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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 neuroendrocrine cells that centrally regulate the reproductive axis in mammals by projecting to the median eminence, where they secrete the decapeptide 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

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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 cribriform 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.

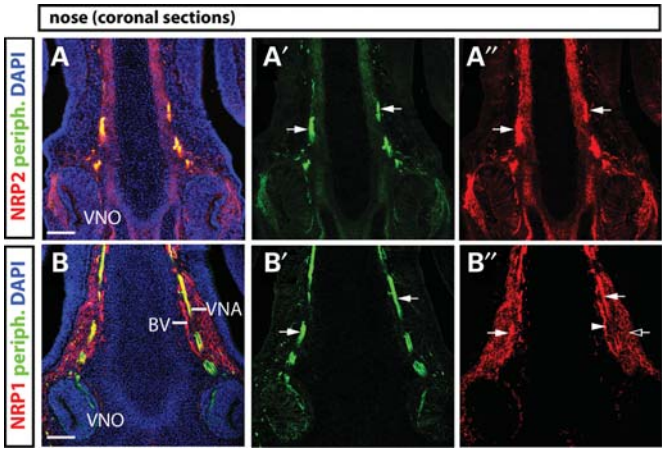

Figure 1. Expression of NRP2 and NRP1 on vomeronasal axons. Coronal sections taken from E14.5 mouse heads show that NRP2 and NRP1 localize to peripherin-positive vomeronasal axons in the nasal compartment. Arrows indicate the vomeronasal nerve; the arrowhead in B″ indicates a NRP1-positive blood vessel; the clear arrow in B″ indicates NRP1 expression in tissues surrounding the vomeronasal nerve. VNA, vomeronasal axons; VNO, vomeronasal organ; BV, blood vessels. Scale bars: 150 μm.

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.

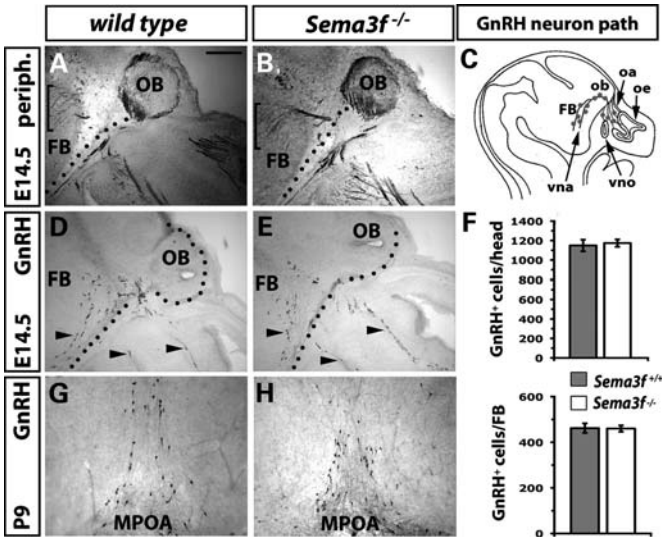

Figure 2. Normal vomeronasal axon (VNA) targeting and GnRH neuron migration in *Sema3f*-null mutants. (**A** and **B**) Sagittal sections of E14.5 mouse heads from wild-type (A) and *Sema3f*-null mutant (B) littermates were immunolabelled for peripherin to reveal the caudal vomeronasal nerve in the forebrain (FB); note that the nerve enters the FB normally and then defasciculates, like in wild-types (bracket). (**C**) Schematic drawing of an embryonic mouse head illustrating the migration of GnRH neurons (grey dots) from the olfactory epithelium (OE) and vomeronasal organ (VNO) along olfactory and vomeronasal axons. (**D**–**H**) Sagittal sections of E14.5 mouse heads (D,E) and coronal sections of postnatal day (P) 9 mouse heads (G,H) were immunolabelled for GnRH to visualize migrating neurons in the nose and FB (examples are indicated with arrowheads). Note that GnRH neurons follow a normal path of migration in the mutants at E14.5 (the boundary of the nasal compartment and FB is indicated with a black dotted line) and are present at normal numbers in the postnatal medial preoptic area (MPOA). (**F**) The quantitation of the total GnRH neuron number in the entire head and the number of GnRH neurons in the FB confirmed that there is no difference between wild-types and mutants at E14.5. Scale bars: 100 μm in A,B,D,E,G,H.

