Semaphorin 4D regulates gonadotropin hormone–releasing hormone-1 neuronal migration through PlexinB1–Met complex

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In mammals, reproduction is dependent on specific neurons secreting the neuropeptide gonadotropin hormone–releasing hormone-1 (GnRH-1). These cells originate during embryonic development in the olfactory placode and migrate into the forebrain, where they become integral members of the hypothalamic–pituitary–gonadal axis. This migratory process is regulated by a wide range of guidance cues, which allow GnRH-1 cells to travel over long distances to reach their appropriate destinations. The Semaphorin4D (Sema4D) receptor, PlexinB1, is highly expressed in the developing olfactory placode, but its function in this context is still unknown. Here, we demonstrate that PlexinB1-deficient mice exhibit a migratory defect of GnRH-1 neurons, resulting in reduction of this cell population in the adult brain. Moreover, Sema4D promotes directional migration in GnRH-1 cells by coupling PlexinB1 with activation of the Met tyrosine kinase (hepatocyte growth factor receptor). This work identifies a function for PlexinB1 during brain development and provides evidence that Sema4D controls migration of GnRH-1 neurons.
high-affinity receptors, PlexinB1 and PlexinB2, are highly expressed in the developing olfactory structures (Perala et al., 2005; Deng et al., 2007), their functions in the development of the olfactory and GnRH-1 systems are still unknown. Here, we find that Sema4D expression is present along the GnRH-1 migratory route, with a peak of expression in the hypothalamic target area, and that GnRH-1 cells express the Sema4D receptor PlexinB1, but not PlexinB2, in a temporal window associated with their migratory process. Analysis of PlexinB1-deficient mice revealed a migratory defect of GnRH-1 cells, leading to reduced size of this neuronal population in adult brains. Using different experimental approaches, we demonstrated that Sema4D promotes the migratory activity of immortalized GnRH-1 cells through the activation of PlexinB1 and the associated Met receptor.

Collectively, our data reveal a novel function of Sema4D in the development of GnRH-1 neurons and identify the PlexinB1–Met receptor complex as a fundamental asset for neuronal cell migration and guidance.

Accumulating evidence shows that semaphorins can repel or attract a wide range of neuronal and nonneuronal cells depending on the cellular targets and on the expression of different subunits of the receptor complexes (Giordano et al., 2002; Pasterkamp et al., 2003; Conrotto et al., 2005; Swiercz et al., 2004, 2007; Chen et al., 2008).

It has been shown that the main transducing semaphorin receptors belong to the plexin family (Tran et al., 2007). Plexins can associate with other membrane receptors, leading to activation of different biological programs (Giordano et al., 2002; Conrotto et al., 2004, 2005; Swiercz et al., 2004, 2007). In fact, we have previously shown that Semaphorin4D (Sema4D), other than being a collapsing signal for axonal growth cones (Swiercz et al., 2002), may also induce chemotaxis of epithelial and endothelial cells, and that it functions as a proangiogenic factor through coupling its receptor PlexinB1 with the Met tyrosine kinase (Giordano et al., 2002; Conrotto et al., 2004, 2005). Yet, the potential role of Sema4D in regulating neuronal cell migration has not been investigated so far. Moreover, although Sema4D high-affinity receptors, PlexinB1 and PlexinB2, are highly expressed in the developing olfactory structures (Perala et al., 2005; Deng et al., 2007), their functions in the development of the olfactory and GnRH-1 systems are still unknown.

Here, we find that Sema4D expression is present along the GnRH-1 migratory route, with a peak of expression in the hypothalamic target area, and that GnRH-1 cells express the Sema4D receptor PlexinB1, but not PlexinB2, in a temporal window associated with their migratory process. Analysis of PlexinB1-deficient mice revealed a migratory defect of GnRH-1 cells, leading to reduced size of this neuronal population in adult brains. Using different experimental approaches, we demonstrated that Sema4D promotes the migratory activity of immortalized GnRH-1 cells through the activation of PlexinB1 and the associated Met receptor.

Collectively, our data reveal a novel function of Sema4D in the development of GnRH-1 neurons and identify the PlexinB1–Met receptor complex as a fundamental asset for neuronal cell migration and guidance.
Results

Sema4D/PlexinB1 expression in the developing nasal regions

Sema4D is a membrane-bound semaphorin that is also proteolytically released in diffusible form in the extracellular space. To gain further insights into the function of this molecule in the developing GnRH-1–olfactory systems, we determined the spatiotemporal expression pattern of Sema4D protein during mouse embryonic development. At embryonic day 12.5 (E12.5), a robust Sema4D immunoreactivity was detectable in the rostral aspect of the developing forebrain (Fig. 1 a, see high-magnification view in box 1) and in the basal forebrain (Fig. 1 a, see high-magnification view in box 2), which, respectively, represent the intermediate and final targets of GnRH-1 migratory process (Fig. 1 a). Sema4D was also found to be expressed in the same regions at E14.5 and E17.5 (unpublished data). Inclusion of a staining control (cntr) confirmed the specificity of the Sema4D staining (Fig. 1 b; no primary antibody).

It was previously found that the PlexinB1 transcript is highly expressed in the developing vomeronasal organ (VNO) and olfactory epithelium (OE; Perala et al., 2005). Immunohistochemistry for PlexinB1 and GnRH-1 was performed on consecutive sagittal sections of mouse embryos. At E12.5, the PlexinB1 expression pattern paralleled GnRH-1 neuronal distribution in the nasal region (Fig. 1, c–f). Indeed, GnRH-1–expressing cells migrating through the nasal mesenchyme (Fig. 1 e) likely coincide with those positive for PlexinB1 (Fig. 1 f). At this developmental stage, the receptor was also expressed in the developing OE and in the presumptive VNO, as demonstrated by simultaneous PlexinB1/βIII tubulin immunofluorescence experiments (Fig. 1, g and h). βIII tubulin is a neuron-specific marker that strongly labels the early olfactory and vomeronasal structures during embryogenesis (De Carlos et al., 1996; Roskams et al., 1998). Double-labeled cells and fibers emerging from the VNO were also detected (Fig. 1 h, arrowheads).

