Ephrin-B1 and ephrin-B2 mediate EphB-dependent presynaptic development via syntenin-1

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The development of central nervous system synapses requires precise coordination between presynaptic and postsynaptic components. The EphB family controls postsynaptic development by interacting with glutamate receptors and regulating dendritic filopodia motility, but how EphBs induce the formation of presynaptic specializations is less well understood. Here, we show that knockdown of presynaptic ephrin-B1, ephrin-B2, or syntenin-1, but not ephrin-B3, prevents EphB-dependent presynaptic development. Ephrin-B1, ephrin-B2, and syntenin-1 are clustered together with presynaptic markers, suggesting that these molecules function jointly in presynaptic development. Knockdown of ephrin-B1 or ephrin-B2 reduces the number of synaptic specializations and the colocalization of syntenin-1 with synaptic markers. Simultaneous knockdown of ephrin-B1 and ephrin-B2 suggests that they function independently in the formation of synaptic contacts, but act together to recruit syntenin-1 to presynaptic terminals. Taken together, these results demonstrate that ephrin-B1 and ephrin-B2 function with EphB to mediate presynaptic development via syntenin-1.

coculture | cortex | Eph | excitatory synapse | synaptogenesis

The EphB family of receptor tyrosine kinases directs postsynaptic development by interacting with NMDA- and AMPA-type glutamate receptors, controlling dendritic filopodia motility, and regulating spine formation (1–3). EphBs can also signal *trans*-synaptically to induce presynaptic development, suggesting that EphB receptors are capable of coordinating the development of both presynaptic and postsynaptic compartments (2). However, the mechanisms by which EphBs induce presynaptic development are not well described. The ligands for EphBs are the ephrin-Bs, a family of three (ephrin-B1–B3) transmembrane molecules that, in addition to "forward" signaling through the activation of EphBs, can also signal in the "reverse" direction through intracellular phosphotyrosines and a C-terminal PDZ-binding domain. Ephrin-Bs have recently been shown to regulate presynaptic development in the *Xenopus* tectum (4) and are expressed in mouse cortex (5–8). Although it is thought that ephrin-Bs might have unique functions at the synapse (9–11), whether particular ephrin-Bs interact with postsynaptic EphBs to regulate synapse development in the mammalian CNS, and what the downstream mechanisms are that mediate this process, are not known.

The syntenin family consists of two (syntenin-1 and syntenin-2) tandem PDZ domain-containing proteins implicated in a number of cellular processes such as trafficking, signaling, and cancer metastasis (12). Initially identified as binding partners for the heparan sulfate proteoglycan syndecan (13), syntenins are comprised mainly of two PDZ domains that enable self-association and interactions with a number of synaptically localized transmembrane molecules such as glutamate receptors, β-neurexin, SynCAM, and ephrin-Bs (14–19). In addition, syntenin-1 may regulate the organization of presynaptic active zones through interactions with the ERC/CAST family of active zone molecules (20).

Here, we show that two members of the ephrin-B family (ephrin-B1 and ephrin-B2) function to mediate EphB-dependent presynaptic development via PDZ-binding domain-dependent interaction with syntenin-1. Simultaneous knockdown of ephrin-B1 and ephrin-B2 suggest that these molecules function independently in the formation of synapses, but function together in the localization of syntenin-1 to synaptic specializations.

Results

Ephrin-B Family Members Are Required for EphB2-Dependent Presynaptic Development. A presynaptic role for ephrin-Bs has been suggested by the finding that EphB-expressing non-neuronal cells can induce presynaptic development (2). To determine whether EphB-dependent presynaptic induction is mediated by specific presynaptic ephrin-B family members, we asked whether non-neuronal cells expressing EphB2 could induce presynaptic specializations when ephrin-B expression is reduced in axons by RNAi-mediated knockdown. We generated constructs encoding 19-nt shRNAs targeting individual ephrin-B family members and confirmed that these constructs were capable of reducing the expression of the target molecule (Fig. 1*F* and Fig. S1). We transfected shRNA constructs into days in vitro (DIV) 3 cortical neurons along with a GFP-tagged version of the presynaptic vesicle marker synaptophysin (syn-GFP) to label transfected axons. At DIV9, transfected neurons were cocultured with HEK293T cells expressing either FLAG epitope-tagged EphB2 (fEphB2) or red fluorescent protein (RFP) and fixed 16–18 h later. Because our transfection efficiency in neurons was low (<1%), expression of syn-GFP revealed easily identifiable stretches of axons with discrete puncta of syn-GFP that colocalized with the excitatory presynaptic marker VGlut1. Labeled HEK293T cells were scattered throughout the culture and occasionally found to be contacting a syn-GFP-expressing axon. To determine the effect of transfected HEK293T cells on presynaptic development, we compared the linear density of syn-GFP in the stretch of axon contacting the HEK293T cells to the density in the adjacent axon region (see *SI Text*).

In control neurons coexpressing syn-GFP with the shRNA vector control, the density of syn-GFP in axon regions contacting RFP-expressing HEK293T cells was similar to that in adjacent regions, resulting in a density ratio near 1.0 (Fig. 1 *A* and *B*). However, consistent with our previous findings (2), syn-GFP puncta density increased by ≈1.5-fold underneath EphB2-expressing HEK293T cells (Fig. 1 *A* and *B*). These results confirm that EphB2-expressing HEK293T cells can induce presynaptic differentiation in segments of single axons.

