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Semaphorin 4C and 4G are ligands of Plexin-B2 required in cerebellar development

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Abstract

Semaphorins and Plexins are cognate ligand-receptor families that regulate important steps during nervous system development. The Plexin-B2 receptor is critically involved in neural tube closure and cerebellar granule cell development, however, its specific ligands have only been suggested by in vitro studies. Here, we show by in vivo and in vitro analyses that the two Semaphorin-4 family members Sema4C and Sema4G are likely to be in vivo ligands of Plexin-B2. The Sema4C and Sema4G genes are expressed in the developing cerebellar cortex, and Sema4C and Sema4G proteins specifically bind to Plexin-B2 expressing cerebellar granule cells. To further elucidate their in vivo function, we have generated and analyzed Sema4C and Sema4G knock-out mouse mutants. Like Plexin-B2−/− mutants, Sema4C−/− mutants reveal exencephaly and subsequent neonatal lethality with partial penetrance. Sema4C−/− mutants that bypass exencephaly are viable and fertile, but display distinctive defects of the cerebellar granule cell layer, including gaps in rostral lobules, fusions of caudal lobules, and ectopic granule cells in the molecular layer. In addition to neuronal defects, we observed in Sema4C−/− mutants also ventral skin pigmentation defects that are similar to those found in Plexin-B2−/− mutants. The Sema4G gene deletion causes no overt phenotype by itself, but combined deletion of Sema4C and Sema4G revealed an enhanced cerebellar phenotype. However, Sema4C/Sema4G double mutants showed overall less severe cerebellar phenotypes than Plexin-B2−/− mutants, indicating that further ligands of Plexin-B2 exist. In explant cultures of the developing cerebellar cortex, Sema4C promoted migration of

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cerebellar granule cell precursors in a Plexin-B2-dependent manner, supporting the model that a reduced migration rate of granule cell precursors is the basis for the cerebellar defects of Sema4C −/− and Sema4C/Sema4G mutants.

Keywords

Cerebellum; lobule; granule cell migration; Semaphorin; Plexin

Introduction

The cerebellum is a prominent part of the vertebrate hindbrain that coordinates posture, locomotion, and a wide range of routine and skilled motor activities. The cells of the cerebellum originate from two different progenitor zones (Altman and Bayer, 1997; Goldowitz and Hamre, 1998). The ventricular zone gives rise to Purkinje cells, Bergmann glia, interneurons, and the neurons of the deep nuclei. In contrast, the upper rhombic lip gives rise to granule cell precursors (GCPs) that migrate rostrally over the cerebellar anlage to form the external granule layer (EGL), which is a heavily proliferating progenitor zone that generates the entire population of cerebellar granule cells. Newborn postmitotic GCPs migrate for a short distance tangentially in the lower EGL, before turning radially inward to settle in the internal granule cell layer (IGL) (Komuro and Yacubova, 2003; Millen and Gleeson, 2008; Sillitoe and Joyner, 2007).

Semaphorins were originally identified as molecules that mediate axon repulsion in insects and vertebrates (Kolodkin et al., 1992; Luo et al., 1993). It was later discovered that Semaphorins are also important regulators of several other biological processes, such as dendrite formation, neural crest migration, vascular development, and activation of the immune response (for reviews, see (Suzuki et al., 2008; Tran et al., 2007; Yazdani and Terman, 2006)). Several Semaphorins have also been described to be involved in tumor formation, acting as regulators of angiogenesis, metastasis, or cell survival (Neufeld and Kessler, 2008).

The mammalian genome contains 19 Semaphorins, which are grouped into classes 3 through 7 (Semaphorin-Nomenclature-Committee, 1999). The main signaling receptors for Semaphorins are Plexins, which are grouped into classes A through D (Tamagnone et al., 1999). The protein structures of Semaphorins and Plexins are characterized by a common large extracellular domain, the “Sema” domain, which mediates binding between ligands and receptors (Gherardi et al., 2004; Janssen et al., 2010). Class 4 Semaphorins (Sema4s) are thought to be ligands for Plexin-B receptors, but apart from the binding of Sema4D to Plexin-B1 (Tamagnone et al., 1999) and of Sema4C to Plexin-B2 (Deng et al., 2007), specific ligand-receptor pairings between the six mammalian Sema4s (Sema4A–D, F, G) and the three Plexin-Bs (B1–B3) are only poorly understood.

The physiological function of Sema4 family members has been well studied for their role in regulation of the immune response (Suzuki et al., 2008). In neural development, it has been shown that Sema4D plays a key role as a migration promoting factor for a class of hormone secreting neurons of the hypothalamus (Giacobini et al., 2008). A large body of data about Sema4 function has been gathered from cell culture based studies. For example, in cultures of cortical neurons, Sema4D activates Plexin-B1 to regulate the growth and branching of axons and dendrites (Oinuma et al., 2004; Swiercz et al., 2002; Uesugi et al., 2009; Vodrazka et al., 2009). In recent years, several additional molecules that are involved in Plexin-B signaling have been identified, but their in vivo relevance remains to be tested. For example, Plexin-Bs can form complexes with the receptor tyrosine kinases Met or ErbB2,

which can determine the outcome of Plexin-B activation (Giordano et al., 2002; Swiercz et al., 2008). The downstream signaling of Plexin-Bs involves small GTPases of the Ras and Rho families that modulate cytoskeletal architecture and integrin adhesion (Zhou et al., 2008). Plexin-B1 and -B2 are widely expressed in the developing nervous system, while Plexin-B3 is restricted to postnatal oligodendrocytes (Perälä et al., 2005; Worzfeld et al., 2004). Targeted deletion of the Plexin-B1 and Plexin-B3 genes in mice resulted in non-detectable or only subtle phenotypes (Deng et al., 2007; Fazzari et al., 2007; Giacobini et al., 2008; Worzfeld et al., 2009). The knockout of Plexin-B2 leads to exencephaly and neonatal lethality on an inbred C57BL/6 genetic background (Deng et al., 2007; Friedel et al., 2007). On an outbred background, surviving Plexin-B2 mutants displayed a severe disruption of the cerebellar cortex that was caused by a disturbed migration of cerebellar granule cell precursors, while overall proliferation and apoptosis rates were largely unchanged (Friedel et al., 2007).

To further elucidate the function of the Plexin-B2 in cerebellar development, we have set out to identify its functional *in vivo* ligands. We have identified here Sema4C and Sema4G as candidate ligands for Plexin-B2 by expression and binding studies. Sema4C and Sema4G share a high degree of sequence homology with each other, and both genes are broadly expressed in the developing nervous system (Inagaki et al., 1995; Li et al., 1999). Interestingly, Sema4C and Plexin-B2 show also largely complementary expression patterns in the adult vascular and endocrine systems (Zielonka et al., 2010). Sema4C has been previously shown to bind to Plexin-B2 in cell culture and to promote migration of granule cells (Deng et al., 2007). However, little is known otherwise about the biological function of these genes in neural development. We have generated mouse mutants for the Sema4C and Sema4G genes, and we provide evidence, based on phenotypic analysis, genetic interaction studies, and migration assays with cerebellar explant cultures, that Sema4C and Sema4G act as ligands of Plexin-B2 to regulate the development of cerebellar granule cells.

Results

Expression of Sema4 and Plexin-B genes in the developing cerebellum

To identify candidate ligands for Plexin-B2 in cerebellar development, we examined the expression of Sema4 genes in the developing cerebellum at postnatal day P10, during the peak period of cerebellar granule cell neurogenesis (Mares et al., 1970) (Fig. 1A). Expression patterns were correlated to cerebellar cell types by comparison with sections that were co-labeled with specific marker proteins (Suppl. Fig. 1). Of the six murine Sema4 genes, *Sema4a*, *Sema4b*, and *Sema4d* are mainly expressed in glial cells: mRNA in situ hybridization signal was detected for *Sema4a* in Bergmann glia, for *Sema4b* in Bergmann glia and astroglia, and for *Sema4d* in Bergmann glia and oligodendroglia. Only *Sema4c* and *Sema4g* are expressed in neurons: *Sema4c* transcripts were detected weakly in granule cells of the EGL and strongly in granule cells of the IGL, as well as in Bergmann glia, and *Sema4g* transcripts were detected in Purkinje cells. *Sema4f* expression was not detectable in the P10 cerebellum. We also surveyed the expression of the putative Sema4 receptors of the Plexin-B family in the cerebellar cortex (Fig. 1B and Suppl. Fig. 1). *Plxnb1* expression was restricted to Bergmann glia. *Plxnb2* appeared to be highly expressed in cerebellar granule cell precursors of the EGL, and at lower levels in the IGL, consistent with previous reports (Friedel et al., 2007). *Plxnb3* expression was found in oligodendroglia. Thus, Plexin-B2 is the only B-type receptor expressed in cerebellar granule cells, and its potential cerebellar ligands comprise five Sema4 members (Sema4A, -B, -C, -D, and -G) (Fig. 1C).

We chose for our study Sema4C as best candidate for a cerebellar Plexin-B2 ligand, since it is the only Sema4 expressed by cerebellar granule cells. A further motivation for our studies was based on previous in vitro findings that had demonstrated Sema4C binding to Plexin-B2

(Deng et al., 2007). In addition, we also chose to examine the cerebellar function of Sema4G, which is phylogenetically the closest relative of Sema4C (Li et al., 1999), and may therefore share some conserved functional roles.

We next analyzed the expression of *Sema4c* and *Sema4g* at earlier time points of cerebellar development. Both genes were robustly expressed in the developing cerebellar cortex between P0 and P6, with patterns that indicate the expression of *Sema4c* in granule cells and Bergmann glia, and of *Sema4g* in Purkinje cells (Fig. 1D). Our data is in agreement with a previous study that had demonstrated Sema4C expression in the developing cerebellar cortex (Deng et al., 2007). In addition, we also utilized the β-galactosidase (β-gal) reporter that is present in the Sema4c targeted trap allele (described in detail further below). Heterozygous Sema4c+/− mice were analyzed for β-gal reporter expression at different developmental time points, confirming the *Sema4c* expression in granule cells and Bergmann glia (Fig. 1E).

