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A Semaphorin 3A Inhibitor Blocks Axonal Chemorepulsion and Enhances Axon Regeneration

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SUMMARY

Secreted semaphorins are a large group of extracellular proteins involved in a variety of processes during development, including neuronal migration and axon guidance. We screened a peptoid combinatorial library to search for semaphorin 3A inhibitors, and identified a peptoid (SICHI: semaphorin Induced *ch*emorepulsion *i*nhibitor) that blocks semaphorin 3A-chemorepulsion and growth-cone collapse in axons at millimolar concentrations. SICHI inhibits the binding of semaphorin 3A to its receptor complex (neuropilin 1/plexin A1) and semaphorin 3A-induced phosphorylation of GSK3. Chemorepulsion induced by semaphorin 3F or netrin 1 is not blocked by SICHI. We also show that SICHI promotes neural regeneration of damaged axons. We suggest that SICHI, a selective inhibitor of semaphorin 3A, is of therapeutic interest for approaches aimed at promoting axonal regeneration and brain repair.

INTRODUCTION

Class III semaphorins are vertebrate-secreted proteins (Anderson et al., 2003) with crucial roles during the development of the nervous system. Most secreted semaphorins mediate axon-growth inhibition at a distance, thereby promoting axonal chemorepulsion in both the central and peripheral nervous systems (CNS and PNS, respectively) (Kolodkin et al., 1993; Luo et al., 1995; Puschel et al., 1995; Van Vactor and Lorenz, 1999). Class III semaphorins act via a receptor complex formed by neuropilins 1/2 as the ligand binding subunit and A-plexins (Deo et al., 2004) as the signal-transducing subunit (Chen et al., 1997, 2000; Feiner et al., 1997; Giger et al., 1998, 2000; He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Nakamura et al., 1998; Renzi et al., 1999; Takahashi et al., 1998). Genetic studies using mutations for either semaphorin or receptor genes, and other experimental studies, indicate a fundamental role of class III semaphorins in the development of neural connections. Class III semaphorins also participate in tissue patterning, cell migration, tumor biology, and heart formation (Chédotal et al., 2005; Klagsbrun et al., 2002; Kruger et al., 2005; Pasterkamp and Verhaagen, 2001). Neuropilin 1 (Np1), a receptor of semaphorin 3A (Sema3A), is also a coreceptor of VEGFR2, which mediates vascular development (Pan et al., 2007; Soker et al., 1998; Suchting et al., 2006). Thus, neuropilins and semaphorins play a role in angiogenesis, both in normal development and in pathological conditions (Guttmann-Raviv et al., 2006; Suchting et al., 2006). Furthermore, Sema3A, and its receptors Np1 and PlexA1 (as well as other semaphorins), have been implicated in tumor progression and metastasis (Guttmann-Raviv et al., 2006; Staton et al., 2007; Suchting et al., 2006; Torres-Vázquez et al., 2004).

In the adult CNS, severed axons fail to regenerate beyond the lesion site, in contrast to those in the peripheral or embryonic nervous system. The failure of axon regeneration is mainly attributable to the environment encountered by injured axons. Inhibition is associated with myelin proteins, such as Nogo-A and MAG, and molecules in the glial scar at the lesion site, including proteoglycans (Bradbury et al., 2002; Bregman et al., 2002; Fontoura et al., 2004; Mingorance et al., 2005; Schnell and Schwab, 1990). However, recent studies that genetically target these molecules or use blockers have shown only modest regeneration, indicating that other inhibitory signals contribute to the failure of axonal regrowth (Bregman et al., 1995; Fontoura et al., 2004; Schmidt and Strittmatter, 2007). It has also been shown that class III semaphorins, including Sema3A, are expressed in the adult brain and spinal cord, where they are regulated by synaptic activity (Pasterkamp and Verhaagen, 2001), and after lesions (De Winter et al., 2002). Overexpression of Sema3A has been demonstrated after anisomorphic injury of the corticospinal tract (De Winter et al., 2002), and in lesions of the lateral olfactory tract or the entorhino-hippocampal pathway (Pasterkamp et al., 1999). The participation of secreted semaphorins in the glial scar, together with that of other inhibitors of axonal growth, has recently been demonstrated in vitro (Shearer et al., 2003).

We attempted to identify small molecules that block semaphorin/neuropilin functions by screening a mixture-based combinatorial library of over 10,000 *N*-alkylglycines trimers (peptoids) (Humet et al., 2003). We searched for products that could block Sema3A-induced axonal chemorepulsion using a tissue-explant assay. We identified an active peptoid, which we then tested in

several functional situations such as axonal chemorepulsion, growth-cone collapse, or GSK-3 phosphorylation. We conclude that the biological function of the peptoid might be linked to its ability to interfere with the semaphorin/neuropilin interaction. We also show that this peptoid reverses semaphorin-mediated axon inhibition in an axotomy model, thereby promoting axon regeneration.

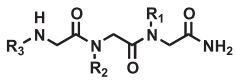
RESULTS

Screening of a Combinatorial Library of Peptoids to Identify Inhibitors of Sema3A

Previous studies have shown that Sema3A acts as a chemorepulsive molecule for embryonic axons in several CNS and PNS regions (Chédotal et al., 1998; Messersmith et al., 1995; Pasterkamp et al., 1998; Shepherd et al., 1996). In the hippocampus, Sema3A causes strong chemorepulsion (Chédotal et al., 1998). In the present study, hippocampal tissue explants cultured over 2–3 days in vitro (DIV) gave rise to radial axons. In contrast, when explants were exposed to aggregates of cells expressing Sema3A, fibers displayed strong chemorepulsion and grew at distal sites in nearly all cases (Figures 1 and 2B). To isolate compounds that might interfere with Sema3A-induced chemorepulsion, we selected a simple, functional in vitro assay. We tested products from a mixture-based combinatorial library composed of trimers of *N*-alkylglycines (peptoids) (see Experimental Procedures) (Humet et al., 2003). This library has two distinctive features: (a) the use of the positional scanning format strategy for its construction; and (b) the selection of diversity includes the use of primary amines bearing an additional tertiary amino moiety; the introduction of these amines affords peptoids containing additional protonable fragments, which could complement the activity pattern and bioavailability of the library contents (Humet et al., 2003). The general structure of the peptoids is shown in Figure 1A. The library is organized into 66 mixtures, each containing 484 molecules, giving rise to a chemical diversity of 10,648 individual compounds. Screening of the 66 mixtures in the above assay identified the preferred position at the three separate sites on the peptoid (R_1, R_2, and R_3) (Figure 1A).

After screening the compounds in the peptoid library using a Sema3A-induced chemorepulsion assay, we observed that 92.4% of the vials were inactive and that only five vial compounds efficiently reversed the Sema3A effects on axonal growth (Figure 1B). Thus, at the R_1 position, the amino substituent identified was 2-(*N*-methyl-2′-pyrrolidinyl)ethyl (denoted as amine 16); at the R_2 position it was 2-(*N*-pyrrolidinyl)ethyl (denoted as amine 6); and at the R_3 position the substituents were amine 16, 2-(*N*-morpholino)ethyl (denoted as amine 18) and 2-(*N*,*N*-diethylamino)ethyl (denoted as amine 22). These results gave an indication of the chemical composition of potentially active molecules. We thus synthesized three individual peptoids containing the different combinations of the above-mentioned amines (i.e., N16-6-16C, N18-6-16C, and N22-6-16C, Figures 1B and 1C) and we then assayed their activity. These experiments in vitro demonstrated that the peptoid N22-6-16C reversed Sema3A-induced chemorepulsion of hippocampal axons most strongly. This compound was purified by preparative reverse-phase high-performance liquid chromatography and referred to as *s*emaphorin-*i*nduced *ch*emorepulsion *i*nhibitor (SICHI) (Figure 1C). Finally, we measured the range of concentrations over which the peptoid is biologically active without reducing cell viability. SICHI was effective at between 0.00001 and 0.0002 μg/μl culture medium (Figure 2). We also determined the IC-50 value (IC_{50} = 0.000039 μg/μl; see Figure S1 available online). Surprisingly, higher peptoid concentrations (up to 0.001 μg/μl) induced weaker effects without apparent cell damage (Figure 2G; see Supplemental Data).

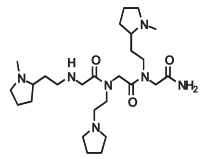
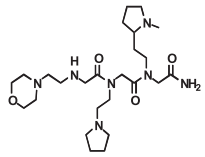
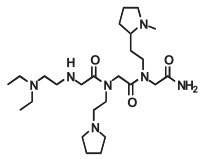
A



B

Sublibraries	Peptoid Mixture	Amines	% Chemorepulsion	Explants
R_3XX	16	16	52.2	23
	18	18	43.5	23
	22	22	46.6	28
$X\ R_2X$	28	6	17.9	28
$XX\ R_1$	60	16	40	20

C

	%Chemorepulsion	Explants	Mixtures
	45.5	22	N16-6-16C
	43.8	22	N18-6-16C
	27.3	22	N22-6-16C

SICHI

Figure 1. Strategy Followed for the Screening of the Library and Identification of SICHI

(A) Structure of the library of peptoids.

