Anchoring and synaptic stability of PSD-95 is driven by ephrin-B3

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Organization of signaling complexes at excitatory synapses by membrane-associated guanylate kinase (MAGUK) proteins regulates synapse development, plasticity, senescence and disease. Post-translational modification of MAGUK family proteins can drive their membrane localization, yet it is unclear how these intracellular proteins are targeted to sites of synaptic contact. Here we show using super-resolution imaging, biochemical approaches and in vivo models that the trans-synaptic organizing protein ephrin-B3 controls the synaptic localization and stability of PSD-95 and links these events to changes in neuronal activity via negative regulation of a newly identified mitogen-associated protein kinase (MAPK)-dependent phosphorylation site on ephrin-B3, Ser332. Unphosphorylated ephrin-B3 was enriched at synapses, and interacted directly with and stabilized PSD-95 at synapses. Activity-induced phosphorylation of Ser332 dispersed ephrin-B3 from synapses, prevented the interaction with PSD-95 and enhanced the turnover of PSD-95. Thus, ephrin-B3 specifies the synaptic localization of PSD-95 and likely links the synaptic stability of PSD-95 to changes in neuronal activity.

Synaptic function requires the dynamic regulation of proteins in the postsynaptic density (PSD). MAGUK scaffold proteins such as PSD-95 cluster in the PSD and interact with synaptic proteins to organize and regulate the synaptic signaling complex. PSD-95 regulates the trafficking and localization of proteins such as AMPA- and NMDA-type glutamate receptors and synaptic signaling molecules to modulate plasticity by linking changes in neuronal activity to changes in gene expression. During development, increases or decreases in PSD-95 expression levels result in formation of higher or lower numbers of excitatory synapses, respectively. Moreover, defects in MAGUK function are linked to cognitive, social and motoric phenotypes found in mouse models and in human diseases such as schizophrenia and intellectual disability. However, despite the importance of PSD-95 at excitatory synapses, little is known about how PSD-95 is organized, recruited and stabilized at synaptic sites.

To control the organization, development and function of excitatory synapses, PSD-95 must be kept in the PSD, yet 20–40% of synaptic PSD-95 turns over every 20–30 min (refs. 8–11). The turnover of PSD-95 is regulated by activity-dependent phosphorylation, PDZ domain–driven protein-protein interactions and post-translational modification of PSD-95 (refs. 11–15). Palmitoylation of PSD-95 in the PSD enhances the membrane localization and retention of PSD-95 but is not sufficient for the accumulation of PSD-95 at synapses.

How might PSD-95 become stabilized at or targeted to synaptic sites? One attractive hypothesis is that PSD-95 could interact with synapse adhesion molecules that initiate synapse formation and maturation. Although several classes of trans-synaptic molecules can interact with PSD-95, none are known to direct PSD-95 to synapses. Thus, a molecule directing the localization of PSD-95 to synapses has yet to be identified.

The ephrin-B family of molecules (ephrin-B1, B2 and B3) can act as synaptic adhesion and signaling proteins and are linked to both pre- and postsynaptic development and function. Ephrin-Bs signal to a number of proteins and bind to and activate Eph receptor tyrosine kinases. One ephrin-B in particular, ephrin-B3, functions postsynaptically to control the density of excitatory synapses through a competitive mechanism that negatively regulates the MAPK pathway by a direct interaction with ERK kinase. Like that of PSD-95, the level of ephrin-B3 expression in a neuron controls the density of synapses, such that neurons with higher levels of ephrin-B3 have a competitive advantage and receive more synapses than neurons expressing less ephrin-B3 (ref. 22). However, whether PSD-95 and ephrin-B3 function in the same pathway is unknown.

Here, using a combination of high-resolution imaging, biochemistry and fluorescence recovery after photobleaching (FRAP), we show that ephrin-B3 controls the synaptic localization and turnover of PSD-95 by interacting directly with PSD-95 at synapses in vitro and in vivo. Notably, ephrin-B3 dependent regulation of PSD-95 is modulated by neuronal activity and sensory deprivation through a negative regulatory mechanism that relies on the phosphorylation of ephrin-B3 at Ser332 by MAPK kinase signaling. Thus, we provide the first example, to our knowledge, of a trans-synaptic signal that controls the synaptic localization of PSD-95.

