Published in final edited form as:

Brain Res. 2015 August 7; 1616: 157–165. doi:10.1016/j.brainres.2015.05.005.

Positive Regulation of Neocortical Synapse Formation by the Plexin-D1 Receptor

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Abstract

Synapse formation is a critical process during neural development and is coordinated by multiple signals. Several lines of evidence implicate the Plexin-D1 receptor in synaptogenesis. Studies have shown that Plexin-D1 signaling is involved in synaptic specificity and synapse formation in spinal cord and striatum. Expression of Plexin-D1 and its principal neural ligand, Sema3E, by neocortical neurons is temporally and spatially regulated, reaching the highest level at the time of synaptogenesis in mice. In this study, we examined the function of Plexin-D1 in synapse formation by primary neocortical neurons *in vitro*. A novel, automated image analysis method was developed to quantitate synapse formation under baseline conditions and with manipulation of Plexin-D1 levels. shRNA and overexpression manipulations caused opposite changes, with reduction resulting in less synapse formation, an effect distinct from that reported in the striatum. The data indicate that Plexin-D1 operates in a cell context-specific fashion, mediating different synaptogenic outcomes depending upon neuron type.

Keywords

synaptogenesis; image analysis; intracortical; mouse; in vitro

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Contribution of authors

Study concept and design: F.W, P.L. Acquisition and analysis of data: F.W, K.L.E. Interpretation of data and preparation of the manuscript: F.W., K.L.E., P.L. Obtained funding: P.L.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

1. Introduction

Alterations in neocortical circuits have been implicated in many neurodevelopmental disorders, such as schizophrenia (Fitzsimmons et al., 2013; Horváth and Mirnics, 2014; Lewis et al., 2012; Piper et al., 2012; Ripke et al., 2014) and autism spectrum disorders (ASD; Berg and Geschwind, 2012; Parikshak et al., 2013; Rudie et al., 2012; Scott-Van Zeeland et al., 2010; Willsey et al., 2013). An understanding of the molecular underpinnings that govern neocortical circuit formation will thus provide insights not only into the development of normal brain circuitry, but also the pathology underlying many neurodevelopmental and psychiatric disorders. During synapse formation, a neuron is required to identify specific synaptic partners among a myriad of potential targets that are often located in close proximity, or even adjacent, to each other (Akins and Biederer, 2006; Kolodkin and Tessier-Lavigne, 2011). The molecular mechanisms underlying the establishment of synaptic specificity in the mammalian neocortex is less well understood compared to invertebrates or other vertebrate neural systems (Arber, 2012; Fox et al., 2007; Kurusu et al., 2008; McLaughlin and Leary, 2005; McLaughlin et al., 2014; Shen et al., 2004; Sieburth et al., 2005). It is likely, however, that neocortical neurons have adopted general strategies, in terms of utilizing attractive and repulsive cues, that are similar to those employed in these systems to establish appropriate connectivity (Sanes and Zipursky, 2010).

There is accumulating evidence that molecules initially identified for their role in axon guidance also are involved directly in synapse formation (Sanes and Yamagata, 2009; Zipursky and Sanes, 2010). For example, EphA/Ephrin signaling contributes to the synaptic specificity of retinotectal projections by matching gradients of ligand and receptor (Flanagan, 2006). Semaphorin/plexin interactions have also been implicated in mediating synaptic specificity in multiple neuronal circuits (Ding et al., 2011; Fukuhara et al., 2013; Low et al., 2008; Pecho-Vrieseling et al., 2009). Early studies showed that Plexin-D1 mediates repulsive interactions during axon path-finding and sensory afferent connectivity with spinal motor neurons, with this response mediated by Sema3E (Chauvet et al., 2007; Pecho-Vrieseling et al., 2009). Similarly, Sema3E/Plexin-D1 inhibits dorsal thalamic input onto striatal medium spiny neurons (Ding et al., 2011). Together, these data suggest that Plexin-D1 interactions inhibit the formation of inappropriate synapses. In the rodent neocortex, Plexin-D1 and Sema3E are expressed in a complementary patterns (Watakabe et al., 2006). Specifically, Plexin-D1 is expressed by neurons located in layers II/III and Va, and Sema3E by layer Vb and VI neurons, suggesting that interactions between Plexin-D1 and Sema3E might contribute to establishing specificity of interlaminar synapse formation within the neocortex. In order to first determine whether Plexin-D1 can actively regulate synapse development in the neocortex, we used a primary neuronal culture system to examine the function of Plexin-D1 in neocortical synapse formation. The analysis of such neurobiological material can be labor-intensive because of the need to manually determine elements light microscopically as synapses. Thus, to facilitate analysis of synapse number, we developed an automated macro plug-in for Image J that we validated through comparison of data collected by the automated program and manual analysis. Surprisingly, the developmental analysis found that, in contrast to other brain regions, Plexin-D1 facilitates, rather than inhibits, synapse formation by neocortical neurons *in vitro*. The data suggest that