(B) Inhibition of the chemorepulsion induced by Sema3A by the library peptoid mixtures.

(C) Structure of the three defined peptoids identified after library deconvolution as the most active inhibitors of the chemorepulsion induced by Sema3A. SICHI is among these peptoids.

Biological Activity of SICHI Is Specific for Sema3A

The findings reported above showed that SICHI blocks axonal chemorepulsion induced by recombinant Sema3A. We examined whether SICHI also blocked chemorepulsion caused by endogenous, tissue-expressed semaphorins. During hippocampal development, Sema3A expression in the entorhinal cortex (EC)

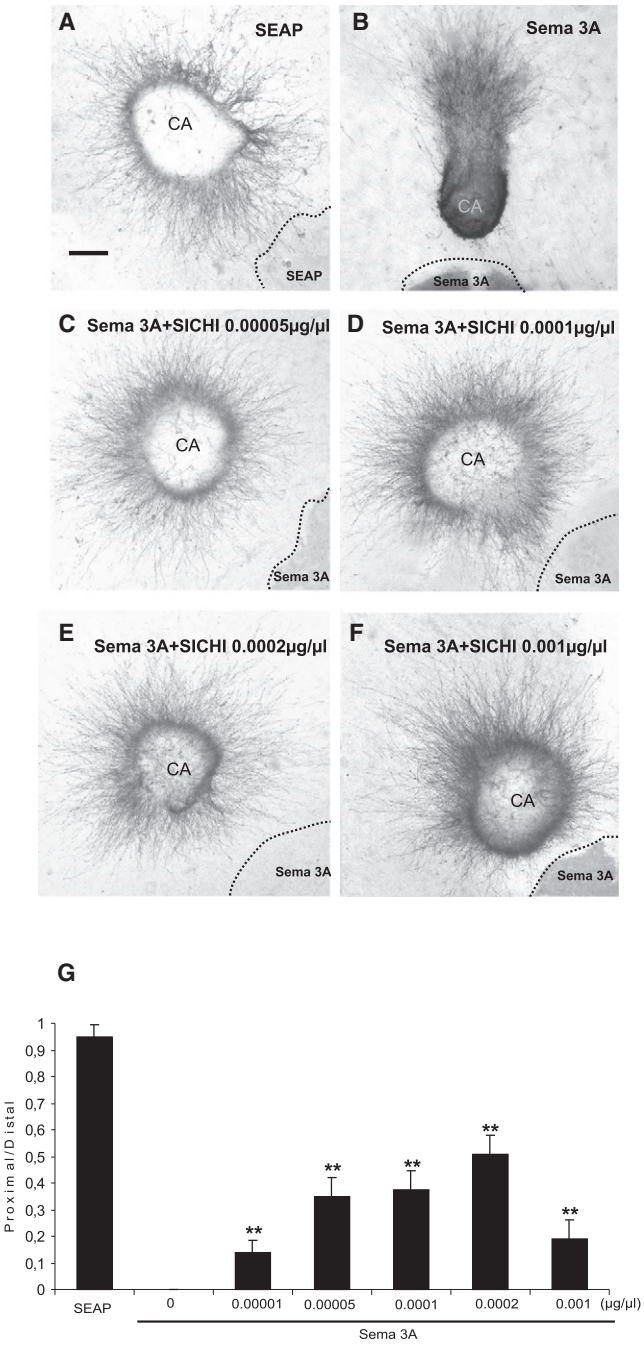


Figure 2. SICHI Inhibits the Chemorepulsion Induced by Sema3A

(A) Hippocampal explants cocultured in collagen gels with control COS cells exhibited redial axonal growth.

(B) Hippocampal axons exhibit strong chemorepulsion when cocultured with aggregates of Sema3A-expressing cells.

(C–F) Sema3A-induced chemorepulsion is blocked when explants are cultured in the presence of SICHI (0.00005–0.0001 µg/µl).

(G) Quantification of repulsion experiments: histograms showing the proximal/distal values obtained, where P is the density of axons on the proximal side, and D represents the density of axons in the distal quadrant. Note that chemorepulsion is impaired in the presence of different concentrations of SICHI. Data represent the means and standard error of the mean (± SEM) of 24 explants from four independent experiments.

inhibits axonal growth, thereby preventing the entrance of these axons to the EC (Chédotal et al., 1998; Pasterkamp et al., 1998; Pozas et al., 2001; Shepherd et al., 1996). We thus cocultured at a distance explants from the entorhinal cortex (EC) and the hippocampus in either the presence or absence of SICHI (0.00001 and 0.0002 µg/µl). After 2–3 DIV the explants were fixed with paraformaldehyde and a small crystal of the lipophilic tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was injected into the hippocampal explants. Fluorescence microscopic examination revealed that EC explants inhibited hippocampal axonal growth in nearly all the cocultures (Figures 3A and 3C). In contrast, incubation with SICHI reversed this effect, thereby yielding a radial pattern of axonal growth (Figures 3B and 3C).

In addition to Sema3A, Sema 3F and netrin 1 are also axonal chemorepellents (Alcántara et al., 2000; Chédotal et al., 1998; Colamarino and Tessier-Lavigne, 1995). We examined whether SICHI was specific to Sema3A or whether it interfered with the inhibition caused by these other chemorepellents. Similar assays in vitro, in which hippocampal explants were exposed to Sema3F-producing cells, showed that SICHI did not block this chemorepulsion (Figures 3D–3F). Cerebellar explants exposed to netrin 1-cell aggregates also showed similar patterns of chemorepulsion both in the presence and in the absence of SICHI (Figures 3G–3I). We thus conclude that SICHI blocks Sema3A-induced chemorepulsion, but not that due to Sema 3F or netrin 1 and that the biological activity of SICHI is associated with the Sema3A signaling pathway.

SICHI Inhibits Sema3A-Induced Growth-Cone Collapse by Blocking GSK-3 Activation

An early step in axonal growth inhibition is growth-cone collapse (Gallo and Letourneau, 2004). Under normal conditions, axonal growth cones show large, triangular axonal endings bearing numerous filopodia and lamellipodia (Figure S2A). In contrast, most growth cones incubated with Sema3A for 30–45 min showed round-tipped or "pencil-like" shapes. They also lacked filopodia and lamellipodia, which is characteristic of axonal growth-cone collapse (Figure S2b, see also Goshima et al., 1995; He and Tessier-Lavigne, 1997; Jin and Strittmatter, 1997). Sema3A-induced axonal growth-cone collapse was also blocked by incubation with SICHI (0.0001–0.0002 µg/µl) (Figures S2c–S2e). These results indicate that SICHI inhibits Sema3A-induced chemorepulsion by blocking growth-cone collapse.

Sema3A-induced growth-cone collapse relies on a complex signaling cascade that requires local activation of the kinase GSK-3 in growth cones. The activity of GSK-3 depends on serine phosphorylation, and Sema3A activates GSK-3 by decreasing its phosphorylation (Bhat et al., 2000; Eickholt et al., 2002; Hughes et al., 1993). To determine whether SICHI altered GSK-3 phosphorylation, cultures were treated with Sema3A in the presence of SICHI. Treatment with SICHI alone or SEAP did not affect GSK-3 serine phosphorylation (Figures 4A and 4E). In contrast to controls, Sema3A induced a rapid decrease in the local pool of P-Ser-GSK-3 (Figure 4B). However, in

**p < 0.01 (Student's test). The p values ranged from 0.0035 to 0.000017. Scale bars in A–F represent 50 µm.

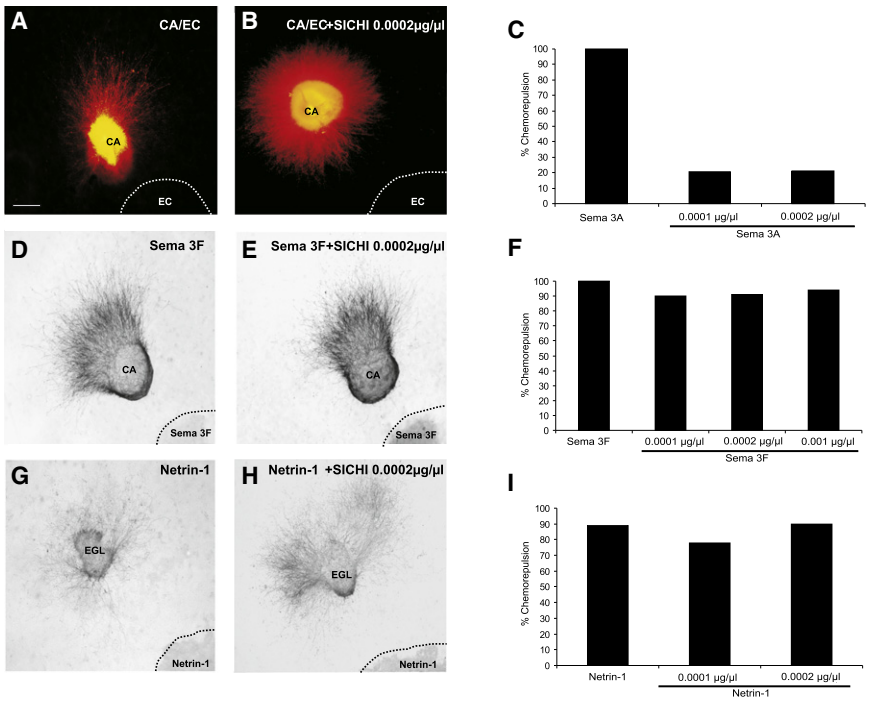


Figure 3. SICHI Specifically Inhibits Chemorepulsion Induced by Sema3A

(A–C) In control assays, hippocampal CA explants cocultured with entorhinal cortical explants showed axonal repulsion (A). In the presence of SICHI (0.0002 µg/µl), hippocampal CA axons grew radially (B). Histogram showing the percentage of hippocampal explants showing chemorepulsion in control cocultures and after treatment with SICHI (C).