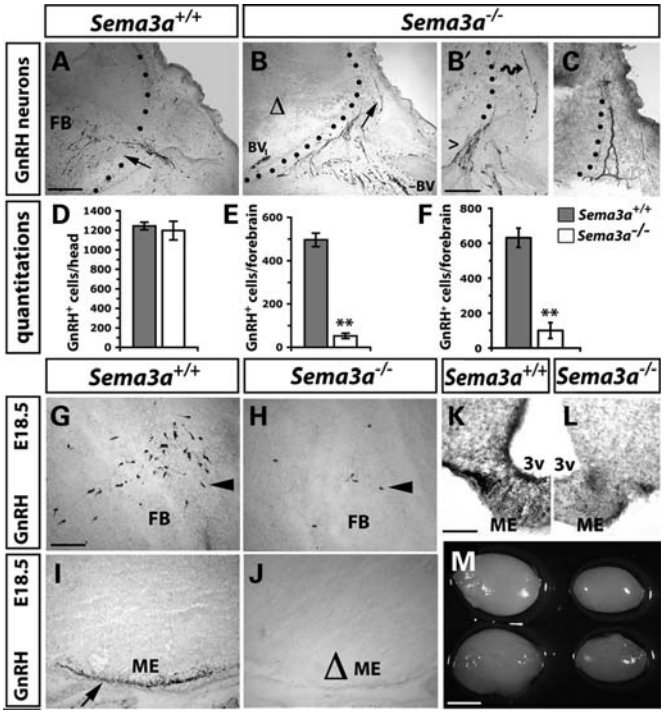

Figure 3. Abnormal GnRH neuron migration in *Sema3a*-null mutants decreases the number of GnRH neuron projections to the median eminence (ME) and impairs gonadal development. (**A** and **B**) Sagittal sections of E14.5 heads from $Sema3a^{+/+}$ and $Sema3a^{-/-}$ littermate embryos were immunolabelled for GnRH to reveal migrating neurons in the nasal compartment and forebrain (FB). The direction of migration of GnRH neurons is indicated with arrows, the lack of GnRH neurons in the mutant FB with △. The boundary of each FB is indicated with a black dotted line and the cribriform plate as CP. Non-specific blood vessel (BV) labelling caused by endogenous peroxidase activity in blood cells. (**B′**) A higher magnification of (B) illustrates ectopic GnRH neurons at the level of the cribriform plate (open arrowhead) and outside the brain and adjacent to the meninges (wavy arrow). (**C**) A corresponding region of a different $Sema3a^{-/-}$ mutant was immunolabelled for peripherin to reveal ectopic vomeronasal axons in the area where ectopic GnRH neurons accumulated. (**D**–**F**) Quantitation of GnRH neuron number in the entire head at E14.5 (D), in the FB at E14.5 (E) and in the FB at E18.5 (F) of $Sema3a^{+/+}$ and $Sema3a^{-/-}$ littermates. (**G**–**J**) Sagittal sections of E18.5 wild-type and *Sema3a*-null mutant littermate heads were immunolabelled for GnRH to visualize GnRH neurons in the FB (examples indicated with arrowheads in **G** and **H**) and their axonal projections in the ME (**I** and **J**). (**K** and **L**) Coronal sections of the hypothalamus from adult wild-type and *Sema3a*-null mutant littermates were immunolabelled to visualize GnRH-positive axons projecting to the ME (the third ventricle is indicated with 3v). (**M**) Pairs of testes from adult littermates were photographed side-by-side to demonstrate their different size in wild-type and *Sema3a*-null mutants. Scale bars: 100 μm in A,B; 200 μm in B′,C and G–J; 300 μm in K,L,M.

