The vesicular SNARE Synaptobrevin is required for Semaphorin 3A axonal repulsion

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Atractive and repulsive molecules such as Semaphorins (Sema) trigger rapid responses that control the navigation of axonal growth cones. The role of vesicular traffic in axonal guidance is still largely unknown. The exocytic vesicular soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor (SNARE) Synaptobrevin 2 (Syb2) is known for mediating neurotransmitter release in mature neurons, but its potential role in axonal guidance remains elusive. Here we show that Syb2 is required for Sema3A-dependent repulsion but not Sema3C-dependent attraction in cultured neurons and in the mouse brain. Syb2 associated with Neuropilin 1 and Plexin A1, two essential components of the Sema3A receptor, via its juxta-transmembrane domain. Sema3A receptor and Syb2 colocalize in endosomal membranes. Moreover, upon Sema3A treatment, Syb2-deficient neurons failed to collapse and transport Plexin A1 to cell bodies. Reconstitution of Sema3A receptor in nonneuronal cells revealed that Sema3A further inhibited the exocytosis of Syb2. Therefore, Sema3A-mediated signaling and axonal repulsion require Syb2-dependent vesicular traffic.

Introduction

The guidance of axonal and dendritic growth cones to their specific cellular target is essential for the establishment of functional neural circuits during development (Tessier-Lavigne and Goodman, 1996). Attractive and repulsive external guidance cues bind to receptors, which activates intracellular signaling pathways and reshapes the growth cone, allowing for positive and negative turning, respectively. Several types of endosomal vesicles have been identified in growth cones, but the role of exocytosis and endocytosis in growth and navigation is still largely unknown (Pfenninger, 2009). Exocytosis involves the fusion of intracellular vesicles with the plasma membrane and relies on soluble SNARE proteins located on both vesicular and plasma membranes. The synaptic vesicular SNARE Synaptobrevin 2 (Syb2; also called VAMP2) is the target of several clostridial neurotoxins, including tetanus neurotoxin (TeNT), which blocks neurotransmitter release (Schiavo et al., 2000). Knockout of Syb2 causes an almost complete loss of spontaneous and evoked release of neurotransmitters leading to postnatal death, but has not been associated with a developmental defect in the mouse brain (Schoch et al., 2001). However, there is evidence suggesting...
that Syb2 may play a role in the development of cultured neurons on specific substrates. Syb2 was recently shown to be required for axon formation in neurons cultured on poly-lysine but not laminin (Gupton and Gertler, 2010). TeNT inhibits calcium-triggered positive turning in neurons grown on an artificial L1 substrate but has no effect on calcium-triggered negative turning in neurons grown on laminin. The later result may suggest that exocytosis is required for attraction, but not repulsion (Tojima et al., 2007), and draw attention to Syb2 as a candidate gene for axonal guidance (Tojima et al., 2011). Previous studies using Semaphorin 3A (Sema3A) have hinted at a complex view. Indeed, repulsion mediated by Sema3A induces massive endocytosis of membrane (Fournier et al., 2000) and of its receptor (Castellani et al., 2004; Piper et al., 2005). After Sema3A-induced endocytosis of its receptor, resensitization requires reexpression and/or recycling of this receptor to the growth cone surface (Piper et al., 2005). Thus, axon guidance may depend on a regulation of both exocytosis and endocytosis.

As alluded to in the previous paragraph, it is not clear whether Syb2 functions in axonal guidance and, if so, how. Here, we took advantage of clonal radial neurotoxins and genetic invalidation to demonstrate that Syb2 is required for Sema3A-mediated repulsion, but not for Sema3C-mediated attraction, in vitro and in vivo. We found that Syb2 associates with Neuropilin 1 (Nrp1) and Plexin A1 (PlexA1), two essential components of the Sema3A receptor. This interaction involves Syb2 juxtaparazmembrane domain, which suggests a strong biochemical link during vesicular traffic between Syb2 and the Sema3A receptor. We further show that Sema3A inhibits the exocytosis of Syb2. Our findings thus demonstrate that Sema3A repulsion depends on Syb2-dependent vesicular traffic.

