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Class 4 Semaphorins and Plexin-B receptors regulate GABAergic and glutamatergic synapse development in the mammalian hippocampus

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Abstract

To understand how proper circuit formation and function is established in the mammalian brain, it is necessary to define the genes and signaling pathways that instruct excitatory and inhibitory synapse development. We previously demonstrated that the ligand-receptor pair, Sema4D and Plexin-B1, regulates inhibitory synapse development on an unprecedentedly fast time-scale while having no effect on excitatory synapse development. Here, we report previously undescribed synaptogenic roles for Sema4A and Plexin-B2 and provide new insight into Sema4D and Plexin-B1 regulation of synapse development in rodent hippocampus. First, we show that *Sema4a*, *Sema4d*, *Plxnb1*, and *Plxnb2* have distinct and overlapping expression patterns in neurons and glia in the developing hippocampus. Second, we describe a requirement for Plexin-B1 in both the presynaptic axon of inhibitory interneurons as well as the postsynaptic dendrites of excitatory neurons for Sema4D-dependent inhibitory synapse development. Third, we define a new synaptogenic activity for Sema4A in mediating inhibitory and excitatory synapse development. Specifically, we demonstrate that Sema4A signals through the same pathway as Sema4D, via the postsynaptic Plexin-B1 receptor, to promote inhibitory synapse development. However, Sema4A also signals through the Plexin-B2 receptor to promote excitatory synapse development. Our results shed new light on the molecular cues that promote the development of either inhibitory or excitatory synapses in the mammalian hippocampus.

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Author Contributions

J.E.M. and S.P. designed the research experiments. J.E.M. and D.G. performed experiments and analyzed the data. J.E.M. and S.P. wrote and edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Keywords

Sema4D; Sema4A; Plexin-B1; Plexin-B2; Synapse development

Introduction

Communication between neurons is orchestrated mainly by excitatory, glutamatergic and inhibitory, GABAergic chemical synapses in the mammalian central nervous system (CNS). While previous work applied biochemical, genetic, and functional approaches to identify molecules that are localized to and function at excitatory synapses (Budnik, 1996; Washbourne et al., 2004; Dalva et al., 2007; Paradis et al., 2007; Shen and Cowan, 2010; Dieterich and Kreutz, 2016), far less is known regarding the molecules that are localized to inhibitory synapses. The process of excitatory synapse development begins when a presynaptic axon comes in contact with postsynaptic dendrite, thereby leading to the recruitment and stabilization of presynaptic active zone machinery (McAllister, 2007; Okabe, 2013; Van Vactor and Sigrist, 2017). This is followed by the localization of postsynaptic components, including neurotransmitter receptors, signaling molecules, and scaffolding proteins to the postsynaptic specialization (Sanes and Lichtman, 2001; Harris and Littleton, 2015). At the molecular level, cell adhesion molecules and transmembrane ligand – receptor pairs play a role in both excitatory and inhibitory synapse development, including Semaphorin ligands and receptors (Shen and Scheiffele, 2010; Kuzirian and Paradis, 2011; Koropouli and Kolodkin, 2014). In the case of Semaphorin signaling, it remains to be determined which family members regulate development of which types of synapses, and where these proteins are expressed to mediate this function.

The Semaphorin family of proteins was initially discovered as repulsive axon guidance molecules (Kolodkin et al., 1993; Swiercz et al., 2002) and primarily signals through the Plexin and Neuropilin family of receptors (Winberg et al., 1998; Tamagnone et al., 1999; Pascoe et al., 2015). The family is grouped into eight classes based on sequence homology and domain structures, and a key feature of the family is the highly conserved extracellular, N-terminus Semaphorin (Sema) domain. Semaphorins can either be secreted (e.g. Class 3 Semaphorins) or membrane bound (e.g. Class 4 Semaphorins) (Elhabazi et al., 2001; Yazdani and Terman, 2006). Interestingly, it has been reported that the extracellular domains of some membrane-bound Semaphorins are cleaved and released from the cell surface (Wang et al., 2001; Nkyimbeng-Takwi and Chapoval, 2011; Nakatsuji et al., 2012), suggesting the possibility that these proteins could signal in a non-cell autonomous manner. Semaphorin ligands and their receptors have now been implicated in a number of developmental processes, including: retinal lamination, neuronal migration, dendrite morphogenesis, lymphocyte specification and signaling, cell migration, and vascular and heart morphogenesis (Tran et al., 2007; Suzuki et al., 2008; Matsuoka et al., 2011; Jongbloets and Pasterkamp, 2014).

The Plexin receptors are transmembrane proteins that contain an extracellular Sema domain and an intracellular GTPase activating protein (GAP) domain. Engagement of a Plexin-B receptor by a Semaphorin ligand leads to activation of the Plexin-B intracellular GAP

domain causing cytoskeletal rearrangement via inactivation of Rap1 and potentially other Ras-like GTPases (Wang et al., 2012b; Worzfeld et al., 2014). It is well-established that homodimers of Sema4D bind to monomeric Plexin-B1, thereby inducing its dimerization, C-terminal conformational change, and subsequent activation of GAP activity (Siebold and Jones, 2013; Wang et al., 2013). Interestingly, while Plexin-B1 can form strong homodimers via its transmembrane region, the Plexin-B2 receptor is thought to function as a monomer (Zhang et al., 2015).

Our previous studies implicated two members of the Class 4 Semaphorin ligands in synapse development (Paradis et al., 2007; Kuzirian et al., 2013; Raissi et al., 2013). We demonstrated that Sema4B is required for both inhibitory and excitatory synapse development, whereas Sema4D is a unique regulator of inhibitory synapse development (Paradis et al., 2007; Kuzirian et al., 2013; Raissi et al., 2013). Sema4D signals through its cognate receptor, Plexin-B1, to regulate inhibitory synapse development, while having no effect on excitatory synapse development. Further, Sema4D is enriched in synaptosome preparations isolated from rat hippocampi containing components of both inhibitory and excitatory synapses (Raissi et al., 2013), suggesting a synaptic localization. These data complement a recent study from another group, which used a novel proteomics approach to discover that both Sema4A and Plexin-B2 are localized to the glutamatergic synaptic cleft (Loh et al., 2016). Thus, we sought to determine if Sema4A and Plexin-B2 also function in the development of excitatory and/or inhibitory synapses.

The identity of the specific cell types in which Class 4 Semaphorins and Plexin-B receptors function to regulate synapse development in the hippocampus is not well described, making it difficult to formulate hypotheses about where these molecules function to mediate synapse development. Transcriptional profiling experiments indicated that *Sema4a*, *Sema4d*, *Plxnb1*, and *Plxnb2* are expressed in both neurons and glia cells in the cerebral cortex, with the highest expression found in glia (Zhang et al., 2014; Zeisel et al., 2015). However, both our studies and others demonstrate that Semaphorins and Plexins, expressed in neurons, function to regulate synapse development (Paradis et al., 2007; O'Connor et al., 2009; Tran et al., 2009; Ding et al., 2011; Kuzirian et al., 2013; Raissi et al., 2013; Duan et al., 2014; Uesaka and Kano, 2018).

Moreover, while treatment with the soluble extracellular domain of Sema4D can promote the development of inhibitory synapses in a Plexin-B1 dependent manner (Kuzirian et al., 2013), the identity of the neuronal subtype(s) which require the function of Plexin-B1 for this process remains an open question. Interestingly, a recent study suggested a potential presynaptic site of action for Plexin-B1, as treatment of organotypic hippocampal slices with the soluble extracellular domain of Sema4D stabilizes presynaptic boutons of GFP-labeled GABAergic axons (Frias et al., 2018). Our previous work also suggested a potential postsynaptic function for Plexin-B1, as treatment of cultured hippocampal neurons with soluble extracellular domain of Sema4D triggers the rapid addition of new postsynaptic assemblies of gephyrin scaffolding proteins, through the splitting of pre-existing assemblies (Kuzirian et al., 2013). Given these data, we sought to determine if the Plexin-B1 receptor functions in the presynaptic neuron, the postsynaptic neuron, or both to regulate Sema4Ddependent inhibitory synapse development.

In this study, we explored if and/or where Sema4A, Sema4D, Plexin-B1, and Plexin-B2 function to modulate synapse development, and determined which specific ligand—receptor interactions mediated this process. First, we evaluated the expression patterns of these genes in the mammalian hippocampus by fluorescent *in situ* hybridization (FISH), in order to ascertain in which hippocampal neuronal subtypes these genes may function to mediate synapse development. Next, we investigated if these Class 4 Semaphorins and Plexin-Bs are either necessary and/or sufficient in specific synaptic compartments, by manipulating gene expression in either dissociated neuronal cultures or co-cultures of HEK293T cells and neurons. Lastly, we defined a new role for Sema4A and Plexin-B2 as regulators of both inhibitory and excitatory synapse development. Our data shed new light on the manner by which cell-cell signaling between Semaphorin ligands and Plexin receptors function in the formation of synapses and thereby the developing nervous system.

Results

Plxnb1, *Plxnb2*, *Sema4a*, and *Sema4d* mRNA expression in the developing hippocampus.

To determine the mRNA expression pattern of *Plxnb1, Plxnb2, Sema4a,* and *Sema4d* in the early postnatal hippocampus, we used a quantitative fluorescent *in situ* hybridization (FISH) technique that allows single molecule detection of multiple RNA targets simultaneously. To identify different cell types in hippocampus, we utilized probes that detect the following mRNAs: *Gad1* + *Gad2* (encoding Glutamate Decarboxylase 1 and 2, respectively*)* to identify inhibitory interneurons, *Slc17a7* (encoding Vesicular Glutamate Transporter 1) to identify excitatory neurons, and *Aldh1l1* (encoding Aldehyde Dehydrogenase 1 family, member L1) to identify astrocytes (Cahoy et al., 2008; Hrvatin et al., 2018). A DAPI stain was used to identify cell nuclei. As a negative control, we employed a probe that recognizes *DapB,* the *Bacillus subtilis* enzyme Dihydrodipicolinate reductase, which is absent from the mammalian genome.

Coronal sections of postnatal day 14 (P14) wildtype mouse brains containing the hippocampus were probed for a gene of interest and a cell type specific marker simultaneously (e.g. *Plxnb1* and *Aldh1l1*; Fig. 1). We validated our approach in three ways: First, we did not observe *DapB* mRNA puncta signal above our threshold in any cells of the hippocampus (Fig. S1*A*). Second, we determined that the percentage of total DAPI+ cells labeled as inhibitory interneurons, excitatory neurons, and astrocytes are in agreement with published studies (Fig. S1*A*) (Bekkers, 2011; Chamberland and Topolnik, 2012; Degen et al., 2012; Seifert and Steinhauser, 2017). Third, we confirmed that the signal obtained from the *Plxnb1, Plxnb2, Sema4a,* and *Sema4d* probes in cerebellum was in agreement with published studies describing the expression of these genes in that brain region ((Fig. S2) (Maier et al., 2011)).

Our hippocampal expression analyses of the *Plxnb1, Plxnb2, Sema4a,* and *Sema4d* genes reveal that they have both overlapping and distinct expression patterns in hippocampal sub-regions. Specifically, *Plxnb1* is expressed in a greater percentage of cells in the Dentate Gyrus (DG) than in the CA1 or CA3 sub-regions (Fig. 1*A,E*; Fig. S1*B,C*). Closer investigation shows that *Plxnb1* is enriched in the DG subgranular zone (SGZ), which is a neurogenic region of the hippocampus (Fig. S1*B*). In contrast, *Plxnb2* has a broader

expression pattern and is expressed both in the SGZ and the granule cell layer (GCL) of the DG. Interestingly, *Plxnb2* is expressed in about half of the cells in each hippocampal sub-regions (approximately 50% of all DAPI+ cells in CA1, CA3, and DG (Fig. 1*B,F*; Fig. S1*D*)).

Our data also demonstrate that *Sema4a* mRNA is expressed throughout all 3 major hippocampal sub-regions, but its expression is highest within CA3 (approximately 60% of all DAPI+ cells in CA3) and lowest within DG (less than 30% of all DAPI+ cells in DG (Fig. 1*C,G*; Fig. S1*E*)), while *Sema4d* mRNA is expressed in a similar percentage of cells throughout all hippocampal sub-regions (approximately 45% of all DAPI+ cells in CA1, CA3, and DG (Fig. 1*D,H*; Fig. S1*F*)). This data suggests that *Plxnb1* and *Sema4a* expression is more confined to distinct hippocampal sub-regions, while *Plxnb2* and *Sema4d* are expressed more broadly throughout all hippocampal sub-regions.