RESULTS

Ephrin-B3 and PSD-95 interact at synapses

Both PSD-95 and ephrin-B3 are found at synapses, and the overexpression or knockdown of either molecule results in similar synaptic phenotypes, suggesting that they might be found in the same biochemical complex. To test whether ephrin-B3 and PSD-95...
colocalize at synapses in the same dendritic spine, we conducted two-color super-resolution imaging using stimulated emission depletion (STED; ~80 nm resolution; Leica SP5 CW STED confocal) microscopy of tdTomato-transfected cortical neurons at 20–22 days in vitro (DIV)\(^{23}\). Four-color images with two-color STED (endogenous PSD-95 (Neuromab) and ephrin-B3 (Invitrogen)) and two-color conventional confocal (endogenous vesicular glutamate transporter 1 (vGlut1) (Millipore) and tdTomato) were collected to visualize the localization of these proteins in single spines (Fig. 1a). Consistent with the location of the epitopes of the antibodies to PSD-95 and ephrin-B3 (Online Methods) and the reported organization of PSD-95 and ephrin-B3 (refs. 21,24,25), STED imaging resolved discrete puncta of both ephrin-B3 and PSD-95 in many spines. Most vGlut1\(^+\) spines (~80% of 248 spines) had clusters of both ephrin-B3 and PSD-95 (Fig. 1b,c; 162 PSD-95+ spines out of 202 vGlut1+ spines). Puncta of either ephrin-B3 or PSD-95 selectively coimmunoprecipitated with ephrin-B3 from synaptosomes isolated from P21 wild-type brains (j) despite the efficient immunoprecipitation (k) and presence (l) of each of the three ephrin-B family members (eB1–3). Representative western blots of three independent experiments from at least three wild-type and Ephb3\(^{−/−}\) animals are shown.

Figure 1 PSD-95 is in a complex with ephrin-B3 at synapses. (a) STED imaging of PSD-95 and ephrin-B3 at synapses. DIV21 tdTomato-transfected rat cortical neurons were labeled with the indicated antibodies. PSD-95 and ephrin-B3 were imaged at ~80 nm resolution using STED. VGlut1 and tdTomato were imaged at conventional resolution (~250 nm) using confocal optics. Images are high contrast examples of PSD-95, ephrin-B3 and VGlut1 immunostaining. Arrows indicate PSD-95, ephrin-B3 and VGlut1 coclusters in dendritic spines. Scale bar, 2 µm. (b) PSD-95, ephrin-B3 and VGlut1 are found in most (~80%) of spines (248 spines on 15 neurons, two separate experiments). (c) PSD-95–ephrin-B3 coclusters are found predominantly in vGlut1+ spines (162 PSD-95+ spines out of 202 vGlut1+ spines). Single PSD-95 (14 of 202 vGlut1+ spines) or ephrin-B3 (20 of 202 vGlut1+ spines) clusters are rarely found at synapses. (d–f) Coimmunoprecipitation (co-IP) of ephrin-B3 and PSD-95 from P21 wild-type and Ephb3\(^{−/−}\) mouse whole brain lysates. Levels of ephrin-B3 immunoprecipitated using an ephrin-B3 antibody and asked whether PSD-95 coimmunoprecipitated with ephrin-B3. (g) Controls were made (Fig. 1g and Supplementary Fig. 1a) and purified synaptosomes were made (Fig. 1i and Supplementary Fig. 1b) from postnatal day (P) 21 wild-type and ephrin-B3 null (Ephb3\(^{−/−}\)) mouse brains. We then immunoprecipitated ephrin-B3 using an ephrin-B3 antibody and asked whether PSD-95 coimmunoprecipitated with ephrin-B3. Ephrin-B3 immunoprecipitated from both whole brain lysates and synaptosomes from wild-type animals (Fig. 1h) and appeared as a doublet on blots, likely owing to post-translational modification\(^{26}\). These bands were absent in blots of lysates and immunoprecipitates from Ephb3\(^{−/−}\) animals, indicating antibody specificity (Fig. 1h). Consistent with the idea that ephrin-B3 and PSD-95 interact only at synaptic sites, PSD-95 coimmunoprecipitated with ephrin-B3 only from synaptosomes isolated from brains of wild-type animals (Fig. 1g and Supplementary Fig. 1b); we failed to detect the coimmunoprecipitation of PSD-95 in whole-brain lysates or from Ephb3\(^{−/−}\) mice (Fig. 1d,g and Supplementary Fig. 1a,b). These data indicate that ephrin-B3 and PSD-95 are found in the same biochemical complex selectively at synapses in vivo.