To further confirm that PlexinB1 was expressed along the developing olfactory axons, double-label immunofluorescence was performed for PlexinB1 (Fig. 1 i) and neural cell adhesion molecule (NCAM; Fig. 1 j), another marker of the olfactory–vomeronasal system (Calof and Chikaraishi, 1989). PlexinB1 and NCAM expressions overlapped along olfactory axon bundles crossing the nasal mesenchyme at E12.5, as shown by single confocal planes (Fig. 1 i, inset). Hence, the PlexinB1 receptor demarcated the olfactory system and the migratory GnRH-1 cell population. Because of low signal-to-noise levels in the developing brain, we were unable to detect specific immunoreactivity for PlexinB1 along the vomeronasal caudal nerve that GnRH-1 cells follow into the ventral forebrain (Yoshida et al., 1995) nor in GnRH-1 neurons located in the brain at later developmental stages (E14.5 and E17.5; unpublished data).

The developmental expression patterns of Sema4D and PlexinB1 are consistent with a role of Sema4D as guidance cue for migratory GnRH-1 cells and/or developing olfactory axons.

Primary GnRH-1 neurons express PlexinB1 but not PlexinB2 during their migratory process

It has been previously shown that the migration of GnRH-1 neurons observed in vivo is consistently recapitulated in nasal explants ex vivo (Fig. 2, a and b; Fueshko and Wray, 1994). We obtained nasal explants and isolated single GnRH-1 cells at 3, 4.5, 10, and 28 d in vitro (div). From 3 to 7 div, GnRH-1 cells display high cell motility and migrate off the explant tissue mass as associated to the olfactory axons (Fig. 2, a and b); whereas, at 10 and 28 div, GnRH-1 cells display characteristics of postmigratory and fully differentiated neurons (Fueshko and Wray, 1994). The expression pattern of PlexinB1 and PlexinB2 transcripts, the only known Sema4D receptors expressed in the central nervous system, was examined within individual GnRH-1 neurons (Fig. 2, c and d). A higher percentage of GnRH-1 neurons...
expressed PlexinB1 between 3 and 4.5 div compared with 10 and 28 div ($P < 0.005$; Fig. 2 e), whereas all examined cells were negative for PlexinB2 (Fig. 2 e). These results indicate that PlexinB1 is expressed by GnRH-1 cells in a temporal window limited to their migratory phase.

**Sema4D binds the developing olfactory-vomeronasal system and migratory GnRH-1 cells**

To assay whether cells in the olfactory–GnRH-1 system could interact with Sema4D, we performed binding studies by incubating mouse embryonic sections with Sema4D recombinant protein fused to AP (Sema4D-AP; Barberis et al., 2004). In brain sections derived from E14.5 PlexinB1+/+ mice, Sema4D-AP protein tightly bound to the VNO, OE, and olfactory/vomeronasal axons (Fig. 3, c and d), whereas no background binding was observed when Sema4D-AP incubation was omitted (Fig. 3, a and b). To determine the relative contribution of PlexinB1 and PlexinB2 in Sema4D binding, we performed the same experiments on sections of PlexinB1-null mice (Fazzari et al., 2007). Interestingly, the AP signal was much weaker in PlexinB1-deficient embryos (Fazzari et al., 2007) compared with wild-type (WT) animals (Fig. 3, f and g), which indicates that Sema4D binds the developing VNO–olfactory system, mainly through PlexinB1 receptor, even though a residual binding to PlexinB2 is also detectable. Moreover, an intense AP positivity was observed in clusters of cells emerging from the VNO in WT embryos (Fig. 3 d, arrow, see inset). We confirmed that these elements were indeed migratory GnRH-1 cells by double-staining an adjacent section for GnRH-1 and βIII tubulin (Fig. 3 e, arrows, see inset). Instead, in PlexinB1 knockout (KO) embryos, Sema4D was bound, though lightly, to the VNO cells and fibers, but not to migratory cell clusters (Fig. 3, g and h, arrows).

**Altered migratory process of GnRH-1 neurons in PlexinB1−/− mice**

To investigate the functional relevance of PlexinB1 in the development of the GnRH-1 neuroendocrine compartment, we examined the phenotype of PlexinB1-deficient mice as compared with WT. We analyzed the GnRH-1 cell distribution in the nasal compartment and in the brain of E14.5 embryos (Fig. 4, a and b) and of postnatal day 3 (P3) mice (Fig. 4, c and d). At E14.5, PlexinB1-null mice revealed a mild but statistically significant accumulation of GnRH-1 neurons in the nasal compartment (Fig. 4 b). Concomitantly, in PlexinB1−/− embryos, fewer GnRH-1 cells reached the brain compared with their WT counterparts (Fig. 4 b), in spite of a similar total number of GnRH-1–positive cells (WT: $1180 ± 56$, $n = 13$; PlexinB1−/−: $1318 ± 69$, $n = 10$; $t$ test, $P = 0.1$).
The migratory defect was more evident at P3. Brains of P3 PlexinB1−/− mice (n = 3) contained 20% fewer cells (P < 0.05) dispersed in the ventral forebrain compared with WT littermates (n = 4; Fig. 4, d–f, arrows). However, a 16% accumulation of GnRH-1 cells was observed at the level of the olfactory bulb in null animals (Fig. 4 f, arrowheads, see inset). The total number of GnRH-1–positive cells was not statistically different between WT mice and mutants (WT: 870 ± 92; PlexinB1−/−: 777 ± 84; t test, P = 0.5). The development of GnRH-1 and the olfactory systems are intimately entwined (Wray, 2002). If the course of the olfactory axons is disrupted, so is the migratory process of GnRH-1 cells. Evaluation with peripherin (Fuensko and Wray, 1994) immunohistochemistry of the olfactory/vomeronasal axons, along which the GnRH-1 neurons migrate, revealed no abnormalities in these transgenic mice (unpublished data).