Results and discussion

Sema3A-mediated repulsion but not Sema3C-mediated attraction requires Syb2
To determine the function of Syb2 in axonal guidance, we analyzed the guidance responses of cortical explants treated with botulinum neurotoxin D (BoNT/D; Schiavo et al., 2000) and cortical explants from Syb2 knockout mice (Schoch et al., 2001). The explants were embedded in plasma matrix facing an aggregate of cells stably secreting Sema3C, Sema3A, or mock. After 24 h of culture, we analyzed both the ratio of proximal over distal axonal length as well as the turning angles of the axons in the area perpendicular to the gradient (Fig. 1 A; Bagnard et al., 1998).

In 3D culture systems, Syb2 is not required for axonal growth of both cortical and dorsal root ganglion (DRG) neurons, as shown by BoNT/D and genetic invalidation (Fig. S1). However, we found a striking requirement of Syb2 for Sema3A-induced repulsion, but not for Sema3C-induced attraction in E15 cortical explants (Fig. 1). In the control condition, Sema3C induced an increase of axonal length in the proximal area and an attractive turning of the axons toward the source as described previously (Bagnard et al., 1998). BoNT/D treatment fully cleaved Syb2 (Fig. S1) but did not affect Sema3C-induced attraction (Fig. 1, B–E). On the contrary, Sema3A decreased axonal length in the proximal area and repelled the axons away from the source in control explants, but did not have any effect in BoNT/D-treated explants (Fig. 1, B–E). To further demonstrate the need for a functional Syb2 in Sema3A repulsion but not Sema3C attraction, we performed guidance assays in Syb2+/+, Syb2−/−, and Syb2−/− explants. We found that the absence of Syb2 in −/− cortical explants abolished the repulsive effects of Sema3A, whereas it had no effect on Sema3C-mediated attraction (Fig. 1, F–I). These results demonstrate that Syb2 is required for the repulsion induced by Sema3A but not for growth or the attraction mediated by Sema3C in cortical axons ex vivo.

We then asked whether Syb2 controls the repulsive response to Sema3A by affecting the acute chemotactic responses of axonal growth cones from isolated neurons in a well-defined concentration gradient. We first cultured dissociated embryonic day 15 (E15) cortical neurons grown in microchambers framed by two reservoirs to produce a linear gradient. We then generated a Sema3A or Sema3C gradient and observed axonal growth cone turning 3 h after the onset of the gradient by time-lapse video microscopy, and quantified the results as described previously (Yam et al., 2009). We found that cortical axons respond to Sema3C by turning toward the source whether or not Syb2 was acutely inactivated by BoNT/D (Fig. 2, A and B). However, Sema3A repulsive turning was abolished by treatment with BoNT/D (Fig. 2, A and B). These experiments further show that Syb2 is not necessary for Sema3C-induced attraction, but is essential for Sema3A-induced repulsion of isolated cortical axonal growth cones.

Sema3A-mediated repulsion requires Syb in DRG
Genetic invalidation of Sema3A induces profound defasciculation of peripheral nerves (Kitsukawa et al., 1997). However, these nerves express both Syb1 and Syb2 (Trimble et al., 1990), which, with Cellubrevin, are functionally interchangeable v-SNAREs (Deák et al., 2006; Liu et al., 2011). Thus, both Sybs1 and -2 would need to be inactivated to obliterate their function in peripheral nerves. To this end, we cultured DRG explants embedded in collagen 3D matrix facing an aggregate of cells secreting Sema3A or mock (Ben-Zvi et al., 2008), and we treated them with BoNT/D, to remove both Syb1 and Syb2. BoNT/D-treated DRG axons were not repelled by Sema3A (Fig. S2, A–C), which confirms our observations in cortical explants. Not surprisingly, in toto neurofilament immunostaining in Syb2−/− did not reveal a massive defasciculation of primary sensory fibers around the eyes nor in the trunk and limbs (Fig. S2 D), unlike what was observed in Sema3A−/− and Nrp1−/− mice (Kitsukawa et al., 1997). In light of the evidence of redundant function of Syb1 and Syb2 in peripheral nerves, these results suggest that the lack of guidance defect in peripheral nerves in Syb2−/− is likely caused by a compensatory expression of Syb1.
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Syb2 knockout embryos display a disorganized corpus callosum