cell type-specific outcomes of Plexin-D1 signaling involved in synapse development may provide a mechanism for meeting circuit-specific requirements to form connections correctly.

2. Results

2.1 Validation of semi-automated synapse analysis

An in vitro assay comprising primary neocortical neurons isolated from embryonic tissue was used to examine the role of the Plexin-D1 receptor in synapse formation. We developed a semi-automated, open access method, which, when used in combination with Image J, facilitated the analysis of synapse density on GFP-transfected neurons *in vitro*. A subfield of the proximal dendritic tree of GFP-labeled neurons (Fig. 1A) was imaged sequentially using three-channel imaging (synapsin-1, PSD-95 and GFP). For each fluorescent channel, a signal threshold was determined manually and applied to all images from a single culturing session. For each image, the custom Image-J plug-in first counted the number of PSD-95 clusters that have congruent signal with GFP-labeled dendrites. Those PSD-95 clusters that co-localized with synapsin-1 were then defined as a synapse (Fig. 1B, C). Manual counts of synapse number on GFP-labeled dendrites in images of 10 randomly selected neurons validated the automated analysis ($r^2=0.98$; Fig. 1D).

2.2 *plxnd1* overexpression increases synapse density in neocortical neurons

In the spinal cord and striatum, Plexin-D1 inhibits synapse formation (Ding et al., 2011; Fukuhara et al., 2013). To determine whether Plexin-D1 has a similar role in the neocortex, primary cultures of neocortical neurons from wild type mice were transfected prior to the start of synaptogenesis (5 days in vitro [DIV]) with human *plxnd1* cDNA or a control plasmid. Immunostaining for Plexin-D1 shows that the *plxnd1* cDNA resulted in increased expression of the protein compared to untransfected neurons in the same culture (Fig. 2). The effects of increased expression of Plexin-D1 on synapse density was determined just after the peak of synaptogenesis (14 DIV), with data obtained from 3 independent culturing sessions. A multiple regression analysis was used to explore the relationship between synapse density and Plexin-D1 expression, with treatment and culture session as predictor variables. Session was not predictive of outcome, but treatment was found to be statistically significant [$F = 15.46$; $p < 0.001$], indicating that the level of Plexin-D1 expression correlated with synapse density. Specifically, overexpression of Plexin-D1 led to an increase in synapse formation by neocortical neurons in vitro (Fig. 3A, B; control, 2.06 [95% CI: 1.45, 2.61]; *plxnd1* overexpression, 3.07 [95% CI: 2.43, 3.75]).