To investigate whether the delay in the migratory process would be reflected in the size of the GnRH-1 neuronal population in adult animals, we collected alternating coronal sections from adult mutants and WT mice and examined them for GnRH-1 expression by immunohistochemistry. Our analysis showed that although in WT animals many GnRH-1–immunoreactive fibers innervated the median eminence, where these cells release the hormone into the pituitary portal capillary system, the density of these fibers was significantly reduced in PlexinB1−/− animals (Fig. 4 g). Moreover, a marked reduction in the total number of GnRH-1 neurons in the brains of PlexinB1−/− mice was detected (Fig. 4 h).

**Sema4D induces Met activation in immortalized GnRH-1 cells**

The manipulation of the GnRH-1 migratory system and functional studies on these neurons have been challenging because of their limited number (800 in mice and 1,000–2,000 in primates) and widely dispersed distribution in the olfactory system. The generation of immortalized GnRH-1 neurons has permitted the study of more immature, migratory neurons (NLT and GN11 cell lines; Radovick et al., 1991). In particular, GN11 cells display a remarkable motility in vitro, and they have been largely used to investigate the molecular mechanisms controlling the directional migration of GnRH-1 neurons (Giacobini et al., 2002; Cariboni et al., 2005, 2007).

We previously demonstrated that migratory GnRH-1 cells (both primary GnRH-1 neurons [Giacobini et al., 2007] and GN11 cells [Giacobini et al., 2002]) express Met and are functionally regulated by its ligand HGF. To exploit these cells for functional studies in response to Sema4D, we first verified that...
that intervening lanes have been spliced out.

were performed at least three times with similar results. The black lines indicate of the immunoprecipitated protein is shown on the bottom. Experiments 25-nM Sema4D stimulation did not activate Met. The control of the amount tyrosine phosphorylation at 1.25 and 5 nM concentrations, whereas tiphosphotyrosine antibodies showed that Sema4D treatment induces Met immunoprecipitation (IP) with anti-Met antibody. Probing the blot with an- containing Sema4D (at the indicated concentrations), and subjected to 30-min stimulation with Sema4D. (d) GN11 cells were treated for 30 min with 5 nM Sema4D (derived from Sema4D-expressing cells). Cell lysates were analyzed by WB with antibodies against Met (top) and PlexinB1 (bot- tom). GN11 cells endogenously express both receptors. We detected two specific bands corresponding to single-chain and cleaved heterodimeric forms of PlexinB1, as described previously in human and murine cells (Artigiani et al., 2003; Fazzari et al., 2007). (c) GN11 cells were treated for 30 min with 5 nM Sema4D (derived from Sema4D-expressing cells). Cell lysates were immunoprecipitated using anti-PlexinB1 or rabbit anti–mouse Ig (RaMig) as a control. Western blots were then probed with anti-Met antibodies. Data show that endogenous Met and PlexinB1 receptors bas- sally coprecipitate in GN11 cells, and this association is increased after a 30-min stimulation with Sema4D. (d) GN11 cells were treated for 30 min with 0.5 nM HGF (50 ng/ml) or with semipurified conditioned medium containing Sema4D (at the indicated concentrations), and subjected to immunoprecipitation (IP) with anti-Met antibody. Probing the blot with an-tiphosphotyrosine antibodies showed that Sema4D treatment induces Met tyrosine phosphorylation at 1.25 and 5 nM concentrations, whereas 25-nM Sema4D stimulation did not activate Met. The control of the amount of the immunoprecipitated protein is shown on the bottom. Experiments were performed at least three times with similar results. The black lines indicate that intervening lanes have been spliced out.

GN11 cells retained coexpression of Met and PlexinB1 recep- tors, as seen for their counterparts in vivo.

Double-labeling experiments followed by confocal micros- copy analysis showed that Met and PlexinB1 receptors are distri- buted throughout the cell surface of GN11 cells (Fig. 5 a) and often colocalize in dot-like clusters, as shown by single confoca- l plane images (Fig. 5 a, merge). Western blot analysis confirmed Met and PlexinB1 coexpression in GN11 cells (Fig. 5 b), and coimmunoprecipitation studies demonstrated PlexinB1–Met asso- ciation in a molecular complex that increased upon Sema4D stimulation (Fig. 5 c). Moreover, when GN11 cells were treated for 30 min with either HGF (0.5 nM) or soluble Sema4D (0.5, 1.25, 5, and 25 nM), the Met receptor became tyrosine phosphorylated, which indicates activation of its signal transduction (Fig. 5 d). Interestingly, as known for other receptors, we observed a bell-shaped curve of tyrosine kinase activation in response to increasing concentrations of Sema4D (Fig. 5 d).

Figure 5. Met and PlexinB1 associate in a complex, and Sema4D acti- vates Met in GN11 cells. (a) Double immunofluorescence was performed on GN11 cells using antibodies to Met (green) and PlexinB1 (red) receptors. Confocal microscopy analysis revealed that a fraction of Met and PlexinB1 colocalize in dot-like clusters on the cell surface (see yellow dots in merged images). The far right panel is an enlarged view of the box region. Bar: (left three panels) 10 μm; (far right) 3 μm. (b) GN11 cell whole lysates were analyzed by WB with antibodies against Met (top) and PlexinB1 (bot- tom). GN11 cells endogenously express both receptors. We detected two specific bands corresponding to single-chain and cleaved heterodimeric forms of PlexinB1, as described previously in human and murine cells (Artigiani et al., 2003; Fazzari et al., 2007). Interestingly, we observed that GN11 cells de- veloped membrane ruffles and lamellipodial extensions within 10 min of Sema4D treatment, as visualized by F-actin staining (Fig. 6 a). Moreover, in treated cultures, cells extended a leading edge with actin ruffles (Fig. 6 a, arrow), a typical sign of local Rac activation, and depolymerized actin fibers at the rear edge (Fig. 6 a). These data indicated that Sema4D stimulation triggers cytoskele- tal remodeling that is typical of migratory cells. To assess whether Sema4D can actually regulate the directional migration of GN11 cells, we performed microchemotaxis assays using Boyden’s chamber. As shown in Fig. 6 b, soluble Sema4D induced a clear chemoattraction on GN11 cells as compared with cntr conditions. Sema4D exerted its effect in a concentration-dependent manner, with maximal response at 5 nM (Fig. 6 b). To elucidate whether the effect of Sema4D on GN11 motility was directional chemo- taxis or simply the induction of random motility (i.e., chemokine- sis), the cells were exposed to the same concentration of Sema4D (5 nM), added either in the lower chamber only, or in both upper and lower compartments of the Boyden’s chamber. We observed a clear directional chemotaxis when the cells were exposed to a gradient of Sema4D diffusing from the lower chamber but only a modest induction of random cell motility when the factor was evenly distributed in both compartments (Fig. 6 b).