Sema4C and Sema4G bind to Plexin-B2 in cell culture and on tissue sections

To determine whether Sema4C and Sema4G proteins can bind to Plexin-B2, we first performed an in vitro binding assay. COS cells were transiently transfected with expression plasmids encoding Plexin-B1, -B2, or -B3. The transfected cells were overlaid with recombinant fusion proteins containing the ectodomains of Sema4C, Sema4G, or Sema4D fused to an alkaline phosphatase (AP) reporter (Fig. 2A). The correct full-length expression of the Plexin-B and Sema4-AP proteins was confirmed by Western blot analysis (Fig. 2B, C). We observed robust binding of Sema4C to Plexin-B2, and a weaker binding to Plexin-B1 (Fig. 2A). No binding to Plexin-B3 was observed. These findings correspond to earlier reports on binding of Sema4C-AP to Plexin-Bs (Deng et al., 2007). Sema4G bound to Plexin-B2, but not to Plexin-B1 or -B3. Sema4D revealed robust binding to both Plexin-B1 and -B2, as has been reported previously (Masuda et al., 2004;Tamagnone et al., 1999), and weaker binding to Plexin-B3.

We next utilized cerebellar sections from wild type and Plexin-B2 mutant mice at P10 to investigate the binding specificity of Sema4-AP proteins to cerebellar tissue (Fig. 2D). Since our previously reported Plexin-B2 mutant allele contains an AP reporter that would obscure binding studies with AP-fusion proteins (Friedel et al., 2007), we employed a novel mutant allele for Plexin-B2 that was generated by the European Conditional Mouse Mutagenesis (EUCOMM) program (Suppl. Fig. 2). Both Sema4C-AP and Sema4G-AP bound strongly to the EGL, and with reduced intensity also to the IGL. In contrast, binding of Sema4C-AP and Sema4G-AP to sections of Plexin-B2−/− mutants was virtually absent. These data suggest that Sema4C and Sema4G bind in the cerebellum specifically to Plexin-B2 expressing granule cells. In contrast, Sema4D-AP protein bound both in wild type and in Plexin-B2 mutant sections to structures in the molecular layer and in the white matter, suggesting that Sema4D binds in the cerebellum mainly to receptors other than Plexin-B2.

Defects in Sema4C and Sema4G mutant mice

To study the in vivo role of Sema4C or Sema4G in cerebellar development, we generated mutant mouse lines for these genes. A mutation for the Sema4C gene was generated by “targeted trapping” (Friedel et al., 2005), which is based on the targeted insertion of a promoterless gene trap cassette by homologous recombination (Fig. 3A). The transmembrane-βgeo cassette of the vector creates a fusion protein with the N-terminal part of the trapped gene, which is sequestered in an intracellular compartment and prevented from reaching the cell surface, resulting in a functional null mutation ((Mitchell et al., 2001; Skarnes et al., 1995); see Methods for details). Absence of Sema4C wild type protein in

homozygous mutants was confirmed by Western Blot analysis with an antibody directed against the C-terminal part of Sema4C (Fig. 3B).

We first analyzed the Sema4C mutation on a C57BL/6 genetic background. About one third of *Sema4c*−/− embryos on C57BL/6 background developed exencephaly, a failure of neural tube closure that leads to neonatal lethality (Suppl. Table 1). This penetrance is lower than that of the Plexin-B2 mutation on C57BL/6 background, in which more than 95% of *Plxnb2*−/− embryos were affected by exencephaly (Friedel et al., 2007). *Sema4c*−/−mutants that bypassed exencephaly were viable and fertile, and showed no obvious behavioral defects.

Interestingly, all *Sema4c*−/− mutants on C57BL/6 background displayed distinct pigmentation defects: white patches along the ventral midline, and white distal fore and hind limbs (Fig. 3C). This phenotype mirrors the pigmentation defects that were observed in Plexin-B2 mutants that had been outcrossed from C57BL/6 to CD-1 for one generation (data not shown), and in Plexin-B2 mutants generated by the EUCOMM program (see Suppl. Fig. 2 for details), suggesting a direct ligand-receptor pairing of Sema4C and Plexin-B2 in pigmentation development.

We next investigated the consequences of ablating Sema4C for the morphogenesis of the cerebellum on a C57BL/6 genetic background. The mouse cerebellum is organized at the vermis in ten distinct lobuli, numbered I – X from rostral to caudal (see (Sillitoe and Joyner, 2007) for review) (Fig. 4A). We utilized the β-gal reporter that is present in the Sema4C mutant allele to visualize the morphology of the cerebellar granule cell layers. The most prominent cerebellar phenotype in adult *Sema4c*−/− mutants and also to a lesser degree in *Sema4c*+/− mutants was a fusion between the rostral lobules VIII and IX. This phenotype was present with variable intensity, ranging from a thin band of granule cells located at the fusion line between the two lobules (scored as “weak” fusion, see Fig. 4B and C as examples), to a continuous bridge of granule cells connecting the IGLs of the two lobules (scored as “strong” fusion, see Fig. 4D as example). We observed fusion defects between lobules VIII and IX in 27% of *Sema4c*+/− mutants and in 80% of *Sema4c*−/− mutants (Table 1).

Fusion defects of lobules VIII/IX were already detectable during postnatal cerebellar development (Fig. 4E). The precise cellular defects causing cerebellar lobule fusions are not known. In FGF9 and integrin pathway mutants, phenotypically similar fusions defects are associated with a reduced basal lamina in cerebellar fissures and a distorted Bergmann glia lattice (Belvindrah et al., 2006;Blaess et al., 2004;Lin et al., 2009;Mills et al., 2006). In *Sema4c*−/− mutants, a disrupted basal lamina was detected at the site of lobule fusion (Fig. 4F), and fusion areas showed defects in the architecture of all major cerebellar cortical cell types, as revealed by immunohistochemical labeling for the granule cell marker Pax6, the Bergmann glia marker GFAP, and the Purkinje cell marker Calbindin (Fig. 4G–I).

Sema4C mutants displayed several cerebellar malformations that are analogous to mild forms of the defects seen in Plexin-B2 mutants (Friedel et al., 2007). These are disruption of the IGL of rostral lobule II by “gaps” (Fig. 4J–L), and small clusters of ectopic granule cells in the molecular layer, mainly at a subpial positions (Fig. 4M, N). The penetrance of these defects in *Sema4c*−/− mice was 50% for gaps in lobule II and 60% for ectopic granule cell clusters (Table 1).

These phenotypes occurred with lower penetrance also in *Sema4c*+/− mutants (Table 1), indicating a potential haploinsufficiency effect in heterozygous *Sema4c*+/− mutants (a dominant negative effect of the Sema4C mutant protein is less likely, as fusion proteins of the secretory trap vector are not expressed at the cell surface (Skarnes et al., 1995)). In

agreement with a haploinsufficiency model, the level of Sema4C protein that was detected in *Sema4c*+/− animals is about 50% of wild type levels (see Fig. 3B).

To examine the influence of the genetic background on the cerebellar phenotype, we conducted Sema4C mutant analysis also on a mixed CD-1 outbred background. We observed all the phenotypes that were found on C57BL/6 also on the CD-1 background, but with reduced severity and frequencies (Suppl. Table 2), suggesting that genetic modifiers present in this mixed background can to some extent compensate for the loss of Sema4C.

We next generated a Sema4G mutation by targeted deletion of exon 1 and 2 of the Sema4G gene (Suppl. Fig. 3). Sema4G mutants on C57BL/6 background displayed normal embryonic development, and were viable and fertile with no overt phenotypes. No cerebellar abnormalities were detected in *Sema4g*−/− mice (Suppl. Fig. 3).

Enhanced cerebellar defects in Sema4C/Sema4G double mutants

To investigate the possibility that Sema4C and Sema4G act in parallel as ligands for Plexin-B2, we generated the respective double mutants on a C57BL/6 background. Deleting one or two copies of the Sema4G gene together with either a heterozygous or homozygous mutation of Sema4C enhanced the penetrance and the severity of the cerebellar phenotypes (Table 1; Fig. 5A–H). For example, the homozygous deletion of Sema4G increased the penetrance of strong lobule VIII/IX fusions for *Sema4c*+/− mutants from 9% to 25% and for *Sema4c*−/− mutants from 70% to 100% (Table 1). Furthermore, deleting Sema4G increased the frequency of gaps in lobule II for *Sema4c*+/− mutants from 20% to 50% and for *Sema4c* −/− mutants from 50% to 67% (Table 1). Removing one or both copies of the Sema4G gene in *Sema4c*−/− mutants resulted in disruption of the IGL of lobule X (Fig. 5D, H), a defect that was not found in mice singly mutant for Sema4C. Thus, double mutations for Sema4C and Sema4G result in enhanced cerebellar phenotypes, suggesting that they act in parallel as ligands for Plexin-B2. However, since the phenotype of the combined deletion of the Sema4C and Sema4G genes is less severe than that of the Plexin-B2 mutation (Friedel et al., 2007), it is likely that other ligands of Plexin-B2 exist in cerebellar development, which remain yet to be identified.

Genetic interaction of Sema4C and Plexin-B2

We next attempted to find genetic evidence that Plexin-B2 and Sema4C act in a common pathway. Heterozygous *Plxnb2*+/− mutants on a C57BL/6 genetic background show a phenotypically normal cerebellar morphology (Friedel et al., 2007). We utilized this heterozygous receptor mutation as a sensitized background for interaction studies with mutations of Sema4C and Sema4G (due to its neonatal lethality, we could not use the *Plxnb2*−/− mutation for interaction studies). When we removed one copy of the Sema4C gene on a *Plxnb2*+/− background (*Sema4c*+/−; *Plxnb2*+/−), no cerebellar abnormalities beyond those observed in *Sema4c*+/− animals were detectable (data not shown). When both copies of Sema4C on a *Plxnb2*+/− background were removed (*Sema4c* −/−; *Plxnb2*+/−), we could detect lobule VIII/IX fusion defects similar to those observed in animals completely deficient for Sema4C (Fig. 5I). Additionally, however, the IGL of lobules II and III were disrupted with a severity that exceeds that detected in *Sema4c*−/− or *Sema4c*−/−; *Sema4g*−/− mutants (Fig. 5J, K). Furthermore, we observed in *Sema4c*−/−; *Plxnb2*+/− mutants a gap in lobule X (Fig. 5L), a defect that was absent in *Sema4c*−/− single mutants.

Thus, combining a homozygous *Sema4c*−/− mutation with a heterozygous *Plxnb2*+/− mutation resulted in cerebellar phenotypes that are stronger than the sum of the individual phenotypes, consistent with the model that Sema4 proteins and Plexin-B2 act as ligands and receptor in a common genetic pathway. We also generated compound mutants carrying a

Sema4g+/− or −/− mutation in combination with a *Plxnb2*+/− mutation, however, no cerebellar phenotypes were observed (data not shown), suggesting that Sema4C plays a more dominant role than Sema4G in mediating Plexin-B2-dependent cerebellar morphogenesis.