(D–F) Hippocampal explants have strong chemorepulsion after incubation with Sema3F-expressing cells alone (D) or in combination with SICHI (E). Histogram showing that Sema3F-induced chemorepulsion does not decrease after SICHI treatment (F).

(G–I) Cocultures of postnatal EGL explants and netrin-1-expressing cells show axonal repulsion in both control assays (G) and after incubation with SICHI (H). The histogram summarizes the percentages of chemorepulsion (I).

Scale bar = 50 µm (A, B, D, E, G, H).

cultures treated with SICHI, no such loss of P-Ser-GSK-3 was induced by Sema3A (Figures 4C–4E). These results correlate with the decreased number of collapsed growth cones observed after SICHI incubation (Figure S2). We thus conclude that SICHI interferes with the Sema3A-induced signaling cascade at the level of growth cones.

SICHI Interferes with Sema3A Binding to Neuropilin 1/Plexin A1 Receptor Complex

The Np1/PlexA1 receptor complex mediates Sema3A binding and axonal chemorepulsion (De Winter et al., 2002; Rohm et al., 2000; Takahashi et al., 1999). To examine whether the peptoid interferes with the binding of Sema3A to its receptors, we transfected COS1 cells with cDNA vectors encoding Np1 and PlexA1 and mapped Sema3A-AP binding by measuring alkaline phosphatase activity (Rohm et al., 2000). In agreement with previous studies (Rohm et al., 2000), the Np1/PlexA1 receptor complex had higher affinity for Sema3A than for Np1 alone (Figures 5A–5C), whereas PlexA1 alone showed no affinity for Sema3A (Takahashi et al., 1999). Incubation with SICHI dramatically decreased the binding of Sema3A-AP to both Np1/PlexA1 complexes and to Np1 alone (Figures 5D–5H). We conclude that SICHI interferes with the binding of Sema3A to its endogenous receptors.

SICHI Promotes Axonal Regeneration of Lesioned Cortical Axons in Organotypic Cultures

The above results show that SICHI interferes with the effects of Sema3A on developing axons. However, class III semaphorins, like other axonal inhibitors, can inhibit regeneration in adult nervous tissue (De Winter et al., 2002; Luo et al., 1993; Pasterkamp et al., 1999; Shearer et al., 2003). We thus examined whether SICHI promotes axonal regeneration. We used an in vitro axotomy mode (cocultures with entorhino-hippocampal organotypic slices) in which the cytoarchitecture of the nervous tissue is preserved and the entorhino-hippocampal pathway develops as it does in vivo (Del Rio et al., 1997). In this system, after resection of the EHP at 15 DIV, axons do not regenerate spontaneously (Del Rio et al., 2002; Mingorance et al., 2005). This is therefore a valid way to test potential promoters of neural regeneration in vitro. First, we examined whether Sema3A or Np1 expression was altered after axotomy. In situ hybridization experiments showed that both Sema3A and Np1 transcripts were upregulated soon after axotomy in the target hippocampus and in the EC (Figure S3). Although expression of Sema3A remained high 7–10 days after axotomy, expression of Np1 mRNAs tended to return to control levels in the EC (Figures S3B, S3C, S3E, and S3F). Increased expression of Sema3A protein was corroborated by western blot after axotomy (Figures S3G and S3H).

These findings indicate that up-regulation of the Sema3A signaling system is involved in the failure of the lesioned EHP to regenerate. To assess this possibility, we used organotypic slice cultures from a *Sema3A* knock-out mouse strain (Behar et al., 1996). After 15 DIV, the EHP was resectioned as above and slices were cultured for a further 15 days, after which the EHP was traced by injections with the anterograde tracer biocytin (Figure 6A). In agreement with previous studies (Del Rio et al., 1997), very few axons regrew in control *wild-type* slices (Figures 6B, 6C, and 6F). The number of regenerating axons increased in cocultures from *Sema3A*-deficient slices (Figures 6D–6F).

These findings show that genetic ablation of the *Sema3A* gene promotes axonal regeneration, thereby validating the Sema3A signaling pathway as a potential target for axonal repair. We then postulated that interfering with Sema3A function by using SICHI might allow regeneration of axotomized axons. Entorhino-hippocampal cultures were established and axotomized after 15 DIV as above. Slices were cultured for a further 15 days, in which SICHI was added daily (see Del Rio et al., 2002) and the

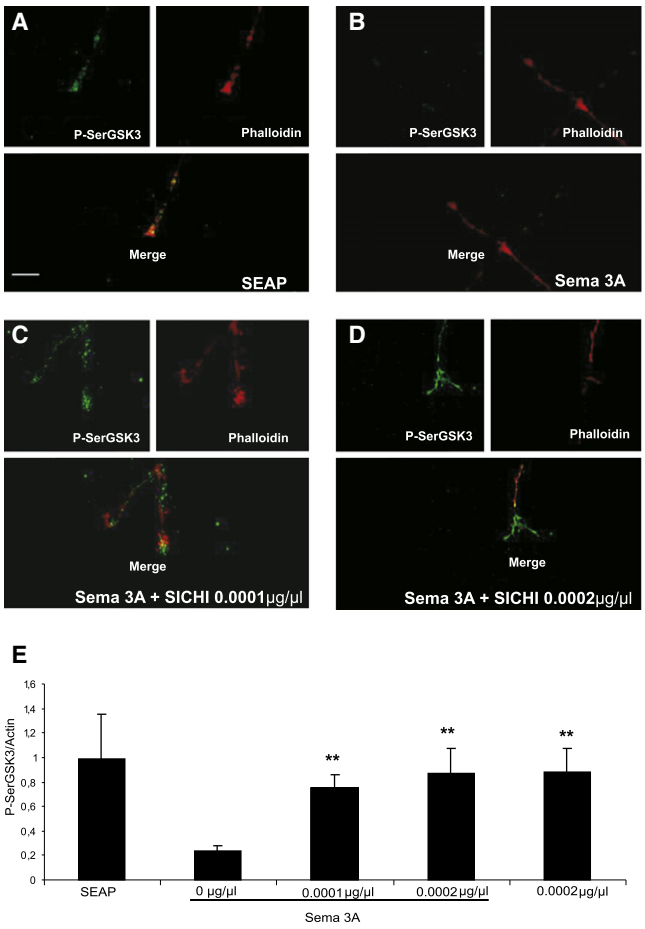


Figure 4. Sema3A-Induced Changes in P-SerGSK3 Expression in Growth Cones Are Blocked by SICHI

(A–D) P-SerGSK3 expression levels in growth cones incubated with control medium (SEAP) (A), with Sema3A (B), with Sema3A and SICHI (0.0001 µg/µl and 0.0002 µg/µl) (C and D). Incubation with SICHI blocks Sema3A-induced loss of P-SerGSK3.

(E) Histogram showing quantification of P-SerGSK3 staining levels versus rhodamine-phalloidin staining (labeled actin) in growth cones incubated under different conditions. (Mean ± SEM, **$p < 0.01$; Student's t test). The p values ranged from 0.0072 to 0.0084.

Scale bars (A–D) = 10 µm.

EHP was traced with biocytin (Figure 7). The results showed that SICHI incubation at concentrations of 0.001–0.0002 µg/µl increased 10-fold the number of regenerating axons growing into the denervated hippocampus (Figures 7C and 7D). Interestingly, many regenerating axons targeted the correct termination layer, the stratum lacunosum-moleculare, and terminated in growth-cone endings. As for embryonic explants, organotypic slices appeared healthy after peptoid incubation (Figure 7). The regeneration of axons following SICHI treatment was similar to that observed in slices lacking Sema3A expression (compare Figure 6 with Figure 7). Taken together, our data indicate that Sema3A signaling is a valuable target for the promotion of axonal regeneration. The peptoid SICHI might be used to interfere with the biological actions of Sema3A in both developing and mature nervous tissue.