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

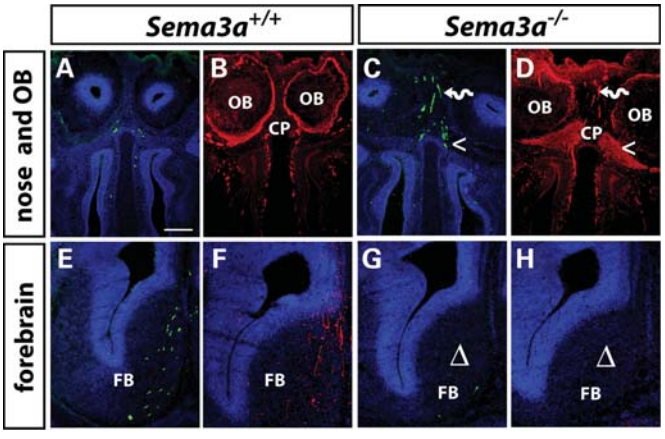

Figure 4. Aberrant olfactory/vomeronasal axon patterning and GnRH neuron migration in *Sema3a* null mice. (**A–H**) Contiguous coronal sections of E14.5 mouse heads were immunolabelled to reveal GnRH neurons (green) and olfactory/vomeronasal axons (red) in the nasal compartment/olfactory bulb and the basal forebrain (FB) of wild-type and $Sema3a^{-/-}$ littermates. 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 cribriform 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.

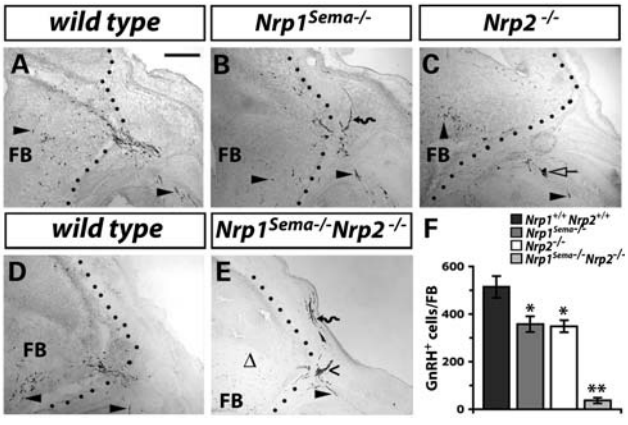

Figure 5. GnRH neurons migrate abnormally in single $Nrp1^{Sema-/-}$ and $Nrp2^{-/-}$ mutants, and are affected more severely in compound $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ mice. (**A–E**) Sagittal sections of E14.5 mouse heads were immunolabelled for GnRH to reveal migrating neurons in the nasal compartment 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 $Nrp1^{Sema-/-}$ 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 cribriform 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 ascertain 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 complete, and confirmed that the number of these cells in the forebrain 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). Consistent 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 (compare Fig. 3K with L). Accordingly, the testes of adult mutants were smaller than those of wild-type littermates (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 semaphorin signalling through NRP1 would phenocopy the vomeronasal axon and, therefore, the GnRH neuron defect of *Sema3a*-null mutants. Thus, we analysed GnRH neuron development 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 [$Nrp1^{Sema}$ mice; (16)]. The previously reported similarity of axon-patterning defects between $Nrp1^{Sema-/-}$ and $Sema3a^{-/-}$ mice in the cranial and spinal nerves had already established that this *Nrp1* allele is effective in disrupting SEMA3A signalling through NRP1, but that it leaves the vascular NRP1 functions intact [e.g. (16–18)]. Therefore, using $Nrp1^{Sema}$ mice circumvents the mid-gestation embryonic lethality of full *Nrp1*-null mutants that is caused by cardiovascular defects (16).