Sema4C and Sema4G promote granule cell precursor migration in a Plexin-B2-dependent manner

Cerebellar granule cell precursors (GCPs) deficient for Plexin-B2 show defects in their migratory behavior in vivo (Friedel et al., 2007). We therefore investigated whether Sema4C and Sema4G are able to regulate the migration of GCPs in a Plexin-B2-dependent fashion. For this purpose, we conducted a transwell migration assay with dissociated GCPs of wild type and *Plxnb2*−/− mice (Fig. 6). When stromal cell line-derived factor-1β (SDF-1β) or brain derived neurotrophic factor (BDNF), both known promoters of GCP migration (Borghesani et al., 2002; Ma et al., 1998), were added to the lower chamber of the assay system, granule cells of both genotypes showed robustly enhanced migration through the transwell membrane. Interestingly, both Sema4C and Sema4G elicited a strong migratory response on wild type GCPs. No effect, however, was seen, when these molecules were added to *Plxnb2*−/− mutant GCPs, indicating that the migration promoting effects of Sema4C and Sema4G are mediated by Plexin-B2. In contrast, Sema4D elicited neither on wild type nor on mutant GCPs a migratory effect.

Sema4C and Plexin-B2 promote migration of cerebellar granule cell in explant cultures

In order to analyze the promoting effect of Sema4C on the migration of GCPs in an experimental paradigm that mimics more closely cerebellar development, we prepared EGL microexplant cultures. In microexplant cultures, GCPs differentiate synchronously, and migrate radially from the explant in a manner that parallels their in vivo tangential migration in the lower EGL (Kawaji et al., 2004; Nagata and Nakatsuji, 1990). We focused our analyses on Sema4C, since it had revealed a more prominent role than Sema4G in cerebellar mutant analyses. We first quantified the rate of GCP migration out of explants from wild type, *Sema4c*+/−, and *Sema4c*−/− animals (Fig. 7A, B). *Sema4c*−/− explants showed a significantly reduced number of GCPs outside of explants when compared to *Sema4c*+/− or wild type explants, supporting the model of Sema4C as a migration-promoting factor. The fact that cells migrating out of explants are almost exclusively GCPs that are in extensive cell-cell contact with each other (Kawaji et al., 2004), suggests that Sema4C acts possibly as paracrine factor from granule cell to granule cell.

We next asked if exogenous Sema4C as substrate would be able to promote granule cell migration from explants. For this purpose, we coated culture dishes with laminin and Sema4C-AP or Sema4D-AP protein before the plating of explants. Wild type explants revealed a significant increase in their migration rate on Sema4C-AP coated dishes, but not on control or Sema4D-AP coated substrates (Fig. 7C, D).

We also tested EGL explants of *Plxnb2*−/− mutant mice (Fig. 7C, E). *Plxnb2*−/− explants displayed a reduced GCP migration rate compared to wild type explants when plated on laminin alone. The reduced migration rate of *Plxnb2*−/− explants was not altered when Sema4C protein was additionally presented as a substrate, indicating that Plexin-B2 is a receptor for the migration promoting effect of Sema4C.

Discussion

Dissecting the Plexin-Semaphorin signaling network is important for understanding how the nervous system is built up. Here we provide evidence that Sema4C and Sema4G are

functional in vivo ligands for Plexin-B2 regulating the migratory properties of cerebellar granule cells during development.

Developmental functions of Sema4C and Sema4G

The mutation of Sema4C alone or of Sema4C/Sema4G together result in neural phenotypes that are qualitatively similar, albeit less severe than those reported for Plexin-B2 mutant mice (Friedel et al., 2007). For example, exencephaly occurs in Sema4C mutants with lower frequency than in Plexin-B2 mutants, and defects in rostral cerebellar lobulation and positioning of granule cells similarly occur in *Sema4c*−/− mutants with less severity and lower penetrance. This indicates that further ligand(s) are required for Plexin-B2 function in neural development. These ligands could be other Sema4 members, such as Sema4A or Sema4B, which are expressed in radial Bergmann glia, and are therefore in direct contact with migrating granule cells. However, Sema4A/Sema4D knockout mutants, as well as a Sema4B gene trap mutant, have not revealed any cerebellar phenotypes ((Friedel et al., 2007); Supp. Fig. 4). Further elucidation of the combined function of Sema4 members in the cerebellum will require mutants carrying mutations in multiple Sema4 genes. Alternatively, Plexin-B receptors may also act as homophilic ligands, as in vitro studies have suggested homophilic binding of Plexin receptors to each other (Hartwig et al., 2005; Ohta et al., 1995).

The Sema4C and Plexin-B2 phenotypes do not entirely overlap in regard to cerebellar topology: *Sema4c*−/− mutants show striking fusions of the caudal lobules VIII and IX, which was not a prominent defect in Plexin-B2 mutants. A possible explanation for this discrepancy may be the fact that the Plexin-B2 mutation was only studied on a CD-1 outbred background, since exencephaly had precluded postnatal analysis on C57BL/6 (Friedel et al., 2007). The C57BL/6 background may be predisposed to lobule VIII/IX fusion defects, which is indicated by the finding that weak lobule VIII/IX fusions can be found in some colonies of C57BL/6 wild type mice (Tanaka and Marunouchi, 2005). In agreement with this notion, we have observed more severe lobule fusion defects of Sema4C and Sema4C/Sema4G mutants on C57BL/6 than on CD-1 outbred background.

It is not known why certain lobules of the cerebellum are more sensitive to genetic disruptions than others. Our in situ expression data does not reveal a localized expression of Sema-4 or Plexin-B2 in certain lobules, which could explain the lobule-specific defects. Rather, the specific localization of phenotypes may be either a result of the complex dynamics of EGL growth and lobule folding (Sillitoe and Joyner, 2007), which may put some cerebellar regions under higher constraints regarding cell motility. Alternatively, the cerebellum may be subdivided in regions of distinctive molecular coding, as has been suggested by recent studies on functional domains of engrailed proteins in the cerebellum (Sgaier et al., 2007), and these regions may display different requirements for Plexin-B function.

The Sema4G mutation by itself did not cause a detectable cerebellar phenotype. However, loss of Sema4G gene function enhances the phenotype of the Sema4C mutation, and Sema4G promoted the migration of GCPs in a transwell migration assay, indicating that both Sema4C and Sema4G act redundantly as ligands for Plexin-B2 on GCPs, although with Sema4C playing a more dominant role. Sema4G is expressed by Purkinje cells, and therefore may promote migration of GCPs by its localization on Purkinje cell dendritic arbors.

As an alternative to an activity as transmembrane ligands, Sema4C and Sema4G may potentially also act as secreted proteins. Such a mechanism has been demonstrated for the extracellular domain of Sema4D, which is shed by proteolytic cleavage from the surface of

lymphocytes (Elhabazi et al., 2001; Wang et al., 2001). In analogy, Sema4C and Sema4G may also promote migration of GCPs in form of secreted ligands. It is interesting to note in this context that Sema4C has been recently reported to be a substrate for the protease BACE1 (Hemming et al., 2009). However, no evidence exists so far for *in vivo* shedding of Sema4C or Sema4G, and this model will require future experimental examination.

In respect to the pigmentation abnormalities seen in Sema4C and Plexin-B2 homozygous mutants, it is tempting to speculate that these might be caused by disturbances in the development of melanocytes, which are derivatives of the neural crest lineage (Barsh, 1996). Future studies will reveal if a direct Sema4C Plexin-B2 ligand-receptor interaction is involved in one of the steps of melanocyte development, such as proliferation, migration, or differentiation.

How do the cerebellar phenotypes in Sema4C/G mutants relate to known functions of Sema4 genes?

We have provided here the first description of the in vivo functions of the Sema4C and Sema4G genes. Our findings are in agreement with several in vitro studies that describe a cell migration-promoting effect for the Sema4D/Plexin-B1 pathway, such as for the migration of endothelial cells (Basile et al., 2007) or liver epithelial cells (Giordano et al., 2002). In addition, it has also been shown that Sema4D can promote the migration of cortical precursor cells through the Plexin-B2 receptor (Hirschberg et al., 2010). However, Sema4D can also inhibit cell migration, as has been shown for a breast carcinoma cell line (Swiercz et al., 2008). These seemingly opposing activities of Sema4s on cell migration highlight the diverse roles of Sema4s in different biological contexts, and may be explained by different components of co-receptors in different cell lines that dictate the outcome of Plexin-B activation (Swiercz et al., 2008). Other morphogenetic roles of Sema4 proteins include the regulation of axon guidance, dendritic branching, and uretric duct growth; in these processes Sema4 proteins can also have positive or negative regulatory roles depending on the cellular context (Korostylev et al., 2008; Masuda et al., 2004; Saito et al., 2009; Vodrazka et al., 2009; Xiao et al., 2003; Zhou et al., 2008).

Sema4C and Sema4G, which are widely expressed in the developing nervous system, may have other morphogenetic functions beyond the promotion of GCP migration. Interestingly, the Sema4C protein has been detected in zones of adult neurogenesis, where it is upregulated during recovery from ischemic stroke (Wu et al., 2009). The further elucidation of the functions of Sema4C/4G proteins will require a more detailed understanding of the cellular and molecular responses upon Plexin activation.

How do Semaphorins orchestrate the development of cerebellar granule cells?

Lobule fusion defects that are similar to the lobule VIII/IX defects of Sema4C mutants have been described for FGF9, integrin-β1, and integrin-linked kinase mutants (Belvindrah et al., 2006; Blaess et al., 2004; Lin et al., 2009; Mills et al., 2006). It has been suggested that the phenotype of these mutants are caused by a primary defect in radial Bergmann glia development, which then leads to a breakdown of the basal membrane and subsequent fusions of granule cell layers (Belvindrah et al., 2006; Lin et al., 2009). The FGF9 and integrin phenotypes are, however, different from the Sema4C mutants in that they affect the entire cerebellum instead of specific lobules. Although we cannot rule out the possibility that some aspects of Sema4C/4G phenotypes may be caused by Bergmann glia defects, our data supports a model that the Sema4C/4G phenotypes are mainly a consequence of primary defects in cerebellar granule cell migration.