During embryonic development, HGF expression has been docu- mented in the nasal mesenchyme and in the developing olfactory bulb (Thewke and Seeds, 1996; Giacobini et al., 2007), with a spatiotemporal pattern that mimics Sema4D localization. Therefore, we examined if these two ligands could functionally cooperate or act in parallel in biological assays. We treated GN11 cells with optimal doses of Sema4D (5 nM) and HGF (0.5 nM), either alone or in combination. An additive effect for Met activa- tion was observed when the two molecules were added together (Fig. 6 c). Moreover, the migratory response of GN11 cells in chemotaxis experiments was significantly increased, even though they were not additive, compared with the single ligand stimula- tions, which supports the notion that these molecules can act in parallel to induce neuronal cell migration through direct (HGF) and indirect (Sema4D) activation of Met (Fig. 6, d and e).

Sema4D induces chemotaxis of GnRH-1 cells

Semaphorins are known to regulate cytoskeletal dynamics (Tran et al., 2007). Interestingly, we observed that GN11 cells de- voped membrane ruffles and lamellipodial extensions within 10 min of Sema4D treatment, as visualized by F-actin staining (Fig. 6 a). Moreover, in treated cultures, cells extended a leading edge with actin ruffles (Fig. 6 a, arrow), a typical sign of local Rac activation, and depolymerized actin fibers at the rear edge (Fig. 6 a). These data indicated that Sema4D stimulation triggers cytoskele- tal remodeling that is typical of migratory cells. To assess whether Sema4D can actually regulate the directional migration of GN11 cells, we performed microchemotaxis assays using Boyden’s chamber. As shown in Fig. 6 b, soluble Sema4D induced a clear chemoattraction on GN11 cells as compared with cntr conditions. Sema4D exerted its effect in a concentration-dependent manner, with maximal response at 5 nM (Fig. 6 b). To elucidate whether the effect of Sema4D on GN11 motility was directional chemo- taxis or simply the induction of random motility (i.e., chemokine- sis), the cells were exposed to the same concentration of Sema4D (5 nM), added either in the lower chamber only, or in both upper and lower compartments of the Boyden’s chamber. We observed a clear directional chemotaxis when the cells were exposed to a gradient of Sema4D diffusing from the lower chamber but only a modest induction of random cell motility when the factor was evenly distributed in both compartments (Fig. 6 b).

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Sema4D-induced migration is mediated by Met

To evaluate whether Met is functionally required for Sema4D- induced motogenic effect, we performed three-dimensional matrix assays by coculturing for 72-h aggregates of GN11 cells together with aggregates of Sema4D-secreting or mock-transfected COS cells in the presence or absence of the specific Met kinase inhibitor.
the aggregate in the presence of a source of Sema4D, as compared with mock treatment (Fig. 7, b and c). Moreover, asymmetrical directional migration of GN11 cells pointed to a chemotactic activity of Sema4D (Fig. 7, b and c). Such an attractive effect was prevented when the Met inhibitor PHA was applied to the culture medium (Fig. 7, b and c). These results were further confirmed by blocking Met activation with RNA interference technology. GN11 cells were infected with a lentiviral construct expressing both a Met-specific short hairpin RNA (shRNA) sequence (shRNA Met; Pennacchietti et al., 2003) and the marker GFP, under control of a separate promoter. As a specificity control, we used a construct expressing a mismatched Met shRNA sequence (shRNA cntr). Infection efficiency was confirmed by visualization of GFP expression under a fluorescent microscope (Fig. 8, a and b), and Met silencing was verified by Western blot analysis (Fig. 8 c). As shown in Fig. 8 c, infection with Met shRNA impaired the expression of Met in these cells, whereas cntr shRNA did not decrease Met expression compared with GN11 WT cells (WT). PlexinB1 expression was not affected after infection (Fig. 8 c). To determine the biological significance of Met in Sema4D-mediated GN11 cell migration, we performed migration assays using the Boyden’s chamber. As expected, cntr cells migrated toward a source of HGF as well as of Sema4D (Fig. 8 d). In contrast, Met-silenced cells displayed impaired ability in responding to either of the two chemoattractive factors (Fig. 8 d). These results show that Sema4D-induced motogenic activity of GnRH-1 cells is mediated by PlexinB1–Met complex.

### Discussion

Although initially characterized as repulsive neuronal guidance cues, semaphorins are now considered versatile signals regulating cell migration (Casazza et al., 2007; Zhou et al., 2008). Semaphorin receptors falling into the PlexinB subfamily are expressed in striking patterns in the developing nervous system (Worzfeld et al., 2004; Peralta et al., 2005; Pasterkamp et al., 2007); however, their functional relevance in vivo is poorly understood (Deng et al., 2007; Fazzari et al., 2007; Frieled et al., 2007).