Immunohistochemical analysis of sagittal sections of E14.5 $Nrp1^{Sema-/-}$ mutants demonstrated a mild defect in the patterning 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; $Nrp1^{+/+}$ 509 ± 44 versus $Nrp1^{Sema-/-}$ 349 ± 26; $n = 4$ each; $P < 0.05$). The overall number of GnRH neurons in the head of the $Nrp1^{Sema-/-}$ mutants was normal ($Nrp1^{+/+}$ 1265 ± 75 versus $Nrp1^{Sema-/-}$ 1211 ± 48; $n = 3$ each). Because the severity of the vomeronasal axon defect of *Sema3a*-null mice was not recapitulated in $Nrp1^{Sema-/-}$ 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 $Nrp1^{Sema-/-}$ and $Nrp2^{-/-}$ mutants with compound $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ mice, which lack all semaphorin signalling through neuropilins and survive to birth (16). As previously shown (9), we found that $Nrp2^{-/-}$ mutants contained small aggregates of GnRH neurons within the nasal compartment at E14.5 (clear arrow in Fig. 5C). Accordingly, the number of GnRH neurons in the forebrain was reduced ($Nrp2^{+/+}$ 525 ± 53 versus $Nrp2^{-/-}$ 358 ± 33; $n = 4$ each; $P < 0.05$). The level of reduction was similar to that seen in $Nrp1^{Sema-/-}$ mutants (Fig. 5F). In contrast, compound receptor null mutants almost completely lacked GnRH neurons in the basal forebrain at E14.5 (Fig. 5D–F; $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ 37 ± 6 versus wild type 509 ± 40; $n = 3$; $P < 0.01$). Instead of entering the forebrain, most GnRH-positive neurons in these animals accumulated in the nose or in the tissue that surrounds the forebrain between the olfactory bulbs (Fig. 4E). However, the total number of GnRH neurons per head was normal ($Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ 1258 ± 221 versus wild-type 1265 ± 75; $n = 3$ each).

Immunofluorescence staining of coronal sections from $Nrp1^{Sema-/-}Nrp2^{-/-}$ embryos confirmed that only few GnRH neurons had reached the forebrain at E14.5, while there were many in the forebrain of wild-type littermates at this time (compare Fig. 6F with A). They also confirmed that GnRH neurons in mutants had accumulated in an area outside the forebrain that is normally devoid of such cells (compare Fig. 6G with B). While wild-types contained peripherin-positive axons in the nose that were projecting normally through the cribriform plate and olfactory bulb (Fig. 6C), $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ mutants contained ectopic axons in the area between the olfactory bulbs, similar to *Sema3a*-null mutants (Fig. 6H and I). The comparison of adjacent sections from $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ mutants labelled with antibodies for GnRH and peripherin correlated the position of ectopic neurons with axons in the same area (e.g. wavy arrow in Fig. 6I and J; double immunofluorescence labelling was not possible with the antibodies available). GnRH neuron distribution is, therefore, affected more severely in $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ mutants than in single neuropilin mutants. Moreover, the phenotype of these compound mutant mice is similar in severity to that of *Sema3a*-null mice (compare Fig. 3B with E). This similarity suggests that SEMA3A signals with partial redundancy through NRP1 and NRP2 to pattern the vomeronasal axons that guide GnRH neurons into the brain. The fact that compound, but not single neuropilin null mutants phenocopied the SEMA3A phenotype also confirmed that $Nrp1^{Sema-/-}$ mutants are not simply hypomorphic with respect to SEMA3A signalling.

SEMA3A binds to tissue from $Nrp1^{Sema-/-}$ or $Nrp2^{-/-}$ mutants, but not to tissue from compound neuropilin mutants

Our *in vivo* analysis implied that SEMA3A could signal through both NRP1 and NRP2 to guide vomeronasal axons.

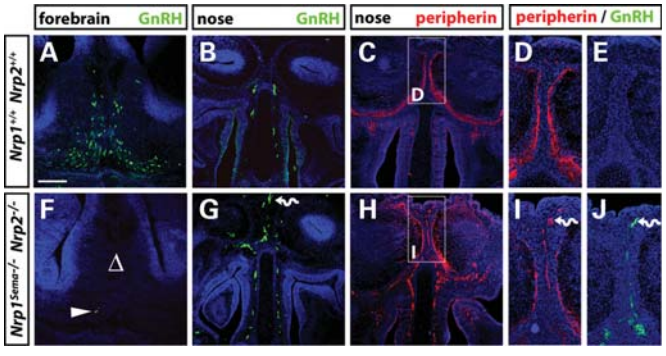

Figure 6. (**A**–**J**) Distribution of GnRH neurons and VNAs in compound $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ and wild-type littermates. Coronal sections of E14.5 mouse heads were immunolabelled to reveal GnRH neurons (green) or olfactory/vomeronasal axons (red) in the basal FB (**A** and **F**) and in the nasal compartment (**B**–**J**). In wild-types, many GnRH neurons were found in the FB (A), but were sparse at the cribriform plate at this stage (B). In $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$ mutants, few GnRH neurons had migrated into the FB (△ and arrowhead in **F**). Instead, GnRH neurons accumulated at the cribriform plate or underneath the meninges (wavy arrow in **G**). The location of ectopic GnRH neurons (wavy arrow in **J**) correlated with the presence of excess peripherin-positive axons, present between the olfactory bulbs (wavy arrow in **I**). Boxed areas in **C** and **H** are shown at higher magnification in **D** and I, respectively. Scale bar: 100 μm.