We went on to test if Syb2 inactivation would generate a defect in Sema3A-mediated guidance in the forebrain in vivo. The corpus callosum is a region where Sema3A and Sema3C play a role in axon guidance. Indeed, mutations in the Sema-binding domain of Nrp1, coreceptor of Sema3A with PlexA1 (Takahashi et al., 1999), prevent both Sema3A and Sema3C activation and generate misguidance of Nrp1-positive fibers in the corpus callosum (Hatanaka et al., 2009; Piper et al., 2009). Furthermore, a possible reduction in Nrp1-expressing axons observed in the corpus callosum of Sema3A−/− mice (Piper et al., 2009) may be caused by defasciculation of Nrp1-positive fibers. Thus, the lack of Sema3A repulsion in Syb2-deficient cortical neurons may generate a defect in Nrp1-positive fibers in the corpus callosum. To investigate this issue, we analyzed the
Figure 2. **Syb2 is required for Sema3A-mediated repulsion, but not Sema3C-mediated attraction, in dissociated neurons.** (A) E15 dissociated cortical neurons were grown in Ibidi microchambers in medium with or without BoNT/D. At t = 0 h, 3 µg/ml of control, Sema3C-Fc, or Sema3A-Fc medium was added at the top of the chamber. Axons were allowed to grow and turn for 3 h. Bar, 10 µm. (B) Mean values ± SEM (error bars) of axonal turning in each condition (control, n = 136; control + BoNT/D, n = 91; Sema3C, n = 41; Sema3C + BoNT/D, n = 39; Sema3A, n = 81; Sema3A + BoNT/D, n = 105 axons). **, P < 0.005 by Student’s t test.