Besides the Sema4/Plexin-B pathway, also the Sema6/Plexin-A pathway is involved in cerebellar development. In particular, Sema6A and Plexin-A2 act as a ligand-receptor pair that regulates the migration of postmitotic GCPs that have left the proliferative zone of the upper EGL and migrate tangentially in the lower EGL (Kerjan et al., 2005; Renaud et al., 2008). The control of Plexin-A2 on GCP migration is exerted through regulation of nucleosome translocation (Renaud et al., 2008). Interestingly, Plexin-B2 is highly expressed in the upper EGL, while Sema6A and Plexin-A2 are expressed in the lower EGL. Thus, it has been suggested that Plexin-As regulate cell migration of postmitotic GCPs in the lower EGL, while Plexin-B2 acts mainly on the migration of proliferating GCPs in the upper EGL (Chedotal, 2010).

In summary, our results suggest the following model for Sema4C/Sema4G function during cerebellar development. Plexin-B2 is expressed by GCPs in the upper EGL, and its activation by Sema4C/4G promotes the migration of GCPs to populate the entire EGL. An impaired rostro-caudal GCP migration during development is likely to manifest in the adult cerebellum in reduced numbers of granule cells in rostral areas and increased numbers in caudal areas. Our model thus helps to explain why defects that are associated with deficiencies of granule cells, such as gaps in the IGL, occur mainly in rostral lobules and defects associated with excess of granule cells, such as subpial ectopic clusters, occur in caudal lobules. Other mechanisms, though, are needed to explain other neural defects, including the fusions of caudal lobules, the disruption of lobule X, and the neural tube closure defects. Further studies will help explain the full range of defects seen in the mutants, and will also help to identify downstream effectors of Plexin-B2 to elucidate how the Semaphorin/Plexin network regulates neural development.

Material and methods

Mouse genetics

The Sema4C gene was mutated by the targeted trapping method (Friedel et al., 2005). A targeting vector was constructed by flanking a secretory trap cassette (Leighton et al., 2001) with 5’ and 3’ homology arms of 5 kb and 3 kb size, respectively. Correct homologous recombination in E14Tg2a mouse embryonic stem cells resulted in the insertion of the trap cassette between exons 12 and 13 of the Sema4C gene (exon 1 counted as the exon containing the start codon), which is upstream of the exons encoding the transmembrane (TM) and intracellular domains of Sema4C. The bicistronic mutant transcript encodes two proteins: a fusion of the extracellular domain of the Sema4C protein to the TM and β-geo domains of the trap vector, and placental alkaline phosphatase (PLAP). The Sema4C genotype was determined by a three primer multiplex PCR using the following primers: TGGTGTGGCTTACCCTGTGCTTTG (genomic forward), AGAAAGGAGCCAGGTTGTTCTGCA (genomic reverse), and ACTTCCGGAGCGGATCTCAAACTC (vector reverse), which amplified a 620 bp wild type and a 430 bp mutant fragment.

For the mutation of the Sema4G gene, a targeting vector was constructed by replacing a 350 bp fragment containing the first and second exons of the Sema4G gene with a neomycin resistance cassette. The vector was flanked with a Herpes simplex virus thymidine kinase (HSV-*TK*) gene that was used to select against random integration. The Sema4G genotype was determined by a three primer multiplex PCR using the following primers: ATCACACCCTGGACTTTGAACCC (genomic forward), TTTCCCTTTCTGATGACACTTGC (genomic reverse), and CAATCCATCTTGTTCAATGGCCGA (vector reverse), which amplified a 450 bp wild type and a 304 bp mutant fragment.

The Plexin-B2 targeted trap mutant mouse line (*Plxnb2*−/−) has been described previously (Friedel et al., 2007). The EUCOMM Plexin-B2 mutation (abbreviated as *Plxnb2*^{Euc1a}) was generated by targeting C57BL/6N embryonic stem cells with a vector that contained an Engrailed2 splice acceptor, a β-gal reporter, a T2A viral self-cleavage peptide, a neomycin resistance gene, and a polyadenylation signal (www.eucomm.org; see Suppl. Fig. 2 for details). The *Plxnb2*^{EUC1a} genotype was determined by a three primer multiplex PCR using the following primers: TACTAGGATCAGAGGTCATCG (genomic forward), GCTTTGGTGTCAACTCCCAAG (genomic reverse), and CACAACGGGTTCTTCTGTTAGTCC (vector reverse), which amplified a 723 bp wild type and a 526 bp mutant fragment.

The Plexin-B2, Sema4C, and Sema4G mutants that were used for experimental studies on C57BL/6 background had been backcrossed for at least 5 generations to C57BL/6N wild type mice (Charles River, Germany). The Plexin-B2 EUCOMM mutation was maintained as coisogenic line by backcrossing to C57BL/6N wildtype mice. For mutant analysis on mixed CD-1 outbred background, mice were outcrossed to CD-1 mice (Charles River, Germany).

Official allele symbols (Mouse Genome Informatics Database, The Jackson Laboratory): Sema4C targeted trap mutation: *Sema4c*^{tm1Matl}; Sema4G mutation: *Sema4g*^{tm1Kik}; Plexin-B2 targeted trap: *Plxnb2*^{tm1Matl}; Plexin-B2 EUCOMM mutation: *Plxnb2*$^{tm1a(EUCOMM)Wtsi}$.

In situ hybridization

Digoxigenin-labeled riboprobes for Sema4 and Plexin-B genes were transcribed from 2 kb cDNA fragments that had been generated by reverse transcription-PCR and cloned into the pCRII vector (Invitrogen). In situ hybridizations were performed on floating vibratome sections as described (Dolan et al., 2007). Briefly, 70 μm vibratome sections were collected in phosphate buffered saline (PBS) and subsequently treated with a methanol series, permeabilized with RIPA-buffer (150mM NaCl, 50mM Tris-HCl pH 8.0, 1mM EDTA, 1% Nonident-P40, 0.5% sodium deoxycholate, 0.1% SDS), and postfixed with 4% paraformaldehyde / 0.2% glutaraldehyde in PBS for 40 min. Hybridizations were performed over night at 65°C, and probes were detected with an alkaline phosphatase conjugated anti-digoxigenin antibody (Roche). Hybridizations with sense probes were included in all experiments to control the nonspecific background signals.

Immunohistochemistry and β-galactosidase staining

For immunohistochemistry, brains were perfused and postfixed over night with 4% paraformaldehyde in 0.1 M phosphate buffer. Immunohistochemistry was performed on vibratome (100 μm) or microtome sections (40 μm). Sections were incubated with antibodies against calbindin (1:500, Chemicon), $GABA_A$ receptor α6 subunit (1:250, Chemicon), glial fibrillary astrocytic protein (GFAP; 1:80, Sigma), laminin (1:60, Sigma), Olig2 (1:100, Chemicon), S100β (1:250, Abcam), and Pax6 (polyclonal 1: 250, Santa Cruz Biotechnology), followed by species-specific secondary antibodies (Jackson Immuno Research). Sections were counterstained with DAPI and mounted with Aqua-Poly/Mount (Polyscienes). Histochemical stainings for the β-galactosidase reporter were performed on 100 μm vibratome sections as described previously (Leighton et al., 2001). All comparisons were done on sagittal sections of the cerebellar vermis region (up to 500 μm lateral from the medial midline), which was identified by using the cerebellar peduncles as landmarks for beginning and end of section series.

Western Blot analysis

Antibodies against the following peptides or proteins were used for protein detection on Western blot membranes: VSV (1:150,000, Sigma), placental alkaline phosphatase (1:200,

Santa Cruz Biotechnology), Sema4C (1:500, BD Bioscience), Plexin-B2 (1:200, Santa Cruz Biotechnology).

Alkaline phosphatase (AP) binding assays

For the production of secreted AP-tagged fusion proteins, COS cells were transiently transfected with expression plasmids for murine Sema4C-AP (a gift of Dr. Strittmatter, Yale University), Sema4D-AP, and Sema4G-AP with a Fugene 6 reagent (Roche). The supernatants of transfected cells were collected, concentrated 10 fold by Centriprep Ultracel YM-50 (Millipore) spin columns, and then normalized to equal activities by measurement of the AP-activity. For cell binding assays, COS cells in 6 well plates were transiently transfected with expression plasmids for human Plexin-B1 (a gift of Dr. Tamagnone, University of Turin), murine Plexin-B2, murine Plexin-B3, or EGFP as a control. Binding and detection were performed as described (Flanagan et al., 2000). Binding of Sema4-AP proteins on sections was performed with fresh frozen sections as described (Renaud et al., 2008).

Transwell migration assay

Dissociated primary GCPs were purified from mouse cerebella as described (Wechsler-Reya and Scott, 1999). Briefly, dissected P5 cerebella were incubated in PBS with papain (20U, Sigma) and DNaseI (10mg/ml, Sigma) for 30 min at 37°C. After trituration and centrifugation, cells were resuspended at 1×10^6 cells/ml in Neurobasal medium (Invitrogen) supplemented with sodium pyruvate, B27 (Invitrogen), and N-acetyl cysteine. For the migration assay, 250 µl of cell suspension were seeded to the upper chamber of poly-L-lysin and laminin coated PET membranes (Greiner Bio-One ThinCert, Germany) with 3 µm pore size in a 24-well plate format. Lower chambers were filled with 200 µl DMEM culture media conditioned with Sema4C-AP, Sema4D-AP, or Sema4G-AP protein, or SDF-1α (100 ng/ml, PeproTech) or BDNF (100 ng/ml, PeproTech). After incubation over night, cells on the upper surface of the membrane were scraped of, and the granule cells that had migrated to the lower surface of the membrane were fixed and stained with DAPI. Five representative photomicrographs were taken at 40x magnification of each membrane and the cells were quantified.

Cerebellar explant cultures

Brains of postnatal day 5 mice (P5) were sectioned and the cerebellum was dissected out and freed from meninges in cold 0.5mg/ml glucose. Cerebellar parasagittal slices (300µm thick) were cut on tissue chopper and separated with needles. Explants were placed on poly-L-lysin/laminin coated coverslips and incubated in 4 well plates with 200 µl of Neurobasal medium (Invitrogen) containing N2 Supplement and BSA. In some cases, coverslips were additionally coated with Sema4C-AP or Sema4D-AP protein. After 48h cultures were fixed and stained with DAPI and rhodamine phalloidin. Quantitative analysis of neuronal migration rate was performed with ImageJ software on photomicrographs of DAPI-stained explants. Concentric rings of 100 µm width were delineated around the explant, and the intensity of pixels in each ring was measured. Values were normalized to the experimental condition that yielded the highest rate of migration (wild type explants on Sema4C substrate = 100%).