We also sought to determine if *Plxnb1, Plxnb2, Sema4a,* or *Sema4d* were expressed exclusively in a specific neuronal subtype, thus we quantified the percentage of cells that co-express a particular cell marker and either *Plxnb1* (Fig. 1*A*), *Plxnb2* (Fig. 1*B*), *Sema4a* (Fig. 1*C*), *or Sema4d* (Fig. 1*D*). We found that all four of these genes are expressed in a subset of inhibitory interneurons, excitatory neurons, and glia. We also found that expression of *Plxnb1*, *Plxnb2*, and *Sema4d* in neurons is greatest in DG compared to other hippocampal sub-regions (Fig. S1*G,I*). In contrast, the percentage of neurons that express *Sema4a* is greatest in CA3 (Fig. S1*G,I*). Our data also demonstrate that *Plxnb1*, *Plxnb2*, *Sema4a*, and *Sema4d* are well-expressed in astrocytes throughout all hippocampal sub-regions (Fig. S1*H*), in agreement with previous transcriptome studies (Zhang et al., 2014; Zeisel et al., 2015). Nonetheless our findings demonstrate that *Plxnb1, Plxnb2, Sema4a,* and *Sema4d* mRNAs are also expressed in neurons, thus we sought to determine if and how these genes function in specific neuronal sub-types to regulate synapse development.

Sema4D is sufficient to cluster and organize presynaptic GABAergic components.

Previously, we demonstrated that Sema4D is enriched at synapses and is required in postsynaptic excitatory neurons to regulate GABAergic synapse development (Paradis et al., 2007; Raissi et al., 2013). We hypothesized that Sema4D expression in excitatory neurons could also be sufficient to promote organization of these opposing presynaptic GABAergic terminals. To address this question, we took advantage of an established HEK293T cell—neuron coculture assay in which HEK293T cells, transfected with a synaptogenic molecule, are added to cultured neurons allowing for the formation of hemi-synapses between neuronal processes and the HEK293T cells. This coculture assay has been used most notably to investigate the synaptogenic potential of Neuroligin and Neurexin family members (Scheiffele et al., 2000; Graf et al., 2004; Craig and Kang, 2007). For example, expression of Neuroligin-1 in non-neuronal cells promotes the accumulation of neuronal proteins localized to the presynaptic GABAergic terminal in the axons contacting these transfected cells (Graf et al., 2004).

To determine if Sema4D expression in HEK293T cells is sufficient to organize the juxtaposed GABAergic presynaptic terminal, we began by co-transfecting HEK293T cells with a cDNA expressing GFP to identify transfected cells and cDNAs expressing either the

well-known presynaptic organizer, Neuroligin-1 (NL-1), or well-known postsynaptic organizer of GABAergic hemi-synapses, Neurexin 1β (Graf et al., 2004). The transfected HEK293T cells were gently trypsinized 2–3 days after transfection, and then added to rat hippocampal neuronal cultures that had been growing for 8 days in vitro (DIV). After 16–24 hours of coculture, cells were fixed and immunostained with antibodies that recognize presynaptic and postsynaptic markers of GABAergic synapses, Glutamate decarboxylase (GAD65) and the gamma2 subunit of the $GABA_A$ receptor ($GABA_AR_{\gamma 2}$), respectively. Formation of a hemi-synapse onto a given HEK293T cell was defined as the overlap of presynaptic GAD65 puncta and the GFP-expressing HEK293T cell (Fig. 2*A*), or postsynaptic $GABA_AR_{\gamma 2}$ puncta and the GFP-expressing HEK293T cell (Fig. 2*B*). Colocalization of both presynaptic and postsynaptic puncta coinciding with a GFP-expressing HEK293T cell were presumed to be neuron-neuron synapses and were excluded from the analysis.

We began by asking whether expression of Sema4A, Sema4D, Plexin-B1, or Plexin-B2 in HEK293T cells was sufficient cluster GAD65 in neurons, thereby inducing the formation of presynaptic hemi-synapses. We found that only Sema4D was sufficient to cluster GAD65 in presynaptic axons contacting the HEK293T cells expressing this protein (Fig. 2*A*). These data are consistent with a model whereby Sema4D, expressed in the postsynaptic excitatory neuron, promotes GABAergic synapse formation, perhaps via a trans-synaptic interaction with the Plexin-B1 receptor expressed in the presynaptic inhibitory interneuron. This model is supported by our analysis of *Plxnb1* mRNA expression (Fig. 1*A*), which demonstrates that this gene is expressed in interneurons that form presynaptic GABAergic terminals onto these excitatory neurons.

Next, we asked if Plexin-B1 expression in HEK293T cells was sufficient to induce formation of postsynaptic hemi-synapses, as detected by clustering of $GABA_AR_{\gamma 2}$ in dendrites contacting these cells. We found that Plexin-B1 expression was not sufficient to induce the formation of hemi-synapses, in contrast to the ability of the known postsynaptic organizer Neurexin 1β (Fig. 2*B*). We similarly assayed the capabilities of Sema4A, Sema4D, and Plexin-B2 to form $GABA_AR_{\gamma 2}$ hemi-synapses. None of these proteins induced postsynaptic hemi-synapse formation when expressed in HEK293T cells, either. Thus, we conclude that while Sema4D can induce clustering of presynaptic GAD65 and the formation of hemi-synapses, its receptor Plexin-B1 does not have similar organizing capabilities for either presynaptic or postsynaptic components of GABAergic synapses.

Plexin-B1 expression in presynaptic GABAergic axons is required for Sema4D-mediated clustering of GAD65

Our finding that Sema4D can induce the formation of presynaptic GABAergic hemi-synapses, taken together with our previous study demonstrating that Plexin-B1 is required for Sema4D-mediated GABAergic synapse development (Kuzirian et al., 2013), suggests that Plexin-B1 may function in inhibitory neurons to mediate Sema4D-dependent GABAergic synapse formation. Thus, we sought to determine if Plexin-B1 was required in GABAergic neurons for Sema4D mediated clustering of GAD65 in the HEK293T cell—neuron coculture assay. To answer this question, we cocultured HEK293T cells expressing

an empty vector control, NL-1, or Sema4D with hippocampal neurons that had been growing in culture for 7 DIV, isolated from wildtype or *Plxnb1*−/− littermate mice (Friedel et al., 2005). These cocultures were fixed after 16–24 hours, immunostained, and analyzed as described above. We found that addition of NL-1 expressing HEK293T cells onto wildtype or *Plxnb1*−/− neuronal cultures was sufficient to induce clustering of presynaptic GAD65 in axons contacting the NL-1 expressing cells, regardless of genotype, as expected (Fig. 3*A*). However, when Sema4D expressing HEK293T cells were added to cultures of *Plxnb1*−/− neurons, the density of GAD65 presynaptic hemi-synapses was similar to that of empty vector control HEK293T cells. This is in contrast to the effect of addition of Sema4D expressing HEK293T cells onto hippocampal neuronal cultures derived from wildtype littermates, in which we observed an increase in GAD65 cluster density, similar to our previous experiment using wildtype rat hippocampal neurons (Fig. 2*A*). These data demonstrate that Sema4D-dependent induction of presynaptic GABAergic hemi-synapses requires the expression of the Plexin-B1 receptor in inhibitory interneurons. This finding, taken together with our previous studies (Paradis et al., 2007; Kuzirian et al., 2013; Raissi et al., 2013) suggest a model whereby Sema4D expressed in the postsynaptic excitatory neuron interacts with Plexin-B1 expressed on the presynaptic axon of inhibitory interneurons to mediate GABAergic synapse development.

Sema4A mediates the development of GABAergic synapses

While the HEK293T cell—neuron coculture assay tests the sufficiency of a molecule to promote the clustering of synaptic components in a contrived system, it does not test the requirement of a particular gene for synapse development. Therefore we sought to determine if Sema4A is required for proper GABAergic synapse development. To begin, we first developed a short hairpin RNA (shRNA) that efficiently knocks down Sema4A expression (Fig. S3*A*). The shRNA targeting Sema4A or an empty vector control was transiently co-transfected into cultured rat hippocampal neurons at 4 DIV along with a plasmid expressing GFP to mark transfected cells. To quantify synapse density, 14 DIV hippocampal cultures were fixed and immunostained with antibodies that recognize presynaptic and postsynaptic markers of GABAergic synapses, GAD65 and $GABA_AR_{\gamma 2}$, respectively. In this assay, a synapse was defined as the overlap of both presynaptic and postsynaptic puncta onto a given GFP-expressing neuron (Paradis et al., 2007). In our analysis, we excluded any GFP-positive neurons that were also immunostained for GAD65 in the soma, as they were presumed to be inhibitory neurons; our cultures typically contain about 25% inhibitory GABAergic neurons and 75% excitatory neurons. Additionally, it is important to note that the transfection efficiency is approximately 10% of the neurons in the culture, therefore the vast majority of presynaptic contacts formed onto a given postsynaptic transfected neuron are from non-transfected cells. Further, we focused our imaging and analysis exclusively on the neuronal cell soma and dendritic arbor. Thus, this experimental paradigm allows us to specifically assay a cell autonomous requirement for the targeted gene in the postsynaptic excitatory neuron.

Using this assay, we found that RNAi-mediated knockdown of Sema4A decreases the density of GABAergic synapses formed onto the transfected excitatory neuron compared to controls (Fig. 4*A*). Importantly, this effect could be rescued by co-transfection of the shRNA

and a RNAi-resistant Sema4A cDNA, in which three silent mutations were introduced into the region of the Sema4A cDNA targeted by the shRNA. Thus, this exogenously expressed Sema4A is resistant to RNAi-mediated knockdown (Fig. S3*A*). The ability to rescue Sema4A knockdown with an RNAi-resistant cDNA demonstrates that the decreased GABAergic synapse density is due to Sema4A knockdown and is not due to an off-target effect of the shRNA. These data also demonstrate that Sema4A is required in postsynaptic excitatory neurons for the proper development of GABAergic synapses.

Since Sema4A is required for proper GABAergic synapse development, we next sought to determine if bath application of soluble Sema4A protein could also promote the development of GABAergic synapses in dissociated neuronal cultures. Therefore, we treated 13 DIV cultured rat hippocampal neurons overnight (12–16hrs) with either the extracellular domain of mouse Sema4A (amino acids 32–683) conjugated to the Fc region of Human IgG_1 or Fc control protein. As a positive control, we treated a subset of our cultures with mouse Sema4D (amino acids 24–711) conjugated to the Fc region of Mouse IgG_{2A}, as we previously demonstrated that treatment with Sema4D-Fc increases GABAergic synapse density (Kuzirian et al., 2013). These treated cultures were fixed on 14 DIV and immunostained with antibodies that recognize microtubule associated protein-2 (MAP2) to visualize dendrites, as well as presynaptic and postsynaptic markers of GABAergic synapses, GAD65 and $GABA_AR_{\gamma 2}$ respectively. We quantified synapse density as the overlap of presynaptic and postsynaptic puncta onto a population of dendrites labeled by MAP2. Our data show that treatment with Sema4A-Fc leads to an increase in the density of dendritic GABAergic synapses compared to Fc treated controls (Fig. 4*B*).

There are multiple GABAergic interneuron classes, including parvalbumin-expressing cells, somatostatin-expressing cells, vasoactive intestinal peptide-expressing cells, and neurogliaform cells (Kawaguchi and Kubota, 1997). Further, different classes of interneurons form presynaptic contacts onto excitatory pyramidal neurons at distinct locations. For example, in the hippocampus, the axons of parvalbumin positive interneurons form synapses primarily onto the somatic region of pyramidal cells, while somatostatin positive interneurons synapse onto dendrites (Ribak et al., 1990; Katona et al., 1999). To determine if Sema4A-Fc mediates the formation of synapses from a distinct interneuron class, we quantified the density of GABAergic synapses on either the dendrites (Fig. 4*B*) or cell somas (Fig. 4*C*) of excitatory neurons. Our results demonstrate that Sema4A-Fc treatment promotes GABAergic synapse formation onto both dendrites and somas of excitatory neurons, similar to Sema4D-Fc treatment (Kuzirian et al., 2013). Taken together, these results suggest that Sema4A acts as a general mediator of GABAergic synapse development, and does not function at a step that specifies GABAergic synapse development between excitatory neurons and a specific class of interneurons.

Sema4A and Sema4D function in the same pathway to regulate GABAergic synapse development

Given our observations that both Sema4A and Sema4D are necessary for proper GABAergic synapse development (Fig. 4*A*; (Paradis et al., 2007)), and that application of soluble Sema4A-Fc and Sema4D-Fc to the media of hippocampal cultures can promote the

development of GABAergic synapses (Fig. 4*B*; (Kuzirian et al., 2013)), we sought to determine if Sema4A and Sema4D signal through the same pathway to mediate this effect.

First, we used shRNAs to knockdown the expression of Sema4A and Sema4D individually or together in cultured hippocampal neurons, similar to synapse assays described above (e.g. Fig. 4*A*). We found that simultaneous knockdown of Sema4A and Sema4D in the same cell does not further decrease GABAergic synapse density when compared to knockdown of each gene individually (Fig. 5*A*). Next, we asked if simultaneous application of Sema4A-Fc and Sema4D-Fc to cultured neurons resulted in a synergistic increase in GABAergic synapse density. We found that treatment with both Sema4A-Fc and Sema4D-Fc together had no additive effect on GABAergic synapse density (Fig. 5*B*). Taken together, these data suggest that Sema4A and Sema4D function in the same signaling pathway to regulate GABAergic synaptogenesis.