We demonstrate here that Sema4D is robustly expressed along the GnRH-1 migratory route during mouse embryonic development, with higher levels in the final target areas. We found that the Sema4D receptor, PlexinB1, is expressed in migratory GnRH-1 cells exiting the olfactory neuroepithelium. These data were confirmed by single-cell RT-PCR analysis on primary GnRH-1 neurons isolated from nasal explants. PlexinB1 expression correlated with the migratory stage of these cells, being higher at 3 and 4.5 div and down-regulated in postmigratory GnRH-1 neurons (10 and 28 div). Recent work has shown a key requirement for PlexinB2 but not PlexinB1 in the patterning of the vertebrate nervous system in vivo (Deng et al., 2007). Our single-cell RT-PCR data provide evidence that GnRH-1 cells do not express PlexinB2 at any developmental stage analyzed, thus excluding a possible direct involvement of this receptor in the cellular response mediated by Sema4D. We further showed that Sema4D binds the developing VNO/OE epithelia, and that PlexinB1–Sema4D binding is much stronger than PlexinB2–Sema4D in these regions. Moreover, we demonstrated that in PlexinB1+/− animals,
Sema4D-AP–positive cell clusters migrating off the developing VNO are indeed GnRH-1 neurons.

Additional semaphorins and semaphorin receptors may play a role in this system. Recently, the Sema7A receptor, PlexinC1, was found to be expressed in migratory GnRH-1 cells in rats (Pasterkamp et al., 2007). Cariboni et al. (2007) reported a direct role for Sema3F as a repellent for the migration of GnRH-1 cells both in vivo and in vitro. Here, we find that PlexinB1–null mice show deficits consistent with reduced migration of GnRH-1 neurons. The development and organization of the olfactory axonal scaffold in PlexinB1−/− mice did not show abnormalities, which suggests that the migratory defect might be cell autonomous rather than dependent on alterations of the olfactory axonal pathway. In brains of adult mutant animals, the population of GnRH-1 cells was significantly reduced, by approximately one third compared with WT, and the median eminence was significantly reduced, by approximately one third compared with PlexinB1+/+ littermates. Consistently, the median eminence of these mice also appeared less innervated by GnRH-1 fibers as compared with WT. Our single-cell RT-PCR data revealed that GnRH-1 neurons differ in their PlexinB1 expression as a function of age. This might explain why only a fraction of neurons are affected by the lack of PlexinB1 gene. It is important to emphasize that a high degree of heterogeneity within the GnRH-1 neuron population has been documented extensively during the last years (Herbison et al., 2007; Schwarting et al., 2007). This seems to warrant the fact that no single genetic mutation may totally prevent these neurons from reaching their final destinations (Schwarting et al., 2007). Therefore, it is likely that failure of puberty in mammals may only occur when the majority of GnRH-1 neurons are affected by multigenic mutations (Herbison et al., 2007; Kim et al., 2007).
It is possible that in PlexinB1 mutant animals, the GnRH-1 cells that do not reach the final target areas at the right time may be eliminated by cell death. However, because of the small size of the GnRH-1 neuronal population and the limited temporal window during which apoptosis takes place, it was not possible to determine if this was the case. In other mutant mice, such as neuropilin 2−/−, netrin1−/−, and ephrin3–5−/− mice, there also were reported losses in immunoreactive GnRH-1 neuron numbers, but it was not feasible to determine the cause (Schwarting et al., 2004; Gamble et al., 2005; Carlbom et al., 2007).

In our study, we have also analyzed whether mice deficient for Sema4D would display a GnRH-1 phenotype similar to that seen in PlexinB1 KO mice. Sema4D-null mice do not show gross abnormalities in other tissues other than the immune system (Shi et al., 2000). Our quantitative analysis of the total number of GnRH-1 cells in adult brains revealed no differences in the size of this neuronal population between WT and mutant mice (WT: 654 ± 16.5, n = 4; Sema4D−/−: 660 ± 24, n = 3; t test, P = 0.8), which suggests that the absence of this semaphorin could be compensated by alternative, as yet unidentified, ligands for PlexinB1, or by the potentiation of other synergistic morphogenetic signals such as netrins, SDF-1, and HGF (Schwarting et al., 2001, 2004, 2006; Giacobini et al., 2007).

Sema4D-induced signaling pathways can use different mechanisms to elicit either repulsion/inhibition of cell motility or promigratory/chemoattractive responses (Zhou et al., 2008). In PC12 cells, Sema4D activation of PlexinB1 suppresses cell migration through inhibition of R-Ras and, subsequently, of β1-integrin activity (Onuma et al., 2006). In other cellular systems, Sema4D effects seem to be gated through the association of the receptor PlexinB1 with tyrosine kinases such as Met, Ron, and ErbB2 (Giordano et al., 2002; Conrotto et al., 2004; Swiercz et al., 2004; Conrotto et al., 2005; Swiercz et al., 2007). Our data demonstrate for the first time in a neuronal a structural and functional association of PlexinB1 and Met receptors (holoreceptor complex) in GN11 cells. Moreover, Sema4D stimulation increases the holoreceptor complex formation. We observed a bell-shaped curve of Met tyrosine kinase activation in response to increasing concentrations of Sema4D, which parallels the bell-shaped curve of Sema4D-stimulated cell motility. One possible explanation for this observation is that Sema4D may be released by cells in monomeric and homodimeric forms (Elhabazi et al., 2001), this last being generally preferred when the semaphorin is explained, then frozen and stored (−80°C) until processing for immunocytochemistry. PlexinB1−/−, Sema4D−/−, and their WT littermates have been described previously (Shi et al., 2000; Fazzari et al., 2007). Sema4D KO mice (Shi et al., 2000) were provided by H. Kikutani (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Adult mice were anesthetized with an intraperitoneal injection of 200 mg/kg ketamine and perfused with 4% PFA.

Materials and methods

Animals
Experiments were conducted in accordance with current European Union and Italian law, under authorization of the Italian Ministry of Health, No. 66/99-A. CD1 embryos (Charles River Laboratories) were harvested at E11.5, E12.5, E14.5, and E17.5 (plug day, E0.5) and used for RNA isolation, or fixed overnight at 4% PFA in 0.1 M phosphate buffer, pH 7.4, cryoprotected, then frozen and stored (−80°C) until processing for immunocytochemistry. PlexinB1−/−, Sema4D−/−, and their WT littermates have been described previously (Shi et al., 2000; Fazzari et al., 2007). Sema4D KO mice (Shi et al., 2000) were provided by H. Kikutani (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Adult mice were anesthetized with an intraperitoneal injection of 200 mg/kg ketamine and perfused with 4% PFA.