Statistical analysis

For all statistical analysis, the significance was evaluated by ANOVA. All data represented as mean + SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

EGL	external granule cell layer
IGL	internal granule cell layer
GCP	granule cell precursor

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References

Altman, J.; Bayer, SA. The Cerebellar System. Boca Raton: CRC Press; 1997.

Barsh GS. The genetics of pigmentation: from fancy genes to complex traits. Trends Genet 1996;12:299–305. [PubMed: 8783939]

Basile JR, Gavard J, Gutkind JS. Plexin-B1 utilizes RhoA and Rho kinase to promote the integrin-dependent activation of Akt and ERK and endothelial cell motility. The Journal of biological chemistry 2007;282:34888–34895. [PubMed: 17855350]

Belvindrah R, Nalbant P, Ding S, Wu C, Bokoch GM, Muller U. Integrin-linked kinase regulates Bergmann glial differentiation during cerebellar development. Molecular and cellular neurosciences 2006;33:109–125. [PubMed: 16914328]

Blaess S, Graus-Porta D, Belvindrah R, Radakovits R, Pons S, Littlewood-Evans A, Senften M, Guo H, Li Y, Miner JH, Reichardt LF, Muller U. Beta1-integrins are critical for cerebellar granule cell precursor proliferation. J Neurosci 2004;24:3402–3412. [PubMed: 15056720]

Borghesani PR, Peyrin JM, Klein R, Rubin J, Carter AR, Schwartz PM, Luster A, Corfas G, Segal RA. BDNF stimulates migration of cerebellar granule cells. Development (Cambridge, England) 2002;129:1435–1442.

Chedotal A. Should I stay or should I go? Becoming a granule cell. Trends in neurosciences 2010;33:163–172. [PubMed: 20138673]

Deng S, Hirschberg A, Worzfeld T, Penachioni JY, Korostylev A, Swiercz JM, Vodrazka P, Mauti O, Stoeckli ET, Tamagnone L, Offermanns S, Kuner R. Plexin-B2, but not Plexin-B1, critically modulates neuronal migration and patterning of the developing nervous system in vivo. J Neurosci 2007;27:6333–6347. [PubMed: 17554007]

Dolan J, Walshe K, Alsbury S, Hokamp K, O'Keeffe S, Okafuji T, Miller SF, Tear G, Mitchell KJ. The extracellular leucine-rich repeat superfamily; a comparative survey and analysis of evolutionary relationships and expression patterns. BMC genomics 2007;8:320. [PubMed: 17868438]

Elhabazi A, Delaire S, Bensussan A, Boumsell L, Bismuth G. Biological activity of soluble CD100. I. The extracellular region of CD100 is released from the surface of T lymphocytes by regulated proteolysis. J Immunol 2001;166:4341–4347. [PubMed: 11254687]

Fazzari P, Penachioni J, Gianola S, Rossi F, Eickholt BJ, Maina F, Alexopoulou L, Sottile A, Comoglio PM, Flavell RA, Tamagnone L. Plexin-B1 plays a redundant role during mouse development and in tumour angiogenesis. BMC developmental biology 2007;7:55. [PubMed: 17519029]

Flanagan JG, Cheng HJ, Feldheim DA, Hattori M, Lu Q, Vanderhaeghen P. Alkaline phosphatase fusions of ligands or receptors as in situ probes for staining of cells, tissues, and embryos. Methods in enzymology 2000;327:19–35. [PubMed: 11044971]

Friedel RH, Kerjan G, Rayburn H, Schuller U, Sotelo C, Tessier-Lavigne M, Chedotal A. Plexin-B2 controls the development of cerebellar granule cells. J Neurosci 2007;27:3921–3932. [PubMed: 17409257]

Friedel RH, Plump A, Lu X, Spilker K, Jolicoeur C, Wong K, Venkatesh TR, Yaron A, Hynes M, Chen B, Okada A, McConnell SK, Rayburn H, Tessier-Lavigne M. Gene targeting using a promoterless gene trap vector ("targeted trapping") is an efficient method to mutate a large fraction of genes. Proceedings of the National Academy of Sciences of the United States of America 2005;102:13188–13193. [PubMed: 16129827]

Gherardi E, Love CA, Esnouf RM, Jones EY. The sema domain. Current opinion in structural biology 2004;14:669–678. [PubMed: 15582390]

Giacobini P, Messina A, Morello F, Ferraris N, Corso S, Penachioni J, Giordano S, Tamagnone L, Fasolo A. Semaphorin 4D regulates gonadotropin hormone-releasing hormone-1 neuronal migration through PlexinB1-Met complex. The Journal of cell biology 2008;183:555–566. [PubMed: 18981235]

Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D, Tamagnone L, Comoglio PM. The semaphorin 4D receptor controls invasive growth by coupling with Met. Nature cell biology 2002;4:720–724.

Goldowitz D, Hamre K. The cells and molecules that make a cerebellum. Trends in neurosciences 1998;21:375–382. [PubMed: 9735945]

Hartwig C, Veske A, Krejcova S, Rosenberger G, Finckh U. Plexin B3 promotes neurite outgrowth, interacts homophilically, and interacts with Rin. BMC Neurosci 2005;6:53. [PubMed: 16122393]

Hemming ML, Elias JE, Gygi SP, Selkoe DJ. dentification of beta-secretase (BACE1) substrates using quantitative proteomics. PloS one 2009;4:e8477. [PubMed: 20041192]

Hirschberg A, Deng S, Korostylev A, Paldy E, Costa MR, Worzfeld T, Vodrazka P, Wizenmann A, Gotz M, Offermanns S, Kuner R. Gene deletion mutants reveal a role for semaphorin receptors of the plexin-B family in mechanisms underlying corticogenesis. Molecular and cellular biology 2010;30:764–780. [PubMed: 19948886]

Inagaki S, Furuyama T, Iwahashi Y. Identification of a member of mouse semaphorin family. FEBS letters 1995;370:269–272. [PubMed: 7656991]

Janssen BJ, Robinson RA, Perez-Branguli F, Bell CH, Mitchell KJ, Siebold C, Jones EY. Structural basis of semaphorin-plexin signalling. Nature. 2010

Kawaji K, Umeshima H, Eiraku M, Hirano T, Kengaku M. Dual phases of migration of cerebellar granule cells guided by axonal and dendritic leading processes. Molecular and cellular neurosciences 2004;25:228–240. [PubMed: 15019940]

Kerjan G, Dolan J, Haumaitre C, Schneider-Maunoury S, Fujisawa H, Mitchell KJ, Chedotal A. The transmembrane semaphorin Sema6A controls cerebellar granule cell migration. Nature neuroscience 2005;8:1516–1524.

Kolodkin AL, Matthes DJ, O'Connor TP, Patel NH, Admon A, Bentley D, Goodman CS. Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron 1992;9:831–845. [PubMed: 1418998]

Komuro H, Yacubova E. Recent advances in cerebellar granule cell migration. Cell Mol Life Sci 2003;60:1084–1098. [PubMed: 12861377]

Korostylev A, Worzfeld T, Deng S, Friedel RH, Swiercz JM, Vodrazka P, Maier V, Hirschberg A, Ohoka Y, Inagaki S, Offermanns S, Kuner R. A functional role for semaphorin 4D/plexin B1 interactions in epithelial branching morphogenesis during organogenesis. Development (Cambridge, England) 2008;135:3333–3343.

Leighton PA, Mitchell KJ, Goodrich LV, Lu X, Pinson K, Scherz P, Skarnes WC, Tessier-Lavigne M. Defining brain wiring patterns and mechanisms through gene trapping in mice. Nature 2001;410:174–179. [PubMed: 11242070]

Li H, Wu DK, Sullivan SL. Characterization and expression of sema4g, a novel member of the semaphorin gene family. Mechanisms of development 1999;87:169–173. [PubMed: 10495281]

Lin Y, Chen L, Lin C, Luo Y, Tsai RY, Wang F. Neuron-derived FGF9 is essential for scaffold formation of Bergmann radial fibers and migration of granule neurons in the cerebellum. Developmental biology 2009;329:44–54. [PubMed: 19232523]

Luo Y, Raible D, Raper JA. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 1993;75:217–227. [PubMed: 8402908]

Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 1998;95:9448–9453. [PubMed: 9689100]

Mares V, Lodin Z, Srajer J. The cellular kinetics of the developing mouse cerebellum. I. The generation cycle, growth fraction and rate of proliferation of the external granular layer. Brain Res 1970;23:323–342. [PubMed: 5478301]

Masuda K, Furuyama T, Takahara M, Fujioka S, Kurinami H, Inagaki S. Sema4D stimulates axonal outgrowth of embryonic DRG sensory neurones. Genes Cells 2004;9:821–829. [PubMed: 15330859]

Millen KJ, Gleeson JG. Cerebellar development and disease. Current opinion in neurobiology 2008;18:12–19. [PubMed: 18513948]

Mills J, Niewmierzycka A, Oloumi A, Rico B, St-Arnaud R, Mackenzie IR, Mawji NM, Wilson J, Reichardt LF, Dedhar S. Critical role of integrin-linked kinase in granule cell precursor proliferation and cerebellar development. J Neurosci 2006;26:830–840. [PubMed: 16421303]

Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, Goodrich LV, Lu X, Avery BJ, Tate P, Dill K, Pangilinan E, Wakenight P, Tessier-Lavigne M, Skarnes WC. Functional analysis of secreted and transmembrane proteins critical to mouse development. Nature genetics 2001;28:241–249. [PubMed: 11431694]

Nagata I, Nakatsuji N. Granule cell behavior on laminin in cerebellar microexplant cultures. Brain research 1990;52:63–73. [PubMed: 2331801]

Neufeld G, Kessler O. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nat Rev Cancer 2008;8:632–645. [PubMed: 18580951]

Ohta K, Mizutani A, Kawakami A, Murakami Y, Kasuya Y, Takagi S, Tanaka H, Fujisawa H. Plexin: a novel neuronal cell surface molecule that mediates cell adhesion via a homophilic binding mechanism in the presence of calcium ions. Neuron 1995;14:1189–1199. [PubMed: 7605632]

Oinuma I, Ishikawa Y, Katoh H, Negishi M. The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. Science (New York, N.Y 2004;305:862–865.

Perälä NM, Immonen T, Sariola H. The expression of plexins during mouse embryogenesis. Gene Expr Patterns 2005;5:355–362. [PubMed: 15661641]

Renaud J, Kerjan G, Sumita I, Zagar Y, Georget V, Kim D, Fouquet C, Suda K, Sanbo M, Suto F, Ackerman SL, Mitchell KJ, Fujisawa H, Chedotal A. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. Nature neuroscience 2008;11:440–449.