To test whether this process is mediated by presynaptic ephrin-Bs, neurons were cotransfected with syn-GFP and shRNA constructs targeting each ephrin-B family member (Fig. 1*F* and Fig. S1). In axons from neurons transfected with shRNA targeting ephrin-B3, HEK293T cells expressing EphB2 caused a significant increase in syn-GFP density similar to that seen in control neurons (Fig. 1 *A*

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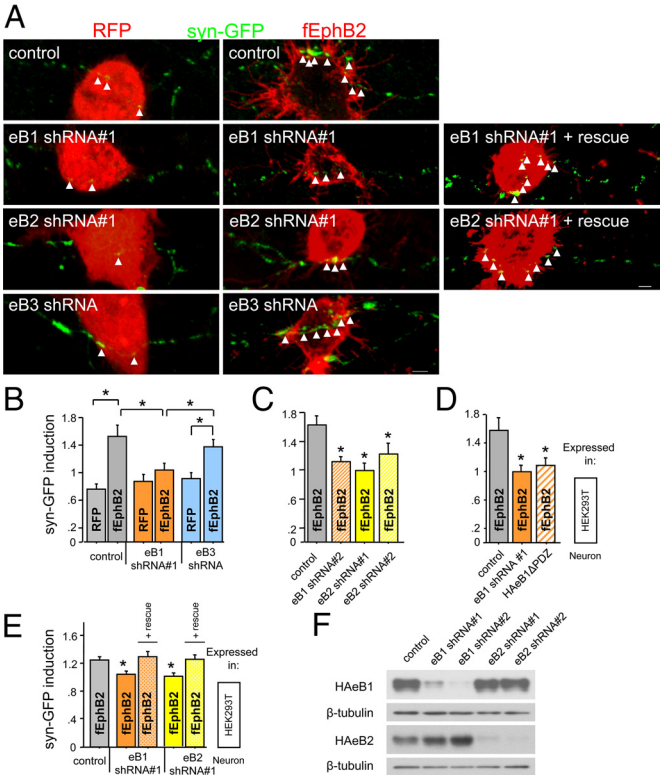

Fig. 1. Ephrin-B1 and ephrin-B2 are required for EphB2-dependent presynaptic development. (*A*) Representative images of DIV10 cortical neuron axons transfected with syn-GFP and shRNA constructs at DIV3 and cocultured with HEK293T cells transfected with RFP or FLAG-EphB2 (fEphB2). Arrowheads indicate syn-GFP puncta colocalized with HEK293T cells. (Scale bar: 3 μm.) (*B*) Quantification of fold increase in syn-GFP puncta density in axon segments contacting HEK293T cells expressing fEphB2 or control constructs compared with adjacent axon segments (syn-GFP induction) for neurons transfected with indicated shRNA constructs: vector control (RFP: $n = 19$; fEphB2: $n = 29$), ephrin-B1 shRNA#1 (RFP: $n = 14$; fEphB2: $n = 29$), ephrin-B3 shRNA (RFP: $n = 21$; fEphB2: $n = 30$). (*C*) Quantification of axonal syn-GFP induction: vector control ($n = 40$), ephrin-B1 shRNA#2 ($n = 47$), ephrin-B2 shRNA#1 ($n = 24$), or ephrin-B2 shRNA#2 ($n = 20$). (*D*) Quantification of axonal syn-GFP induction: vector control ($n = 25$), ephrin-B1 shRNA#1 ($n = 26$); HA-ephrin-B1ΔPDZ ($n = 24$). (*E*) Quantification of axonal syn-GFP induction: vector control ($n = 50$), ephrin-B1 shRNA#1 ($n = 25$), ephrin-B1 shRNA#1 + rescue ($n = 27$), ephrin-B2 shRNA#1 ($n = 30$), or ephrin-B2 shRNA#2 + rescue ($n = 28$). (*F*) Western blots of lysates from HEK293T cells transfected with HA-ephrin-B1 or HA-ephrin-B2 plus vector control, ephrin-B1 shRNA#1, ephrin-B1 shRNA#2, ephrin-B2 shRNA#1, or ephrin-B2 shRNA#2 and probed for HA and β-tubulin. Error bars indicate SEM. *, $P < 0.04$.

and *B*). However, in axons from neurons expressing shRNA targeting ephrin-B1 or ephrin-B2, HEK293T cells expressing EphB2 failed to induce presynaptic vesicle clustering (Fig. 1 *A*–*C*). To confirm that the effect of these shRNAs are specific, we determined that blockade of EphB2-induced presynaptic differentiation can be rescued by coexpressing ephrin-B1 or ephrin-B2 shRNAs with constructs encoding the appropriate molecule rendered insensitive to knockdown (Fig. 1 *A* and *E*). These results suggest that EphB-dependent presynaptic differentiation is controlled by ephrin-B1 and ephrin-B2.