DISCUSSION

Sema3A is a strong axonal chemorepellent that induces growth cone collapse and might inhibit CNS regeneration (Fan and Raper, 1995; Luo et al., 1993; Messersmith et al., 1995; Pasterkamp et al., 1999; Shearer et al., 2003; De Winter et al., 2002). Several strategies have been followed to reverse semaphorin functions. These include pharmacological interference with intracellular semaphorin signaling, and extrinsic attempts to block the semaphorin/neuropilin interaction with soluble ectodomains of Np1-2 receptors (Goshima et al., 1999) or with a complementary peptide (Williams et al., 2005), and the blockage of the Np1-2 receptor with antibodies (Shearer et al., 2003). Moreover, Hanbali et al. (2004) report a synthetic neurotrophic compound that promotes cortical axon outgrowth in vitro in the presence of Sema3A. However, neurotrophic factors supplied in the culture medium might also promote axonal growth, and it is difficult to discern whether these effects were due to the increase in axonal outgrowth or to the inhibition of chemorepulsion. A compound isolated from the fermentation broth of a fungal strain, *Penicillum sp*, reverses Sema3A-induced growth cone collapse of dorsal root ganglion (DRG) neurons in vitro by reducing receptor binding. Furthermore, this compound also accelerates nerve regeneration in vivo in a rat model of olfactory nerve axotomy (Kikuchi et al., 2003) and promotes regeneration of lesioned cortico-spinal axons in the spinal cord (Kaneko et al., 2006).

Here, we have developed a new strategy to isolate Sema3A blockers by screening a peptoid combinatorial library. Peptoids are a family of synthetic molecules with a wide variety of biological activities, which renders them attractive for pharmaceutical innovation (Simon et al., 1992; Zuckermann et al., 1992). The various activities associated with *N*-trialkylglycines, together with their proteolytic resistance and low-to-moderate toxicity make them good candidates for hit identification. Peptoids have been successfully used to identify high-affinity ligands for membrane receptors of disruptors of macromolecular complexes (Heizmann et al., 1999) and more recently, for protein-protein interactions (Malet et al., 2006). Furthermore, *N*-trialkylglycines have been used to identify anti-inflammatory, analgesic, and neuroprotectant compounds with activity in vivo (García-Martínez et al., 2002; Montoliu et al., 2002; Planells-Cases et al., 2002), and the structural simplicity of *N*-trialkylglycines renders them labile to structural manipulation and, therefore, lead-like property optimization.

A synthetic neurotrophic compound (tCFA15) counteracts the inhibitory action of Sema3A and other myelin-associated inhibitors (Hanbali et al., 2004). However, the reversion of Sema3A effects induced by this compound was moderate, and its action appears to be attributable to an overall neurotrophic potentiation of axonal growth capabilities, rather than to the specific blockade of Sema3A signaling. Another study has identified peptides, mimicking the MAM Neuropilin domain and Sema3A binding domain, that reduce growth-cone collapse at high concentrations (Williams et al., 2005). However, as they are susceptible to protease activity and the difficulties encountered for introducing the adequate chemical modifications for converting a peptide into a pharmacologically friendly molecule, it seems difficult that these peptides could be used as therapeutic

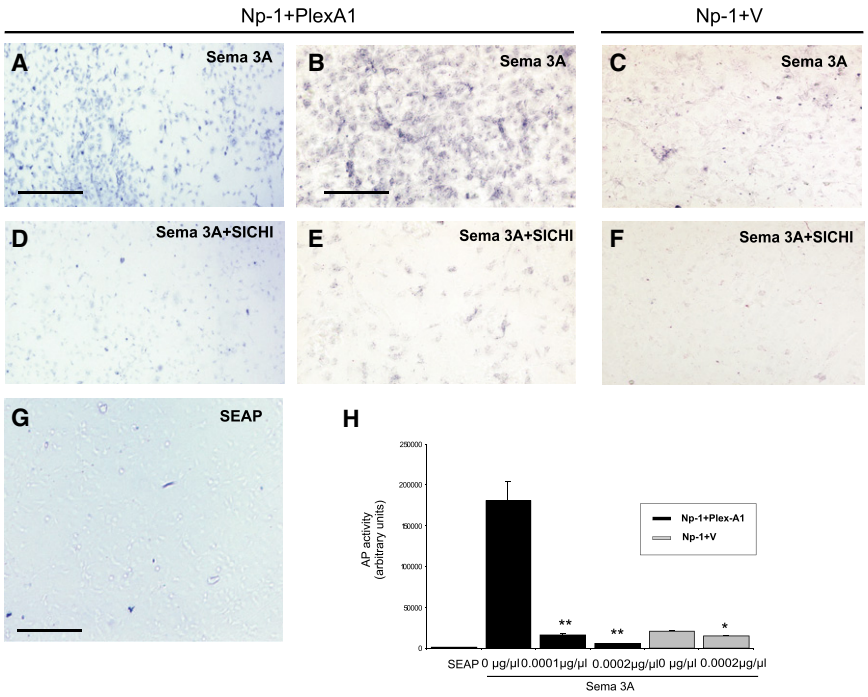


Figure 5. SICHI Blocks Sema3A-AP Binding to Np1/PlexA1 Receptor Complexes

Binding of Sema3A-AP to Np1/PlexA1 receptor complexes expressed in COS cells; binding of proteins was visualized using alkaline phosphatase histochemistry. Treatments with Sema3A. The strong binding of Sema3A to Np1/PlexA1 receptor complexes (A and B) decreased dramatically after incubation with SICHI (D and E).

(C and F) The low binding of Sema3A-AP with Np1 is not altered by incubation with SICHI.

(G) Control binding assay with SEAP on Np1/PlexA1 expressing cells showing no histochemical signals.

(H) Histogram representing Sema3A-AP-bound under different conditions, expressed in arbitrary units. (Mean ± SEM, *$p < 0.05$, **$p < 0.01$; Student's t test). The p values ranged from 0.020 to 1×10^{-9}.

Scale bars represent 70 μm in A, C, D, F, G, and 35 μm in B and E.

tools. Like SICHI, SM-2162689 (xanthofulvin) appears to be fairly specific for Sema3A signaling: it inhibits binding of Sema3A to its receptors and promotes regeneration of axotomized neurons (Kikuchi et al., 2003; Kaneko et al., 2006). However, from the chemical point of view, xanthofulvin has a more complex structure than SICHI. This means that its availability would be limited by microorganism production and further isolation and purification. Moreover, the synthetic complexity of this molecule indicates that an optimization program would be difficult. Conversely, trimers of *N*-alkylglycine, such as SICHI, can be synthesized in a matter of hours and, interestingly for a hit compound, they offer a broad variety of structural optimization strategies (e.g., Mondragón et al., 2008). For these reasons we believe that SICHI not only constitutes an advantageous pharmacological tool for studies on semaphorin inhibition, but also opens the field for the future development of improved molecules.

The present study shows that SICHI consistently inhibits Sema3A biological functions. Thus, the peptoid not only blocks axonal chemorepulsion after exposure to recombinant Sema3A, but also inhibits endogenous chemorepulsion (e.g., from the entorhinal cortex; see Figures 3A–3C). Furthermore, SICHI blocks Sema3A-induced growth cone collapse. Finally, chemorepulsion induced by Sema 3F or netrin 1 was not affected by SICHI, thereby reinforcing the specificity of the peptoid.

Our findings show that SICHI strongly decreases the binding of recombinant Sema3A to the Np1/PlexA1 receptor complex (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997), suggesting that SICHI interacts with these receptors. The present data showing that SICHI blocks Sema3A-induced GSK3 de-phosphorylation also support the idea that SICHI interferes with the Sema3A signaling cascade (Eickholt et al., 2002; Zhou et al., 2004). In this respect, the structure of SICHI is worth mentioning. As shown in Figure 1, all three residues found on the peptoid backbone contain an additional tertiary amino moiety. In physiological conditions, the molecule would be expected to be extensively protonated, which would suggest a role of positive charges in the inhibitory activity. This feature could be used in future efforts to optimize the structure of SICHI. Our results showed that SICHI inhibits Sema3A chemorepulsion in a dose-dependent manner. We found that low (0.00001 μg/μl) or high (0.01 μg/μl) doses did not affect responses to Sema3A. This indicates that SICHI might be nontoxic, and thus has potential therapeutic uses.

Axonal regeneration after injury to the CNS requires not only the survival of injured neurons but also the functional re-establishment of synaptic connections. Axons in the adult mammalian CNS exhibit poor regeneration after injury. The inhibitory activity is mainly associated with components of CNS myelin and molecules in the glial scar at the lesion site (He and Koprivica, 2004; Schwab and Bartholdi, 1996; Silver and Miller, 2004). Recent studies have shown that three myelin proteins (myelin-associated glycoprotein, Nogo-A, and oligodendrocyte myelin glycoprotein) account for most of the inhibitory activity of CNS myelin (Schwab and Bartholdi, 1996; Silver and Miller, 2004). The inhibitory activity of these proteins might be mediated by common receptor complexes that consist of the ligand-binding Nogo-66 receptor and its coreceptors p75/TROY and Lingo-1 (Wang et al., 2002a, 2002b; Shao et al., 2005). However, recent studies that genetically target these molecules have shown only modest regeneration, suggesting that other inhibitory signals contribute to the failure of axonal regrowth (Dimou et al., 2006; Fry et al., 2007; Schnell and Schwab, 1990). The present study, which shows that axotomy upregulates Sema3A and Np1 expression in axotomized tissues, is consistent with others (De Winter et al., 2002; Pasterkamp et al., 1998; Shearer et al., 2003), and implicates semaphorins in the failure of the adult CNS to regenerate. Indeed, our data demonstrate that either genetic ablation of the Sema3A gene or functional blockade of Sema3A/Np1 by incubation with the specific peptoid SICHI dramatically enhances axonal regeneration in an in vitro organotypic model in vitro. Furthermore, SICHI promoted