Figure 3. **Syb2−/− embryos display morphological defects similar to loss of Sema3A.** (A) E18 brain from Syb2+/+ and Syb2−/− embryos were cut in 100-µm slices and stained with Nrp1 (red), L1 (green), and DAPI (blue; Syb2+/+, n = 3; Syb2−/−, n = 4 embryos). Bar, 500 µm. (B, left) Schematic view of loss of compaction of Nrp1-positive fibers in Syb2−/−. (B, right) Profile of Nrp1 fluorescence measured in corpus callosum of Syb2+/+ and Syb2−/− embryos (2–3 slices quantified for each embryo). ***, P < 0.0005 by two-way analysis of variance test.
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We tested whether or not Syb2, Nrp1, and PlexA1 interact in developing neurons. We reasoned on the basis that Syb2 interacts with other vesicular proteins including Synaptophysin (Syp) and V-ATPase (Edelmann et al., 1995; Galli et al., 1996) that are sorted together into synaptic vesicles in mature neurons. Here, we found that Syb2 coimmunoprecipitated Nrp1 and PlexA1 in E15 brain extracts, but not in adult brain extracts (Fig. 4 A). In addition, Syb2 coimmunoprecipitated Syp, but not L1, N-cadherin, or Syntaxin 1 (Stx1), a target SNARE partner of Syb2 in neurotransmitter release, which suggests that Syb2–Nrp1–PlexA1 interaction is not a mere result of detergent solubilization (Fig. 4 A). Tetanus insensitive–vesicle associated membrane protein (TI-VAMP)/VAMP7, another v-SNARE expressed in neurons, did not coimmunoprecipitate Nrp1 and coimmunoprecipitated only small amounts of PlexA1. Further more, Stx1 did not coimmunoprecipitate Nrp1 or PlexA1, which provides additional evidence for the specificity of the interaction with Syb2 (Fig. S3 A). The reverse experiment using Nrp1 and PlexA1 as bait failed to coimmunoprecipitate Syb2, most likely because of the fact that the available tested antibodies could not recognize the tripartite complex because the Sema3A receptor has a specific conformation when bound to Syb2. To circumvent this problem and further identify which domain of Syb2 interacts with Nrp1 and PlexA1, we first tested whether or not Syb2, Nrp1, and PlexA1 interact in developing neurons. We reasoned on the basis that Syb2 interacts with other vesicular proteins including Synaptophysin (Syp) and V-ATPase (Edelmann et al., 1995; Galli et al., 1996) that are sorted together into synaptic vesicles in mature neurons. Here, we found that Syb2 coimmunoprecipitated Nrp1 and PlexA1 in E15 brain extracts, but not in adult brain extracts (Fig. 4, A and B). In addition, Syb2 coimmunoprecipitated Syp, but not L1, N-cadherin, or Syntaxin 1 (Stx1), a target SNARE partner of Syb2 in neurotransmitter release, which suggests that Syb2–Nrp1–PlexA1 interaction is not a mere result of detergent solubilization (Fig. 4 A). Tetanus insensitive–vesicle associated membrane protein (TI-VAMP)/VAMP7, another v-SNARE expressed in neurons, did not coimmunoprecipitate Nrp1 and coimmunoprecipitated only small amounts of PlexA1. Furthermore, Stx1 did not coimmunoprecipitate Nrp1 or PlexA1, which provides additional evidence for the specificity of the interaction with Syb2 (Fig. S3 A). The reverse experiment using Nrp1 and PlexA1 as bait failed to coimmunoprecipitate Syb2, most likely because of the fact that the available tested antibodies could not recognize the tripartite complex because the Sema3A receptor has a specific conformation when bound to Syb2. To circumvent this problem and further identify which domain of Syb2 interacts with Nrp1 and PlexA1, we first tested whether or not Syb2, Nrp1, and PlexA1 interact in developing neurons. We reasoned on the basis that Syb2 interacts with other vesicular proteins including Synaptophysin (Syp) and V-ATPase (Edelmann et al., 1995; Galli et al., 1996) that are sorted together into synaptic vesicles in mature neurons. Here, we found that Syb2 coimmunoprecipitated Nrp1 and PlexA1 in E15 brain extracts, but not in adult brain extracts (Fig. 4, A and B). In addition, Syb2 coimmunoprecipitated Syp, but not L1, N-cadherin, or Syntaxin 1 (Stx1), a target SNARE partner of Syb2 in neurotransmitter release, which suggests that Syb2–Nrp1–PlexA1 interaction is not a mere result of detergent solubilization (Fig. 4 A). Tetanus insensitive–vesicle associated membrane protein (TI-VAMP)/VAMP7, another v-SNARE expressed in neurons, did not coimmunoprecipitate Nrp1 and coimmunoprecipitated only small amounts of PlexA1. Furthermore, Stx1 did not coimmunoprecipitate Nrp1 or PlexA1, which provides additional evidence for the specificity of the interaction with Syb2 (Fig. S3 A). The reverse experiment using Nrp1 and PlexA1 as bait failed to coimmunoprecipitate Syb2, most likely because of the fact that the available tested antibodies could not recognize the tripartite complex because the Sema3A receptor has a specific conformation when bound to Syb2. To circumvent this problem and further identify which domain of
Figure 5. Sema3A-induced repulsion requires Syb2-dependent traffic of its receptor Nrp1/PlexA1. (A) E15 dissociated neurons from Syb2+/+ and Syb2−/− embryos were grown on poly-L-lysine and laminin coverslips. At DIV2, 3 µg/ml of control or Sema3A-Fc was added to the medium. After 3 h of treatment, neurons were fixed and immunostained for MAP2 (green) and Tau (red). (B) The graph displays mean ± SEM values (error bars) of axonal length (%). (C) Axonal length (%). (D) Table showing the average intensity of PlexA1 in soma (%). (E) Average intensity of PlexA1 in soma (%). (F) Average intensity of PlexA1 in soma (%). (G) Time course of exocytosis. (H) Graph showing exocytosis (% initial activity). (I) Diagram illustrating the Sema3A receptor with t-SNAREs and v-SNARE Syb2.
Syb2 mediates the interaction with Nrp1 and PlexA1, we reconstituted the complex in heterologous HEK-293T cells by expressing tagged Nrp1, PlexA1, and Syb2. GFP-Syb2, bearing GFP at the N-terminal cytosolic end, or Syb2–Super Ecliptic Phluorin (Syb2-SEP), bearing a pH-sensitive GFP at the C-terminal luminal extremity, were cleaved or not with cotransfected wild-type or mutant TeNT light chain, respectively (Fig. 4 C). The interaction between Nrp1, PlexA1, and full-length Syb2 was demonstrated by coimmunoprecipitation using each of the respective tags (Fig. 4 D). Although this interaction was maintained in the TeNT-cleaved Syb2-SEP, it was abolished in the TeNT-cleaved GFP-Syb2, showing that Syb2 interacts with Nrp1 and PlexA1 through its transmembrane and linker domain (Fig. 4 D). Additionally, Syb2 and Nrp1 strongly colocalized in intracellular membranes, particularly early endosomes (EEA1 staining) and clathrin-coated pits (AP2 labeling), when coexpressed with PlexA1 in Cos7 cells (Fig. S3 B). These results suggest that (a) the recognition between Syb2 and Nrp1/PlexA1 is highly specific, and the v-SNARE is part of a complex excluding its t-SNARE partner Stx1; and (b) Syb2 and the Sema3A receptor interact and traffic together in endocytic and/or recycling vesicles. Moreover, several single transmembrane domain proteins, including Nrp1 and Syb2, were shown to share similar structural features in their transmembrane regions susceptible to induce homo- or heterodimerization (Hubert et al., 2010). Our results showing that Syb2 interacts with Nrp1/PlexA1 through its juxta-transmembrane domain suggest that this region may play a pivotal role in sorting together Syb2, Nrp1, and PlexA1. Additional work will be required to further characterize the function of these transmembrane domains in the co-sorting of Syb2 and Sema3A receptors.