Presynaptic assembly is mediated in part by protein–protein interactions with multidomain scaffolding molecules, many of which contain multiple PDZ domains (21). To test whether the ephrin-B PDZ-binding domain is required for EphB-dependent presynaptic development, we coexpressed syn-GFP with HA-tagged ephrin-B1 lacking the PDZ-binding domain (HAeB1ΔPDZ) in DIV3 neurons. Because the known intracellular signaling domains are highly conserved, overexpression of intracellular mutants such as HAeB1ΔPDZ are thought to act as dominant negatives and block PDZ-binding domain-dependent signaling through all ephrin-B subtypes (22, 23). In DIV9 axons coexpressing HAeB1ΔPDZ and syn-GFP, EphB2-expressing HEK293T cells failed to induce an increase in syn-GFP density similar to that seen with knockdown of ephrin-B1 or ephrin-B2 (Fig. 1*D*). These results indicate that, similar to other molecules that mediate presynaptic development (1, 24), EphB-dependent presynaptic differentiation likely relies on protein–protein interactions with the ephrin-B PDZ-binding domain.

Syntenin-1 Is Required for EphB-Dependent Presynaptic Development. Interactions between the ephrin-B PDZ-binding domain and the tandem PDZ domain-containing protein syntenin-1 have been demonstrated by GST pull-down (15, 16), yeast two-hybrid assay (14, 20, 25), and X-ray crystallography (26). Because a recent report (20) demonstrated that syntenin-1 participates in the organization of presynaptic terminals through interactions with ERC/CAST family members, we hypothesized that ephrin-B may recruit presynaptic vesicles downstream of EphB by interacting with syntenin-1. To test whether EphB-dependent presynaptic induction is caused by syntenin-1 PDZ domain interactions, we generated a syntenin-1 molecule lacking the second PDZ domain (syntenin-1ΔPDZ2). The second PDZ domain of syntenin-1 is required to bind ephrin-B (15, 16, 20, 26), and we confirmed that syntenin-1ΔPDZ2 cannot bind ephrin-B1 by coimmunoprecipitation. Because this mutant cannot interact with ephrin-Bs, we predicted that it might act in a dominant negative fashion, similar to ephrin-B1ΔPDZ. We found that overexpression of syntenin-1ΔPDZ2 blocked the ability of EphB2-expressing HEK293T cells to induce an increase in syn-GFP in underlying axons (Fig. 2 *A* and *B*), suggesting that EphB-dependent presynaptic recruitment depends on PDZ domain interactions between ephrin-Bs and syntenin-1.

To confirm the role of PDZ proteins in EphB-dependent presynaptic induction, we generated shRNA constructs targeting syntenin-1 and GRIP1 (Fig. 2*D*), a synaptically localized PDZ protein that can also interact with ephrin-B (15, 27). Although GRIP1 is primarily thought to function postsynaptically, GRIP1 protein has also been identified in axons (28). In axons expressing GRIP1 shRNA, EphB2-expressing HEK293T cells induced a significant increase in syn-GFP density, suggesting that GRIP1 is not involved in this process (Fig. 2 *A* and *B*). However, in axons expressing either of two unique syntenin-1-targeting shRNAs, fEphB2-expressing HEK293T cells failed to induce an increase in syn-GFP density (Fig. 2 *A* and *B*). Knockdown of syntenin-1 had no effect on the ability of ephrin-B1 or ephrin-B2 to bind exogenously applied EphB2-Fc, suggesting that ephrin-Bs were still found at the cell surface (Fig. S2). However, syntenin-1 knockdown did cause a decrease in the colocalization of both ephrin-B1 and EphB2-Fc with syn-GFP, consistent with a model in which syntenin-1 links ephrin-Bs to the presynaptic complex (Fig. S2). The effects of syntenin-1 knockdown were rescued by transfecting an shRNA targeting syntenin-1 together with a knockdown-insensitive syntenin-1 molecule (Fig. 2 *A* and *C*), demonstrating that the effects of syntenin-1 shRNA constructs are specific. Taken together, these results demonstrate that EphB-dependent presynaptic development is likely mediated by ephrin-B1 and ephrin-B2, which can recruit presynaptic machinery through PDZ domain interactions with syntenin-1.

Localization of Ephrin-B Subtypes and Syntenin-1 in Cultured Cortical Neurons. To begin to address how ephrin-Bs and syntenin function together to regulate synapse development, we immunostained mature DIV21–30 cultures for ephrin-B1, ephrin-B2, and syntenin-1 along with synaptic markers to determine the distribution of these molecules in cortical neurons. We have previously reported that ephrin-B1 and ephrin-B3 are colocalized with excitatory presynaptic and postsynaptic markers (2). To determine how this