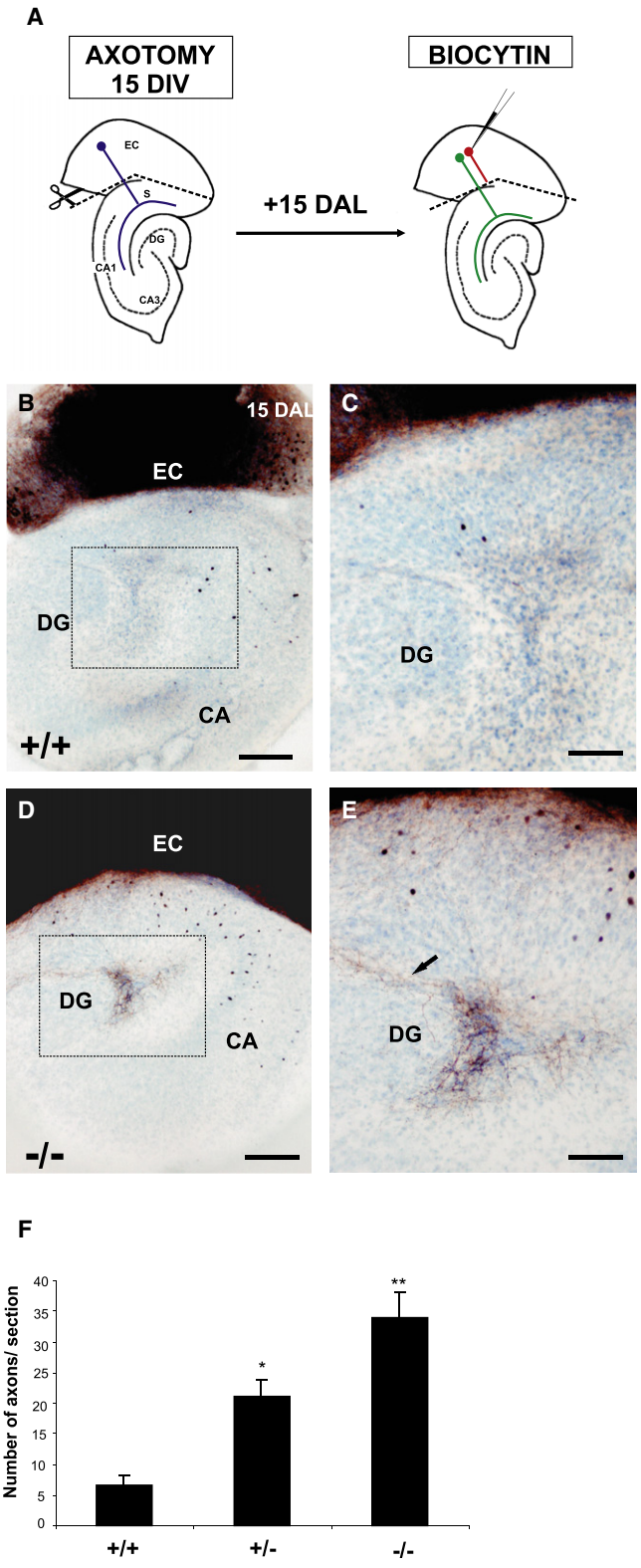


Figure 6. Increased Regrowth of Entorhino-Hippocampal Fibers in Organotypic Cultures from Sema3A Mutant Newborn Mice

(A) Schematic diagram illustrating the in vitro axotomy model used. The EHP was axotomized at 15 DIV and traced with biocytin after an additional 15 day period.

(B and C) Organotypic cultures from Sema3A wild-type slices showing that entorhinal axons do not regenerate after axotomy.

(D and E) Organotypic cultures from Sema3A mutant slices showing that substantial entorhinal axons reinnervate specifically the stratum lacunosum-moleculare (SLM) and molecular layer (ML), after in vitro axotomy. Panels C and E are high magnifications of panels B and D.

(F) Histogram showing densities of regenerating axons under different conditions. *$p < 0.017$, **$p < 0.007$; Student's t test.

Scale bar represents 300 μm in A and C, and 60 μm in B and D.

axonal regeneration in a target-specific way, so the majority of regenerating entorhinal axons correctly targeted the stratum lacunosum-moleculare. Together, our findings indicate that SICHI can not only block Sema3A in developing neurons, but also promote axonal regeneration in the CNS.

SIGNIFICANCE

In the adult CNS, severed axons fail to regenerate beyond the lesion site, in contrast to those in the peripheral or embryonic nervous system. The failure of axon regeneration is attributable to the environment encountered by injured axons, including semaphorins. Sema3A is a strong axonal chemorepellent that induces growth cone collapse and might inhibit CNS regeneration. By using a combinatorial screening strategy, we identified a stable *N*-alkylglycine peptoid (SICHI) that specifically blocks Sema3A biological functions, including chemorepulsion, in both the developing and the adult brain. Moreover, the biological activity of SICHI is specific for Sema3A, because this compound does not affect Sema 3F or netrin 1 chemorepulsion. Moreover, SICHI application enhanced the regeneration of lesioned axons in slice cultures, indicating that Sema3A signaling is a valuable target for the promotion of axonal regeneration. Given the crucial involvement of Sema3A in CNS regeneration (Kaneko et al., 2006) and other human pathologies (Ieda et al., 2007), we suggest that SICHI offers great potential for chemical optimization (Mondragón et al., 2008). Thus, the development of conformationally more restricted derivatives could lead to therapeutic approaches for diseases related to semaphorin function and axonal regeneration (Koprivica et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

OF1 embryos (E14–E15) and postnatal (P0–P6) mice (Criffa-Credo, Lyon, France) were used. The mating day was considered embryonic day 0 (E0) and the day of birth postnatal day 0 (P0). Mice were maintained and killed in accordance with accepted animal care and use protocols. All procedures involving animals and their care were approved by the Ethics Committee of the University of Barcelona and were conducted according to institutional guidelines that are in compliance with regional (Generalitat de Catalunya decree 214/1997, DOGC 2450) and international (*Guide for the Care and Use of Laboratory Animals*, National Institutes of Health, 85-23, 1985) laws and policies.

Peptoid Library Screening

A library of peptoids containing 10,648 compounds was synthesized by using the positional scanning format in solid phase (Humet et al., 2003). The library was organized into 66 controlled mixtures and divided into three sublibraries (OXX, XOX, and XXO, where O represents a defined diversity position and X a pre-equilibrated mixture of all 22 commercially available amines used to

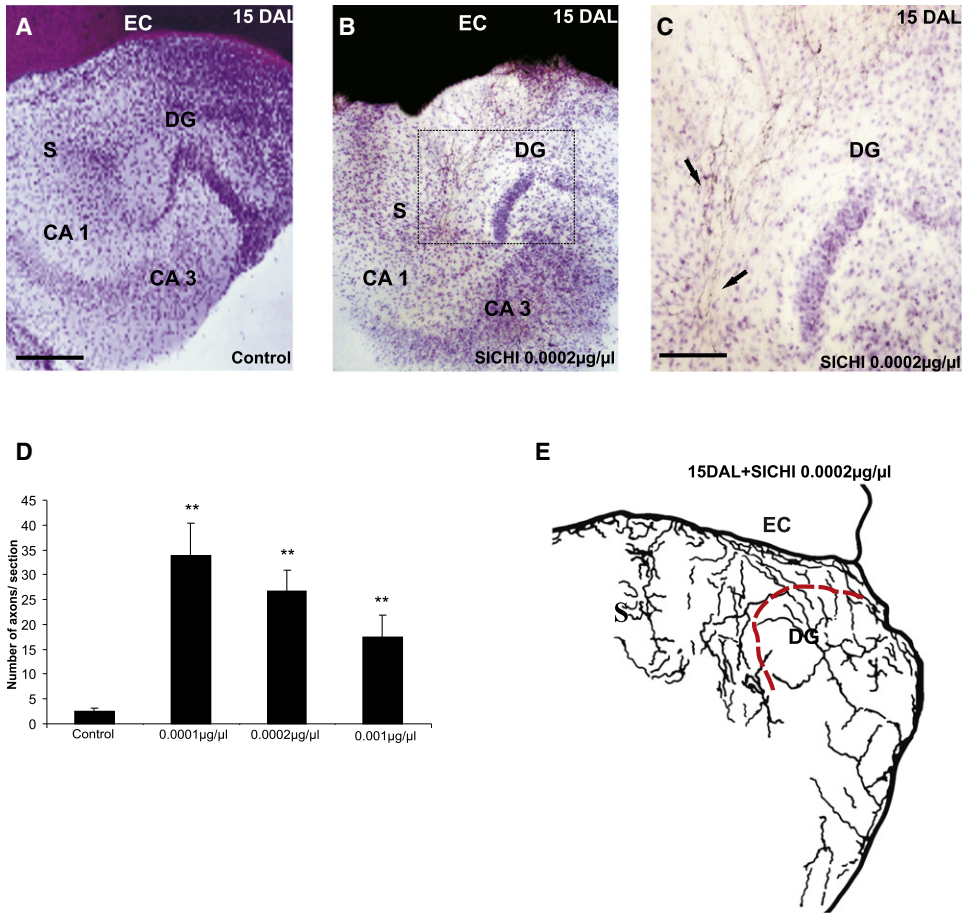


Figure 7. SICHI Promotes the Regeneration of the Perforant Pathway after Axotomy

(A–C) The entorhino-hippocampal pathway was axotomized as above, and the EHP was traced with biocytin 15 DIV after axotomy. In control conditions very few axons regenerate (A). Incubation with SICHI dramatically increases the number of regenerating axons (B). High magnification of the boxed area shown in C, showing robust regeneration of axotomized entorhinal axons.