To test whether ephrin-B3 and PSD-95 interaction at synapses is specific to ephrin-B3 or whether it is a general feature of the ephrin-B family of proteins, we immunoprecipitated each of the three ephrin-B family members from cortical synaptosomes and tested whether PSD-95 coimmunoprecipitated (Fig. 1j–l and Supplementary Fig. 1c). Despite their high sequence homology with ephrin-B3 (Fig. 2a), ephrin-B1 and ephrin-B2 did not coimmunoprecipitate PSD-95 from synaptosomes (Fig. 1j). Similarly, in an in vitro binding assay, ephrin-B1 and ephrin-B2 bound PSD-95-GST much less well than ephrin-B3 (Supplementary Fig. 1d–f). These experiments demonstrate that the interaction with PSD-95 is specific to ephrin-B3 and occurs at...
Distinct MAPK domains on ephrin-B3 regulate PSD-95 binding

Ephrin-B3 binds extracellular signal–regulated kinase 2 (ERK2) through an ERK binding D-domain in its juxtamembrane region. This domain is unique to ephrin-B3 and enables ephrin-B3 to act as a negative regulator of MAPK signaling by inhibiting the nuclear translocation of ERK2 (Fig. 2a)\(^2\). In addition, we identified a putative MAPK phosphorylation consensus site at Ser332 (PPQSPP) immediately upstream of the ephrin-B3 PDZ binding domain (Fig. 2a).

To test whether ERK signaling might regulate the ability of ephrin-B3 to interact with PSD-95, we used our previously characterized ephrin-B3 D-domain (L293A) mutant that disrupts the interaction with ERK2 and leads to more active ERK signaling\(^3,4\), as well as ephrin-B3 mutants of Ser332 that either mimic phosphorylation (S332D) or render ephrin-B3 non-phosphorylatable (S332A). The wild-type mutant Flag-tagged ephrin-B3 (Flag-eB3) constructs were tested for their ability to coimmunoprecipitate PSD-95-GFP (Fig. 2b and Supplementary Fig. 2a,b). To account for differences in the expression of each Flag-ephrin-B3 construct, the amount of PSD-95-GFP coimmunoprecipitated was normalized to the amount of the Flag-ephrin-B3 protein expressed in the lysates of that condition (Fig. 2c). Consistent with our findings in vivo, PSD-95-GFP coimmunoprecipitated with wild-type Flag-eB3 when coexpressed in HEK293T cells (Fig. 2b,c and Supplementary Fig. 2a,b). In contrast, the Flag-eB3 D-domain mutant (L293A) exhibited significantly reduced coimmunoprecipitation of PSD-95-GFP compared to wild-type Flag-eB3 (Fig. 2b,c and Supplementary Fig. 2a,b, \(P < 0.01\), ANOVA), indicating that the ERK-binding D-domain is important for ephrin-B3–PSD-95 interaction. Moreover, significantly less PSD-95-GFP coimmunoprecipitated with the Flag-eB3 mutant that mimics Ser332 phosphorylation (Flag-eB3-S332D) than with wild-type Flag-eB3 (Fig. 2b,c and Supplementary Fig. 2a,b, \(P < 0.05\), ANOVA). Mutation of Ser332 to a non-phosphorylatable alanine residue (Flag-eB3-S332A) did not significantly alter the coimmunoprecipitation of PSD-95-GFP compared to wild-type Flag-eB3 (Fig. 2b,c and Supplementary Fig. 2a,b, \(P = 0.8739\), ANOVA). Thus, the mutations in ephrin-B3 that result in or that simulate increased ERK signaling also resulted in reduced association with PSD-95-GFP, suggesting that ERK signaling negatively regulates the ephrin-B3–PSD-95 interaction.