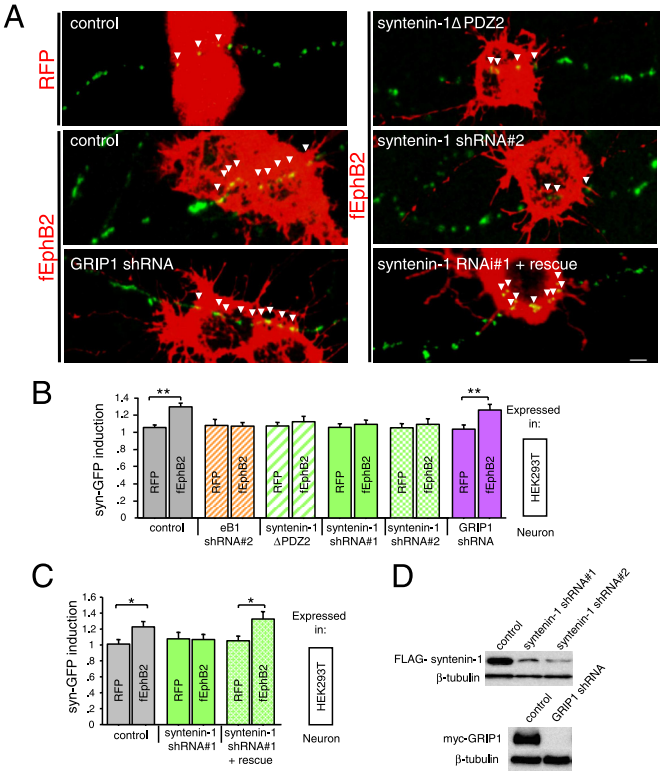

Fig. 2. Syntenin-1 is required for EphB-dependent presynaptic development. (*A*) Representative images of DIV10 cortical neuron axons transfected with syn-GFP and indicated constructs and cocultured with HEK293T cells transfected with RFP or fEphB2. Arrowheads indicate syn-GFP puncta colocalized with HEK293T cells. (Scale bar: 3 μm.) (*B*) Quantification of axonal syn-GFP induction for neurons transfected with the indicated shRNA constructs: vector control (RFP: $n = 76$; fEphB2: $n = 76$), ephrin-B1 shRNA#2 (RFP: $n = 26$; fEphB2: $n = 33$), syntenin-1ΔPDZ2 (RFP: $n = 32$; fEphB2: $n = 35$), syntenin-1 shRNA#1 (RFP: $n = 30$; fEphB2: $n = 29$), syntenin-1 shRNA#2 (RFP: $n = 27$; fEphB2: $n = 30$), or GRIP1 shRNA (RFP: $n = 43$; fEphB2: $n = 47$). (*C*) Quantification of axonal syn-GFP induction: vector control (RFP: $n = 27$; fEphB2: $n = 31$), syntenin-1 shRNA#1 (RFP: $n = 31$; fEphB2: $n = 32$), or syntenin-1 shRNA#1 + rescue (RFP: $n = 27$; fEphB2: $n = 30$). (*D*) Western blots of lysates from HEK293T cells transfected with FLAG-syntenin-1 or myc-GRIP1 plus vector control, syntenin-1 shRNA#1, syntenin-1 shRNA#2, or GRIP1 shRNA and probed for FLAG or myc and β-tubulin. Error bars indicate SEM. **, $P < 0.002$; *, $P < 0.04$.

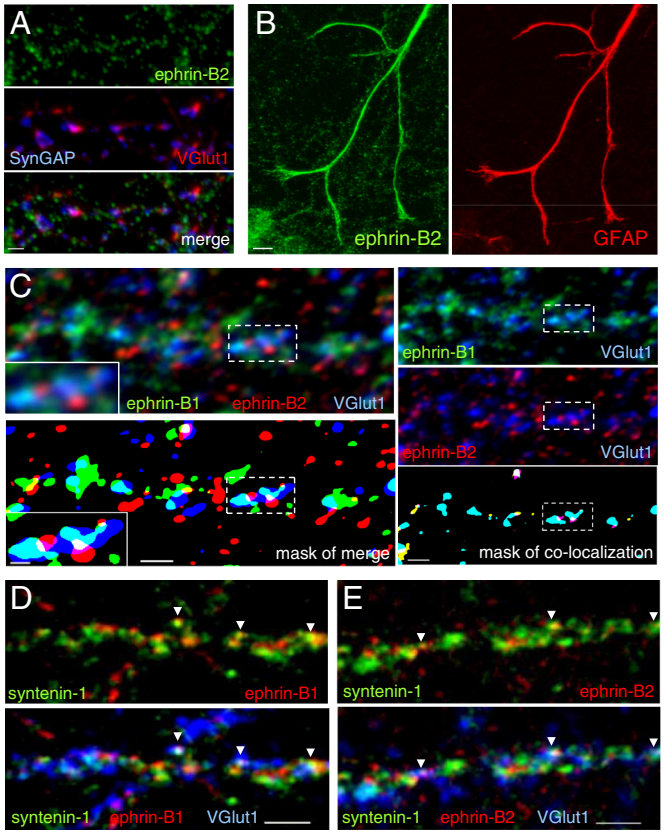

Fig. 3. Localization of ephrin-B1, ephrin-B2, and syntenin-1 in cultured cortical neurons. (*A*) Representative image of DIV21 neurons stained for ephrin-B2 (green), SynGAP (blue), and VGlut1 (red). (Scale bar: 3 μm.) (*B*) Representative image of DIV21 neurons stained for ephrin-B2 (green) and GFAP (red). (Scale bar: 10 μm.) (*C*) Representative image of DIV30 neurons stained for ephrin-B1 (green), ephrin-B2 (red), and VGlut1 (blue). See *SI Text* for staining details. Mask of merge is RGB image created from binary masks of the three individual channels. Image shown is of only the colocalized pixels with all other pixels removed. Colocalization in masks is indicated by: ephrin-B1 and ephrin-B2, yellow; ephrin-B1 and VGlut1, cyan; ephrin-B2 and VGlut1, magenta. (Scale bar: 3 μm.) (*Inset*) High-magnification image of boxed region. (Scale bar: 1 μm.) (*D* and *E*) Representative image of DIV21 neurons stained for syntenin-1 (green), VGlut1 (blue) and either ephrin-B1 (*D*) or ephrin-B2 (*E*) (red). Arrowheads indicate triple colocalization. (Scale bar: 3 μm.)