We therefore asked next whether SEMA3A was able to bind NRP2 *in situ*. 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 $Nrp1^{Sema-/-}$ 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 $Nrp1^{Sema-/-}$ or $Nrp2^{-/-}$ 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 $Nrp1^{Sema-/-}$ or $Nrp2^{-/-}$ mutants (Fig. 7H). Together, these findings confirm that the $Nrp1^{Sema}$ allele is not hypomorphic with respect to SEMA3A binding,

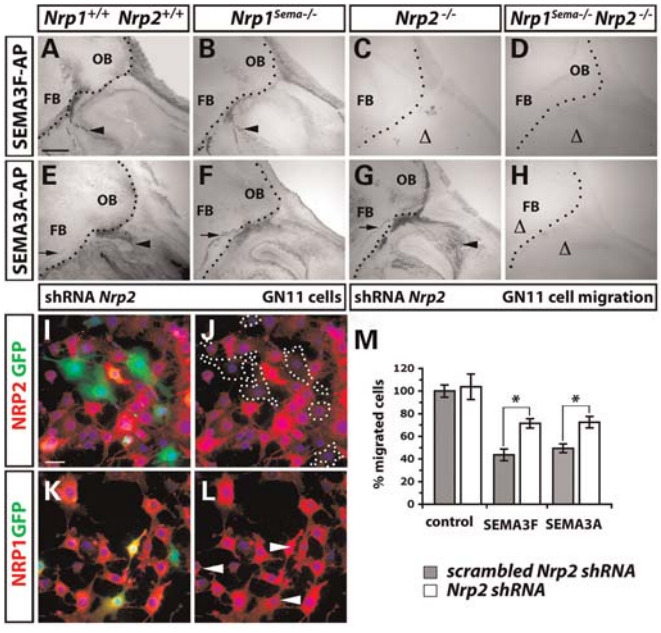

Figure 7. NRP2 serves as a SEMA3A receptor *in vivo* and *in vitro*. (**A**–**H**) Alkaline phosphatase (AP)-ligand binding assay on tissues from neuropilin mutants. Sagittal sections of E14.5 mouse heads of the indicated genotypes were incubated with SEMA3F-AP or SEMA3A-AP ligand. As expected, SEMA3F-AP bound olfactory axons in wild-type mice and $Nrp1^{Sema-/-}$ mutants (arrowheads in **A** and **B**), but not in *Nrp2*-null mutants or compound neuropilin mutants (△ 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 $Nrp1^{Sema-/-}$ 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 (△ in **H**). The FB boundary is indicated with a black dotted line. (**I**–**M**) *Nrp2* shRNA reduces the responsiveness of GN11 cells to SEMA3F and SEMA3A in the Boyden chamber chemotactic assay. The immunofluorescent staining of GN11 cells for NRP2 (red in **I** and **J**) 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 I 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 vivo* 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 nerves 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*$^{-/-}$ or *Nrp1*$^{Sema-/-}$ 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