To further examine the nature of the ephrin-B3–PSD-95 interaction and the importance of the ephrin-B3 intracellular domain for its regulation, we performed in vitro binding experiments using a PSD-95-GST fusion protein and the cytosolic domains of ephrin-B3. Flag-tagged wild-type and mutant ephrin-B3 cytosolic domain proteins were made by in vitro translation (Invitrogen), purified using Flag antibody–coated beads and then incubated with 160 ng of PSD-95-GST. Both the wild-type and S332A ephrin-B3 mutant intracellular domains pulled down PSD-95-GST (Fig. 2d,e and Supplementary Fig. 2c). These constructs also depleted PSD-95-GST from the column flow-through (data not shown). In contrast, the S332D ephrin-B3 mutant failed to pull down PSD-95-GST (Fig. 2d,e and Supplementary Fig. 2c, \(P < 0.05\), ANOVA). These findings indicate that the ephrin-B3 intracellular domain binds directly to PSD-95 and that this interaction requires Ser332.

Ser332 in ephrin-B3 controls the localization of PSD-95

To better understand the role of the Ser332 residue in ephrin-B3, we used a super-resolution imaging and a molecular replacement strategy to ask whether mutation of Ser332 might alter the synaptic localization of ephrin-B3 and PSD-95 (ref. 22). The impact of Ser332 on the synaptic localization of PSD-95 was analyzed in neurons rescued with short hairpin RNA–resistant wild-type, phosphomimetic (S332D) or non-phosphorylatable (S332A) Flag-eB3 constructs. Effects on synaptic localization of ephrin-B3 (Flag) and endogenous PSD-95 were analyzed by STED imaging (~80 nm resolution) at DIV21, when neurons have many dendritic spines and mature synapses\(^27\) (Fig. 2f). Synaptic sites were defined by the presence of vGlut1 staining (~250 nm resolution; Fig. 2f), and the expression level of Flag-eB3 rescue constructs was the same for all conditions (Supplementary Fig. 2d–f). As expected from imaging of endogenous ephrin-B3 (Fig. 1), wild-type Flag-eB3 localized to dendritic spines (Fig. 2f,g; >80% of 89 total spines) and was enriched in dendritic spines with both PSD-95 and vGlut1 (Fig. 2f,h; 76% of 65 vGlut1+ spines).

Mutations to ephrin-B3 (Flag-eB3-S332D rescue) that rendered it unable to interact with PSD-95 resulted in significantly reduced localization of both endogenous PSD-95 and Flag-eB3-S332D to spines (Fig. 2f,g; \(P < 0.0001\), ANOVA). Moreover, expression of Flag-eB3-S332D resulted in a 50% reduction of ephrin-B3–PSD-95 coclusters in vGlut1+ spines (Fig. 2h; 41% out of 248 vGlut1+ spines, \(P < 0.0001\), ANOVA). Although the number of synapses on Flag-eB3-S332D–transfected neurons was reduced (Fig. 2i and Supplementary Fig. 2d,e; \(P < 0.0001\), ANOVA), the proportion of spines associated with presynaptic terminals (vGlut1+ spines) was similar to control (Fig. 2g; 78% of 318 total spines, \(P = 0.91\), ANOVA). These findings suggest that phosphorylation of Ser332 reduces the synaptic localization of both ephrin-B3 and PSD-95.

In contrast, the Flag-eB3-S332A rescue mutant was enriched in dendritic spines and often found at synaptic sites (Fig. 2f). The proportion of spines containing Flag-eB3-S332A and endogenous PSD-95 coclusters was significantly higher than the proportion in neurons transfected with the non-PSD-95 binding Flag-eB3-S332D phosphomimetic mutant (Fig. 2g; >80% of 247 spines, \(P < 0.0001\), ANOVA). Notably, expression of Flag-eB3-S332A led to a small but significant increase in the proportion of vGlut1+ spines (Fig. 2g; 89% of 247 total spines, \(P = 0.0168\), ANOVA), suggesting that in neurons the unphosphorylated form of ephrin-B3 may act dominantly to recruit PSD-95 and ephrin-B3 to synaptic sites. Ephrin-B3 might then act in a trans-synaptic fashion to induce presynaptic maturation\(^22\). Consistent with this, we observed significantly more colocalization of Flag-eB3-S332A and PSD-95 in vGlut1+ spines than in neurons transfected with Flag-eB3-S332D (Fig. 2h; 78% of 207 vGlut1+ spines, \(P < 0.0001\), ANOVA). These results indicate that the synaptic localization of ephrin-B3 and PSD-95 and the PSD-95–ephrin-B3 interaction are likely mediated by ephrin-B3 that is not phosphorylated at Ser332.

