regulation of fertility in all mammalian species. These cells originate in the nasal placode and develop coordinately with olfactory and vomeronasal tissues to ensure that cues, provided by pheromones and other odours, influence reproductive function throughout adult life. GnRH neurons initially migrate along olfactory and vomeronasal axons in the nasal compartment towards the cribriform plate, and then follow the caudal branch of the vomeronasal nerve to enter the forebrain and reach the hypothalamus. Accordingly, loss of function for molecules that control olfactory or vomeronasal axon-patterning perturbs GnRH neuron migration, leading to delayed or absence of pubertal maturation and infertility (9,24,25). Reports are beginning to emerge (26) of transgenic mouse models that recapitulate the anatomical defects described for the human foetus with X-linked KS, in which GnRH neurons were trapped in a tangle of axons on the dorsal surface of the cribriform plate, outside the forebrain (5). However, the lack of a definable genetic cause for the majority of subjects with KS suggests that unidentified guidance pathways regulate
olfactory/vomeronasal axonal growth and GnRH neuron migration to ensure normal reproductive function.

We previously showed that the class 3 semaphorin receptor NRP2 is important in the fasciculation of the olfactory/vomeronasal axons in the nasal compartment and, therefore, in the migration of GnRH neurons (9). In a more recent work, Giacobini and colleagues (8) reported that the transmembrane SEMA4D and its receptor, PlexinB1, are critical for the guidance of migrating GnRH neurons. However, the lack of a strong phenotype in the knockout mice analysed in these two studies suggested that other member(s) of the semaphorin-signalling pathway might also be involved in this process. Contrary to the prevailing view that redundant signals guarantee the establishment of GnRH neuronal system, our present results suggest that SEMA3A is absolutely essential for targeting of the vomeronasal axons and for the migration of GnRH neurons into the forebrain. Thus, mice lacking SEMA3A not only have abnormal innervation of the olfactory bulbs (11), but also show impairment of the developing GnRH neuronal system, with vomeronasal axons and GnRH neurons accumulating at the level of the cribriform plate or mistargeted towards the meningeal tissue. Surprisingly, we found that SEMA3A exerted its effects by signalling through its conventional receptor NRP1 and, additionally, NRP2, which was originally thought to lack the ability to bind SEMA3A [e.g. (14,15,19,21)]. Thus, vomeronasal axon guidance and GnRH neuron migration were affected only mildly in either Nrp2<sup>-/-</sup> or Nrp1<sup>Sema3a<sup>-/-</sup></sup> single mutants, but both processes were affected with similar severity in compound receptor mutants and Sema3a-null mice.

Consistent with this genetic evidence for redundancy between NRP1 and NRP2 with respect to SEMA3A signalling, our ligand-binding studies demonstrated that SEMA3A binds to both NRP1 and NRP2 in situ. The observation that NRP2 helps to organize the axons that guide GnRH neurons by serving as a SEMA3A receptor was also corroborated by the absence of obvious defects in olfactory/vomeronasal axon-patterning and GnRH neuron migration in mice lacking SEMA3F, the predominant NRP2 ligand in other models of axon guidance. We also found that interfering with NRP2 impairs the chemorepulsive response of GN11 cells to SEMA3A signalling, suggesting that a role for NRP2 in SEMA3A signalling is not exclusive to vomeronasal axons. Our findings agree with a recent study in cancer cell lines, which had raised the possibility that SEMA3A binds NRP2 to modulate cell migration (22). With respect to its ability to signal through both NRP1 and NRP2 in vivo, SEMA3A is therefore similar to SEMA3C, which signals redundantly through either NRP1 or NRP2 to control cardiac neural crest cell behaviour (16,27,28).

Strikingly, the phenotype of Sema3a-null mutants corresponds in severity and morphological appearance to the defect observed in a foetus affected by X-linked KS, in which GnRH neurons accumulated on the dorsal surface of the cribriform plate, outside the forebrain (5). Consistent with this similarity, Sema3a-null males that survive the early postnatal period develop into adults with small testes (Fig. 3) and have very poor fertility (J. Vieira and C. Ruhrberg, unpublished observations). Similar to loss of SEMA3A, the mutation of Prok2, one of the genes mutated in autosomal forms of KS, severely reduces the number of GnRH neurons in the adult hypothalamus, and GnRH neurons were found to be trapped in the tangle of olfactory axons after crossing the cribriform plate at E13.5 (26).