localization compares for ephrin-B2, we stained DIV21 cortical neurons for ephrin-B2 and the presynaptic and postsynaptic markers VGlut1 and SynGAP (29). Ephrin-B2 staining was found throughout cortical neuron cultures. However, in contrast to the highly synaptic staining observed for ephrin-B1 and ephrin-B3, the pattern of ephrin-B2 staining consisted of smaller puncta, some of which were colocalized with synaptic puncta (Fig. 3*A*). Consistent with a previous report (30), we also observed a few cells with intense ephrin-B2 staining that were positive for the glial marker GFAP (Fig. 3*B*). We next directly compared the synaptic localization of ephrin-B1 and ephrin-B2 by costaining DIV30 cultures for these molecules and VGlut1. Similar to previous observations, ephrin-B1 was highly colocalized with VGlut1 (≈45%), whereas ephrin-B2 was found in small puncta that were also colocalized with VGlut1 (≈23%; see Table S1, Fig. 3*C*, and Fig. S3). In addition, many synaptic puncta colocalized with both ephrin-B1 and ephrin-B2, and there was a significant association of these two molecules at presynaptic sites ($P < 0.0001$; Pearson's χ^2 test) (Fig. 3*C* and Table S1). Interestingly, we often observed several small ephrin-B2 puncta surrounding and adjacent to ephrin-B1-positive VGlut1 puncta (Fig. 3*C Insets*). Thus, both ephrin-B1 and ephrin-B2 colocalize with synaptic markers, but the staining pattern is different from that for each ephrin-B protein.

The ephrin-B PDZ-binding domain can bind the tandem PDZ protein syntenin-1 (14–17, 20, 25, 26), and our findings that knockdown of syntenin-1 blocks EphB-dependent presynaptic development suggests a model in which ephrin-Bs interact with the presynaptic machinery via syntenin-1. To further address the relationship between syntenin, ephrin-Bs, and the presynaptic machinery, we coimmunostained mature DIV21–30 neurons for syntenin-1, the excitatory presynaptic marker VGlut1, and either ephrin-B1 or ephrin-B2. Consistent with previous studies (14, 20), we found that syntenin-1 is localized to presynaptic specializations (Fig. 3 *D* and *E*). In addition, we found that syntenin-1 is enriched at VGlut1-positive presynaptic puncta containing ephrin-B1 or ephrin-B2 ($P < 0.0001$; Pearson's χ^2 test) (Fig. 3 *D* and *E* and Table S1). These results demonstrate that ephrin-B1, ephrin-B2, and syntenin-1 are associated at presynaptic specializations.

Presynaptic Ephrin-B1 and Ephrin-B2 Are Required for the Development of Synapses and Recruitment of Syntenin-1. To test whether ephrin-Bs regulate the formation of synaptic specializations, we examined the