2.3 Reduced Plexin-D1 expression decreases synapse density in neocortical neurons

Based on our overexpression data, we predicted that decreased expression of Plexin-D1 would lead to a decrease in synaptic density. In the next set of studies, we therefore reduced expression of Plexin-D1 in cultures of neocortical neurons using both knockdown and knockout approaches. For both approaches, a multiple linear regression analysis was performed to explore the relationship between synapse density as the outcome, with treatment and experiment session as predictor variables. For knockdown experiments, neurons from wild type mice were transfected at 5 DIV with either *plxnd1* shRNA or vector

alone and synapse density determined at 14 DIV, with data obtained from 2 independent culturing sessions. Prior to these experiments, the efficacy of four *plxnd1* short-hairpin (sh)RNAs in reducing *plxnd1* expression had been determined following transfection of Neuro2A neuroblastoma cells, which express *plxnd1*, using quantitative RT-PCR. Despite similar transfection efficiencies, knockdown of *plxnd1* varied across the shRNA constructs (Fig. 4). The most efficient *plxnd1* shRNA - #75, which produced a ~70% reduction in transcript, was then used in all subsequent experiments. Again, session was not predictive of outcome. The multiple regression analysis was found to be statistically significant [$F = 12.50$; $p < 0.001$]. Synaptic density was reduced by approximately 50% following *plxnd1* shRNA transfection compared to vector alone ($p < 0.01$; Fig. 5A, B; control, 3.36 [95% CI: 2.75, 4.30]; *plxnd1* shRNA, 1.60 [95% CI: 1.24, 2.07]). Knockdown mediated by shRNA, however, can be confounded by off-target effects (Jackson et al., 2006). In a second approach, we deleted *plxnd1* in select neurons in primary neocortical cultures derived from *Plxnd1^{fx/fx}* mice, with data obtained from 3 independent culturing sessions. At 5 DIV, neurons were transfected with 1) GFP alone (control), 2) GFP and *cre* (*plxnd1* knockout), or 3) GFP, *cre*, and *hPlxnd1* cDNA (rescue). Immunostaining confirmed expression of Cre recombinase in transfected neurons (data not shown). At 14 DIV, consistent with the knockdown experiments, culturing session was not a predictive variable, and the multiple regression analysis was found to be statistically significant for treatment ($F=12.63$, $p < 0.001$). There was a significant reduction in synaptic density in neurons in which *plxnd1* had been deleted compared to control neurons ($p < 0.01$; Fig. 6A, B; control, 1.74 [95% CI: 1.37, 2.24]; *plxnd1* knockdown, 0.67 [95% CI: 0.52, 0.89]). Co-transfection with *hPlxnd1* cDNA rescued the phenotype, with synapse density statistically indistinguishable from that observed in control neurons ($p = 0.692$, Fig. 6A, B; rescue, 1.52 [95% CI: 1.17, 1.84]). Together with the overexpression data, these experiments indicate that Plexin-D1 signaling has a positive modulatory effect on synapse formation in neocortical neurons.

3. Discussion

In the present study, we use complementary overexpression and knockdown/knockout approaches, combined with a newly-developed automated analysis of synapse number, to show that increasing levels of Plexin-D1 expression positively influence the number of synapses formed on neocortical neurons *in vitro*. Together with previous studies, which have demonstrated that Plexin-D1 signaling negatively regulates synapse formation in the spinal cord and striatum (Ding et al., 2011; Fukuhara et al., 2013; Pecho-Vrieseling et al., 2009), our data suggest that Plexin-D1 can mediate bifunctional outcomes in regulating synapse formation, similar to that previously described for axon guidance (Chauvet et al., 2007).

3.1 Semi-automated synapse analysis

There are some important methodological considerations with respect to the use of our semi-automated analysis of synapse density. 1) This analysis requires that the entire dendritic tree of discrete neurons is labeled by a synapse-independent marker, such as transfected GFP, and that the dendritic trees of labeled neurons that are analyzed for synapse density do not overlap. In the current study, we achieved this by cotransfection of GFP under low-efficiency transfection conditions, which typically resulted in transfection of between 20 –