Saito Y, Oinuma I, Fujimoto S, Negishi M. Plexin-B1 is a GTPase activating protein for M-Ras, remodelling dendrite morphology. EMBO reports 2009;10:614–621. [PubMed: 19444311]

Semaphorin-Nomenclature-Committee. Unified nomenclature for the semaphorins/collapsins. Cell 1999;97:551–552. [PubMed: 10367884]

Sgaier SK, Lao Z, Villanueva MP, Berenshteyn F, Stephen D, Turnbull RK, Joyner AL. Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrailed proteins. Development (Cambridge, England) 2007;134:2325–2335.

Sillitoe RV, Joyner AL. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. Annual review of cell and developmental biology 2007;23:549–577.

Skarnes WC, Moss JE, Hurtley SM, Beddington RS. Capturing genes encoding membrane and secreted proteins important for mouse development. Proceedings of the National Academy of Sciences of the United States of America 1995;92:6592–6596. [PubMed: 7604039]

Suzuki K, Kumanogoh A, Kikutani H. Semaphorins and their receptors in immune cell interactions. Nature immunology 2008;9:17–23. [PubMed: 18087252]

Swiercz JM, Kuner R, Behrens J, Offermanns S. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. Neuron 2002;35:51–63. [PubMed: 12123608]

Swiercz JM, Worzfeld T, Offermanns S. ErbB-2 and met reciprocally regulate cellular signaling via plexin-B1. The Journal of biological chemistry 2008;283:1893–1901. [PubMed: 18025083]

Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 1999;99:71–80. [PubMed: 10520995]

Tanaka M, Marunouchi T. Abnormality in the cerebellar folial pattern of C57BL/6J mice. Neuroscience letters 2005;390:182–186. [PubMed: 16139954]

Tran TS, Kolodkin AL, Bharadwaj R. Semaphorin regulation of cellular morphology. Annual review of cell and developmental biology 2007;23:263–292.

Uesugi K, Oinuma I, Katoh H, Negishi M. Different requirement for Rnd GTPases of R-Ras GAP activity of Plexin-C1 and Plexin-D1. The Journal of biological chemistry 2009;284:6743–6751. [PubMed: 19136556]

Vodrazka P, Korostylev A, Hirschberg A, Swiercz JM, Worzfeld T, Deng S, Fazzari P, Tamagnone L, Offermanns S, Kuner R. The semaphorin 4D-plexin-B signalling complex regulates dendritic and axonal complexity in developing neurons via diverse pathways. The European journal of neuroscience 2009;30:1193–1208. [PubMed: 19788569]

Wang X, Kumanogoh A, Watanabe C, Shi W, Yoshida K, Kikutani H. Functional soluble CD100/Sema4D released from activated lymphocytes: possible role in normal and pathologic immune responses. Blood 2001;97:3498–3504. [PubMed: 11369643]

Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron 1999;22:103–114. [PubMed: 10027293]

Worzfeld T, Puschel AW, Offermanns S, Kuner R. Plexin-B family members demonstrate non-redundant expression patterns in the developing mouse nervous system: an anatomical basis for morphogenetic effects of Sema4D during development. The European journal of neuroscience 2004;19:2622–2632. [PubMed: 15147296]

Worzfeld T, Rauch P, Karram K, Trotter J, Kuner R, Offermanns S. Mice lacking Plexin-B3 display normal CNS morphology and behaviour. Molecular and cellular neurosciences 2009;42:372–381. [PubMed: 19699796]

Wu H, Fan J, Zhu L, Liu S, Wu Y, Zhao T, Wu Y, Ding X, Fan W, Fan M. Sema4C Expression in Neural Stem/Progenitor Cells and in Adult Neurogenesis Induced by Cerebral Ischemia. J Mol Neurosci. 2009

Xiao T, Shoji W, Zhou W, Su F, Kuwada JY. Transmembrane sema4E guides branchiomotor axons to their targets in zebrafish. J Neurosci 2003;23:4190–4198. [PubMed: 12764107]

Yazdani U, Terman JR. The semaphorins. Genome biology 2006;7:211. [PubMed: 16584533]

Zhou Y, Gunput RA, Pasterkamp RJ. Semaphorin signaling: progress made and promises ahead. Trends in biochemical sciences 2008;33:161–170. [PubMed: 18374575]

Zielonka M, Xia J, Friedel RH, Offermanns S, Worzfeld T. A systematic expression analysis implicates Plexin-B2 and its ligand Sema4C in the regulation of the vascular and endocrine system. Experimental cell research 2010;316:2477–2486. [PubMed: 20478304]

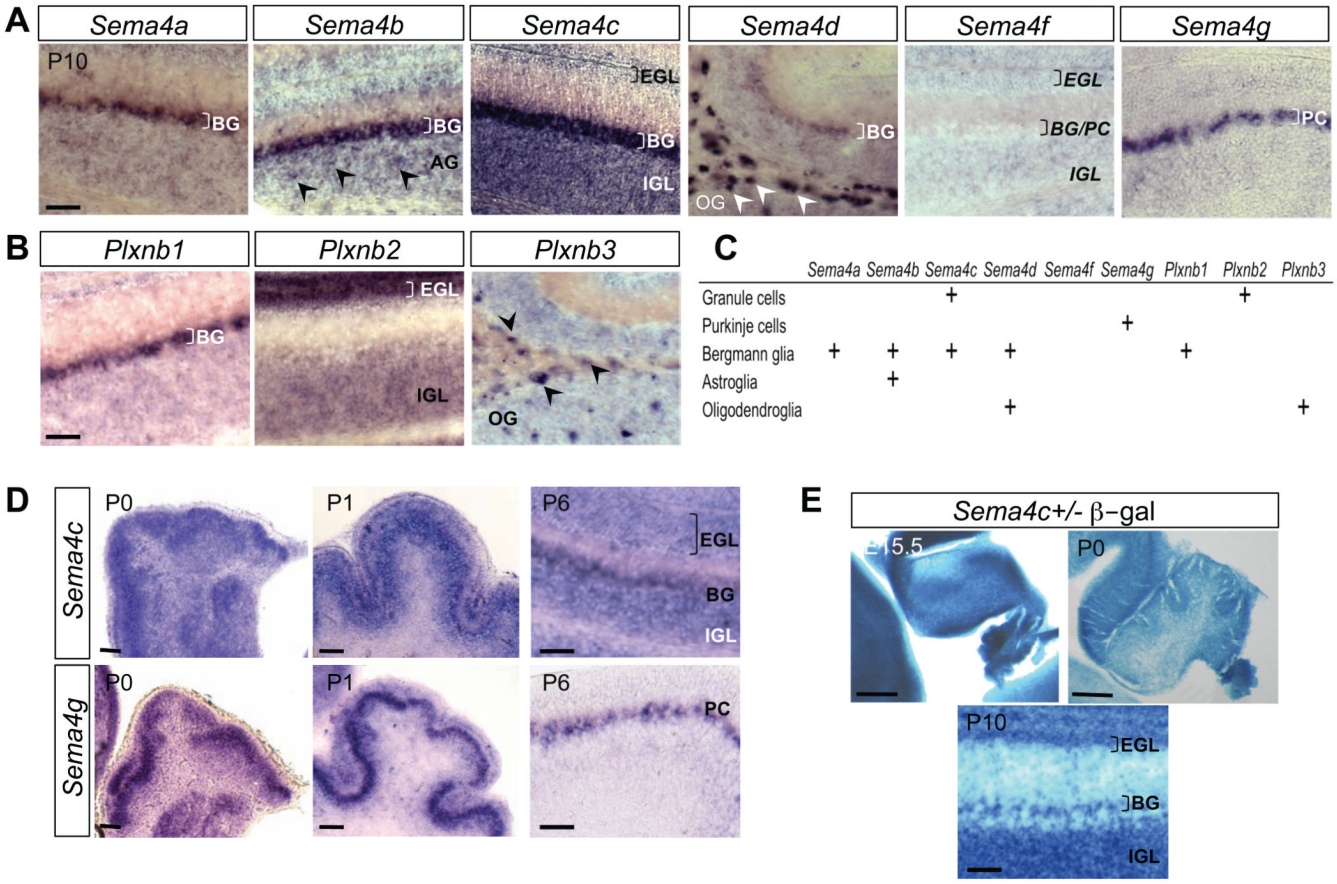

Fig. 1. Expression of Sema4 and Plexin-B genes in the developing cerebellum

(A) Expression of Sema4 genes in the cerebellar cortex at P10 as revealed by mRNA in situ hybridization. The section for *Sema4d* comprises parts of the cerebellar white matter. *Sema4a* corresponded in its expression pattern to Bergmann glia cells, *Sema4b* to Bergmann glia and astroglia, *Sema4c* to granule cells and Bergmann glia, *Sema4d* to oligodendroglia and Bergmann glia, and *Sema4g* to Purkinje cells. *Sema4f* mRNA was not detectable in the developing cerebellum.

(B) The *Plxnb1* expression pattern corresponded to Bergmann glia cells, the *Plxnb2* pattern to granule cells, and the *Plxnb3* pattern to oligodendroglia. The section for *Plexinb3* comprises parts of the cerebellar white matter.

(C) Summary of Sema4 and Plexin-B expression patterns in the developing cerebellum.

(D) In situ hybridization for Sema4c and Sema4g expression at postnatal days P0, P1, and P6. Sema4c gene expression was found in the granule cell and Bergmann glia layers, and Sema4g gene expression was found in the Purkinje cell layer.

(E) X-gal stainings for the β-galactosidase reporter of *Sema4c*+/− animals on cerebellar sections at E15.5, P0, and P10.

BG, Bergmann glia; AG, astroglia; EGL, external granule cell layer; IGL, Internal granule cell layer; PC, Purkinje cells; OG, oligodendroglia.

Scale bars in (A, B): 25μm; (D): for P0 and P1 100 μm, for P6 25 μm; (E): for E15.5 and P0 250 μm, for P10 25 μm.