Figure 8. (A–C) Schematic illustration of GnRH neurons and olfactory/vomeronasal axons in wild-type mice and the mutants examined in this study; green dots indicate neurons that migrate normally, red dots indicate ectopic neurons. (D and E) Schematic illustration of the novel aspects of netrin-1/DCC signalling uncovered in this study. Conventional pathway: SEMA3A and 3F are thought to control axon/soma guidance by binding to neuronal cell surface receptors composed of a ligand-binding subunit of the neuropilin family (NRP1 or NRP2) and a signal transduction subunit of the A-type Plexin family (PLXNA). Unconventional pathway: while NRP1 was previously thought to be the exclusive SEMA3A receptor, we show here that NRP2 acts as an additional SEMA3A receptor in the patterning of the caudal VNAs (VNc).

In summary, we have demonstrated that SEMA3A is the key player in semaphorin signalling during GnRH neuron development. Lack of this signalling pathway impairs the positioning of GnRH neurons in the hypothalamus, leading to infertility. These observations are central to our understanding of the molecular mechanisms of the neuroendocrine control of sexual
development and behaviour, and are likely to shed light into the aetiology of KS. They also have wider biological significance as they uncover the unexpected versatility of SEMA3A acting through a novel receptor combination (Fig. 8).

MATERIALS AND METHODS

Animals

All animal procedures were performed in accordance with institutional and UK Home Office guidelines. To obtain mouse embryos of defined gestational ages, mice were mated in the evening, and the morning of vaginal plug formation was counted as 0.5 days post coitum (dpc). To stage-match embryos within a litter or between litters from different matings, we compared facial and limb development. The following mouse strains were used: mice with a heterozygous null mutation in the Sema3a (33) or Sema3f (10) genes; mice lacking semaphorin signalling through one or both neuropilins [Nrp1Sema−/−, Nrp2−/− and Nrp1Sema−/− Nrp2−/−; (16); NRPI null mice (20)]. For genotyping, genomic DNA from tissue biopsies was analysed with the polymerase chain reaction using Megamix Blue reaction mix (Microzone) and oligonucleotide primers specific for the gene-targeted loci.

Immunofluorescence labelling

Formaldehyde-fixed cryosections or GN11 cells were blocked with phosphate-buffered saline (PBS) containing 10% normal goat serum and 0.2% TritonX-100 or, for primary goat antibodies, with serum-free protein block (DAKO). Samples were immunostained for immunofluorescence, as described above. The remaining cells were used for chemotaxis experiments using a Boyden chamber. For these experiments, cells were treated with conditioned medium from untransfected COS7 cells or COS7 cells transfected with expression vectors for SEMA3A or SEMA3F, as described previously (9). After 3 h, cells that had migrated through the membrane separating the two compartments of the chamber were stained using the Diff-Quick kit (Biomap, Italy) and counted in three random fields per well. Data were expressed as fold-decrease of migrated cells in Nrp2 shRNA- relative to control shRNA-transfected cells.

Chemomigration assays

Subconfluent GN11 cells were transfected with a bicistronic vector expressing GFP and Nrp2 or scrambled shRNA (23) using Lipofectamine2000 (Invitrogen). The transfecion efficiency was calculated 48 h post-transfection by counting the number of GFP-positive cells in 10 random fields imaged with a 20 × objective; the NRP2 expression level was evaluated by immunofluorescence, as described above. The remaining cells were used for chemotaxis experiments using a Boyden chamber. For these experiments, cells were treated with conditioned medium from untransfected COS7 cells or COS7 cells transfected with expression vectors for SEMA3A or SEMA3F, as described previously (9). After 3 h, cells that had migrated through the membrane separating the two compartments of the chamber were stained using the Diff-Quick kit (Biomap, Italy) and counted in three random fields per well. Data were expressed as fold-decrease of migrated cells in Nrp2 shRNA- relative to control shRNA-transfected cells.

Statistical analysis

For all experiments, we calculated the mean of at least three independent samples. Data are expressed as mean ± standard error of the mean; error bars represent the standard error of the mean. To determine the statistical significance, we used a paired t-test; P-value of <0.05 was considered significant and indicated with an asterisk; a P-value of <0.01 was indicated with two asterisks. Statistical analysis was performed using Prism4 software (GraphPad Software, San Diego, CA, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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