(C and D) Histogram showing the densities of regenerating axons in different conditions (mean ± SEM). **$p < 0.01$; Student's t test. The p values ranged from 0.00067 to 0.000022.

(E) Camera lucida drawing illustrating regenerating axons in a SICHI-treated axotomized culture. The red dashed line indicates the hippocampal fissure. Areas CA1 and CA3 are shown; DG, dentate gyrus; S, subiculum; EC, entorhinal cortex.

Scale bars represent 300 µm in A, 60 µm in B, and 30 µm in C.

introduce the desired chemical diversity). Briefly, starting from Rink amide resin (Rapp Polymer 0.7 mEq) the eight-step synthetic pathway involved the initial release of the Fmoc-protecting group. Then successive steps of acylation with chloroacetyl chloride followed by the corresponding amination of the chloromethyl intermediate using the particular primary amine, or the equimolecular mixture of the 22 amines, were performed, as appropriate. All these reactions were carried out in duplicate. Finally, the products were released from the resin by a trifluoroacetic acid/dichloromethane/water mixture, solvents were evaporated, and the residues were lyophilized and dissolved in 10% dimethyl sulfoxide at a concentration of 10 mg/ml for screening. We used 1 mg/ml for each assay. The library was screened by assays on cultures in collagen gel to identify active compounds.

Explant Cultures and Cocultures

Embryonic brains (E14–E16) were dissected out and sectioned at 250–350 µm with a tissue chopper (Mickle Laboratory, UK). Selected slices were further dissected with fine tungsten needles to isolate CA1 and CA3 areas of the hippocampus proper, and the entorhinal cortex. Explants of CA1 and CA3 were confronted at a distance (200–600 µm) with COS1 cell aggregates transfected with Sema3A-AP, sema3F-Ap, or netrin 1 cDNA containing vectors (PsecTag1) (Chédotal et al., 1998). Semaphorin expression in transfected cells was tested by western blot. Explants and cell aggregates were embedded in rat-tail collagen gel, and cultured in neurobasal medium supplemented with L-glutamine, $NaHCO_3$, D-glucose, and B27 supplement (GIBCOLife Technologies, Merelknge) for 72 hr in a 5% CO_2, 95% humidly incubator at 37°C.

We then tested the 66 mixtures in the peptoid library. The final dimethyl sulfoxide (DMSO) concentration was below 1% and DMSO controls showed no effect on explant cultures. Subsequent library deconvolution identified three molecules that were individually synthesized and tested. Purified SICHI was selected for further studies as the best inhibitor of these compounds. The structure of SICHI was confirmed by analytical (high-resolution mass spectrometry) and spectroscopic (^1H and ^{13}C nuclear magnetic resonance) methods, and details of the chemistry involved in the synthesis of this compound will be published elsewhere. We added SICHI (10 ng/µl) to explant cultures and cocultures.

Explants were fixed in 4% paraformaldehyde for 1 hr. Cocultures of the entorhinal cortex and hippocampus were injected with a small crystal of lipophilic tracer DiI (Molecular Probes) in CA1 and CA3. After 4–6 days in the dark, the explants were examined under rhodamine fluorescent optics. In addition, cocultures with aggregates of transfected COS cells were immunostained

with monoclonal anti-β-tubulin III antibodies 2. A total of 1980 explants were cocultured.

Collapse Assays

Glass coverslips (10 mm Ø) were coated with laminin (5 μg/ml, 1 hr) and poly-D-lysine (10 μg/ml, 2 hr). After washing, CA1-3 explants were placed in the same medium as above. After 2–3 DIV, their growth cones were visible. Explants were incubated with 20% conditioned medium containing recombinant Sema3A or control media, and including SICHI at the concentration shown above, for 30–45 min. Cultures were then fixed in 4% paraformaldehyde, stained with phalloidin-rhodamine (Sigma, Poole, Dorset, UK), and observed by confocal microscopy (Olympus). For quantification, a total of 50 growth cones were counted for each explant (a total of 216 explants were used).

Binding Assay

We used AP-Sema3A as a ligand. This AP-semaphorin fusion protein was harvested from the conditioned media of transiently transfected COS cells. Conditioned medium was concentrated using Ultrafree-15 30KD (Millipore). COS cells were transfected with Np1 and PlexA1 in poly-L-lysine-coated 24-well plates. Cells transfected with the expression vector pBK-CMV were used to measure nonspecific binding. We added SICHI with the ligand 48 hr before transfection, except for control, at 37°C. Cells were immediately fixed in methanol at −80°C for 5 min. Endogenous phosphatase was heat-inactivated at 65°C for 45 min. Bound alkaline phosphatase was detected by precipitation of insoluble reaction product after incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) 5 (18 μg/ml) and nitroblue tetrazolium (NBT) 6 (34 μg/ml) Sigma 7. The intensity of the reaction product was determined for 92 cells from digital images using an image analysis program.

Immunocytochemical Methods

Previous studies have identified a requirement for GSK3 activity in the Sema3A signal transduction pathway. We used the collapse assay as described above and explants were then fixed for immunocytochemical procedures. Mouse monoclonal antibody against P- (Ser 21) GSK3 (Upstate Biotechnology) (1:300) was incubated overnight at 4°C. Bound antibody was visualized using fluorescein goat-antimouse Alexa fluor (1:300, Molecular Probes) for 2 hr at room temperature, and phalloidin. A total of 2500 growth cones were analyzed.

In Situ Hybridization

In situ hybridization was performed on free-floating sections, as described elsewhere (Alcántara et al., 1998). Sections were permeabilized in 0.2% Triton X-100 (15 min), treated with 2% H_2O_2 (15 min), deproteinized with 0.2 N HCl (10 min), fixed in 4% PFA (10 min), and blocked in 0.2% glycine (5 min). Thereafter, sections were prehybridized at 60°C for 3 hr in a solution containing 50% formamide, 10% dextran sulfate, 5X Denhardt's solution, 0.62 M NaCl, 10 mM EDTA, 20 mM Pipes (pH 6.8), 50 mM DTT, 250 μg/ml yeast t-RNA, and 250 μg/ml denatured salmon sperm DNA. *Sema3A* (Messersmith et al., 1995) and *Np1* (He and Tessier-Lavigne, 1997) riboprobes were labeled with digoxigenin-d-UTP (Boehringer-Mannheim) by in vitro transcription. Antisense Sema3A and Np1 riboprobes were transcribed using T3 polymerase (Ambion) and the corresponding sense riboprobes were obtained using T7 polymerase (Ambion). Labeled antisense cRNA was added to the prehybridization solution (250–500 ng/ml) and hybridization was carried out at 60°C overnight. Sections were then washed in 2XSSC (30 min, room temperature), digested with 20 mg/ml RNase A (37°C, 1 hr), washed in 0.5XSSC/50% formamide (4 hr, 55°C) and in 0.1XSSC/0.1% sarcosyl (1 hr, 60°C). After rinsing in Tris-buffered saline/0.1% Tween 20 (15 min), sections were blocked in 10% normal goat serum (2 hr) and incubated overnight with an alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim, 1:2000). After washing, sections were developed with NBT and BCIP (Life Technologies), mounted on gelatinized slides, and coverslipped with Mowiol™.

Control hybridizations, including hybridization with sense digoxigenin-labeled riboprobes or RNase A digestion before the hybridization, prevented alkaline phosphatase staining above background levels.

Organotypic Slice Cultures and Regeneration Assays

OF1 mice and mutant mice, generated by replacement of the first coding exon of the Sema3A gene with a neo cassette (Behar et al., 1996), were used. Entorhino-hippocampal organotypic slice cocultures were prepared as described by Stoppini et al. (1991). Two weeks after explantation, the entorhino-hippocampal connections were axotomized by cutting the cocultures from the rhinal fissure to the ventricular side along the entire entorhino-hippocampal interface with a tungsten knife (Del Rio et al., 2002). SICHI was administered thereafter every 48 hr for 2 weeks after the lesion (0.001, 0.002, 0.01 μg/μl), in 38 lesioned cultures. After 15 of treatment, the regeneration of the entorhino-hippocampal connection was accessed/assessed by injecting a small crystal of Biocytin (Sigma) into the entorhinal cortex. Biocytin-labeled cultures were fixed with paraformaldehyde and processed as described elsewhere (Del Rio et al., 1996, 1997). For quantification, the number of biocytin-labeled fibers that crossed a 400 μm segment located at a distance of 75–80 μm from the lesion in the hippocampus, parallel to the lesion interphase, was counted for consecutive sections from each culture (40× oil immersion objective).