**Sema3A signaling requires Syb2 for both endocytosis and exocytosis**

In developing neurons, Syb2 is transported from the Golgi apparatus to the surface of dendrites, being afterward transcytosed to the axon, where it mediates exocytosis (Sampo et al., 2003). Therefore, inactivation of Syb2 in developing neurons could lead to a defective polarization of Nrp1/PlexA1 and/or defective intracellular traffic of Nrp1/PlexA1. The first hypothesis is contradicted by the expression of Nrp1 and PlexA1 at the cell surface in Syb2–/– neurons (Fig. S3 C). When axons encounter an isotopic concentration of Sema3A, they collapse and retract due to massive endocytosis of the membrane and of the Nrp1/PlexA1 receptor in growth cones (Fournier et al., 2000; Piper et al., 2005). To test if Syb2 is involved in Sema3A-induced endocytosis, we measured axonal length and PlexA1 localization in dissociated cortical neurons in the presence or absence of Syb2 upon Sema3A treatment. Sema3A led to a 40% reduction of axonal length of wild-type axons, but had no effect on axonal length in the absence of Syb2 in both Syb2–/– or BoNT/D-treated cortical neurons (Fig. 5, A–C). Furthermore, Sema3A led to both depletion of PlexA1 in growth cones and a 60% increase of its concentration in the cell body in the presence of Syb2, but had no effect in Syb2–/– and BoNT/D-treated cortical neurons (Fig. 5 D–F; and Fig. S3 D). Thus, these data further demonstrate that axons lacking a functional Syb2 are insensitive to Sema3A and suggest that Syb2 is required for the endocytosis, retrograde transport of Nrp1/PlexA1 receptor upon Sema3A activation, and signaling downstream of the Sema3A receptor.

In turn, activation of the Sema3A receptor may be expected to regulate Syb2-dependent secretion. As shown earlier, Sema3A induces the collapse of growth cones, thus reducing cell surface. Decrease in growth cone cell surface complicates proper quantification of the frequency of exocytic events by optical imaging. Therefore, we reconstituted Syb2 and Sema3A receptor in a heterologous system that is not prone to collapse. We imaged Syb2-SEP exocytosis in Cos7 cells in which Nrp1 and PlexA1 were coexpressed to reconstitute Sema3A receptor. We found that control Fc treatment did not affect exocytosis, whereas the number of exocytic events decreased by 50% upon addition of Sema3A (Fig. 5, G and H; and Video 1). As a control, we found no effect of Sema3A on Syb2 exocytosis when we expressed a mutant of Nrp1 lacking its cytosolic domain (Valdembri et al., 2009). This result reveals that Sema3A signaling through Nrp1 and PlexA1 inhibits Syb2 exocytosis.