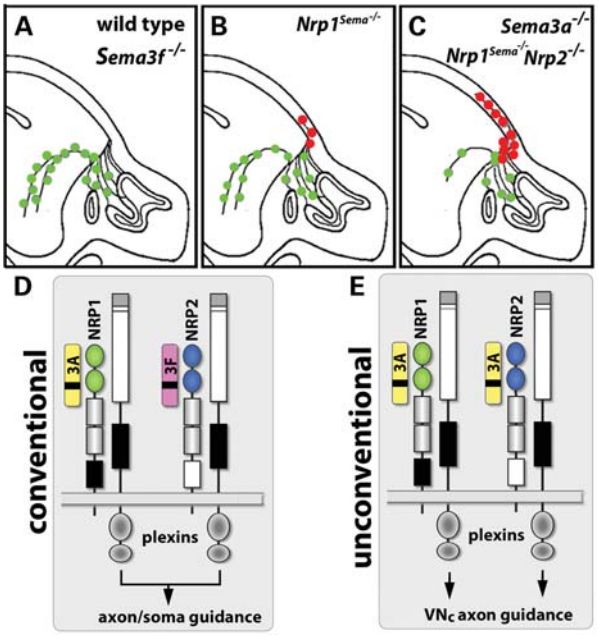

Figure 8. (**A**–**C**) Schematic illustration of GnRH neurons and olfactory/vomeronasal axons in wild-type mice and the mutants examined in this study; green dots indicate neurons that migrate normally, red dots indicate ectopic neurons. (**D** and **E**) Schematic illustration of the novel aspects of neuropilin 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 (VN_c).

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). However, this GnRH neuron abnormality was indirectly caused by defective olfactory bulb formation and not by defective targeting of the olfactory/vomeronasal axons, as in *Sema3a*-null mice. The null mutation of *Fgfr1*, another gene responsible for autosomal KS and additionally some HH cases, also prevents olfactory bulb development in mice, but olfactory sensory axons nevertheless enter the forebrain (29).

Several other mouse models feature defective GnRH neuron migration, but mutations in these genes have not yet been found in patients with KS or HH. For example, loss of lactosamine, the transcription factor EBF2 or SDF1/CXCR4 signalling all cause defective GnRH neuron migration independently of defects in their axonal substrates (30–32). Instead, loss of netrin-1/DCC signalling diverts GnRH neurons away from their normal path to the hypothalamus, because the caudal vomeronasal nerve extends into the medial wall of the cerebral cortex (24).

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 [$Nrp1^{Sema-/-}$, $Nrp2^{-/-}$ and $Nrp1^{Sema-/-}$ $Nrp2^{-/-}$; (16); NRP1 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 with rabbit anti-peripherin (Chemicon) or anti-GnRH (Immunostar), followed by Alexa-conjugated goat anti-rabbit IgG (1:500, Invitrogen), or with goat anti-rat NRP1, NRP2 (1:100, R&D systems), followed by Cy3-conjugated donkey anti-goat Fab fragment (1:200; Jackson Immunoresearch). Nuclei were counterstained with DAPI or Hoechst fluorochrome (Sigma).

Immunoperoxidase labelling

Sagittal cryostat sections of formaldehyde-fixed embryo heads of 25 μm were incubated with hydrogen peroxide to quench endogenous peroxidase activity and then blocked and incubated with primary antibodies as described for immunofluorescence, but followed by biotinylated goat anti-rabbit antibody (Vector Laboratories). Immunoreactivity was visualized with the ABC kit (Vector Laboratories) and 3,3-diaminobenzidine (Sigma). The total number of GnRH neurons per head or per forebrain was determined by counting all GnRH-positive cells in all sagittal sections of each embryo head at high magnification (Supplementary Material, Fig. S3).

AP-fusion protein-binding assays

AP-SEMA3A, AP-SEMA3F and AP-mock were prepared as described (18). Freshly dissected E14.5 heads were snap-frozen in isopentane cooled on dry ice. Cryostat sections were fixed for 5 min in methanol, washed five times with PBS, incubated in PBS containing 10% fetal bovine serum for 30 min and then reacted with AP-fusion protein for 2 h at room temperature (RT). For competition experiments, sections were pre-incubated for 1 h at RT with conditioned medium of COS cells transfected with SEMA3A-myc and SEMA3F-flag vectors (9). Sections were then washed, 5 min each, with PBS, fixed with 4% formaldehyde for 2 min at room temperate and washed again. Endogenous AP was heat-inactivated by incubation at 65°C for 3 h. Tissue-bound heat-stable recombinant AP activity was detected as an insoluble reaction product after incubation with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

Chemomigration assays

Subconfluent GN11 cells were transfected with a bicistronic vector expressing GFP and *Nrp2* or scrambled shRNA (23) using Lipofectamine2000 (Invitrogen). The transfection 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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