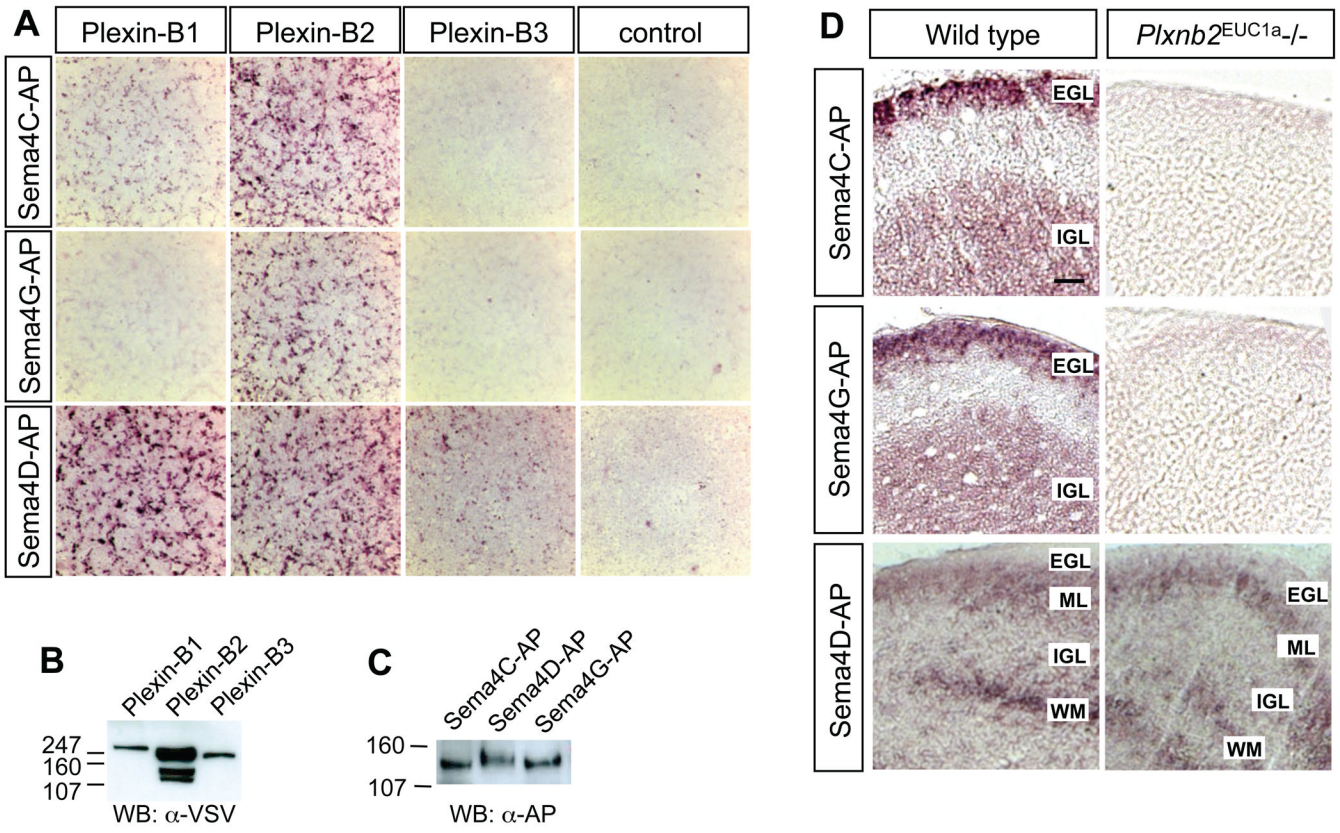

Fig. 2. Sema4C and Sema4G bind to Plexin-B2 *in vitro*

(A) Binding Sema4 ectodomains fused to an alkaline phosphatase (AP) reporter to COS fibroblasts that were transiently transfected with expression plasmids for Plexin-B1, -B2, -B3 or green fluorescent protein (control). Sema4C bound robustly to Plexin-B2 and weaker to Plexin-B1 expressing cells, Sema4G bound to Plexin-B2, and Sema4D to Plexin-B1, -B2, and -B3.

(B) Expression of transfected Plexin-B plasmids was confirmed by Western blot analysis of cell lysates with an antibody directed against a VSV-tag. Predicted sizes: Plexin-B1, 235kDa; Plexin-B2, 207 kDa; Plexin-B3, 213 kDa.

(C) Size of recombinant Sema4-AP constructs was confirmed by Western blot analysis with an antibody directed against the AP reporter. Predicted sizes: Sema4C-AP, 128 kDa; Sema4D-AP, 125 kDa; Sema4G-AP, 127 kDa.

(D) Binding of Sema4-AP proteins on cryosections of P10 cerebella of wild type and *Plxnb2*^{EUC1a} −/−mutants. Sema4C and Sema4G bind robustly to EGL and weaker to IGL on wild type sections. No binding of Sema4C-AP or Sema4G-AP was detectable on *Plxnb2*^{EUC1a}−/− mutant sections. Sema4D-AP binding was detected within the molecular layer and white matter, both on wild type and *Plxnb2*^{EUC1a} mutant sections.

EGL, external granule cell layer; IGL, Internal granule cell layer; ML, molecular layer; WM, white matter. Scale bar in (D): 50μm.

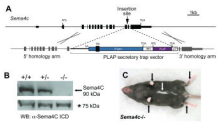

Fig. 3. Mutation of the Sema4C gene

(A) Targeted trapping strategy to mutate the Sema4C gene. The targeting vector contains a secretory trap cassette flanked by 5 kb and 3 kb homology designed to insert the cassette between exons 12 and 13 of the Sema4C gene (exon 1 counted as the exon containing the start codon). SA, splice acceptor; TM, transmembrane domain; ATG, start codon; TGA, Stop codon (see Methods for details).

(B) Western blot analysis of lysates from P5 cerebella with an antibody directed against the intracellular domain (ICD) of Sema4C confirms absence of wild type protein in *Sema4c*−/− mutants (arrow). In wild type and *Sema4c*+/− animals, a polypeptide of 90 kDa, corresponding to the predicted size of Sema4C, was detected. An unspecific band of 75 kDa served to control for loading (asterisk).

(C) *Sema4c*−/− mutants on C57BL/6 background reveal pigmentation defects at the ventral midline and at the distal fore and hind limbs.

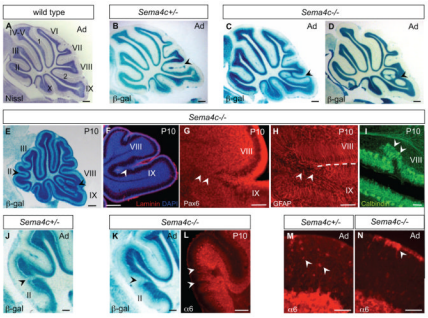

Fig. 4. Cerebellar defects in Sema4C-deficient mice

(A–N) Sagittal cerebellar sections of adult (Ad) or postnatal day 10 (P10) animals on C57BL/6 background, stained for Nissl substance (A), β-galactosidase activity of the Sema4C mutant allele (B–E, J, K), or for immunoreactivity for laminin (F), Pax6 (G), glial fibrillary astrocytic protein (GFAP) (H), calbindin (I), and GABA-A receptor alpha 6 (L–N).

(A) Section of adult wild type mouse. Lobules are indicated by Roman numerals. 1 primary fissure, 2 secondary fissure.

(B) Weak lobule VIII/IX fusion phenotype in Sema4c+/− cerebellum.

(C, D) Cerebella of *Sema4c*−/− animals reveal weak (C) or a strong fusion (D) defects between lobules VIII and IX.

(E) The lobule phenotypes are detectable at P10.

(F) The basal lamina between the fused lobules VIII and IX is disrupted, as shown by absence of laminin immunoreactivity.

(G) Pax6 staining reveals a continuous bridge of granule cells between lobules VIII and IX.

(H) Disrupted radial Bergmann glia palisade. Dotted line indicates fissure between lobule VIII and IX.

(I) Displaced Purkinje cells on section adjacent to lobule VIII/IX fusion.

(J–L) Gaps in the IGL of rostral lobule II of Sema4c +/− and Sema4c−/− animals.

(M, N) Ectopic cerebellar granule cells are found as dispersed cells in the molecular layer of *Sema4c*+/− mice (M) and as subpial clusters of cells in *Sema4c*−/− mice (N).

Scale bars in (A–F): 300 μm; (G–I, M, N): 50 –m; (J–L): 150 –m.

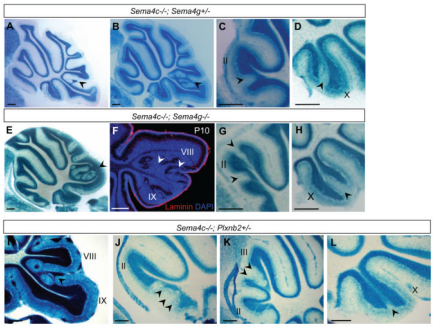

Fig. 5. Genetic interaction of the Sema4C mutation with Sema4G and Plexin-B2

(A–L) Sagittal cerebellar sections of adult animals stained for β-galactosidase activity of the Sema4C mutant allele (A–E, G–L), and P10 section stained for laminin immunoreactivity (F). All mice are on C57BL/6 background.

(A–D) In *Sema4c*−/−; *Sema4g*+/− mice, fusion defects between lobules VIII and IX occurred with weak (A) or strong (B) characteristics. In addition, gaps in the IGL of lobules II (C) and lobule X (D) occurred.

(E–H) Double homozygous *Sema4c*−/−; *Sema4g*−/− mutants reveal strong fusion defects between lobules VIII/IX (E, F), and gaps in the IGL of lobules II (G) and X (H). Absence of laminin staining between the lobules indicates loss of a separating basal lamina (F).

(I) *Sema4c*−/−; *Plxnb2*+/− mutants reveal lobule VIII/IX fusion defects.

(J–L) *Sema4c*−/−; *Plxnb2*+/− mutants reveal severe disruptions in the IGL of rostral lobules II and III (J, K), and gaps in lobule X (L).

Scale bars in (A–L): 300 μm.