We then asked if *Sema4a* and *Sema4d* mRNA are co-expressed in the same neurons in the hippocampus using three-color FISH. Coronal sections of P14 wildtype mouse brains were simultaneously probed for *Sema4a* and *Sema4d* mRNAs in conjunction with *Slc17a7* to identify excitatory neurons. We found that *Sema4a* and *Sema4d* have overlapping yet distinct expression patterns in the rodent hippocampus (Fig. 5*C*). For example, *Sema4a* is expressed in a relatively low percentage of excitatory neurons in the DG sub-region of the hippocampus (12.6 ± 1.9% *Sema4a*+ DG cells/ *Slc17a7+* DG cells), in contrast to the high percentage of excitatory neurons in the DG that express Se*ma4d* (78.8 ± 1.4% *Sema4d*+ DG cells/ *Slc17a7+* DG cells; Fig. S1*I*). Interestingly, both *Sema4a* and *Sema4d* are independently expressed in the majority of excitatory neurons in CA3 (Fig. 5*D,E*; Fig. S1*I*). In total, we found that greater than 40% of all excitatory *Slc17a7*+ neurons combined across all hippocampal sub-regions express both *Sema4a* and *Sema4d*, with the highest percentage of co-expressing cells found in the CA3 sub-region of the hippocampus (Fig. 5*D-G*). These co-expression data are consistent with a model in which Sema4A and Sema4D function in the same signaling pathways to mediate GABAergic synapse development.

Plexin-B1 is required in the postsynaptic neuron to mediate Sema4A and Sema4D-dependent GABAergic synapse development

Sema4D and Sema4A appear to signal through the same pathway, thus we next sought to determine the signaling partner(s) required for their synaptogenic activity. Our previous study showed that Plexin-B1 is required for Sema4D-dependent GABAergic synapse development (Kuzirian et al., 2013). However, because this experiment was performed using hippocampal cultures obtained from the constitutive *Plxnb1* knockout mouse, it failed to ascertain whether Plexin-B1 was required in the presynaptic neuron or the postsynaptic neuron to mediate Sema4D-dependent synapse development. The HEK293T cell—neuron coculture assay implicates Plexin-B1 function in the presynaptic inhibitory interneuron to mediate Sema4D-dependent synapse formation (Fig. 3*A*). However, none of our experiments to date have assayed a requirement for Plexin-B1, or the closely related family member Plexin-B2, as a postsynaptic regulator of GABAergic synapse development.

To address this question we first developed shRNAs that efficiently knock down either Plexin-B1 or Plexin-B2 expression (Fig. S3*B,C*). Next, each shRNA or an empty vector

control was transiently transfected into cultured rat hippocampal neurons as previously described. We found that knockdown of Plexin-B1 in excitatory neurons has no effect on the density of GABAergic synapses formed onto these cells (Fig. 6*A*). These data suggest that Plexin-B1 is not required in the postsynaptic excitatory neuron for proper GABAergic synapse development, and these data are in agreement with our previous study, where GABAergic synapse density in cultured hippocampal neurons obtained from *Plxnb1* knockout mice was not different from wildtype littermate control cultures (Kuzirian et al., 2013). Interestingly, we found that RNAi mediated knockdown of Plexin-B2 decreases GABAergic synapse density (Fig. 6*A*) and that decreased synapse density could be rescued by co-transfection of an RNAi-resistant Plexin-B2 cDNA (Fig. 6*A*; Fig. S3*C*). Taken together, these data suggest that Plexin-B2, but not Plexin-B1, is required in postsynaptic excitatory neurons for proper GABAergic synapse development.

Next, we asked if soluble Sema4D signals through postsynaptic Plexin-B1 or Plexin-B2 receptors to promote GABAergic synapse development. To this end, we sparsely transfected hippocampal neurons with GFP and a shRNA targeting either Plexin-B1, Plexin-B2, or an empty vector control. At 13 DIV, these neurons were treated with Sema4D-Fc overnight and synapse density was quantified by immunostaining as described above. We found that Plexin-B1 knockdown prevents the Sema4D-Fc dependent increase in GABAergic synapse density (Fig. 6*B*). In contrast, Sema4D-Fc treatment is capable of promoting GABAergic synapse development onto neurons in which Plexin-B2 expression has been knocked down by RNAi.

Using a similar strategy, we also sought to determine if Plexin-B1, Plexin-B2, or both receptors were required for Sema4A-Fc dependent GABAergic synapse development. We found that Plexin-B1 expression in the postsynaptic neuron is also required for Sema4A-Fc dependent increases in GABAergic density, while Plexin-B2 in the postsynaptic neuron is dispensable for this process (Fig. 6*C*). These data are consistent with a model whereby soluble Sema4A and Sema4D function in the same pathway to promote the development of GABAergic synapses via the Plexin-B1 receptor, but also suggest that Plexin-B2 is not essential for this process in response to soluble Sema4A or Sema4D. These data, taken together with our HEK293T cell—neuron coculture assay (Fig. 2, 3), demonstrate that Sema4D can signal through the Plexin-B1 receptor expressed in either the presynaptic GABAergic neuron or the postsynaptic excitatory neuron to regulate GABAergic synapse development.

The Plexin-B2 receptor is required for Sema4A-dependent glutamatergic synapse development

Given our findings indicating a novel role for both Sema4A and Plexin-B2 in GABAergic synapse development, we wondered if these two proteins might also function in glutamatergic synapse development. To determine if Sema4A is required for proper glutamatergic synapse development, a Sema4A shRNA or empty vector control construct was transiently co-transfected into cultured rat hippocampal neurons at 4 DIV along with a plasmid expressing GFP to mark transfected cells. At 14 DIV, hippocampal cultures were fixed and immunostained using antibodies that recognize the postsynaptic density protein 95

(PSD95) and Synapsin I, a protein localized to all presynaptic terminals. In this assay, a glutamatergic synapse was defined as the overlap of PSD95 and Synapsin I puncta onto a GFP-expressing neuron. Using this approach we found that RNAi-mediated knockdown of Sema4A significantly decreased the density of glutamatergic synapses (Fig. 7*A*). This effect could be rescued by co-transfection of the shRNA with an RNAi-resistant Sema4A cDNA plasmid, indicating that the observed phenotype is due to decreased Sema4A gene expression. Thus, Sema4A is required in the postsynaptic excitatory neuron for proper glutamatergic synapse development.

Next, we asked if treatment with soluble Sema4A could increase the density of glutamatergic synapses. On 13 DIV, cultured rat hippocampal neurons were treated overnight with either Sema4A-Fc, Sema4D-Fc, or Fc control. These cultures were fixed on 14 DIV and immunostained with antibodies that recognize MAP2, to visualize dendrites, as well as antibodies against Synapsin I and PSD95. We found that treatment with Sema4A-Fc promotes the development of glutamatergic synapses in hippocampal cultures (Fig. 7*B*) while treatment with Sema4D-Fc has no effect on the density of glutamatergic synapses, in agreement with our previous results (Kuzirian et al., 2013). Thus, our data reveal that Sema4A plays a role in both glutamatergic and GABAergic synapse development.

We sought to determine which receptor mediates Sema4A-dependent glutamatergic synapse development. To begin, we asked if Plexin-B1 or Plexin-B2 were required in the postsynaptic neuron for glutamatergic synapse development. Using our RNAi reagents (Fig. S3*B,C*), a shRNA targeting Plexin-B1, Plexin-B2, or an empty vector was transiently transfected into cultured rat hippocampal neurons. As a positive control, RNAi mediated knockdown of an unrelated gene, Rem2, significantly decreased glutamatergic synapse density (Fig 7*C*; (Ghiretti and Paradis, 2011)). Our data show that neither Plexin-B1 nor Plexin-B2 is required in postsynaptic excitatory neurons for the proper development of glutamatergic synapses (Fig. 7*C*).

Next, we asked if either Plexin-B1 or Plexin-B2 were required in the postsynaptic excitatory neuron for synaptic development that is promoted in response to addition of soluble Sema4A to the culture media. To accomplish this goal, we sparsely transfected hippocampal neurons with GFP and a shRNA targeting either Plexin-B1, Plexin-B2, or an empty vector control. At 13 DIV, these neurons were treated with Sema4A-Fc overnight and glutamatergic synapse density was quantified by immunostaining. Our data reveal that Sema4A-Fc treatment promotes glutamatergic synapse development onto neurons in which Plexin-B1 has been knocked down using RNAi (Fig. 7*C*), suggesting that Sema4A does not signal through the Plexin-B1 receptor to promote the development of glutamatergic synapses. In contrast, Plexin-B2 knockdown abolishes the Sema4A-Fc mediated increase in glutamatergic synapse development (Fig. 7*D*). These data demonstrate that soluble Sema4A signals through the Plexin-B2 receptor in postsynaptic excitatory neurons to regulate the development of glutamatergic synapses.

Discussion

In this study, we expand our understanding of Class 4 Semaphorin—Plexin-B signaling pathways that mediate synapse development. Specifically, we demonstrated that Sema4D expression in HEK293T cells is sufficient to induce the formation of presynaptic terminals in the axons of inhibitory interneurons via the Plexin-B1 receptor (Fig. 2*A*). We showed a new requirement for Plexin-B2 in the proper development of inhibitory synapses (Fig. 6*A*); however, the identity of the specific Class 4 Semaphorin ligand(s) that signal through the Plexin-B2 receptor to regulate this process remains unknown. In addition, we established that Sema4A and Sema4D are co-expressed in a subset of hippocampal neurons and appear to signal through a Plexin-B1 dependent pathway to regulate inhibitory synapse development (Figs. 5, 6). Finally, we determined that Sema4A not only regulates inhibitory synaptogenesis (Fig. 4*A*), but also mediates the development of excitatory synapses, in part through the Plexin-B2 receptor (Fig. 7*A*). This is in contrast to Sema4D, which is a specific regulator of inhibitory synaptogenesis. Collectively, our data define new roles for Class 4 Semaphorin—Plexin-B signaling in controlling synapse development.

Our data strongly suggest that Sema4D, expressed in the postsynaptic neuron, binds to Plexin-B1, expressed in the presynaptic GABAergic bouton (Fig. 8*A*), to mediate inhibitory synapse development. We propose a model whereby Sema4D binding induces Plexin-B1 dimerization and activation of its GAP domain (Pascoe et al., 2015), leading to changes in cytoskeletal elements that stabilize the nascent presynaptic bouton. The Sema4D—Plexin-B1 interaction could then generate a trans-synaptic signal that subsequently induces development of the postsynaptic specialization of GABAergic synapses. Our model is also in agreement with a study from the Wierenga group, which recently showed that Sema4D treatment stabilizes inhibitory presynaptic boutons in organotypic hippocampal slices using time-lapse imaging of GFP-labelled GABAergic boutons (Frias et al., 2018).

The broad and overlapping expression patterns of Sema4A and Sema4D in the hippocampus suggest that these proteins could function in both the presynaptic and postsynaptic neuron to mediate inhibitory synapse development. Our functional data is consistent with the possibility that Sema4A and Sema4D signal through their extracellular domains as membrane-bound molecules, soluble cleaved molecules, or both (Fig. 8). Cleavage of the extracellular domain of Sema4A and Sema4D from the cell surface is well-described in the immune system (Kumanogoh and Kikutani, 2003; Toyofuku et al., 2007). In the nervous system, the extracellular domain of Sema4D is also cleaved; however, cleavage is not required for Sema4D to regulate synapse development when expressed in the postsynaptic neuron (Raissi et al., 2013). A role for cleavage of the extracellular domain of Sema4A in synapse development has yet to be investigated, and thus the relevance of Sema4A and Sema4D proteolysis to synapse development remains an open question.

Our data offer a number of additional insights into possible signaling configurations between Sema4A, Sema4D, Plexin-B1 and Plexin-B2 in synapse development (Fig. 8). Importantly, we demonstrate that Plexin-B1 functions in both the presynaptic and postsynaptic neuron to regulate GABAergic synapse formation in response to Class 4 Semaphorins (Figs. 3, 6). One model to explain these results is that Sema4A and Sema4D, expressed in the presynaptic

inhibitory interneuron, engage Plexin-B1 expressed in the postsynaptic neuron (Fig. 8*B*). From the presynaptic neuron, Sema4A and Sema4D could signal either as membrane-bound proteins (Fig. 8*B*), or act in a paracrine manner after cleavage of their extracellular domain from the cell surface (Fig. 8*C*). Alternatively, it could be that the extracellular domains of Sema4A and Sema4D, expressed and cleaved from the postsynaptic neuron, signal in an autocrine manner by engaging Plexin-B1 expressed in the same neuron (Fig. 8*D*). Similarly, it is possible that Sema4A signals in either a paracrine or autocrine manner to regulate excitatory synapse development (Fig. 8*E*). Taken together, our data indicate that multiple Semaphorin ligands and Plexin receptors could simultaneously signal from both the presynaptic and postsynaptic neurons to regulate synapse development.