30 neurons out of several thousand per coverslip. Similar analyses could be applied to cultures established from mice in which discrete neuron subpopulations are labeled *in vivo*, such as reporter- or GFP-labeled transgenic lines. 2) When using the plug-in analysis in combination with knock-down or overexpression of proteins of interest in the labeled cell, analysis is limited to the effect on the postsynaptic neuron. 3) Although both the dendritic and axonal compartments are labeled following transfection with GFP, the fluorescent intensity in axons is much lower than that in dendrites. Thus, by setting the threshold in the GFP channel at the appropriate level, only GFP signal from the dendritic compartment is included in the analyses. 4) After establishing the appropriate size and signal intensity criteria, the automated counts closely match those obtained by counting manually. However, the number of images analyzed in a given time frame is approximately 3-4 times greater using the plug-in. 5) The plug-in is only appropriate for analysis of synapse density *in vitro*. Similar analyses *in vivo* require more sophisticated image-analysis programs, including deconvolution of captured images.

3.2 Regulation of synapse formation by Plexin-D1

Our results suggest that Plexin-D1 signaling in the neocortex plays a supportive role in synapse formation that may be essential for controlling quantitative aspects of cortical connectivity. Plexin-D1 is expressed transiently at high levels in the cortex during early embryonic development (Chauvet et al., 2007; Deck et al., 2013), and protein can be detected on growing axons. Plexin-D1 signaling during this developmental window is thought to influence axon guidance of discrete corticofugal projections, as well as underlying the waiting period of corticothalamic axons in the internal capsule (Chauvet et al., 2007; Deck et al., 2013). Plexin-D1 expression again is present perinatally in rodents and continues to be expressed over the early postnatal period, when cortical circuitry is established (Molyneaux et al., 2009). At this time, Plexin-D1 is preferentially expressed in callosal, rather than corticofugal, neurons, which express the receptor prenatally during pioneer path formation (Deck et al., 2013). Thus, postnatally, later-developing intra- and intercortical connectivity may be mediated positively through Plexin-D1 signaling.

The current study does not address the specific ligand that is interacting with Plexin-D1 in the neocortex. Thus far, the principal ligand identified for Plexin-D1 during axon guidance and synaptic recognition is Sema3E (Ding et al., 2011; Fukuhara et al., 2013; Gu et al., 2005; Oh and Gu, 2013; Pecho-Vrieseling et al., 2009). Sema3E/Plexin-D1 interactions are also important during angiogenesis, principally through their action on endothelial cell migration (Kim et al., 2011; Oh and Gu, 2013). In this system, however, additional ligands for Plexin-D1 have been identified, including other secreted Sema3 ligands or the membrane-bound Sema4A (Bellon et al., 2010; Meda et al., 2012; Toyofuku et al., 2007). These ligands have been mapped in the mouse forebrain during development and, except for Sema3E, do not appear to be expressed in the developing cortex during early postnatal development (P0 – P21; Allen Brain Atlas, <http://developingmouse.brain-map.org/>). This suggests that, in the context of the experiments reported here, Sema3E is the likely ligand for Plexin-D1 on neocortical neurons.

Recent studies in the spinal cord and striatum demonstrate that Plexin-D1/Sema3E signaling inhibits inappropriate synapse formation, thus contributing to synapse specificity during circuit formation (Ding et al., 2011; Fukuhara et al., 2013; Pecho-Vrieseling et al., 2009). We provide evidence that Plexin-D1 has a bifunctional role in synapse development, exhibiting a positive modulatory role in certain cell contexts. Similarly, during axon guidance, Plexin-D1/Sema3E mediates either repulsive or attractive signaling, depending upon the expression of the neuropilin I co-receptor (Chauvet et al., 2007). It is unlikely, however, that neuropilin 1 acts as a switch between positive and inhibitory influences in synapse formation, as mapping studies suggest that it is not expressed in the mouse neocortex over the period of synapse formation (Allen Brain Atlas, <http://developingmouse.brain-map.org/>).