The PSD-95–ephrin-B3 interaction regulates synapse density

Ephrin-B3 regulates synapse density in cortical neurons\(^3,5\). Given the role of PSD-95 in the maturation of excitatory synapses\(^3–5\), we used a molecular replacement strategy to ask whether the ability of ephrin-B3 to interact with PSD-95 might regulate synaptic density. As expected, mutations to ephrin-B3 that reduce the ability of ephrin-B3 to interact with PSD-95 (L293A and S332D) failed to rescue decreased synapse density resulting from ephrin-B3 knockdown, while the wild type or the mutation that promotes this interaction (S332A) rescues synapse density (Fig. 2i and Supplementary Fig. 2d,e; \(P < 0.0001\), ANOVA). Notably, synapse density in Flag-eB3-S332A expressing neurons was significantly higher than both control and wild-type Flag-eB3 rescue conditions (Fig. 2i and Supplementary Fig. 2e, 2f).
by MAPK at Ser332, we raised a specific antibody directed against the phosphorylated form (pSer332; Supplementary Fig. 3a–h; see Online Methods). Using the pSer332-specific antibody, we tested whether ephrin-B3 might undergo neuronal activity-dependent MAPK phosphorylation at Ser332 in cortical neurons (Fig. 3a). DIV14 cortical neurons were depolarized (55 mM KCl) for 1 h, which triggered activation of ERK1/2 and induced a significant 1.92 ± 0.17-fold increase above the tetrodotoxin (TTX)-treated baseline in pSer332 signal (Fig. 3b,c, P < 0.001, ANOVA). The effects of depolarization

Ser332 of ephrin-B3 is necessary and sufficient for binding to PSD-95, and it resides in a putative MAPK phosphorylation motif, PPQSP. To determine whether ephrin-B3 might be phosphorylated

Figure 2 Ephrin-B3 links MAPK signaling to PSD-95–ephrin-B3 interaction and localization. (a) Sequence alignment of the intracellular domains (ICD) of the ephrin-B family (eB1–3). Yellow shading highlights the ERK-binding D-domain in the juxtamembrane region of ephrin-B3; green, a putative MAPK phosphorylation motif. Red boxes indicate the positions of point mutations in the D-domain (L293A) and in the putative MAPK phosphorylation motif (S332A, S332D). (b) Immunoprecipitation (IP), using antibodies to Flag, of HEK 293T lysates transfected with PSD-95-GFP and indicated Flag-tagged ephrin-B3 (Flag-eB3) constructs. WT, wild type. (c) Quantification of communoprecipitation experiments shown in b (ANOVA, F(3,23) = 9.842. Wild-type versus S332D: P = 0.0475, wild-type versus L293A: P = 0.0034, S332A versus S332D: P = 0.0087, S332A versus L293A: P = 0.0006; n = 6 blots from separate transfections). (d) In vitro-generated Flag-tagged wild-type and mutant ephrin-B3 ICDs used to pull down PSD-95-GST. Direct interaction between the two proteins is disrupted by the S332D mutation in the ephrin-B3 ICD. (e) Quantification of pull-down experiments from d (ANOVA, F(2,9) = 17.58, Fisher’s LSD post hoc, P = 0.0176 (wild type versus S332D), P = 0.0257 (S332A versus S332D); n = 4 independent in vitro binding experiments). (f) Representative high contrast STED images of DIV21 neurons transfected with tdTomato, ephrin-B3 (eB3) shRNA and the indicated Flag-eB3 rescue constructs. Arrowheads indicate colocalized Flag-ephrin-B3–PSD-95–vGlut1 clusters. Scale bars, 1 μm; scale bar in middle row applies to bottom as well. (g) Quantification of spine localization of Flag-ephrin-B3 (ANOVA, F(2,88) = 17.58, P = 0.0001), PSD-95 (ANOVA, F(2,88) = 16.01, P = 0.0029) and vGlut1 (ANOVA, F(2,88) = 7.536, P = 0.0168). In wild-type (n = 12 cells, 89 spines), S332D (n = 32 cells, 318 spines) and S332A (n = 36 cells, 247 spines) transfected neurons. (h) Quantification of synaptic PSD-95–ephrin-B3 cocluster localization in vGlut1* dendritic spines in wild-type (n = 12 cells, 65 vGlut1* spines), S332D (n = 32 cells, 248 vGlut1* spines) and S332A (n = 36 cells, 212 vGlut1* spines) transfected neurons. ANOVA, F(2,88) = 20.75, P = 0.0001. (i) Quantification of synaptic density in DIV14 neurons transfected with ephrin-B3 shRNA and indicated ephrin-B3 rescue constructs by measuring the colocalization of PSD-95 and vGlut1 in conventional confocal mode (ANOVA, F(5, 204) = 19.81, Fisher’s LSD, control versus S332A: P = 0.0103, control versus S332D: P = 0.0003, control versus L293A: P < 0.0001, control versus shRNA: P = 0.0015, shRNA versus S332A: P < 0.0001, rescue versus S332A: P = 0.0015, L293A versus S332A and S332D versus S332D: P < 0.0001; control, n = 27 cells; eB3shRNA, n = 28 cells; eB3L293A, n = 42 cells; eB3-S332A, n = 43 cells; eB3-S332D, n = 30 cells). Representative images are shown in Supplementary Figure 1. All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA, Tukey’s post hoc, unless otherwise stated.
were blocked by pretreatment of neurons with MEK inhibitors (U0126, 10 μM; Fig. 3b,c and Supplementary Fig. 4a, or PD98059, 100 μM; Supplementary Fig. 4b–d), suggesting that Ser332 is phosphorylated by ERK–MAPK signaling. Thus, our results demonstrate that Ser332 is phosphorylated through a MAPK-dependent pathway, downstream of neuronal depolarization.