Interestingly, there is precedence for both paracrine and autocrine signaling mechanisms in the regulation of synapse development in the mammalian brain. For example, the secreted Semaphorin ligand, Sema3E, is released from the thalamostriatal projection neurons and specifies the connectivity of synapses onto the direct-pathway medium spiny neurons (MSNs) which express the Plexin-D1 receptor (Ding et al., 2011). In addition, the synaptogenic factor BDNF signals in both an autocrine and a paracrine manner through its receptor TrkB to regulate synapse development and strength (McAllister et al., 1999; Edelmann et al., 2015; Wang et al., 2015).

Further, since the Class 4 Semaphorins and Plexin-B receptors studied here are largely expressed in overlapping sets of cells, including excitatory and inhibitory neurons, we cannot rule out the potential for *cis* interactions between Semaphorins and Plexins in the same cellular membrane. Inhibitory *cis* signaling was previously described in dorsal root ganglion neurons (DRGs) *in vitro*, as *cis* interactions between Sema6A—Plexin-A4 expressed in the same neuron inhibit *trans* signaling by exogenous Sema6A (Haklai-Topper et al., 2010). To date, it is unclear whether Sema4A or Sema4D participates in *cis* signaling with Plexin-B receptors to regulate synapse development.

It is also possible that Sema4A signals through its intracellular C-terminal domain to regulate the development of either inhibitory (Fig. 8*D*) or excitatory (Fig. 8*E*) synapses. In support of this hypothesis, Sema4A was shown to reverse signal through its intracellular C-terminal domain upon engagement by Plexin-B1 in dendritic cells and various cell lines in order to mediate cell migration (Sun et al., 2017). Further, Sema4A functions in retinal pigment epithelial cells to mediate endosomal sorting via its C-terminal domain through a Rab11-dependent mechanism (Toyofuku et al., 2012). Thus one highly speculative possibility is that Sema4A participates in the sorting of synaptic components of the postsynaptic specialization, such as scaffolding proteins and neurotransmitter receptors during synapse development. While our data showed that the Sema4D C-terminal is dispensable for synapse development (Raissi et al., 2013), comparable studies with Sema4A have yet to be performed.

An intriguing finding of our study is the ability of Sema4A to promote the development of inhibitory or excitatory synapses, specifically through the Plexin-B1 or Plexin-B2 receptors, respectively (Fig. 8). How does this signaling specificity occur? One possibility is that Plexin-B1 and Plexin-B2 engage different co-receptors for the development of specific

synaptic types. Both Plexin-B1 and Plexin-B2 interact with receptor tyrosine kinases, c-Met and ErbB2 (Deng et al., 2007; Swiercz et al., 2008; Le et al., 2015), therefore engagement of a particular co-receptor could lead to the activation of divergent downstream intracellular signaling pathways. Another possibility is that although Plexin-B1 and Plexin-B2 both have similar GAP domains, they may regulate the activity of different G-proteins (Oinuma et al., 2003; Azzarelli et al., 2014). In either case, Plexin-B interactions with different co-receptors or G-proteins presumably converge onto actin-based remodeling proteins to affect changes in cytoskeletal elements (Tasaka et al., 2012) and therefore synapse development. Lastly, it is possible that Plexin-B1 and Plexin-B2 achieve signaling specificity by being spatially restricted in the cell; if so, an understanding of the origins of such spatial restriction awaits further investigation.

Ultimately, changes in synapse development lead to changes in circuit connectivity and function. A balance of excitation and inhibition (E/I) must be established for proper circuit function, and disruptions to this E/I balance can lead to neurological disorders such as epilepsy. In fact, the unique and rapid effect of Sema4D on inhibitory synapse development *in vitro* (Kuzirian et al., 2013), recently led us to test its action *in vivo*. We demonstrated that a few hours of Sema4D treatment into the hippocampi of adult mice increases inhibitory synapse density and suppresses seizure activity in two mouse models of epilepsy (Acker et al., 2018). In the future, it will be imperative to fully understand the downstream mechanisms of Sema4D—Plexin-B1 signaling, in conjunction with our growing knowledge of Sema4A and Plexin-B2 in synapse development, in order to identify and create novel anti-seizure therapies.

Materials and Methods

Ethics Statement

All animal procedures were approved by the Brandeis University Institutional Animal Care and Usage Committee, and all experiments were performed in accordance with relevant guidelines and regulations.

Neuronal Cell Culture

Timed matings were set up between heterozygous *PlxnB1*+/− (Friedel et al., 2005) males and females to produce mouse litters containing wildtype and knockout animals for dissociated hippocampal neuronal cultures. Vaginal plug observation was a designation of E0 and hippocampi were dissected at E16. Genotyping of all mice, including all embryos, was performed by PCR. Primary rat hippocampal neurons were isolated from timed pregnant E18.5 female rats that were obtained from Charles River Laboratories.

Dissociated mouse or rat hippocampal neurons were cultured on a feeder layer of astrocytes as described previously (Ghiretti and Paradis, 2011). Briefly, a layer of confluent astrocytes was generated by plating the cells onto 12 mm glass coverslips coated with poly-D-lysine (20 μg/ml) and laminin (3.4 μg/ml) in 24 well plates. Dissociated hippocampal neurons from E18 rat embryos were plated in neurobasal medium with B27 supplementation (Thermo Fisher) at a density of 80,000/well on the astrocyte feeder layer. When astrocytes were

confluent, AraC (Sigma-Aldrich) was added to a final concentration of 5 μM typically 4–24 hrs after neuron plating. Cultured cells were fed every 4–5 days by replacing half the media per well with fresh media. For synapse assays, hippocampal neurons were transfected at 4 DIV by calcium phosphate (Xia et al., 1996) and fixed at 14 DIV. For all experiments in neurons, shRNAs were transfected at a concentration of 33ng/well and RNAi resistant rescue cDNAs were transfected at a concentration of 300ng/well. For treatment of neuronal cultures with soluble Semaphorin extracellular domains, E18 rat hippocampal neurons were plated as described above on glia and were treated at 13 DIV with either Sema4D-Fc, Sema4A-Fc or Fc control (all R&D Systems; 2nM treatments) overnight for 16–24 hours and then fixed and immunostained as below.

Immunostaining

Neurons were fixed and immunostained at 14 DIV for synapse assays. Neurons were quickly washed with 1x PBS and then fixed with 4% paraformaldehyde/4% sucrose for 8 min at room temperature. Coverslips were washed 3 times with 1x PBS for 5 min each and incubated overnight at 4°C in a humidified chamber with primary antibody. All antibody dilutions were prepared in 1x GDB buffer (0.1% gelatin, 0.3% Triton X-100, 4.2% 0.4 M phosphate buffer, and 9% 5 M NaCl). After overnight incubation, coverslips were washed 3 times with 1x PBS for 10 min each and then incubated with appropriate Cy3- and Cy5-conjugated secondary antibodies (1:500 each; Jackson ImmunoResearch Laboratories) in 1x GDB for 2 h at room temperature. Coverslips were then washed 3 times with 1x PBS for 10 min each, dipped in dH_2O, and mounted on glass slides with Aquamount (VWR). The following antibodies were used: ms α GAD65 (1:1000; Millipore Cat# MAB351, RRID:AB_2263126), rb α $GABA_AR_{\gamma 2}$, (1:150; Millipore Cat# AB5559, RRID:AB_177523), rb α Synapsin I (1:1000; Millipore Cat# AB1543, RRID:AB_2200400), ms α PSD95 (1:500; UC Davis/NIH NeuroMab Facility Cat# 75-028, RRID:AB_2307331), ms α MAP2, (1:1000; Sigma-Aldrich Cat# M4403, RRID:AB_477193), ms α 488 (1:1000; Jackson ImmunoResearch Labs Cat# 115-545-003, RRID:AB_2338840), ms α Cy3 (1:500; Jackson ImmunoResearch Labs Cat# 715-165-150, RRID:AB_2340813), and rb α Cy5 (1:500; Thermo Fisher Scientific Cat# A10523, RRID:AB_2534032).

HEK293T cell—Neuron coculture assay

This heterologous cell coculture assay was adapted from previous studies (Scheiffele et al., 2000; Kang et al., 2008). E18 hippocampal rat neurons were cultured in a 24-well plate on glass coverslips that had been coated overnight at 37˚C with Poly-D-lysine (20 μg/ml) and laminin (3.4 μg/ml). Hippocampal neurons were grown in glia conditioned NB/B27 at a density of 250,000/well. For the heterologous cell assays using *PlxnB1−/−* and *PlxnB1+/+* mice, hippocampal neurons were generated from individual E16.5 embryos that were genotyped post-dissociation, cultured on an astrocyte feeder layer, and maintained as described above.

For co-cultures with HEK293T cells and hippocampal neurons, HEK293T were grown in DMEM + 10% FBS and seeded at a density of 300,000/well in a 12 well plate. After 24 hrs, HEK293T cells were transiently transfected with relevant cDNA and a plasmid expressing

GFP. 48 hrs post transfection HEK293T were trypsinized and added at a low density to hippocampal cultures. Co-cultures were fixed after 12–24 hrs and immunostained for ms α GAD65 and rb α $GABA_AR_{\gamma 2}$, and 60x confocal images were acquired. Image acquisition and data analysis was performed as described below.

DNA Plasmids and shRNAs

Sema4A full length cDNA (gift of Dr. Atsushi Kumanogoh) was cloned into a mammalian expression vector containing a CMV promoter, artificial signal sequence, and myc epitope tag at the N-terminus similar to pCMV-SS-myc-Sema4D (Raissi et al., 2013). Full length human pcDNA2-vsv-PlexinB1 and pMT2-PlexinB2-vsv were gifts from Dr. Luca Tamagnone. Neuroligin-1 (Scheiffele et al., 2000) and pCAG-HA-Nrxn1beta AS4 (−) (Addgene plasmid # 59409) (Chih et al., 2006) were gifts from Dr. Peter Scheiffele.

Target sequences for Plexin-B1 and Plexin-B2 shRNAs were chosen from previously derived siRNA sequences [Life technologies; PlexinB1 (4390827) and PlexinB2 (4390771)], and were cloned into the pSuper shRNA expression vector as previously described (Paradis et al., 2007). The targets of the shRNAs are the following sequences: Plexin-B1 5’ GTATATCAACAAGTACTAT 3’ ; Plexin-B2 5’ GCAAGTCCTTCCTTATCAA 3’; Sema4A (42–3n) 5’ ACAAAGCCTTGACCTTCAT 3’. The Sema4D (44–1) shRNA has been well described and validated in Paradis et al., 2007 and Raissi et al., 2013 and targets 5’ AGTGGACCTCCTTCCTAAA 3’.

Validation of shRNAs by Western blotting

shRNAs and cDNA constructs were transiently transfected into HEK293T cells, and after 48–72hrs these cells were harvested in 3x loading buffer + 10% β-mercaptoethanol. Lysates from cells transfected with Plexin-B1 and Plexin-B2 cDNAs were loaded onto a 6% SDS-PAGE gel for electrophoresis, and all other Westerns used a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane at 4°C overnight, blocked in 5% milk/TBST solution for 1 hr, then incubated overnight at 4˚C in primary antibody [anti- VSV-g (1:100; Sigma, V4888); anti- myc (Santa Cruz; sc-40; 1:500)]. The following day the membrane was washed three times for 15 min in TBST and then incubated with secondary antibody [anti- rabbit IR680LT (Licor; 1:10,000); anti-mouse IR800CW (Licor; 1:10,000)] for 2 hr at room temperature. After three 15 minute washes in TBST, fluorescent images were obtained using the Odyssey Western Blotting System (Licor). To assess loading, blots were probed with anti-mouse Tubulin (1:5000; Sigma, T5168) and the secondary antibody anti-mouse IR800CW (1:10,000; Licor) similar to above.

RNAScope Fluorescence in situ hybridization (FISH), imaging, and analysis

Wild type mouse brains (P14) were flash frozen in 2-methylbutane. Brain tissue was then embedded in O.C.T. (Tissue Tek) and sectioned at 20 μm using a cryostat at 22˚C. Coronal or sagittal sections were mounted on charged glass slides (Coverfrost glass green, Fisher) and stored at −80˚C for no more than three months. Mounted sections were fixed in 4% PFA, pretreated for 30 min at room temperature, and probes (Advanced Cell Diagnostics (ACD), Newark, CA) were hybridized for 2 hrs at 40˚C. FISH was performed by following the protocol with the RNAScope® Multiplex Fluorescent Detection Reagent kit (ACD,

320851) (Wang et al., 2012a). A combination of the following probes was used: *PlxnB1*, *PlxnB2*, *Sema4A*, *Sema4D*, *Aldh1l1*, *Gad1*, *Gad2*, *Slc17a7*, and/or a negative control probe *DapB* (See Table 1). Slides were counterstained with DAPI and then mounted using flouromont-G. Sections were imaged using a Ni-E inverted microscope equipped with a Nikon C2 confocal head, and sixteen-bit images of four to five individual regions of CA1, CA3, and DG for each hippocampus (2 hippocampi/ animal) were acquired with a 60x objective. Within each experiment, images were acquired with identical detector settings for laser power, gain, and offset for each probe using the Nikon Elements AR software. Images were acquired as a z-stack (5–8 optical sections and 0.5 μm step size). Maximum intensity projections (MIPs) were created from each stack (FIJI). MIPs of channels were processed into a usable stack, using the StackPackBot (MATLAB; (Cicconet et al., 2017)), and channels were converted into eight-bit files for image analysis (FIJI).