The molecular mechanisms and specific signaling that underlie the effects of Plexin-D1 on synapse formation remain unknown. It has previously been postulated, however, that Plexin-D1/Sema3E signaling may influence synapse formation differently, depending upon whether Plexin-D1 is expressed pre- or post-synaptically (Ding et al., 2011; Fukuhara et al., 2013). In the spinal cord, where Sema3E released by postsynaptic cells modulates the ability of incoming Plexin-D1-expressing axons to form synapses on select neuron populations (Fukuhara et al., 2013; Pecho-Vrieseling et al., 2009), Plexin-D1 signaling likely has a direct effect on synapse formation. In contrast, the current study examines the effects of manipulating postsynaptic expression of Plexin-D1, similar to that reported in the striatum (Ding et al., 2011). Fukuhara and colleagues (Fukuhara et al., 2013) hypothesized that, in this context, the effect of Plexin-D1 signaling may be indirect, potentially “through gating or induction of other recognition systems”. A similar mechanism has been demonstrated in the developing vascular system, where Plexin-D1/Sema3E signaling negatively regulates VEGF-induced Dll4-Notch signaling to influence sprouting and branching of growing vessels (Kim et al., 2011). Thus, the different response of Plexin-D1-expressing thalamic and neocortical neurons may reflect the activation and/or repression of distinct signaling pathways in each population.

What is the role of Plexin-D1 signaling in cortical circuit development *in vivo*? Given the complementary expression pattern of Plexin-D1 and Sema3E in upper and lower layers in neocortex (Watakabe et al., 2006), it is likely that these molecular partners play an important role in the formation of short range and long range intracortical and intercortical synapses. Confirmation of this will require detailed electrophysiological and quantitative ultrastructural studies.

4. Materials & Methods

4.1 Animals

Timed pregnant C57BL/6^J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The *Plxnd1*-floxed mouse (B6.129-Plxnd1^{tm1.1Tmj/J}; (Gu et al., 2005)) was obtained from Dr. Yutaka Yoshida (Cincinnati Children's Hospital Medical Center). The primer sequences for genotyping are: D1-lox-1 5'-ACAGGTGTGTGCTCAAGGCCACCTC-3' and D1-lox-5 5'-CAGCCCTATAGTTCTCCACCAAAGA-3'. All research procedures using mice were approved by the Institutional Animal Care and Use Committee at the University

of Southern California and at Children's Hospital Los Angeles and conformed to NIH guidelines.

4.2 Plasmid and viral constructs

Human *Plxnd1* cDNA, which shares over 80% sequence similarity with mouse *plxnd1* cDNA, was obtained from Dr. Jonathan A. Epstein (University of Pennsylvania) and subcloned into a PCAGGS vector. A V5-epitope tag was added to the amino terminus of the *Plxnd1* coding sequence. PCAGGS-Cre and PCAGGS-eGFP constructs were obtained from Dr. Le Ma (University of Southern California). Four distinct *plxnd1* short-hairpin (sh)RNAs were purchased from Sigma (St Louis, MO). The efficiency of each *plxnd1* shRNA was tested using quantitative RT-PCR (qPCR) following transfection of each construct into Neuro2A neuroblastoma cells, which express endogenous *plxnd1*. Total RNA was isolated from N2A cell pellets using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. cDNA was synthesized with the Roche Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Indianapolis, IN) using oligo dT primers, according to the manufacturer's protocol. qPCR was done in triplicate for each sample using the Power SYBR Green PCR master mix (Life Technologies, Carlsbad, CA) and target-specific primers according to the manufacturer's recommendations. Assays were then analyzed using a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA). The delta cycle threshold (Ct) of target relative to *ACTB* was averaged per sample. The following primers are used: mouse *Plxnd1*: 5'-TGAACACACTGGCCCACTACAAGA-3' and 5'-TGTCCAAGTCTTTCACTCTGCCCA-3'; mouse *Actb*: 5'-CAACGAGCGGTTCCGATG-3' and 5'-GCCACAGGATTCCATACCCA-3'; mouse *Gapdh*: 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-TTGCTGTTGAAGTCGCAGGAG-3'.