Immunocytochemistry
Primary antisera used were against GnRH-1: SW-1, and rabbit (Rb) polyclonal (Wray et al., 1988), provided by S. Wray (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD); PlexinB1: IC-2 (Artigiani et al., 2003), Rb, and H300, Rb, (Santa Cruz Biotechnology Inc.); Sema4D: CD100, mouse monoclonal (BD Biosciences); H300 Rb (Santa Cruz Biotechnology, Inc.) and BMA-12, rat IgG2a (eBioscience, Inc.); peripherin (No. AB1530, Rb, Millipore); NCAM (No. C9672, mouse monoclonal IgG, Sigma-Aldrich); Met (No. SP42, and No. H-190, rabbit polyclonal); and No. B-2, mouse monoclonal IgG; Santa Cruz Biotechnology, Inc.). Immunohistochemistry was performed as described previously (Giacobini et al., 2007). For double immunoperoxidase staining, the chromogen for the first antigen–antibody complex was DAB (brown precipitate; Kramer and Wray, 2000), whereas the chromogen for the second antigen–antibody complex was 5B substrate (blue precipitate; Vector Laboratories). For double-immunofluorescence experiments, Alexa Fluor 488– and Alexa Fluor 568–conjugated secondary antibodies (Invitrogen) were used. F-actin was stained with rhodamine-phalloidin labeled phalloidin (Invitrogen). Fluorescent specimens were mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich).

Image analysis
Images were captured using a microscope (Eclipse 80i; Nikon) and 2x/0.06 NA, 10x/0.30 NA, and 20x/0.50 NA objectives [Nikon] equipped with a digital camera (CX 9000, mbl Bioscience). For the observation coupled with confocal analysis, a laser-scanning Fluoview confocal system (IX70: Olympus) and 10x/0.30 NA, 20x/0.70 NA, and 60x/1.25 NA objectives (Olympus) were used. For the subsequent analysis of the digitized pictures, ImageJ [National Institutes of Health] and Photoshop (Adobe) software were used.

Nasal explants
Embryos were obtained from timed pregnant animals in accordance with current European Union and Italian law, under authorization of the Italian Ministry of Health, No. 66/99-A. Nasal pils of E11.5 staged CD1 embryos (Charles River Laboratories) were isolated under aseptic conditions.

Finally, we demonstrated the requirement for Met in Sema4D-induced directional migration on GN11 cells by three-dimensional coculture experiments in the presence of a Met-specific kinase inhibitor and by chemotaxis assays in which we interfered with Met expression through RNA interference technology. It has now become clear that GnRH-1 migratory process is not only dependent on the expression of specific receptors in these neurons but also on their ability to process guidance information provided by their surroundings. Our results shed light on a novel function for Sema4D as a long-range guidance cue in the GnRH-1 neuronal migration and identify PlexinB1–Met interaction as an essential requirement for Sema4D promigratory effect in this system. These data advance our understanding of the molecular mechanisms controlling the development of GnRH-1 system and may represent an important asset to elucidate the etiology of numerous forms of IHH.
in Gey’s Balanced Salt Solution (Invitrogen) enriched with glucose (Sigma-Aldrich) and maintained at 4°C until plating. Nasal explants were placed onto glass coverslips coated with 10 μl of chicken plasma (Cocalico Biologicals, Inc.). 10 μl thrombin (Sigma-Aldrich) was then added to adhere (thrombin/plasma clot) the explant to the coverslip. Explants were maintained in defined serum-free medium (Fuehsko and Wray, 1994) containing 2.5 mg/ml fungizone (Sigma-Aldrich) at 37°C with 5% CO2 for up to 10 min. For cell stimulation with mock treatment, we used appropriate dilution of elution buffer used to purify the fused Sema4D-GST from Sepharose glutathione chromatography or of concentrated 48-h conditioned media from mock-transfected COS cells.

**Protein analysis**

Except for the immunoprecipitation experiment, cells were lysed and proteins were extracted in boiling extraction buffer (1.25 mM Tris-HCl, pH 6.8, and 2.5% SDS). For immunoprecipitations, cells were lysed with modified RIPA buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM sodium chloride, 10% glycerol, and 1% Triton X-100) in the presence of 1 μg/ml leupeptin, 3 μg/ml aprotinin, 1 μg/ml pepstatin, 2 mM phenylmethylsulphonyl fluoride, and 1 mM sodium orthovanadate. 1–2 μg proteins were immunoprecipitated for 2 h at 4°C with the indicated antibodies. After immunoprecipitation, high-stringency washes were performed (modified RIPA buffer containing 1 M lithium chloride). Western blots were performed according standard methods, and nitrocellulose membranes were probed with [35S]proteins Y20 monoclonal antibody (BD Biosciences), Met (B-2, mouse monoclonal IgG; Santa Cruz Biotechnology Inc., mouse anti-human Met monoclonal antibody; Invitrogen), anti-phospho-Met (P-Met rabbit polyclonal antibody; Cell Signaling Technology), PlexinB1 (A-8, mouse monoclonal IgG; Santa Cruz Biotechnology, Inc.), and mouse monoclonal β-actin (Sigma-Aldrich).

**Boyden’s chamber assay**

The assay was performed using a 48-well Boyden’s microchemotaxis chamber according to manufacturer’s instructions (Neuro Probe). In brief, the cells grown in complete medium until subconfluence were harvested, and the suspension (105 cells/50 μl serum-free DME) was placed in the open-bottom wells of the upper compartment. Each pair of wells was separated by a polyvinylpyrrolidone-free polycarbonate porous membrane (8-μm pores) pre-coated with gelatin (0.2 mg/ml in PBS). The lower chamber of the Boyden’s apparatus was filled with DME FBS 0% in the absence or in the presence of 0.5 nM HGF/scatter factor and 0.5, 2.5, 5, and 10 nM Sema4D.