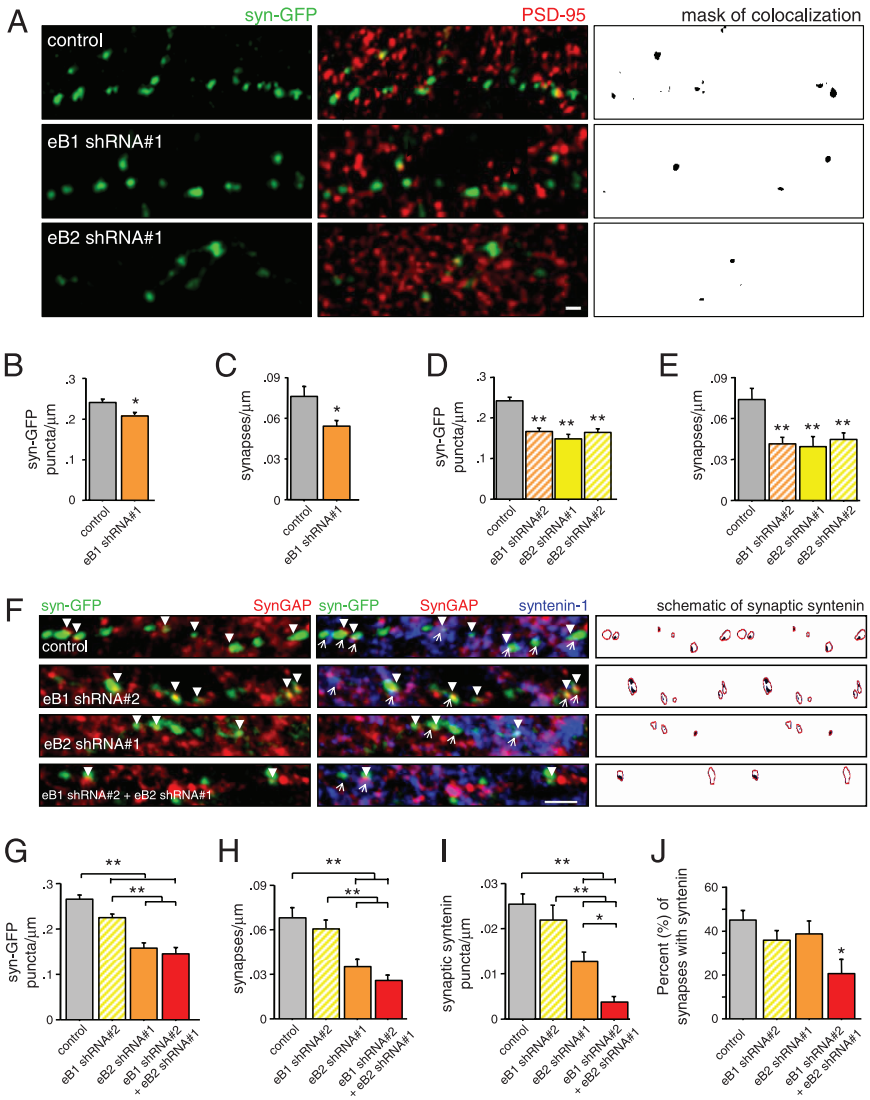

Fig. 4. Presynaptic ephrin-B1 and ephrin-B2 are required for synapse formation and synaptic localization of syntenin-1. (*A*) (*Left* and *Center*) Representative images of DIV21–23 axons transfected at DIV3 with syn-GFP and indicated shRNA construct costained for GFP (green) and PSD-95 (red). (Scale bar: 3 μm.) (*Right*) Mask of colocalization created by identifying colocalized puncta in binary masks from syn-GFP and PSD-95 images. (*B–E*) Quantification of density of syn-GFP puncta (*B* and *D*) and synaptic puncta defined by colocalized syn-GFP and PSD-95 (*C* and *E*) for vector control ($n = 21$) or ephrin-B1 shRNA#1 ($n = 24$) (*B* and *C*) and vector control ($n = 19$), ephrin-B1 shRNA#2 ($n = 19$), ephrin-B2 shRNA#1 ($n = 12$), or ephrin-B2 shRNA#2 ($n = 19$) (*D* and *E*). (*F*) (*Left* and *Center*) Representative images of DIV21 axons transfected at DIV3 with syn-GFP and indicated shRNA construct costained for GFP (green), syn-GAP (red), and syntenin-1 (blue). Arrowheads indicate syanptic puncta identified colocalization of syn-GFP and SynGAP puncta. Arrows indicate colocalized synGAP and syntenin-1 puncta. (Scale bar: 3 μm.) (*Right*) Schematic showing outlines of synaptic syn-GFP puncta and areas of colocalization with syntenin-1 in blue. (*G–J*) Quantification of syn-GFP puncta density (*G*), synaptic puncta identified by colocalization of syn-GFP and SynGAP (*H*), synaptic syntenin-1 (triple colocalized syn-GFP, SynGAP, and syntenin-1) puncta density (*I*), and percentage of synaptic puncta that colocalize with syntenin-1 for neurons transfected with vector control ($n = 30$), ephrin-B1 shRNA2 ($n = 29$), ephrin-B2 shRNA1 ($n = 26$), and ephrin-B1 shRNA2 + ephrin-B2 shRNA1 ($n = 25$) (*J*). Error bars indicate SEM. *, $P < 0.05$; **, $P < 0.003$.

density of synapses in single axons after knockdown of ephrin-B1 or ephrin-B2 in the absence of exogenous stimulation with EphB2-expressing HEK293T cells. EphB-dependent synapse formation occurs between DIV7–14, and robust decreases in synapse number can be seen by knocking down EphB2 from DIV3–21 (3). Therefore, to identify the effects of ephrin-Bs on EphB-dependent synapse development, neurons were cotransfected with ephrin-B shRNA and syn-GFP constructs at DIV3, fixed at DIV21–23, and immunostained for GFP and the postsynaptic marker PSD-95. Synapses were identified as colocalization between syn-GFP and endogenous PSD-95 puncta. Our low transfection efficiencies allowed us to selectively examine the presynaptic role of ephrin-Bs during synapse development. Knockdown of ephrin-B1 or ephrin-B2 with either of two unique shRNA constructs for each led to a significant decrease in the density of both syn-GFP puncta and colocalized synaptic puncta (Fig. 4 *A–E* and Fig. S4). Together, these results demonstrate that reducing the expression of ephrin-B1 or ephrin-B2 results in fewer synapses.

To begin to investigate whether ephrin-B1 and ephrin-B2 function independently in the formation of synaptic contacts, we compared the effects of individual ephrin-B knockdown to simultaneous knockdown of ephrin-B1 and ephrin-B2 in the same axon. This approach is designed to mimic genetic experiments in which a single functional pathway is demonstrated by a more severe phenotype in double hypomorphic mutants than in the single mutants (refs. 31 and 32 and see *SI Text*). To achieve this we used partial shRNA knockdown for both ephrin-B1 and ephrin-B2 in tandem.