4.3 Dissociated neocortical neuron cultures, plasmid transfection and immunohistochemistry

Primary cultures of neocortical neurons were established as described previously from our laboratory (Eagleson et al., 2013), using whole cerebral hemispheres (without the hippocampus and olfactory bulb) harvested from embryonic day (E) 18.5 mice. For each culturing session, tissue from a single litter, including male and female embryos, was pooled. At 5 DIV, neurons were transfected using the Clontech CalPhos Mammalian Transfection Kit (Clontech Laboratories Inc, Mountain View, CA) following a published protocol (Jiang and Chen, 2006). GFP plasmid was co-transfected to visualize transfected neurons. At 14 DIV, the cultures were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min, washed three times with PBS, permeabilized with PBS-0.1% Triton X-100 (PBST) for 10 min, and blocked for 30 minutes with 5% fetal bovine serum in PBST (blocking buffer). Neurons were incubated with primary antibodies in blocking buffer for 2 hours, washed three times with PBS, and then incubated with secondary antibodies for 1 hour. Following three washes with PBS, cells were mounted on slides with Prolong Gold Antifade Reagent (Life Technologies). All washes and incubations were at room temperature. For primary antibodies, we used: chicken anti-GFP antibody (1:500; Abcam, Cambridge, MA.), rabbit anti-Synapsin I antibody (1:200; Millipore), mouse anti-PSD95 antibody (1:100; Millipore), mouse anti-V5 antibody (1:200; Life Technologies), goat anti-

PlexinD1 antibody (1:50; R&D), and mouse anti-Cre antibody (1:500; Sigma). The following Alexa Fluor-conjugated secondary antibodies were used (all at 1:1000; Life Technologies): goat anti-chicken 488, goat anti-rabbit 543, and goat anti-mouse 633.

4.4 Confocal imaging and analysis

Before imaging, coverslips were coded so that the individual performing the quantitative analysis was blind to treatment. For each culture session, transfected neurons with a pyramidal morphology were chosen randomly in each treatment group for analysis and a subfield of the proximal dendritic tree imaged. Images were acquired sequentially using triple-channel (488, 543 and 633) imaging on an Olympus FV-1000 confocal microscope (Olympus America, Center Valley PA) with a 60X oil immersion objective and 1.5X optical zoom. The imaging parameters were adjusted to maintain the signal level within dynamic range and parameters (0.06 to 2 μm^2), which remained unchanged for all images collected. All images collected from a single culture session were imported simultaneously into Image J, converted into TIFF format, then coded and randomized. Images were analyzed using a custom plug-in for Image J software developed in our laboratory (available for free download at: <https://github.com/Pat-Levitt/SynapseCounter>). The density of synapses per neuron was calculated by dividing the synapse number by the area of the dendritic tree analyzed.

4.5 Data analysis

Data were collected from three independent culturing sessions, with the exception of the shRNA study (2 sessions). Sample size varied between studies and is indicated in the figure legends. Because the data were not normally distributed, a nonparametric test was used to determine significance. Values are reported as median, with 95% confidence interval in parentheses. The original data were transformed into ranks. Multiple linear regression analysis was used to model the relation between the outcome, synapse density, and predictor variables of interest, treatment and experiment session. No data were excluded from the analyses. This model accounted for any potential differences in calculated synaptic density values across different experiment sessions. A post-hoc analysis (Tukey test) was used to compare differences between treatment groups. Statistical analyses were performed with the SAS software package (Version 9.3).

4.6 Digital illustrations

Images of primary neurons were acquired using an Olympus Fluoview FV1000 confocal microscope. Graphs were prepared in GraphPad Prism (Version 5.01, GraphPad Software, La Jolla, CA). Images and graphs were converted to TIFF format and imported into Adobe Photoshop CS5 (v. 12.1), where they were cropped, and adjusted for brightness and contrast for preparation of final figures.

Acknowledgements

We thank members of the Levitt laboratory for critical discussions and comments on the studies. This research was supported by the National Institute of Mental Health (R01 MH067842).

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Highlights

- New semi-automated plug-in for quantitative analysis of synapse formation by cultured neurons
- Plexin-D1 signaling can mediate positive aspects of synapse formation in neocortical neurons
- Adjusting levels of Plexin-D1 expression are predictive of increases or decreases in synapse formation

Figure 1.