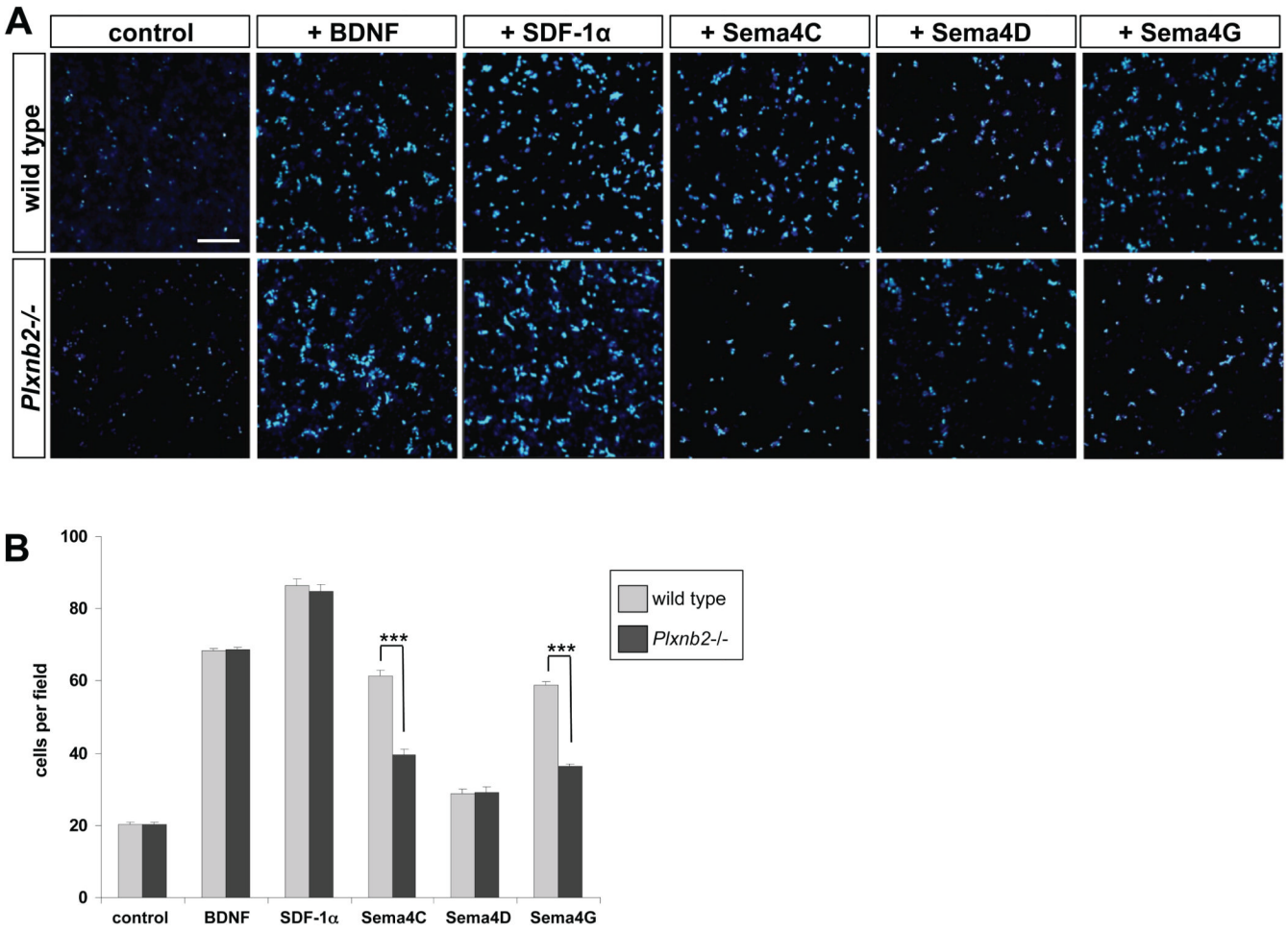

Fig. 6. Sema4C and Sema4G promote granule cell migration in a transwell assay

(A) Representative photomicrographs of DAPI stained transwell membranes, revealing migration rate of GCPs. Dissociated GCPs of wild type and *Plxnb2−/−* mice were seeded on top of transwell membranes, and the lower compartment was conditioned with migration stimulating proteins (BDNF and SDF-1α, or with Sema4C-AP, Sema4D-AP, or Sema4G-AP fusion proteins. Supernatant from cells transfected with a green fluorescent protein plasmid served as control. Cells that had migrated to the lower side of the membrane were stained with DAPI.

(B) Quantification of transwell migration assays. Sema4C and Sema4G promoted migration of GCPs from wild type animals, but not from *Plxnb2−/−* animals. No statistically significant effect was observed for Sema4D.

Significant differences are indicated by three asterisks (t-Test, $p < 0.001$).

Scale bar in (A): 100 µm.

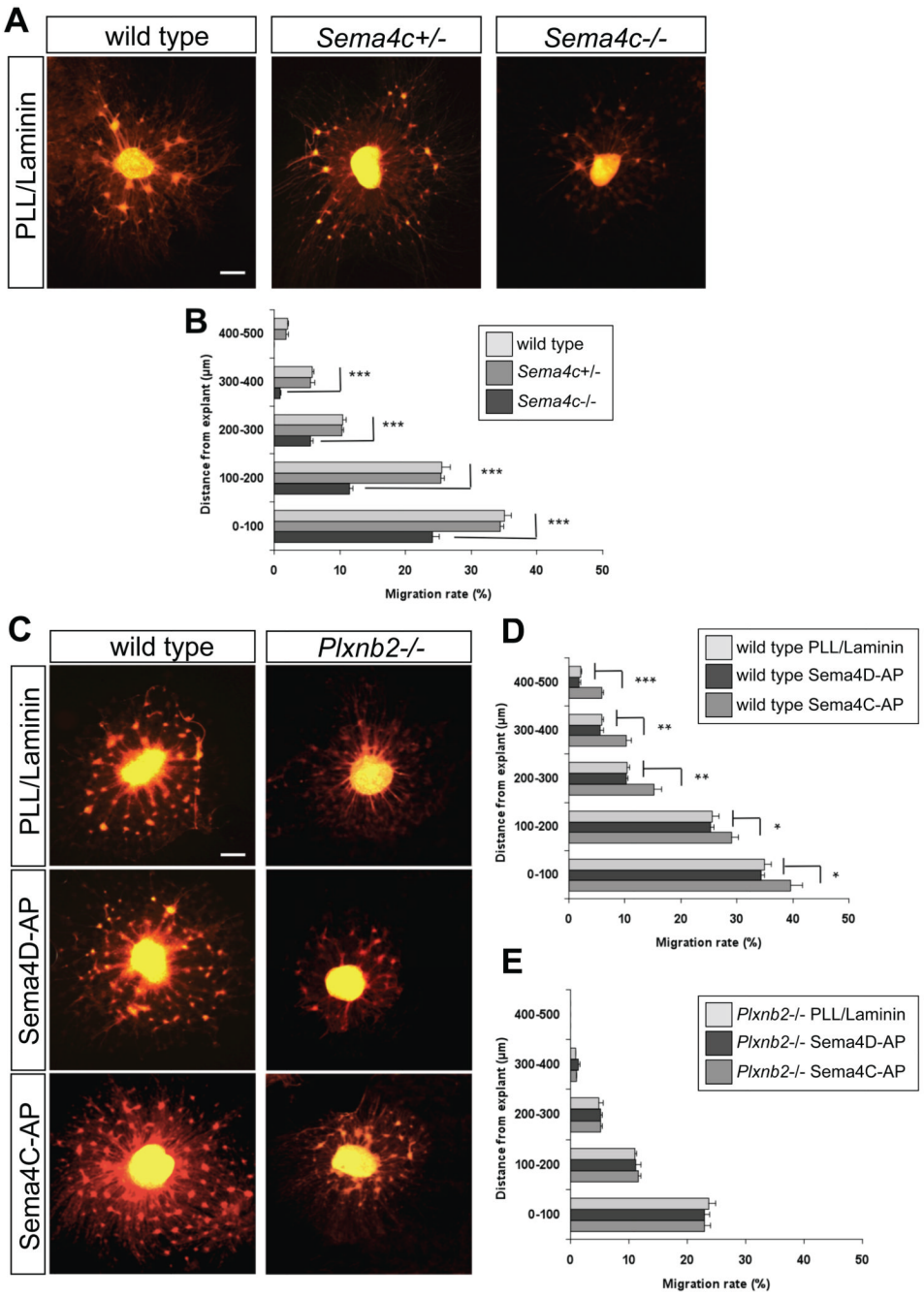

Fig. 7. Granule cell migration of explant cultures is promoted by Sema4C

(A, C) EGL microexplants of wild type, *Sema4c*+/−, *Sema4c*−/−, or *Plxnb2*−/− P5 mice were plated on cell culture dishes, incubated for 72h, and labeled by phalloidin to visualize cell bodies and neurites.

(B, D, E) Quantification of cell migration rates of explants cultures shown in (A, C). Cell nuclei were labeled by DAPI, and fluorescence signal was measured in concentric rings around the explants. Migration rates were normalized to the experimental series that showed the strongest migration rate (the sum of cells from wild type explants on Sema4C-AP substrate in all distance bins (0 to 500 µm) equals 100%).

(A, B) EGL explants of wild type, *Sema4c*+/−, and *Sema4c*−/− animals on PLL/laminin substrate. Explants of Sema4c−/− mice showed reduced migration rates. (4 independent experiments; total explants numbers: wild type n=20, *Sema4c*+/− n= 21, *Sema4c*−/− n=24). (C–E) EGL microexplant cultures of wild type and *Plxnb2*−/− P5 mice were plated on dishes that were coated with either only Poly-L-lysin/Laminin alone, or additionally with Sema4C-AP or Sema4D-AP. Wild type explants showed an enhanced migration rate on Sema4C coated substrate. In contrast, *Plxnb2*−/− explants showed reduced migration rates independent of substrate composition (4 independent experiments; total explants numbers: control n=19, Sema4C-AP n=20, Sema4D-AP n=20).
The significant differences (t-Test) are indicated by one asterisk ($p < 0.02$), two asterisks ($p < 0.002$) or three asterisks ($p < 0.001$).
Scale bar in (A, C): 200 μm.

Table 1

Cerebellar phenotypes of Sema4C and Sema4G mutants (on C57BL/6 background)

Genotype	*n*	Normal cerebellum	Fusion of lobules VIII/IX*		Gap in IGL of lobule II	Gap in IGL of lobule X	Ectopic granule cells in molecular layer
			weak	strong			
Wild type	*17*	100%	0%	0%	0%	0%	0%
Sema4c+/−	*11*	73%	18%	9%	20%	0%	10%
Sema4c−/−	*10*	20%	10%	70%	50%	0%	60%
Sema4c+/−; *Sema4g*+/−	*16*	31%	56%	13%	40%	0%	40%
Sema4c+/−; *Sema4g*−/−	*13*	31%	44%	25%	50%	0%	60%
Sema4c−/−; *Sema4g*+/−	*10*	0%	20%	80%	60%	60%	80%
Sema4c−/−; *Sema4g* −/−	*11*	0%	0%	100%	67%	75%	100%

*Fusions of lobules VIII and IX were scored as “weak” when ectopic granule cells formed a band of cells at the fusion line, and as “strong” when a continuous bridge of granule cells connected the IGL of the two lobules.