To interpret double-knockdown experiments, knockdown of each molecule must be sufficient to create a sensitized background but not to an extent that further changes cannot be observed. Because shRNA-mediated knockdown is incomplete, and the effects we observe on synapse number is partial, it is likely that the effects of single knockdown can be modified by double knockdown. Nevertheless, to ensure that knockdown levels were moderate, we first identified reduced amounts of ephrin-B1 and ephrin-B2 shRNA that generated similar, but decreased, levels of knockdown in non-neuronal cells (Fig. S5 and *SI Text*). The expression of this reduced amount of ephrin-B1 shRNA resulted in a small, but significant, reduction in the number of syn-GFP puncta without a change in the number of colocalized synaptic puncta (Fig. 4 *F–H*), suggesting that this level of ephrin-B1 knockdown creates a sensitized background. The expression of ephrin-B2 shRNA at this reduced level resulted in a significant effect on the number of both syn-GFP puncta and colocalized synaptic puncta, which is also consistent with a sensitized background. Interestingly, the differences between the effects of ephrin-B knockdown suggest that synaptic specializations are more susceptible to changes in the expression level of ephrin-B2 than ephrin-B1. These findings sug-

gest that knockdown of ephrin-B1 and ephrin-B2 with reduced levels of shRNA is suitable for the evaluation of double knockdown.

We next asked whether coexpression of these shRNAs might potentiate the effects on synapse density. When expressed together at these reduced levels, simultaneous knockdown of both ephrin-B1 and ephrin-B2 caused a decrease in synapse density similar to knockdown of ephrin-B2 alone (Fig. 4 *F* and *H*). Thus, the effects of ephrin-B1 and ephrin-B2 shRNAs do not appear to be additive, consistent with ephrin-B1 and ephrin-B2 functioning nonredundantly in the formation of synaptic contacts.

Because syntenin-1 is required for EphB-dependent presynaptic development, and ephrin-Bs are enriched at synaptic specializations containing syntenin-1, we next asked how knockdown of ephrin-Bs specifically affects the localization of syntenin-1 to synapses. To address this question, we expressed ephrin-B1 and ephrin-B2 shRNA, alone or together, and determined the number of synapses that contain syntenin-1. We found that expression of reduced levels of ephrin-B2 shRNA, but not ephrin-B1 shRNA, led to a significant decrease in the number of synaptic puncta containing syntenin-1 (Fig. 4*F*). However, simultaneous knockdown of ephrin-B1 and ephrin-B2 together resulted in a further significant reduction in the number of synapses containing syntenin-1 compared with either ephrin-B1 or ephrin-B2 alone (Fig. 4*F*). These results suggest that both ephrin-B1 and ephrin-B2 are involved in normal syntenin-1 localization, and that ephrin-B1 and ephrin-B2 may function together during the formation of syntenin-1-containing synapses. Because decreases in the density of syntenin-1-containing synapses after ephrin-B knockdown might be caused by the overall loss in synapses number (Fig. 4*H*), we asked how knockdown of ephrin-B1 and/or ephrin-B2 affected the ability of syntenin-1 to localize to the remaining synaptic contacts. For each axon, we determined the proportion of synapses that contain syntenin-1 by dividing the density of syntenin-1-containing synapses (Fig. 4*I*) by the overall density of synaptic contacts (Fig. 4*H*). We found that neither knockdown of ephrin-B1 nor ephrin-B2 led to a decrease in the percentage of synaptic contacts containing syntenin-1 (Fig. 4 *F* and *J*), suggesting that ephrin-B1 and ephrin-B2 each can compensate for the loss of the other at the remaining synapses. However, simultaneous knockdown of ephrin-B1 and ephrin-B2 together resulted in a significant reduction in the percentage of synaptic puncta that contain syntenin-1 (Fig. 4 *F* and *J*). Thus, simultaneous knockdown of both ephrin-B1 and ephrin-B2 results in a synergistic effect on the ability of syntenin-1 to localize to synaptic contacts. Taken together, these results suggest that ephrin-B1 and ephrin-B2 are required for normal numbers of excitatory synapses and appear to function in a partially redundant fashion in the recruitment of syntenin-1 to synaptic specializations.

Discussion

In this study we show that ephrin-B1 and ephrin-B2 are key regulators of EphB-dependent presynaptic development, likely through PDZ domain-dependent interactions with syntenin-1. Ephrin-B1, ephrin-B2, and syntenin-1 colocalize at synaptic contacts, and knockdown of ephrin-B1 or ephrin-B2 leads to a reduction in the number of synaptic contacts. Simultaneous knockdown of both ephrin-B1 and ephrin-B2 suggests that these molecules are required for the synaptic localization of syntenin-1, but function independently in the control of synapse formation. In sum, these results support a model in which excitatory synapse development occurs via a *trans*-synaptic interaction between postsynaptic EphB and specific presynaptic ephrin-Bs (Fig. S6).

Further study will be necessary to elucidate the mechanisms that determine specificity among different ephrin-B family members. Potential mechanisms include differences in signaling, localization, or affinity for EphBs. Differences in signaling or localization could be mediated by domains of the well-conserved juxamembrane regions of ephrin-B1 and ephrin-B2, which diverges in ephrin-B3; affinity differences are possible given that ephrin-B3 has a slightly lower binding affinity for EphBs than ephrin-B1 or ephrin-B2 (33).