Semi-automated analysis of synapse density on GFP-transfected neurons. A portion of the proximal dendritic tree of individual GFP-labeled neurons was imaged (A), and the resulting confocal image pseudocolored: GFP, blue; PSD95, red; synapsin-1, green. Schematic diagram (B) and photomicrograph (C) illustrating the criteria used to classify a synapse on a GFP-labeled dendrite (orange arrows – congruent PSD95 and GFP signal, with at least partial overlap of synapsin-1 and PSD95). Congruent PSD95 and GFP signal (olive arrows) or PSD95 and synapsin-1 signal (light blue arrows) could also be observed, but were not included in the synapse count. (D) Comparison between automated and manual counts of the number of synapses associated with GFP-labeled dendrites in images from 10 neurons. The black line represents the simple linear regression model and the red lines represent the 99% confidence intervals. Scale bar: 10 μ m.

Figure 2.

Transfection of *Plxnd1* cDNA at 5 DIV results in increased expression of Plexin-D1 at 14 DIV. Confocal images illustrating immunostaining of neurons at 14 DIV following *Plxnd1* cDNA over-expression at 5 DIV: white arrowhead, transfected neuron; yellow arrowhead, non-transfected neuron. (A) Transfected neurons could be visualized by the co-transfection of GFP. (B) Plasmid-encoded protein, identified by the V5 epitope tag, was only observed in transfected neurons. (C) Note the increased expression of Plexin-D1 in

transfected, compared to neighboring untransfected, cells. A merged image of all three channels is illustrated in (D). Scale bar: 50 μm .

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Figure 3.

Overexpression of Plexin-D1 increases synaptic density on neocortical neurons *in vitro*. (A) Confocal images illustrating GFP (blue), synapsin-1 (green), PSD95 (red) and merged signal at 14 DIV following transfection with GFP alone (control, left panels) or GFP and *plxnd1* cDNA (right panels) at 5 DIV. Scale bar: 10 μ m. (B) Quantification of synapse density at 14 DIV reveals a significant increase in synapse density in *plxnd1* cDNA-transfected compared to control neurons. N = 115 neurons from 3 independent culturing sessions, ** $p < 0.01$.

Figure 4.

Efficiency of knock-down of *plexind1* expression in N2A cells. Four *plexind1* shRNAs (#74 – 77) were transfected into N2A cells and *plexind1* mRNA levels quantified by q-PCR. Vector alone (Vect) and scrambled shRNA (Scr) were used as controls. Note that shRNA #75 had the largest effect (arrow) and was used in subsequent experiments.

Figure 5. Knock-down of Plexin-D1 expression decreases synaptic density on neocortical neurons in vitro. (A) Confocal images illustrating GFP (blue), synapsin-1 (green), PSD95 (red) and merged signal at 14 DIV following transfection with GFP and shRNA vector (control, left panels) or GFP and *plxnd1* shRNA (right panels) at 5 DIV. Scale bar: 10 μm . B) Quantification of synapse density at 14 DIV reveals a significant decrease in synapse density in *plxnd1* shRNA-transfected compared to control neurons. N = 94 neurons from 2 independent culturing sessions, ** $p < 0.01$.

Figure 6.

Genetic knockout of *plxnd1* expression decreases synaptic density on neocortical neurons *in vitro*. (A) Confocal images illustrating GFP (blue), synapsin-1 (green), PSD95 (red) and merged signal at 14 DIV following transfection at 5 DIV with GFP (control, left panels), GFP and cre recombinase (middle panels), or GFP, cre recombinase and *plxnd1* cDNA (right panels) into neurons isolated from *Plxnd1*-floxed mice. Scale bar: 10 μm . B) Quantification of synapse density at 14 DIV reveals a significant decrease in synapse density in knock-out compared to control neurons. Transfection of *plxnd1* cDNA into knock-out neurons rescued synapse density. N = 165 neurons from 3 independent culturing sessions, **p < 0.01.