All analysis presented here was performed using automated MATLAB software assistant bots (Cicconet et al., 2017; Hrvatin et al., 2018). The automated MATLAB software identifies DAPI stained nuclei, and then counts the number of fluorescent probe puncta per nuclei. Given that the hippocampus has a high cell density, we first trained these bots in nuclear segmentation through use of a stacked Random Forest model (NucleiSegmentationBot), by using at least 10 images containing more than 3,000 nuclei. This Random Forest model trains on parameters such as background signal, nuclei contour, and the nuclei center. A watershed algorithm is then applied to these parameters to identify and split nuclei in close proximity to one another, creating individual nuclei masks, and assuring that fluorescent probe signals are not double-counted. DAPI nuclei masks are grown by 4 pixels (0.41μm/pixel) from the nucleus to account for RNAs that might be present in cell somas. Finally, the SpotsInNucleiBot was used in tandem with the nuclei segmentation training in order to generate the number of fluorescent puncta of up to three different probes per cell. These results were verified by an independent analysis performed on a subset of images using Halo (Indica labs; data not shown).

For the *DapB*, *Aldh1l1*, *Plxnb1*, *Plxnb2*, *Sema4a*, and *Sema4d* genes a cell was defined as expressing a specific RNA if it was found to contain ≥ 4 mRNA puncta; for the *Gad1*/*Gad2* genes ≥ 6 mRNA puncta; and for the *Slc17a7* gene ≥ 8 mRNA puncta. These thresholds were empirically determined based on the density of puncta observed for each probe set. Very rarely were fluorescent puncta observed above the established threshold with the *DapB* probe (0.1 +/− 0.09% *DapB*+ cells/all DAPI+ cells analyzed; Fig. S1A). Glutamatergic neurons are very densely packed in the pyramidal cell layer of the hippocampus, and *Slc17a7* is well-expressed in these neurons. Thus, despite our higher thresholding of *Slc17a7* positive puncta per cell, 9% of excitatory cells (*Slc17a7*+) co-labeled as inhibitory cells (*Gad1*/*Gad2*+), suggesting that these neurons are actually inhibitory and not excitatory. However we also cannot exclude the possibility that the co-expression of these markers (*Slc17a7* and *Gad1*/*Gad2*) might simply be a biological feature. Nonetheless, this “double counting” does not affect our main conclusion as it only slightly increases the number of cells that are classified as excitatory neurons, and this misclassification is uniformly present in all tissue sections.

For visualization purposes, representative large field 10x images of the hippocampus (Figs. 1,5; Fig. S1,S2) were acquired with identical detector settings within an experiment and pairwise stitched together in FIJI (Preibisch et al., 2009). For single cell images (Fig. 5*F*), a Gaussian filter was used to decrease the DAPI+ nuclei noise for representative image visualization only. Statistical analysis was determined by a One-Way ANOVA (fixed factor was sub-region of the hippocampus) followed by a Tukey's *post hoc* Test for significance (Fig. S1; SPSS).

Image acquisition, Data analysis and normalization, and Statistics

All image acquisition and quantification were performed in a blinded manner. Twelve-bit images of immunostained neuronal cultures were acquired on an Olympus Fluoview 300 confocal microscope using a 60x objective, unless otherwise stated. Within each experiment, images were acquired with identical settings for laser power, detector gain, and amplifier offset. Images were acquired as a z-stack (8–14 optical sections and 0.5 μm step size). Maximum intensity projections were created from each stack (FIJI).

For shRNA knockdown synapse assay experiments, synapse density was quantified as the overlap of the GFP filled dendrites, presynaptic antibody marker, and the postsynaptic antibody marker using MetaMorph image analysis software. For Semaphorin treatment experiments, synapse density was quantified on dendrites as overlap of synaptic markers on a MAP2 positive area of neuronal dendrites. For each experiment, a threshold for the Cy3 and Cy5 channels was determined and was then applied across all images. The GFP or MAP2 threshold for each image was determined independently. A binary mask including all pixels above the threshold was created for all channels of each image. The cell body was manually deleted from the GFP mask and the "logical and" function was used to determine regions of colocalization of at least one pixel in size between GFP, Cy3, and Cy5 channels. To calculate synapse density, this number was divided by the area of the neuron as measured by the GFP area minus the cell body. 7–10 images from at least two separate coverslips were acquired and analyzed for each condition within an experiment for at least three biological replicates, unless otherwise stated.

For analysis of somatic synapses, images were processed as previously described above, except the soma area was defined by manually drawing a ROI around the cell soma. Inhibitory synapses were measured within this ROI by the "logical and" function to determine regions of colocalization of at least one pixel in size between GFP, Cy3, and Cy5 channels. Somatic synapse density was calculated by dividing this overlap by the area of the ROI. Only pyramidal somas were analyzed. Images from at least two separate coverslips were acquired and analyzed for each condition within an experiment for at least three biological replicates.

For heterologous cell assay experiments, cluster density was quantified as the overlap of GFP filled HEK293T cell with either the presynaptic antibody marker (Cy3; GAD65) or the postsynaptic antibody marker (Cy5; $GABA_AR_{\gamma 2}$) using MetaMorph image analysis software. For each experiment, a threshold for the Cy3 and Cy5 channels was determined and was then applied across all images. The GFP threshold was determined independently for each image. A binary mask including all pixels above the threshold was created for all

channels of each image. Cy3 puncta co-localized with Cy5 puncta are presumed to be sites of neuron:neuron synapses and not neuron:HEK293T cell hemi-synapses, therefore these puncta were excluded from the analysis. Formation of presynaptic hemi-synapses was quantified by the density of GAD65 puncta on a HEK293T cell area. Formation of postsynaptic hemi-synapses was quantified by the density of $GABA_AR_{\gamma 2}$ puncta on a HEK293T cell area. Greater than 6 images from at least two separate coverslips were acquired and analyzed for each condition within an experiment for at least three biological replicates.

For presentation purposes in Figures 2 and 4–7, data were normalized to the appropriate negative control condition within each experiment to account for variation across experiments, including differences in antibody staining and cell density. Within an experiment, the average value (i.e. synapse density for neuronal cultures experiments, or cluster density for neuron-HEK293T coculture experiments) was obtained for the negative control (e.g. empty vector transfection, Fc control protein treatment, etc.) and experimental conditions. The normalized value for the negative control condition was calculated as the control average value divided by the control average value (i.e. 1), and is indicated by a dotted line at 100%. The normalized value for each experimental condition was calculated as the experimental average value divided by the control average value, and the standard error of this ratio was also calculated (Paradis et al., 2007). These normalized values from each individual experiment were then averaged (total of 2–4 experiments) within each condition and plotted as the percentage relative to control. Error bars are the standard error of this ratio. In some cases the representation of the control value at 100% lacks an error bar since the representation of the error for the control condition is contained within the error bars for the experimental conditions (Paradis et al., 2007).

Statistical analysis was performed on the raw data before normalization of experiments. Statistical significance was determined by a Univariate Two-Way ANOVA (fixed factors were experimental condition and date of experiment) followed by a Tukey's *post hoc* test for significance (SPSS), as indicated in each figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- *Sema4a*, *Sema4d*, *Plxnb1*, & *Plxnb2* are expressed in excitatory and inhibitory neurons
- Sema4D induces the clustering of inhibitory presynaptic components via Plexin-B1
- Sema4A regulates excitatory and inhibitory synapse development
- Sema4D & Sema4A signal through postsynaptic Plexin-B1 for inhibitory synapse development
- Sema4A functions through postsynaptic Plexin-B2 for excitatory synapse development

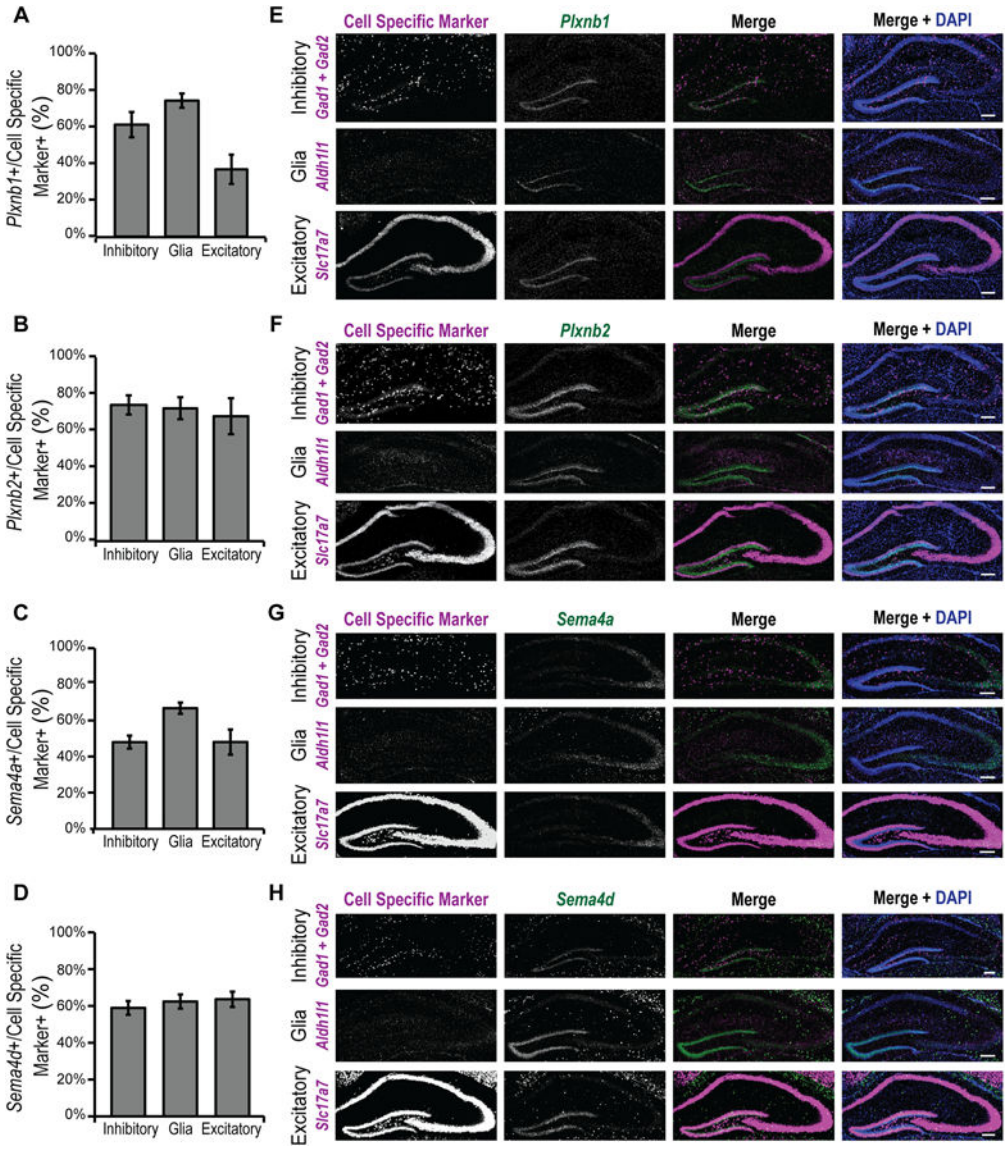

Figure 1. *Plxnb1, Plxnb2, Sema4a,* and *Sema4d* are expressed in inhibitory and excitatory neurons and glia in the developing hippocampus.

(A-D) Percentage of a specific cell type in P14 rat hippocampus that express ***(A)*** *Plxnb1*, ***(B)*** *Plxnb2*, ***(C)*** *Sema4a*, or ***(D)*** *Sema4d* mRNAs using two-color fluorescent *in situ* hybridization (FISH). Percentage is the number of DAPI+ cells positive for an experimental marker/ # DAPI+ cells positive for a cell specific marker. ***(E-H)*** Representative images of P14 hippocampi with probes detecting ***(E)*** *Plxnb1*, ***(F)*** *Plxnb2*, ***(G)*** *Sema4a*, or ***(H)*** *Sema4d* mRNAs and probes detecting mRNA expressed specifically in either inhibitory neurons (*Gad1* + *Gad2*), glia (*Aldh1l1*), or excitatory neurons (*Slc17a7*). Sections are also stained for DAPI (blue). Data are plotted as mean ± SEM. ***(A-H)***: $n \geq 3$ sections, $n \geq 2$ animals. Scale bars, 200 μm.