**Chemokinesis**

Stimulation of increased random cell motility was distinguished from chemotaxis by placing the same concentration of the factor in both the upper and lower wells of the Boyden’s chamber, thereby eliminating the chemical gradient. After 4 h of incubation, cells attached to the upper side of the filter were mechanically removed. Cells that migrated to the lower side were fixed and stained with either DAPI nuclear dye or Diff Quik staining kit (Dade Behring AG) according to the manufacturer’s instructions. The stained cells were photographed and counted.

**Statistical analysis**

For comparison of multiple groups, statistical significance was determined using a two-way analysis of variance (ANOVA); for Gaussian distributed data, followed by Fisher’s least significant difference post-hoc analysis or Kruskal-Wallis (nonparametric) tests. For comparison between paired normally distributed data, a paired t-test was used. The significance level was set at P < 0.05. Data groups are indicated as mean ± SEM (for normally distributed data). A nonparametric unpaired t-test (Wilcoxon-Mann-Whitney) was used to compare percentages of primary GnRH-1 cells at different in vitro stages expressing specific transcripts. The significance level was set at P < 0.01.
References

Artigiani, S., D. Barberis, P. Fazzari, P. Longati, P. Angelini, J.W. van de Loo, P.M. Comoglio, and L. Tamagnone. 2004. Functional regulation of semaphorin receptors by protein convertases. *J. Biol. Chem.* 279:10094–10101.

Barberis, D., S. Artigiani, A. Casazza, S. Corso, S. Giordano, C.A. Love, E.Y. Jones, P.M. Comoglio, and L. Tamagnone. 2004. Plexin signaling hampers integrin-based adhesion, leading to Rho-kinase independent cell rounding, and inhibiting lamellipodia extension and cell motility. *FASEB J.* 18:592–594.

Calo, A.L., and D.M. Chikaraishi. 1989. Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. *Neuron.* 3:115–127.

Cariboni, A., R. hacked, A. Liapi, R. Maggi, A. Goffinet, and J.G. Parnavelas. 2005. Reelin provides an inhibitory signal in the migration of gonadotropin-releasing hormone neurons. *Development.* 132:4709–4718.

Cariboni, A., J. Hickok, S. Rakic, W. Andrews, R. Maggi, S. Tischkau, and P.M. Comoglio. 2007. Neuropilins and their ligands are important in the migration of gonadotropin-releasing hormone neurons. *J. Neurosci.* 27:2387–2395.

Casazza, A., P. Fazzari, and L. Tamagnone. 2007. Semaphorin signals in cell adhesion and cell migration: functional role and molecular mechanisms. *Adv. Exp. Med. Biol.* 600:90–108.

Chen, G., J. Sima, M. Jin, K.Y. Wang, X.J. Xue, W. Zheng, Y.Q. Ding, and X.B. Schwarting. 2006. Silencing the MET oncogene leads to regression of experimental adrenal tumor. *Oncogene.* 25:108–130.

Friedel, R.H., G. Kerjan, H. Rayburn, U. Schuller, C. Serini, P.M. Comoglio, and L. Tamagnone. 2004. Plexin-B1 plays a redundant role during mouse development and in tumour angiogenesis. *MBMC Dev. Biol.* 7:55.

Fueshko, S., and S. Wray. 1994. LHRH cells migrate on peripherin fibers in embryonic olfactory explant cultures: an in vitro model for neurophilic neuronal migration. *Dev. Biol.* 166:331–348.

Gamble, J.A., D.K. Karunadasa, I.R. Pape, M.I. Skynner, M.G. Todman, R.J. Bicknell, J.P. Allen, and A.E. Herbsin. 2005. Dispersion of ephrin signaling associates with disordered axophial migration of the gonadotropin-releasing hormone neurons. *J. Neurosci.* 25:3142–3150.

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Gaurov, S., A. Giangiacomo, M. Fantin, I. Pape, R. Maggi, A. Cariboni, I. Perroteau, and A. Fasio. 2002. Hepatocyte growth factor receptor c-Met facilitates migration of GN-11 immortalized LHRH neurons. *Endocrinology.* 143:3306–3315.

Giacobini, P., A. Mennina, S. Wray, C. Giangiacomo, T. Crepaldi, P. Carmeleit, and A. Fasio. 2007. Hepatocyte growth factor acts as a motogen and guidance signal for gonadotropin hormone-releasing hormone-1 neuronal migration. *J. Neurosci.* 27:341–445.

Giacobini, P., S. Cariboni, and T. Crepaldi. 2006. Focal adhesion kinase is activated by VEGF and NRTN and required for the migration of LHRH neurons. *J. Neurosci.* 26:120–129.

Gonzalez-Martinez, D., B. Loisel, T. Crespi, and P. Potier. 2005. Netrin 1-mediated chemoattraction regulates the migratory pathway of gonadotropin hormone-releasing hormone neurons. *Neuron.* 45:67–78.

Hemmerle, R., S. Giordano, and P. Bouloux. 2007. Diversity in fibroblast growth factor receptor 1 regulation: learning from the investigation of Kallmann syndrome. *Front. Neuroendocrinol.* 28:108–130.

Kim, S.H., Y. Hu, S. Cadman, and P. Bouloux. 2007. Diversity in fibroblast growth factor receptor 1 regulation: learning from the investigation of Kallmann syndrome. *Front. Neuroendocrinol.* 28:108–130.

Kramer, P.R., and S. Wray. 2000. Novel gene expressed in nasal region influences outgrowth of olfactory axons and migration of luteinizing hormone-releasing hormone (LHRH) neurons. *Genes Dev.* 14:1824–1834.

Kramer, P.R., R. Krishnamurthy, P.J. Mitchell, and S. Wray. 2000. Transcription factor activator protein-2 is required for continued luteinizing hormone-releasing hormone expression in the forebrain of developing mice. *Endocrinology.* 141:1823–1838.

Liu, Y., H. Katoch, and M. Negishi. 2006. Semaphorin 4D/Plexin-B1-mediated R-Ras GAP activity inhibits cell migration by regulating β1 integrin activity. *J. Cell Biol.* 173:660–673.