Collectively, these results suggest that Sema3A signaling requires Syb2-dependent traffic. This is in good agreement with the role of Stx1B in Sema3A repulsion (Kabayama et al., 2011). Our results further agree with the dual role of Syb2 in exocytosis and endocytosis of synaptic vesicles (Déak et al., 2004). Given that Syb2 interacts with clathrin assembly lymphoid myeloid leukemia protein (CALM), a clathrin adaptor (Koo et al., 2011), it is tempting to speculate that Syb2 is required for the endocytosis of the Sema3A receptor in growth cones. Then, as shown here, Syb2 and the Sema3A receptor would be sorted together into endosomal vesicles because of an interaction

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**length quantified in each condition (control: Syb2+/–, n = 47; Syb2–/–, n = 79; Sema3A: Syb2+/–, n = 30; Syb2–/–, n = 59 axons). Bar, 10 µm.**

**Mean values ± SEM (error bars) of axonal length quantified in each condition (control, n = 88; control + BoNT/D, n = 101; 3 µg/ml Sema3A, n = 97; 3 µg/ml Sema3A + BoNT/D, n = 99 axons). ***, P < 0.0005 by Student’s t test.**

**Immunofluorescence of PlexA1 and Tau in dissociated cortical neurons cultured from Syb2+/– or Syb2–/– embryos and treated for 30 min with or without 1 µg/ml Sema3A. Bar, 2 µm.**

**Measurement of PlexA1 in soma of cortical neurons (control: Syb2+/–, n = 129; Syb2–/–, n = 109; Sema3A: Syb2+/–, n = 129; Syb2–/–, n = 162 somas in two embryos per genotype).**

**Mean intensity of PlexA1 in soma of cortical neurons (control, n = 41; control + BoNT/D, n = 50; Sema3A, n = 64; Sema3A + BoNT/D, n = 55 somas).**

**Scheme of Syb2-SEP exocytosis protocol. Cos7 cells were cotransfected with PlexA1-FLAG, Syb2-SEP, and Nrp1-mRFP full length or Nrp1ΔCyto-mRFP, and exocytic events of Syb2-SEP were recorded for 3 min.**

**Quantification of Syb2-SEP exocytic events. To evaluate the effect of treatment, exocytic events in the second recording are expressed as a percentage of the first recording for each cell (Nrp1 full length in control, n = 4; and Sema3A condition, n = 8 cells; Nrp1ΔCyto in Sema3A condition, n = 8 cells).**

**Vertical bar, 10 s; horizontal bar, 10 µm. Error bars indicate SEM.**

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**Sema3A induces a local disequilibrium (endocytosis + exocytosis) in the growth cone, generating a local surface shrinkage, and prevents progression of the growth cone in the direction of the source, favoring turning to the opposite direction. Syb2 interacts with Nrp1 and PlexA1 and is required for their traffic.**
mediated by their transmembrane domains. Sema3A further inhibited Syb2-dependent exocytosis when we reconstituted Sema3A receptor in nonneuronal cells. On this basis, we consider that the model proposing that attraction relies on Syb2 exocytosis, whereas repulsion relies on endocytosis (Tojima et al., 2011), needs to be revised. We instead propose that the molecular mechanism of Sema3A repulsion is based on fine tuning of the opposing processes of Syb2-dependent endocytosis and exocytosis (Fig. 5 I). Sema3A would induce a local disequilibrium at the site facing the Sema3A source by simultaneously triggering endocytosis and inhibiting exocytosis. Such a combination of locally increased endocytosis and decreased exocytosis would prevent progression in the direction of the source, favor turning of the growth cone in the opposite direction, and reorient it to Sema3A (Piper et al., 2005). It is possible that TI-VAMP’s role in Netrin1 attraction (Cotrufo et al., 2011) may also be based on a change of exocytosis/endocytosis equilibrium, further generalizing the principle unraveled here.

Therefore we propose that a main function of Syb2 in developing neurons is to traffic Sema3A receptor, allowing for its endocytosis, traffic away from the Sema3A source, and reexpression at the cell surface by exocytosis. It now remains to be tested whether the primary role of Syb2 resides in endocytosis or exocytosis of the Sema3A receptor, and how Sema3A signaling controls Syb2 exocytosis.