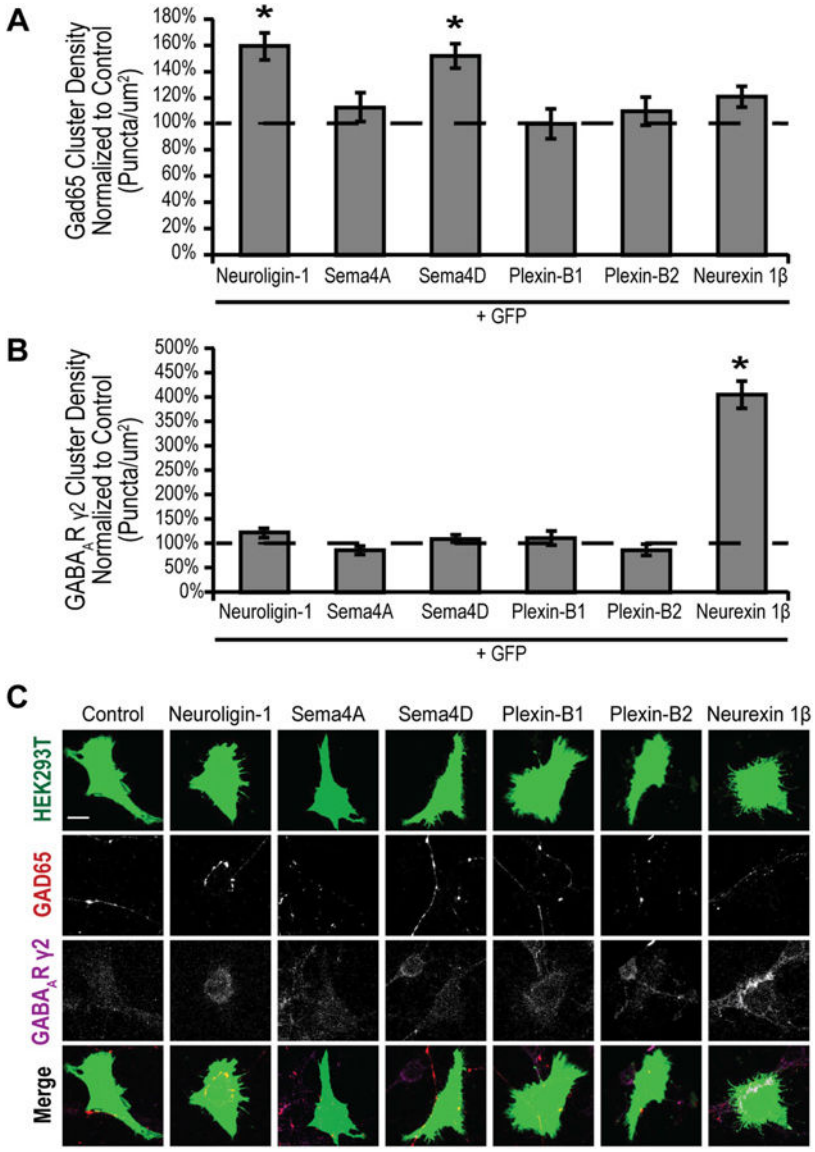

Figure 2. Sema4D is sufficient to cluster presynaptic GABAergic components in the HEK293T cell—neuron coculture assay.

(A) Density of presynaptic GAD65 clusters formed in 9 DIV rat hippocampal neurons upon contact with a HEK293T cell expressing a specific cDNA, normalized to the density of the negative control condition. In this case, the negative control was HEK293T cells co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicates the control condition normalized to itself. ***(B)*** Density of postsynaptic $GABA_AR_{\gamma 2}$ clusters formed in 9 DIV rat hippocampal neurons upon contact with a HEK293T cell expressing a specific cDNA, normalized to the density of the negative control condition. In this case, the negative control was HEK293T cells co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicated the control condition normalized to itself. ***(C)*** Representative images of GFP expressing HEK293T cells cocultured with 9 DIV rat hippocampal neurons immunostained for GABAergic synaptic markers, GAD65 (red) and $GABA_AR_{\gamma 2}$ (magenta). $n > 101$ cells per condition from ≥ 3 experiments/

condition. *$p < 0.05$, Two-Way ANOVA with Tukey's *post hoc* Test. Error bars are ± standard error of the average ratio. Scale bars, 10μm.

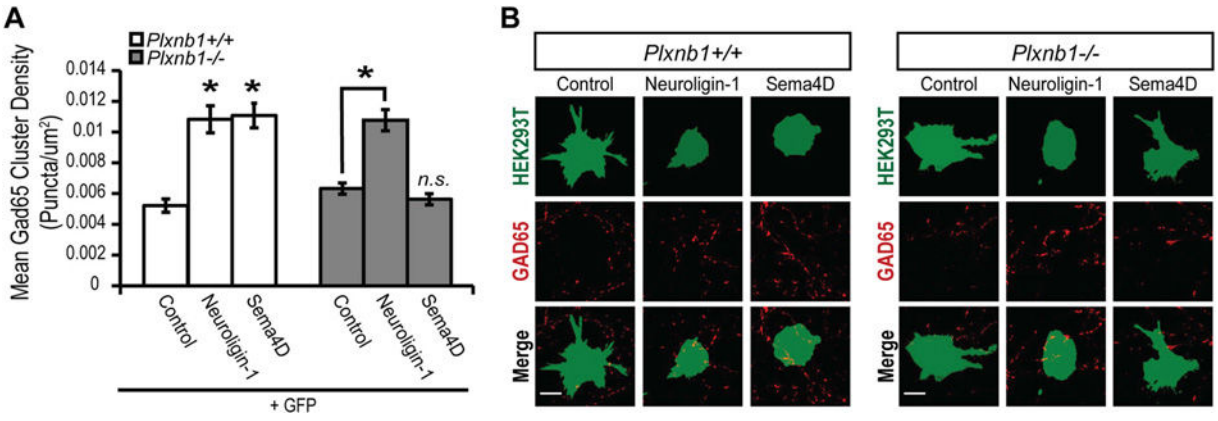

Figure 3. *Plxnb1* is required in the axons of inhibitory neurons for Sema4D mediated clustering of presynaptic GABAergic components.

(A) Mean density of presynaptic GAD65 clusters in 8 DIV *Plxnb1*+/+ (open bars, left) or *Plxnb1*−/− (gray bars, right) mouse hippocampal neurons contacting a HEK293T cell. ***(B)*** Representative images of GFP expressing HEK293T cells cocultured with 8 DIV *Plxnb1*+/+ (left) or *Plxnb1*−/− (right) mouse hippocampal neurons. Cultures were immunostained for GAD65 (red). $n > 80$ cells per condition from three experiments; $*p < 0.05$, Two-Way ANOVA with Tukey *post hoc* test. Data are plotted as mean ± SEM. Scale bars, 10μm.

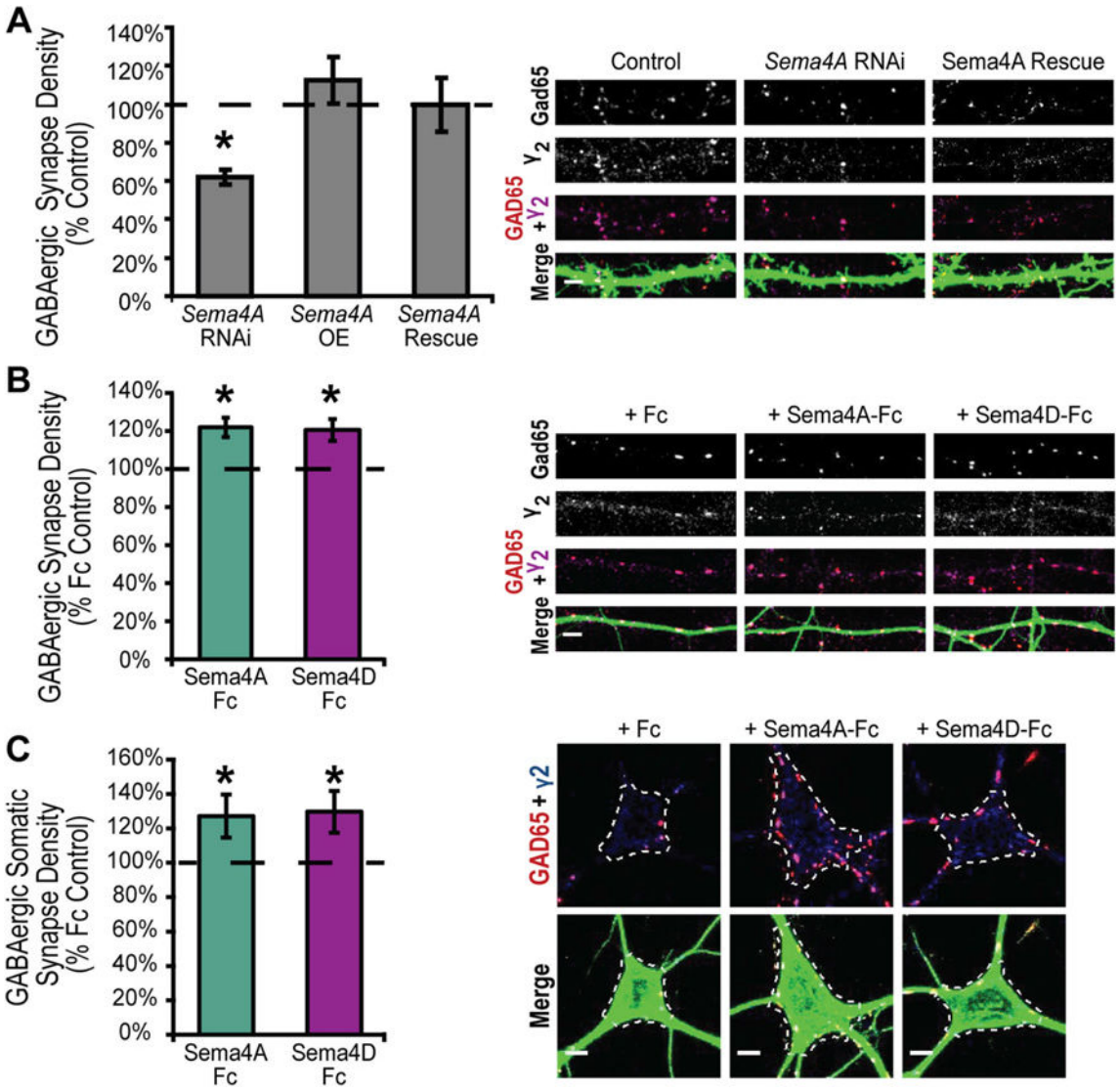

Figure 4. Sema4A is required for proper GABAergic synapse development and the soluble extracellular domain Sema4A promotes the development of GABAergic synapses.

(A) Inhibitory synapse density in 14 DIV hippocampal neurons for a given condition normalized to the synapse density of the negative control condition. Synapse density is defined as the overlap of GAD65/$GABA_AR_{\gamma 2}$ puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP expressing vector and either an empty vector control, a shRNA targeting Sema4A, a myc-tagged Sema4A cDNA, or co-transfection of myc-Sema4A RNAi resistant cDNA with a shRNA targeting Sema4A. In this case, the negative control is neurons co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicates the control condition normalized to itself. *(A, right)* Representative stretches of GFP-transfected dendrite immunostained for GAD65 (red) and $GABA_AR_{\gamma 2}$ (magenta). Scale bars, 5μm. ***(B)*** Inhibitory synapse density in 14 DIV hippocampal neuronal dendrites in response to Sema4A-Fc and Sema4D-Fc treatment normalized to the synapse density of the negative control condition. Synapse density is defined by the overlap of GAD65/$GABA_AR_{\gamma 2}$ puncta on MAP2-positive dendrites. In this

case, the negative control is neurons treated with Fc control. The dotted line at 100% indicates the control condition normalized to itself. *(B, right)* Representative stretches of MAP2-positive dendrite immunostained for GAD65 (red), $GABA_AR_{\gamma 2}$ (magenta), and MAP2 (green). Scale bars, 5μm. ***(C)*** Inhibitory synapse density in 14 DIV hippocampal neuronal somas in response to Sema4A-Fc and Sema4D-Fc treatment normalized to the synapse density of the negative control condition. In this case, the negative control is neurons treated with Fc control. The dotted line at 100% indicates the control condition normalized to itself. *(C, right)* Neuronal somas were traced (white dashed line) and the density of GABAergic synapses was measured (overlap of GFP/GAD65/$GABA_AR_{\gamma 2}$ within the traced areas). Scale bars, 10μm. *(B,C)* For treatments: 2nM Fc control, Sema4A-Fc or Sema4D-Fc were added to 13 DIV hippocampal neurons overnight (12–16hrs). $n \geq 45$ neurons for each condition from at least 2–3 experiments; $^*p < 0.05$, Two-Way ANOVA with Tukey's *post hoc* Test. Error bars are ± standard error of the average ratio.