To study the role of ephrin-Bs in EphB-dependent presynaptic development, we have developed an assay that allows us to simultaneously manipulate both members of a *trans*-synaptic interaction pair. In previous experiments using coculture assays (34), a single molecule of a potential interaction pair was expressed in heterologous cells, and the *trans*-synaptic binding partner was inferred. In contrast, our modified coculture system allows us to evaluate the effects of molecular interactions between pairs of cells that may occur in vivo. In addition, our assay allows us to study the intracellular events downstream of *trans*-synaptic interactions that induce presynaptic maturation. By coculturing heterologous cells expressing fEphB2 with neurons expressing shRNA constructs targeting ephrin-B family members, we provide direct evidence simultaneously implicating both members of a receptor–ligand pair in the *trans*-synaptic control of synapse formation. These findings are validated by our long-term knockdown experiments.

Presynaptic organization is supported by multidomain scaffolding molecules that regulate both structure and signaling at presynaptic terminals, including the PDZ domain-containing proteins Mint, CASK, Piccolo, RIM, and syntenin-1 (24). Syntenin-1 binds directly to the ephrin-B PDZ-binding domain (14–17, 20, 25, 26) and is linked to presynaptic maturation via ERC2/CAST1 (20). ERC2/CAST1 associates with a number of other presynaptic molecules, including RIM, Piccolo, Bassoon, and liprin-α. RIM1 binds the synaptic vesicle protein Rab3A, and ERC2/CAST1 interacts with RIM1 and Piccolo/Bassoon to regulate synaptic transmission (24). Thus, syntenin-1 provides a directly link by which ephrin-B can associate with a protein complex involved in the recruitment and regulation of presynaptic vesicles (Fig. S6).

The degree to which presynaptic development is mediated by specific interactions between synaptogenic factors and particular scaffolding proteins is not well established. The finding that disruption of syntenin-1 blocks EphB-dependent presynaptic development suggests that presynaptic development can be mediated by specific interactions between ephrin-Bs and syntenin-1. This pathway is likely distinct from those involving other PDZ domain interactions such as that between neurexin and Mint/CASK (24). Thus, these results suggest that presynaptic terminals may be organized by independent pathways.

Knockdown of ephrin-B1 or ephrin-B2 alone disrupts EphB-dependent presynaptic development and results in a decrease in the number of synaptic contacts. To test whether ephrin-B1 and ephrin-B2 function together or independently in synaptogenesis, we transfected neurons with shRNAs targeting both of these proteins to induce a partial loss in single neurons. Because we selected shRNA levels that generated a partial loss of function, results from these experiments are interpreted as analogous to genetic experiment using *trans*-heterozygous animals (32). However, while knockdown using shRNAs has often been described as generating a hypomorphic condition, it remains possible that simultaneous use of two shRNAs results in unexpected effects. Therefore, to fully resolve the roles of these proteins additional complex genetic experiments will be needed. Regardless, our simultaneous knockdown experiments suggest that ephrin-B1 and ephrin-B2 likely function independently to control EphB-dependent synapse development. There are several possible explanations that account for these findings. While ephrin-B1 and ephrin-B2 share functional domains, they may coordinate synapse development through distinct pathways. Consistent with this idea, neurons display different sensitivities to the knockdown of ephrin-B1 versus ephrin-B2 for the formation of synaptic contacts, and the staining pattern of ephrin-B1 and ephrin-B2 is different at the level of individual synaptic puncta (Fig. 3*C*). This specificity may be mediated by differences in *trans*-synaptic interactions or by distinct, currently unidentified, functional domains. Alternatively, while ephrin-B1 and ephrin-B2 colocalize at many synapses, they are often found alone. Thus, one

mechanism for the function of the ephrin-Bs in synapse formation might be their localization to different synaptic puncta.

Ephrin-B1 and ephrin-B2 do appear to function together to recruit syntenin-1 to synapses. Evidence for this synergy comes from our findings that combined knockdown of ephrin-B1 and ephrin-B2 lead to a significant further reduction in density and percentage of synaptic specializations that colocalize with syntenin-1. One likely mechanism is the identical PDZ-binding domains found on ephrin-B1 and ephrin-B2, suggesting that they can both bind syntenin-1 with equal affinity. Although more work will be needed to resolve the different roles of ephrin-B1 and ephrin-B2 in synaptic development, our results provide evidence that ephrin-B1 and ephrin-B2 function to mediate EphB-dependent presynaptic maturation via syntenin-1.

Materials and Methods

For detailed methods see *SI Text*.

Cell Culture and Transfection. Primary dissociated cortical neurons were prepared from embryonic day 17 (E17) to E18 rats and transfected at DIV0 or DIV3 as described (2, 3). See *SI Text* for details on the culture conditions for the heterologous cell culture assay.

Expression and shRNA Constructs. Nineteen-nucleotide RNAi sequences were identified for ephrin-B1, ephrin-B2, and ephrin-B3. Sequences used for shRNAs and details for HA-ephrin-B1, HA-ephrin-B2, and FLAG-syntenin-1 constructs are in *SI Text*. Except when noted, 0.75 μg of shRNA construct per well (of 24-well plate) was transfected into neurons.

Western Blot Analysis. See *SI Text* for more details.

Imaging and Analysis. Cultures were fixed and immunostained using methods similar to those described in ref. 1. Significance between experimental conditions was determined by ANOVA, except where noted. Statistical measures were conducted on a per-cell basis, collected from a minimum of three independent experiments. See *SI Text* for details.

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