Materials and methods

Antibodies

The following antibodies were used: Nrp1 (Western blotting [WB], 1/1,000 [Millipore]; immunofluorescence [IF], 1/500; [R&D Systems]), PlexA1 [WB, 1/1,000; IF, 1/500; Abcam], tubulin [IF, 1/10,000; Developmental Studies Hybridoma Bank], AP2 [WB, 1/1,100 [Abcam]]; IF, 1/1,100, Thermo Fisher Scientific], L1 [a gift from F. Rathjen, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany; WB, 1/1,100; IF, 1/200], Syb2 (69.1 clone, a gift from R. Jahn, Max Planck Institute, Göttingen, Germany; WB, 1/1,000; IF, 1/1,000), Syntaxin1 (HPC1 clone, a gift from C. Barnstable, Yale University, New Haven, CT; WB, 1/10,000), Syp (7.2 clone, a gift from R. Jahn, Max Planck Institute, Göttingen, Germany; WB, 1/500), control IgG (Sigma-Aldrich), TI-VAMP (158.2, WB, 1/1,000), Tau (Millipore; IF, 1/1,000), EE1A (Applied Biosystems); IF, 1/500), FLAG (Sigma Aldrich), GFP (Roche) and RFP (Millipore). DAPI was from Invitrogen.

Guidance assays

Explant assays. We are grateful to T. Siddiqui (Stanford University, Stanford, CA) for the generous gift of the Syb2 knockout mouse. E13 and E15 embryos were extracted from Syb2 strain or Swiss mice, and DRG and cortical explants were embedded in front of stably transfected Sema3A, Sema3C, or mock-secreting cells, in 3D matrices, as described previously (Yam et al., 2009).

In toto immunofluorescence. DIV2 E15 neurons or Cos7 cell cultures were fixed for 20 min with 4% PFA; quenched for 20 min in PBS 1x and 50 mM NH₄Cl; permeabilized 4 min in PBS 1x and 0.1% Triton X-100; and blocked for 30 min in PBS 1x and 2.5% fish gelatin. Primary antibodies were incubated either overnight at 4°C or for 2 h at room temperature, in PBS 1x and 0.125% fish gelatin. After washes, secondary antibodies were incubated for 45 min at room temperature before mounting in Prolong medium (Applied Biosystems). For surface staining, DIV2 E15 neurons were processed as described previously without the permeabilization step.

In vivo immunofluorescence. DIV18 brains were fixed overnight in 4% PFA, washed in PBS 1x, and embedded in 3% low-melt agarose for vibratome sectioning. Slices were first permeabilized for 3 h in PBS 1x, 1% Triton X-100, and 0.25% fish gelatin before incubation of primary antibodies for 2-3 d in PBS 1x, 0.1% Triton X-100, and 0.125% fish gelatin. Slices were washed three times for 2 h, then secondary antibodies were incubated overnight at 4°C before mounting of slices in Vectashield medium (Vector Laboratories).

In toto immunofluorescence. E12 embryos were fixed overnight in 4% PFA; washed in PBS 1x; dried in methanol series before being whitened 3 h in 80% methanol, 5% DMSO, and 1% H₂O₂ (Huber et al., 2005); and permeabilized for 3 h in PBS 1x, 1% Triton X-100, and 2% fish gelatin before incubation of primary antibodies for 2-3 d at 4°C in PBS 1x, 0.1% Triton X-100, and 0.125% fish gelatin. Slices were washed three times for 2 h, then secondary antibodies were incubated overnight at 4°C before mounting of slices in Vectashield medium (Vector Laboratories).

Transfection

Cos7 cells were transfected with an Amamax system according to the manufacturer’s instructions (Lonza). HEK-293T cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Time-lapse video microscopy

Syb2-SEP time-lapse video imaging was performed in modified Krebs-Ringer-Hepes buffer (140 mM NaCl, 4.7 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM Hepes, and 5.5 mM glucose, pH 7.4) at 37°C with an inverted epifluorescence microscope (DMI6000B, Leica) modified to use laser illumination. The 488-nm argon ion laser beam was sufficiently expanded to get near uniform illumination. Fluorescent excitation and collection were performed with 63x/1.6 NA Plan-Apochromat oil-immersion objective lenses. Images were acquired every 500 ms, with an integration...
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