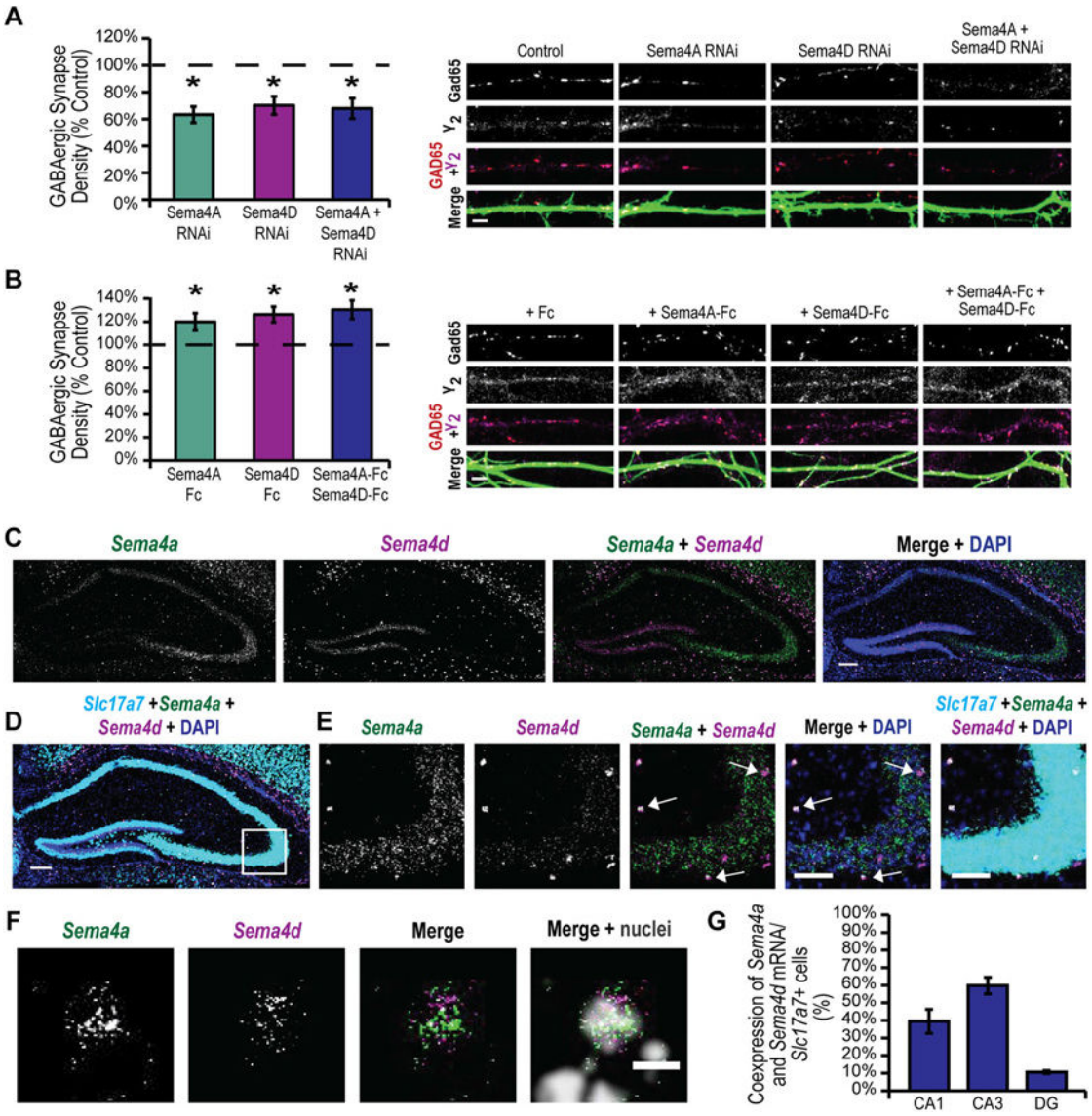

Figure 5. Sema4A and Sema4D are co-expressed and function in the same pathway to regulate GABAergic synapse development.

(A) Inhibitory synapse density in 14 DIV hippocampal neurons for a given condition normalized to the synapse density of the negative control condition. Synapse density is defined as the overlap of GAD65/$GABA_AR_{\gamma 2}$ puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP-expressing vector and either an empty vector control, a shRNA targeting Sema4A, a shRNA targeting Sema4D, or co-transfection of both shRNAs targeting Sema4A and Sema4D simultaneously. In this case, the negative control is neurons co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicates the control condition normalized to itself. *(A, right)* Representative stretches of GFP-transfected dendrite immunostained for GAD65 (red) and $GABA_AR_{\gamma 2}$ (magenta). ***(B)*** Inhibitory synapse density in 14 DIV hippocampal neurons in response to Sema4A-Fc and Sema4D-Fc treatment normalized to the synapse density of the negative control condition. Synapse density is defined by the overlap of GAD65/$GABA_AR_{\gamma 2}$ puncta on MAP2-positive

dendrites. In this case, the negative control is neurons treated with Fc control. The dotted line at 100% indicates the control condition normalized to itself. For treatments: 2nM Fc control, Sema4A-Fc or Sema4D-Fc were added to 13 DIV hippocampal neurons overnight (12–16hrs). *(B, right)* Representative stretches of MAP2-positive dendrite immunostained for GAD65 (red), $GABA_AR_{\gamma 2}$ (magenta), and MAP2 (green). ***(A, B)*** $n \geq 46$ neurons for each condition from at least 3 experiments; $*p < 0.05$, Two-Way ANOVA with Tukey's *post hoc* Test. Error bars are ± standard error of the average ratio. Scale bars, 5μm. ***(C)*** Representative images of P14 hippocampus using probes to detect *Sema4a* (green) and *Sema4d* (magenta) mRNAs by FISH. Sections are also stained for DAPI (blue). Scale bars, 200μm. ***(D)*** Three-color FISH of *Sema4a* (green), *Sema4d* (magenta), *Slc17a7* (excitatory cell marker; cyan), and DAPI (blue) in a large field image of the hippocampus. Scale bars, 200μm. ***(E)*** Boxed region from *(D)*. Arrows point to representative cells with co-expression of both *Sema4a* and *Sema4d*. Scale bars, 100μm. ***(F)*** Co-expression of *Sema4a* and *Sema4d* mRNA in CA1 DAPI+ nuclei. ***(G)*** Percentage of cells with colocalization of *Sema4a*, *Sema4d*, and *Slc17a7* in each specific hippocampal sub-region. Data are plotted as mean ± SEM. ***(C-G)*** Two biological replicates, four hippocampi in total. Scale bars, 10μm.

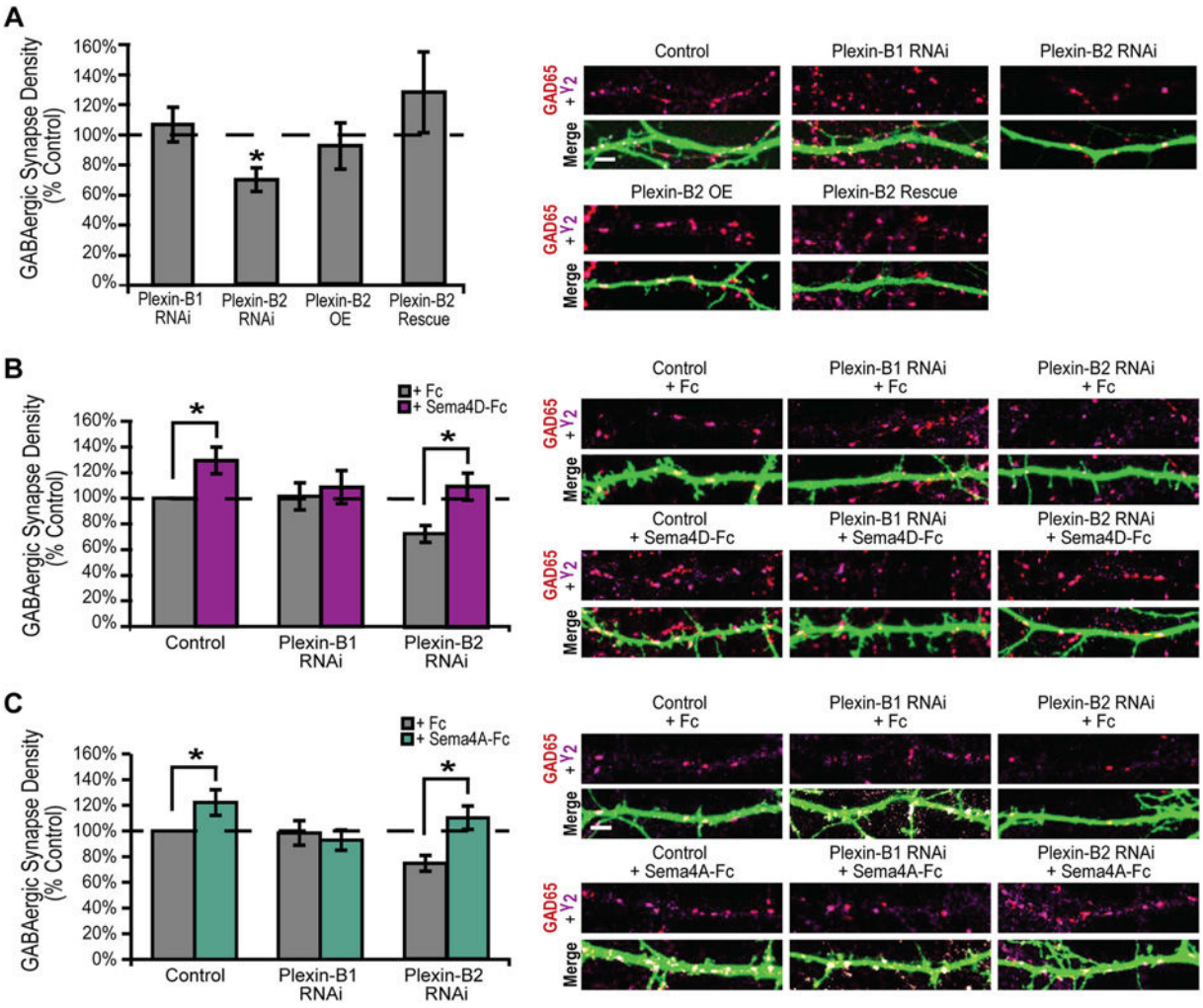

Figure 6. Plexin-B1, but not Plexin-B2, is required in the postsynaptic excitatory neuron for Sema4A-Fc and Sema4D-Fc mediated GABAergic synapse development.

(A) Inhibitory synapse density in 14 DIV hippocampal neurons for a given condition normalized to the synapse density of the negative control condition. Synapse density is defined as the overlap of GAD65/$GABA_AR_{\gamma 2}$ puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP expressing vector and either an empty vector control, a shRNA targeting Plexin-B1, a shRNA targeting Plexin-B2, a VSV-tagged Plexin-B2 cDNA, or co-transfection of VSV-tagged Plexin-B2 RNAi resistant cDNA with a shRNA targeting Plexin-B2. In this case, the negative control is neurons co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicates the control condition normalized to itself. *(A, right)* Representative stretches of GFP-transfected dendrite immunostained for GAD65 (red) and $GABA_AR_{\gamma 2}$ (magenta). ***(B,C)*** Inhibitory synapse density in 14 DIV hippocampal neurons in response to either Sema4D-Fc *(B)* or Sema4A-Fc *(C)* normalized to the synapse density of the negative control condition. Synapse density is defined by the overlap of GAD65/$GABA_AR_{\gamma 2}$ puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP-expressing vector and either an empty vector control, a shRNA targeting Plexin-B1, or a shRNA targeting Plexin-B2 and then were treated with

either Fc control *(B,C)*, Sema4D-Fc *(B)*, or Sema4A-Fc *(C)*. In these cases, the negative control is neurons treated with Fc control and co-transfected with an empty vector and a GFP-expressing vector (gray bar, far left). The dotted line at 100% indicates the control condition normalized to itself. The error for the negative control condition is contained within the error bar for each experimental condition (see Materials and Methods). *(B,C right)* Representative stretches of GFP transfected dendrite immunostained for GAD65 (red) and $GABA_AR_{\gamma 2}$ (magenta). For treatments: 2nM Fc control, Sema4A-Fc or Sema4D-Fc were added to 13 DIV hippocampal neurons overnight (12–16hrs). $n \geq 42$ neurons per condition from at least three experiments; $^*p < 0.05$, Two-Way ANOVA with Tukey's *post hoc* Test. Error bars are ± standard error of the average ratio. Scale bars, 5μm.