Radovick, S., S. Wray, E. Lee, D.K. Nichols, Y. Nakayama, B.D. Weintraub, H. Westphal, G.B. Cutler Jr., and F.E. Wondisford. 1991. Migratory arrest of gonadotropin hormone neuronal migration suggests roles in axon guidance and neuronal migration. *BMC Dev. Biol.* 7:98.

Pasterkamp, R.J., J.J. Peschon, M.K. Spriggs, and A.L. Kolodkin. 2003. Semaphorin 7A promotes axon outgrowth through integrins and MAGPs. *Nature.* 424:398–405.

Pasterkamp, R.J., S.M. Kolk, A.J. Hellemons, and A.L. Kolodkin. 2007. Expression patterns of semaphorin7A and plexinC1 during rat neural development. *Dev. Growth Factor Act.* 17:1–16.

Rutt, B., W. Wehr, and A. Jones. 2005. Disruption of ephrin signaling disrupts gonadotropin hormone neuronal migration. *Dev. Biol.* 297:237–244.

Bicknell, J.P., A.E. Herbison, and A.L. Kolodkin. 2005. Disruption of ephrin signaling disrupts gonadotropin hormone neuronal migration. *Dev. Biol.* 297:237–244.

Schwarting, G.A., D. Raitcheva, E.P. Bless, S.L. Ackerman, and S. Tobet. 2004. Gonadotropin-releasing hormone (GnRH) neuron requirements for puberty, ovulation and fertility. *Endocrinology.* 149:597–604.

Schwarting, G.A., M.E. Wierman, and S.A. Tobet. 2007. Gonadotropin-releasing hormone neuronal migration. *Semin. Reprod. Med.* 25:141–163.

Swiercz, G.A., M. Nisbet, and A.L. Kolodkin. 2003. Expression of plexins during mouse embryogenesis. *Gene Expr. Patterns.* 3:355–362.

Rutten, M., J. van der Meulen, and J. Van Duijn. 2003. Disruption of ephrin signaling disrupts gonadotropin hormone neuronal migration. *Dev. Biol.* 297:237–244.

Stromal cell-derived factor-1 (chemokine C-X-C motif ligand 12) and chemokine C-X-C motif receptor 4 are required for migration of gonadotropin-releasing hormone neurons to the forebrain. *Nat. Cell Biol.* 8:3402–3406.

Roskams, A.J., X. Cai, and G.V. Ronnett. 1998. Expression of neuron-specific betaIII tubulin during olfactory neurogenesis in the embryonic and adult rat. *Neuroscience.* 83:191–200.

Schwarz, G.A., and A.L. Kolodkin. 2003. Origin of luteinizing hormone-releasing hormone neurons. *Nature.* 338:161–164.

Schwarting, G.A., K. Estep, P.E. Bless, N. Ahmad, and S.A. Tobet. 2001. Deleted in colorectal cancer (DCC) regulates the migration of luteinizing hormone-releasing hormone neurons to the basal forebrain. *J. Neurosci.* 21:911–919.

Schwarting, G.A., D. Raitcheva, P.E. Bless, S.L. Ackerman, and S. Tobet. 2004. Ntr1-1 mediated chemoattraction regulates the migratory pathway of LHRH neurons. *Eur. J. Neurosci.* 19:11–20.

Schwarting, G.A., T.R. Henion, J.D. Nugent, B. Caplan, and S. Tobet. 2006. Structural cell-derived factor (chondromatrix G-C-X-C motif ligand 12) and chomokine C-X-C motif receptor 4 are required for migration of gonadotropin-releasing hormone neurons to the forebrain. *J. Neurosci.* 26:6834–6840.

Schwarting, G.A., M.E. Wierman, and S.A. Tobet. 2007. Gonadotropin-releasing hormone neuronal migration. *Semin. Reprod. Med.* 25:305–312.

Shi, W., A. Kumanogoh, C. Watanabe, J. Ichida, X. Wang, T. Yassiu, K. Yawaka, M. Ikawa, M. Okabe, J.R. Parner, et al. 2000. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. *Immunol. 13:633–642.

Swiercz, J.M., R. Kuner, J. Behrens, and S. Offermanns. 2002. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. *Neuron.* 5:355–362.
Swiercz, J.M., R. Kuner, and S. Offermanns. 2004. Plexin-B1/RhoGEF-mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. *J. Cell Biol.* 165:869–880.

Swiercz, J.M., T. Worzfeld, and S. Offermanns. 2007. ERBB-2 and met reciprocally regulate cellular signaling via plexin-B1. *J. Biol. Chem.* 283:1893–1901.

Thewke, D.P., and N.W. Seeds. 1996. Expression of hepatocyte growth factor/scatter factor, its receptor, c-met, and tissue-type plasminogen activator during development of the murine olfactory system. *J. Neurosci.* 16:6933–6944.

Tran, T.S., A.L. Kolodkin, and R. Bharadwaj. 2007. Semaphorin regulation of cellular morphology. *Annu. Rev. Cell Dev. Biol.* 23:263–292.

Wray, S. 2002. Development of gonadotropin-releasing hormone-1 neurons. *Front. Neuroendocrinol.* 23:292–316.

Wray, S., B.H. Gahwiler, and H. Gainer. 1988. Slice cultures of LHRH neurons in the presence and absence of brainstem and pituitary. *Peptides.* 9:1151–1175.

Wray, S., P. Grant, and H. Gainer. 1989. Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc. Natl. Acad. Sci. USA.* 86:8132–8136.

Yoshida, K., S.A. Tobet, J.E. Crandall, T.P. Jimenez, and G.A. Schwarting. 1995. The migration of luteinizing hormone-releasing hormone neurons in the developing rat is associated with a transient, caudal projection of the vomeronasal nerve. *J. Neurosci.* 15:7769–7777.

Zhou, Y., R.A. Gunput, and R.J. Pasterkamp. 2008. Semaphorin signaling: progress made and promises ahead. *Trends Biochem. Sci.* 33:161–170.