Statistics

Data are expressed as mean and standard error of the mean. Statistical significance was evaluated using the Student's t test.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Discussion, Supplemental Experimental Procedures, and three figures and can be found with this article online at http://www.cell.com/chemistry-biology/S1074-5521(09)00173-2.

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REFERENCES

Alcántara, S., Ruiz, M., D'Arcangelo, G., Ezan, F., de Lecea, L., Curran, T., Sotelo, C., and Soriano, E. (1998). Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse. J. Neurosci. *18*, 7779–7799.

Alcántara, S., Ruiz, M., De Castro, F., Soriano, E., and Sotelo, C. (2000). Netrin 1 acts as an attractive or as a repulsive cue for distinct migrating neurons during the development of the cerebellar system. Development *127*, 1359–1372.

Anderson, C.N., Ohta, K., Quick, M.M., Fleming, A., Keynes, R., and Tannahill, D. (2003). Molecular analysis of axon repulsion by the notochord. Development *130*, 1123–1133.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., and Fishman, M.C. (1996). Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature *383*, 525–528.

Bhat, R.V., Shanley, J., Correll, M.P., Fieles, W.E., Keith, R.A., Scott, C.W., and Lee, C.M. (2000). Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. Proc. Natl. Acad. Sci. USA *97*, 11074–11079.

Bradbury, E.J., Moon, L.D., Popat, R.J., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J.W., and McMahon, S.B. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature *416*, 636–640.

Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D., and Schwab, M.E. (1995). Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature *378*, 498–501.

Chédotal, A., Del Rio, J.A., Ruiz, M., He, Z., Borrell, V., de Castro, F., Ezan, F., Goodman, C.S., Tessier-Lavigne, M., Sotelo, C., and Soriano, E. (1998). Semaphorins III and IV repel hippocampal axons via two distinct receptors. Development *125*, 4313–4323.

Chédotal, A., Kerjan, G., and Moreau-Fauvarque, C. (2005). The brain within the tumor: new roles for axon guidance molecules in cancers. Cell Death Differ. *12*, 1044–1056.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547–559.

Chen, H., Bagri, A., Zupicich, J.A., Zou, Y., Stoeckli, E., Pleasure, S.J., Lowenstein, D.H., Skames, W.C., Chedotal, A., and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron *25*, 43–56.

Colamarino, S.A., and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell *81*, 621–629.

Del Rio, J.A., Heimrich, B., Super, H., Borrell, V., Frotscher, M., and Soriano, E. (1996). Differential survival of Cajal-Retzius cells in organotypic cultures of hippocampus and neocortex. J. Neurosci. *16*, 6896–6907.

Del Rio, J.A., Heimrich, B., Borrell, V., Förster, E., Drakew, A., Alcantara, S., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., et al. (1997). A role for Cajal-Retzius cells and reelin in the development of hippocampal connections. Nature *385*, 70–74.

Del Rio, J.A., Solé, M., Borrell, V., Martínez, A., and Soriano, E. (2002). Involvement of Cajal-Retzius cells in robust and layer-specific regeneration of the entorhino-hippocampal pathways. Eur. J. Neurosci. *15*, 1881–1890.

Deo, R.C., Schmidt, E.F., Elhabazi, A., Togashi, H., Burley, S.K., and Strittmatter, S.M. (2004). Structural bases for CRMP function in plexin-dependent semaphorin3A signalling. EMBO J. *23*, 9–22.

De Winter, F., Oudega, M., Lankhorst, A.J., Hamers, F.P., Blits, B., Ruitenberg, M.J., Pasterkamp, R.J., Gispen, W.H., and Verhaagen, J. (2002). Injury-induced class 3 semaphorin expression in the rat spinal cord. Exp. Neurol. *175*, 61–75.

Dimou, L., Schnell, L., Montani, L., Duncan, C., Simonen, M., Schneider, R., Liebscher, T., Gullo, M., and Schwab, M.E. (2006). Nogo-A-deficient mice reveal strain-dependent differences in axonal regeneration. J. Neurosci. *26*, 5591–5603.

Eickholt, B.J., Walsh, F.S., and Doherty, P. (2002). An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signalling. J. Cell Biol. *157*, 211–217.

Fan, J., and Raper, J.A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron *14*, 263–274.

Feiner, L., Koppel, A.M., Kobayashi, H., and Raper, J.A. (1997). Secreted chick semaphorins bind recombinant neuropilin with similar affinities but bind different subsets of neurons in situ. Neuron *19*, 539–545.

Fontoura, P., Ho, P.P., DeVoss, J., Zheng, B., Lee, B.J., Kidd, B.A., Garren, H., Sobel, R.A., Robinson, W.H., Tessier-Lavigne, M., and Steinman, L. (2004). Immunity to the extracellular domain of Nogo-A modulates experimental autoimmune encephalomyelitis. J. Immunol. *173*, 6981–6992.

Fry, E.J., Ho, C., and David, S. (2007). A role for Nogo receptor in macrophage clearance from injured peripheral nerve. Neuron *53*, 649–662.

Gallo, G., and Letourneau, P.C. (2004). Regulation of growth cone actin filaments by guidance cues. J. Neurobiol. *58*, 92–102.

García-Martínez, C., Humet, M., Planells-Cases, R., Gomis, A., Caprini, M., Viana, F., De La Pena, E., Sanchez-Baeza, F., Carbonell, T., De Felipe, C., et al. (2002). Attenuation of thermal nociception and hyperalgesia by VR1 blockers. Proc. Natl. Acad. Sci. USA *99*, 2374–2379.

Giger, R.J., Urquhart, E.R., Gillespie, S.K., Levengood, D.V., Ginty, D.D., and Kolodkin, A.L. (1998). Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron *21*, 1079–1092.

Giger, R.J., Cloutier, J.F., Sahay, A., Prinjha, R.K., Levengood, D.V., Moore, S.E., Pickering, S., Simmons, D., Rastan, S., Walsh, F.S., et al. (2000). Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron *25*, 29–41.

Goshima, Y., Nakamura, F., Strittmatter, P., and Strittmatter, S.M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature *376*, 509–514.

Goshima, Y., Hori, H., Sasaki, Y., Yang, T., Kagoshima-Maezono, M., Li, C., Takenaka, T., Nakamura, F., Takahashi, T., Strittmatter, S.M., et al. (1999). Growth cone neuropilin-1 mediates collapsin-1/Sema III facilitation of antero- and retrograde axoplasmic transport. J. Neurobiol. *39*, 579–589.

Guttmann-Raviv, N., Kessler, O., Shraga-Heled, N., Lange, T., Herzog, Y., and Neufeld, G. (2006). The neuropilins and their role in tumorigenesis and tumor progression. Cancer Lett. *231*, 1–11.

Hanbali, M., Bernard, F., Berton, C., Gatineau, G., Perraut, M., Aunis, D., Luu, B., and Bagnard, D. (2004). Counteraction of axonal growth inhibitory properties of semaphorin 3A and myelin-associated proteins by a synthetic neurotrophic compound. J. Neurochem. *90*, 1423–1431.

He, Z., and Koprivica, V. (2004). The Nogo signaling pathway for regeneration block. Annu. Rev. Neurosci. *27*, 341–368.

He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell *90*, 739–751.

Heizmann, G., Hildebrang, P., Tanner, H., Ketterer, S., Pansky, A., Froidevaux, S., Beglinger, C., and Eberle, A.N. (1999). A combinatorial peptoid library for the identification of novel MSH and GRP/bombesin receptor ligands. J. Recept. Signal Transduct. Res. *19*, 449–466.

Hughes, K., Nikolakaki, E., Plyte, S.E., Totty, N.F., and Woodgett, J.R. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. EMBO J. *12*, 803–808.

Humet, M., Carbonell, T., Masip, I., Sánchez-Baeza, F., Mora, P., Cantón, E., Gobernando, M., Abad, C., Pérez-Payá, E., and Messeguer, A. (2003). A positional scanning combinatorial library of peptoids as a source of biological active molecules: identification of antimicrobials. J. Comb. Chem. *5*, 597–605.

Ieda, M., Kanazawa, H., Kimura, K., Hattori, F., Ieda, Y., Taniguchi, M., Lee, J.K., Matsumura, K., Tomita, Y., Miyoshi, S., et al. (2007). Sema3a maintains normal heart rhythm through sympathetic innervation patterning. Nat. Med. *13*, 604–612.

Jin, Z., and Strittmatter, S.M. (1997). Rac1 mediates collapsin-1-induced growth cone collapse. J. Neurosci. *17*, 6256–6263.

Kaneko, S., Iwanami, A., Nakamura, M., Kishino, A., Kikuchi, K., Shibata, S., Okano, H.J., Ikegami, T., Moriya, A., Konishi, O., et al. (2006). A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. Nat. Med. *12*, 1380–1389.

Kikuchi, K., Kishino, A., Konishi, O., Kumagai, K., Hosotani, N., Saji, I., Nakayama, C., and Kimura, T. (2003). In vitro and in vivo characterization of a novel semaphorin 3A inhibitor, SM-216289 or xanthofulvin. J. Biol. Chem. *278*, 42985–42991.

Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., and Fujisawa, H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron *19*, 995–1005.

Klagsbrun, M., Takashima, S., and Mamluk, R. (2002). The role of neuropilin in vascular and tumor biology. Adv. Exp. Med. Biol. *515*, 33–48.

Kolodkin, A.L., Matthes, D.J., and Goodman, C.S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell *75*, 1389–1399.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., and Ginty, D.D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753–762.

Koprivica, V., Cho, K.S., Park, J.B., Yiu, G., Atwal, J., Gore, B., Kim, J.A., Lin, E., Tessier-Lavigne, M., Chen, D.F., and He, Z. (2005). EGFR activation mediates inhibition of axon regeneration by myelin and chondroitin sulfate proteoglycans. Science *310*, 106–110.

Kruger, R.P., Aurandt, J., and Guan, K. (2005). Semaphorins command cells to move. Nat. Rev. Mol. Cell Biol. *6*, 789–800.

Luo, Y., Raible, D., and Raper, J.A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217–227.

Luo, Y., Shepherd, I., Li, J., Renzi, M.J., Chang, S., and Raper, J.A. (1995). A family of molecules related to collapsin in the embryonic chick nervous system. Neuron *14*, 1131–1140.

Malet, G., Martín, A.G., Orzáez, M., Vicent, M.J., Masip, I., Sanclimens, G., Ferrer-Montiel, A., Mingarro, I., Messeguer, A., Fearnhead, H.O., and Pérez-Payá, E. (2006). Small molecule inhibitors of Apaf-1-related caspase-3/-9 activation that control mitochondrial-dependent apoptosis. Cell Death Differ. *13*, 1523–1532.

Messersmith, E.K., Leonardo, E.D., Shatz, C.J., Tessier-Lavigne, M., Goodman, C.S., and Kolodkin, A.L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron *14*, 949–959.

Mingorance, A., Fontana, X., Soriano, E., and Del Rio, J.A. (2005). Overexpression of myelin-associated glycoprotein after axotomy of the perforant pathway. Mol. Cell. Neurosci. *29*, 471–483.

Mondragón, L., Orzáez, M., Sanclimens, G., Moure, A., Armiñán, A., Sepúlveda, P., Messeguer, A., Vicent, M.J., and Pérez-Payá, E. (2008). Modulation of cellular apoptosis with apoptotic protease-activating factor 1 (Apaf-1) inhibitors. J. Med. Chem. *14*, 521–529.

Montoliu, C., Humet, M., Canales, J.J., Burda, J., Planells-Cases, R., Sánchez-Baeza, F., Carbonell, T., Pérez-Payá, E., Messeguer, A., Ferrer-Montiel, A., and Felipo, V. (2002). Prevention of in vivo excitotoxicity by a family of trialkylglycines. A novel class of neuroprotectants. J. Pharmacol. Exp. Ther. *301*, 29–36.

Nakamura, F., Tanaka, M., Takahashi, T., Kalb, R.G., and Strittmatter, S.M. (1998). Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron *21*, 1093–1100.

Pan, Q., Chanthery, Y., Liang, W.C., Stawicki, S., Mak, J., Rathore, N., Tong, R.K., Kowalski, J., Yee, S.F., Pacheco, G., et al. (2007). Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell *11*, 53–67.

Pasterkamp, R.J., and Verhaagen, J. (2001). Emerging roles for semaphorins in neural regeneration. Brain Res. Brain Res. Rev. *35*, 36–54.

Pasterkamp, R.J., De Winter, F., Giger, R.J., and Verhaagen, J. (1998). Role for semaphorin III and its receptor neuropilin-1 in neuronal regeneration and scar formation? Prog. Brain Res. *117*, 151–170.

Pasterkamp, R.J., Ruitenberg, M.J., and Verhaagen, J. (1999). Semaphorins and their receptors in olfactory axon guidance. Cell Mol Biol. (Noisy-le-grand) *45*, 763–779.

Planells-Cases, R., Montoliu, C., Humet, M., Fernández, A.M., García-Martínez, C., Valera, E., Merino, J.M., Pérez-Payá, E., Messeguer, A., Felipo, V., and Ferrer-Montiel, A. (2002). A novel N-methyl-D-aspartate receptor open channel blocker with in vivo neuroprotectant activity. J. Pharmacol. Exp. Ther. *302*, 163–173.

Pozas, E., Pascual, M., Nguyen Ba-Charvet, K.T., Guijarro, P., Sotelo, C., Chédotal, A., Del Río, J.A., and Soriano, E. (2001). Age-dependent effects of secreted Semaphorins 3A, 3F, and 3E on developing hippocampal axons: in vitro effects and phenotype of Semaphorin 3A (−/−) mice. Mol. Cell. Neurosci. *18*, 26–43.

Puschel, A.W., Adams, R.H., and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron *14*, 941–948.

Renzi, M.J., Feiner, L., Koppel, A.M., and Raper, J.A. (1999). A dominant negative receptor for specific secreted semaphorins is generated by deleting an extracellular domain from neuropilin-1. J. Neurosci. *19*, 7870–7880.

Rohm, B., Ottemeyer, A., Lohrum, M., and Puschel, A.W. (2000). Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. Mech. Dev. *93*, 95–104.

Schmidt, E.F., and Strittmatter, S.M. (2007). The CRMP family of proteins and their role in Sema3A signalling. Adv. Exp. Med. Biol. *600*, 1–11.

Schnell, L., and Schwab, M.E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature *343*, 269–272.

Schwab, M.E., and Bartholdi, D. (1996). Degeneration and regeneration of axons in the lesioned spinal cord. Physiol. Rev. *76*, 319.

Shao, Z., Browning, J.L., Lee, X., Scott, M.L., Shulga-Morskaya, S., Allaire, N., Thill, G., Levesque, M., Sah, D., McCoy, J.M., et al. (2005). TAJ/TROY, an orphan TNF receptor family member, binds nogo-66 receptor 1 and regulates axonal regeneration. Neuron *45*, 353.

Shearer, M.C., Niclou, S.P., Brown, D., Asher, R.A., Holtmaat, A.J., Levine, J.M., Verhaagen, J., and Fawcett, J.W. (2003). The astrocyte/meningeal cell interface is a barrier to neurite outgrowth which can be overcome by manipulation of inhibitory molecules or axonal signalling pathways. Mol. Cell. Neurosci. *24*, 913–925.

Shepherd, I., Luo, Y., Raper, J.A., and Chang, S. (1996). The distribution of collapsin-1 mRNA in the developing chick nervous system. Dev. Biol. *173*, 185–199.

Silver, J., and Miller, J.H. (2004). Regeneration beyond the glial scar. Nat. Rev. Neurosci. *5*, 146.

Simon, R.J., Kania, R.S., Zuckermann, R.N., Huebner, V.D., Jewell, D.A., Banville, S., Ng, S., Wang, L., Rosenberg, S., Marlowe, C.K., et al. (1992). Peptoids: a modular approach to drug discovery. Proc. Natl. Acad. Sci. USA *89*, 9367–9371.

Soker, S., Takashima, S., Miao, H.Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell *20*, 735–745.

Staton, C.A., Kumar, I., Reed, M.W., and Brown, N.J. (2007). Neuropilins in physiological and pathological angiogenesis. J. Pathol. *212*, 237–248.

Stoppini, L., Buchs, P.A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods *37*, 173–182.

Suchting, S., Bicknell, R., and Eichmann, A. (2006). Neuronal clues to vascular guidance. Exp. Cell Res. *312*, 668–675.

Takahashi, T., Nakamura, F., Jin, Z., Kalb, R.G., and Strittmatter, S.M. (1998). Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat. Neurosci. *1*, 487–493.

Takahashi, T., Fournier, A., Nakamura, F., Wang, L.H., Murakami, Y., Kalb, R.G., Fujisawa, H., and Strittmatter, S.M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell *99*, 59–69.

Torres-Vázquez, J., Gitler, A.D., Fraser, S.D., Berk, J.D., Pham, V.N., Fishman, M.C., Childs, S., Epstein, J.A., and Weinstein, B.M. (2004). Semaphorin-plexin signalling guides patterning of the developing vasculate. Dev. Cell *7*, 117–123.

Van Vactor, D.V., and Lorenz, L.J. (1999). Neural development: The semantics of axon guidance. Curr. Biol. *9*, R201–R204.

Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L., and He, Z. (2002a). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature *417*, 941.

Wang, K.C., Kim, J.A., Sivasankaran, R., Segal, R., and He, Z. (2002b). p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature *420*, 74.

Williams, G., Eickholt, B.J., Maison, P., Prinjha, R., Walsh, F.S., and Doherty, P. (2005). A complementary peptide approach applied to the design of novel semaphorin/neuropilin antagonists. J. Neurochem. *92*, 1180–1190.

Zhou, F.Q., Zhou, J., Dedhar, S., Wu, Y.H., and Snider, W.D. (2004). NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. Neuron *42*, 897–912.

Zuckermann, R.N., Kerr, J.M., Kent, S.B.H., and Moos, W.H. (1992). Efficient method for the preparation of peptoids [olgo(N-substituted glycines)] by submonomer solid phase synthesis. J. Am. Chem. Soc. *114*, 10646–10647.