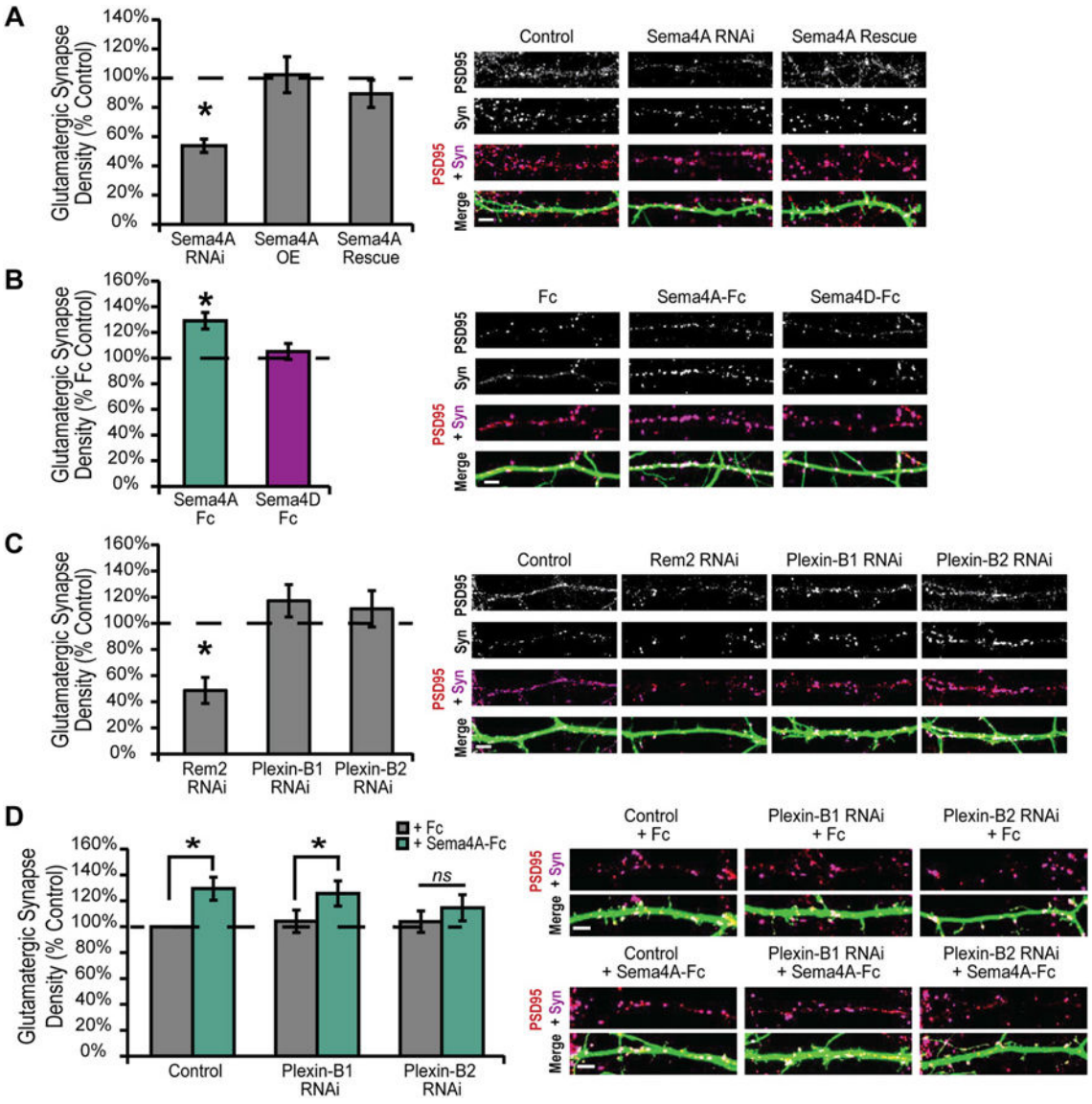

Figure 7. Sema4A regulates the development of glutamatergic synapses through the postsynaptic Plexin-B2 receptor.

(A) Excitatory synapse density in 14 DIV hippocampal neurons for a given condition normalized to the synapse density of the negative control condition. Synapse density is defined as the overlap of PSD95/Synapsin I puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP expressing vector and either an empty vector control, a shRNA targeting Sema4A, a myc-tagged Sema4A cDNA, or co-transfection of myc-Sema4A RNAi resistant cDNA with a shRNA targeting Sema4A. In this case, the negative control is neurons co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicates the control condition normalized to itself. *(A, right)* Representative stretches of GFP-transfected dendrite immunostained for PSD95 (red) and Synapsin I (magenta). ***(B)*** Excitatory synapse density in 14 DIV hippocampal neurons in response to Sema4A-Fc and Sema4D-Fc treatment normalized to the synapse density of the negative control condition. Synapse density is defined by the overlap of PSD95/Synapsin I

puncta on MAP2-positive dendrites. In this case, the negative control is neurons treated with Fc control. The dotted line at 100% indicates the control condition normalized to itself. *(B, right)* Representative stretches of MAP2-positive dendrite immunostained for PSD95 (red), Synapsin I (magenta), and MAP2 (green). ***(C)*** Excitatory synapse density in 14 DIV hippocampal neurons for a given condition normalized to the synapse density of the negative control condition. Synapse density is defined as the overlap of PSD95/Synapsin I puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP expressing vector and either an empty vector control, a shRNA targeting Rem2, a shRNA targeting Plexin-B1, or a shRNA targeting Plexin-B2. In this case, the negative control is neurons co-transfected with an empty vector and a GFP-expressing vector. The dotted line at 100% indicates the control condition normalized to itself. *(A, right)* Representative stretches of GFP-transfected dendrite immunostained for PSD95 (red) and Synapsin I (magenta). ***(D)*** Excitatory synapse density in 14 DIV hippocampal neurons in response to Sema4A-Fc normalized to the synapse density of the negative control condition. Synapse density is defined by the overlap of PSD95/Synapsin I puncta on GFP-transfected neurons. Neurons were co-transfected with a GFP expressing vector and either an empty vector control, a shRNA targeting Plexin-B1, or a shRNA targeting Plexin-B2 and were treated with either Fc control or Sema4A-Fc. In this case, the negative control is neurons treated with Fc control and co-transfected with an empty vector and a GFP-expressing vector (gray bar, far left). The dotted line at 100% indicates the control condition normalized to itself. The error for the negative control condition is contained within the error bar for each experimental condition (see Materials and Methods). *(D, right)* Representative stretches of GFP transfected dendrite immunostained for PSD95 (red) and Synapsin I (magenta). For treatments: 2nM Fc control, Sema4A-Fc or Sema4D-Fc were added to 13 DIV hippocampal neurons overnight (12–16hrs). $n \geq 55$ neurons per condition from three experiments; $n = 44$ neurons for *Rem2* RNAi. *$p < 0.05$, Two-Way ANOVA with Tukey's *post hoc* Test. Error bars are ± standard error of the average ratio. Scale bars, 5μm.

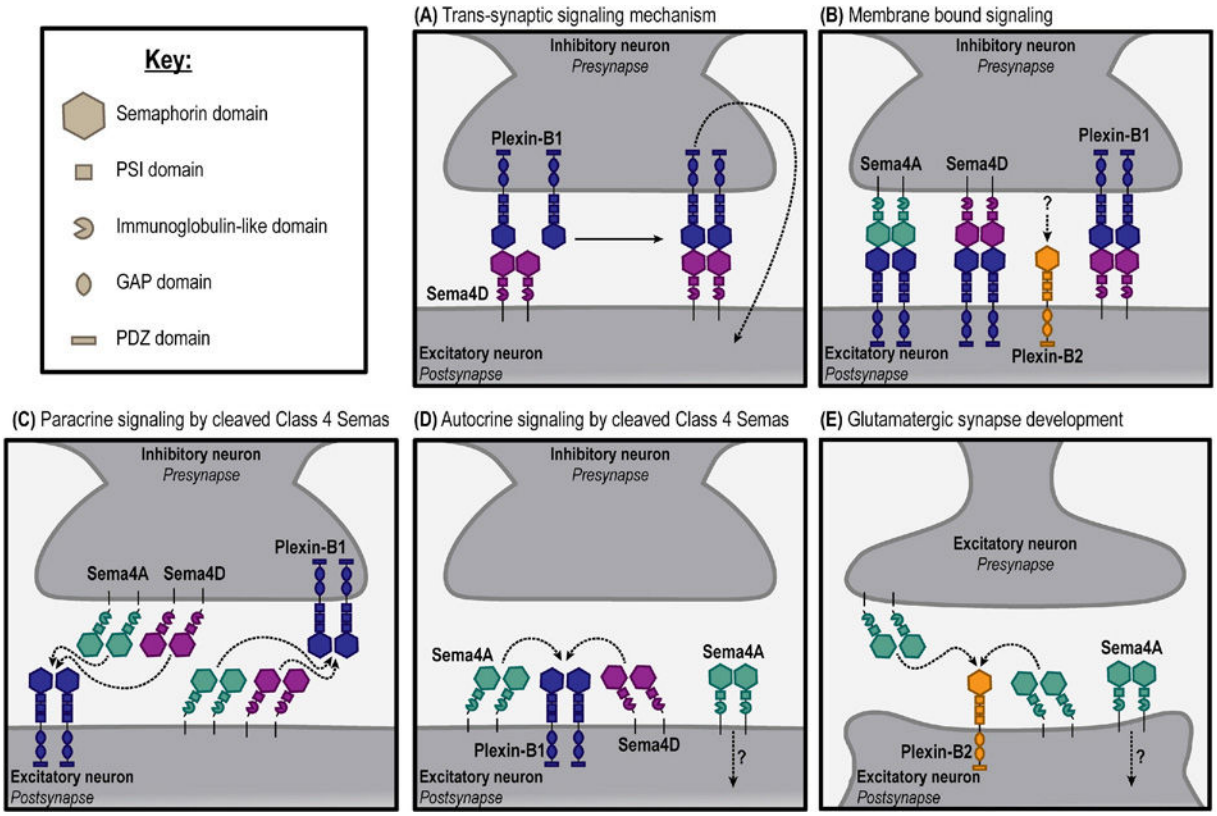

Figure 8. Models of Sema4A, Sema4D, Plexin-B1, and Plexin-B2 signaling configurations that could regulate inhibitory or excitatory synapse development.

(A) Membrane bound Sema4D expressed in the postsynaptic neuron interacts with Plexin-B1 in the presynaptic axon, clustering presynaptic components of inhibitory synapses and possibly generating a trans-synaptic signal that promotes inhibitory synapse development in the postsynaptic neuron. ***(B)*** Membrane bound Sema4A and Sema4D, expressed in the presynaptic inhibitory axon, could activate Plexin-B1 expressed in the postsynaptic neuron to promote inhibitory synapse development. Plexin-B2 expressed in the postsynaptic neuron also regulates inhibitory synapse development, presumably through engagement with a yet to be determined ligand. ***(C)*** The soluble extracellular domains of Sema4A and Sema4D expressed in the presynaptic neuron could be cleaved and signal in a paracrine manner, activating Plexin-B1 in the postsynaptic neuron. Alternatively, cleavage of the extracellular domains of Sema4A and Sema4D expressed in the postsynaptic neuron could allow for signaling in a paracrine manner through presynaptic Plexin-B1. ***(D)*** It is possible that cleavage of the extracellular domains of Sema4A and Sema4D, expressed in the postsynaptic neuron, could signal in an autocrine manner through Plexin-B1 expressed in the same postsynaptic neuron. Another possibility is that Sema4A expressed in the postsynaptic neuron regulates inhibitory synapse development via signaling through its intracellular C-terminal domain. ***(E)*** Sema4A expressed in the postsynaptic neuron also regulates excitatory synapse development, potentially via signaling through its intracellular C-terminal domain. The soluble extracellular domain of Sema4A expressed in either the pre- or postysynaptic membrane could be cleaved to engage in either paracrine or autocrine signaling through the postsynaptic Plexin-B2 receptor. Taken together, it is likely that Sema4A and Sema4D

function as both membrane bound and soluble cleaved proteins to regulate synapse development.

Table 1:

RNAScope probe information

Target	Channel	Accession number	Probe target	Catalog number
Negative Control Probe- DapB	1	EF191515	bp 414 – 862	310043
PlxnB1	1	NM_172775.2	bp 838 – 1716	469241
PlxnB2	1	NM_001159521.2	bp 1207 – 2153	459181
Aldh1l1 (Astrocyte)	2	NM_027406.1	bp 1256 – 2112	405891-C2
Gad1	2	NM_008077.4	bp 62 – 3113	400951-C2
Gad2	2	NM_008078.2	bp 552 – 1506	415071-C2
Gad1	3	NM_008077.4	bp 62 – 3113	400951-C3
Gad2	3	NM_008078.2	bp 552 – 1506	439371-C3
Slc17a7 (Vglut1)	1	NM_182993.2	bp 464 – 1415	416631
Slc17a7 (Vglut1)	3	NM_182993.2	bp 464 – 1415	416631-C3
Sema4A	1	NM_013658.3	bp 935 – 1861	498371
Sema4A	3	NM_013658.3	bp 935 – 1861	498371-C3
Sema4D	3	NM_001281880.1	bp 1645 – 2573	498381-C3