Phosphorylation of Ser332 regulates localization of ephrin-B3

We next asked whether phosphorylation of ephrin-B3 at Ser332 might control the subcellular localization of endogenous ephrin-B3. Consistent with a role for Ser332 in controlling ephrin-B3 localization in DIV21 cultured cortical neurons (Fig. 2f–h), pSer332 ephrin-B3 was preferentially localized to dendritic shafts (Supplementary Fig. 5a,b; P < 0.01, ANOVA). To address whether pSer332 ephrin-B3 might have a similar distribution in vivo, brain sections from wild-type and Efnb3−/− mice were stained with the pSer332 antibody. In sections from wild-type, but not Efnb3−/−, mouse cortex, pSer332 ephrin-B3 was enriched in apical dendrites of many layer 2/3 and 5 pyramidal neurons (Supplementary Fig. 3g). The subcellular localization of phosphorylated ephrin-B3 in vivo was determined by staining P14–15 pyramidal neurons filled with EGFP in cortical brain sections from wild-type and Efnb3−/− mice with the pSer332 antibody (Fig. 3d and Supplementary Fig. 3h). These experiments revealed that pSer332 immunostaining...
Figure 4  Ephrin-B3 regulates subcellular PSD-95 localization. (a) Western blots of nonsynaptic (S1), crude synaptosomal (P2) and pure synaptosomal (syn.) fractions from cortices of P10 and P21 wild-type and Efnb3<sup>−/−</sup> mice probed with the indicated antibodies. Less synaptic enrichment of PSD-95 is observed in synaposomes from Efnb3<sup>−/−</sup> mice. (b) Model and predicted outcomes for the organotypic slice culture experiment. (c,d) Representative two-photon images of cortical pyramidal neurons from wild-type and Efnb3<sup>−/−</sup> organotypic slices transfected with PSD-95-GFP and the indicated ephrin-B3 rescue constructs. Right panels in c and d are high contrast representations of tdTomato and PSD-95-GFP. Filled arrowheads indicate PSD-95-GFP localized to a spine; open arrowheads show PSD-95-GFP localized in the dendritic shaft. PSD-95-GFP is diffusely localized in dendritic shafts of Efnb3<sup>−/−</sup> neurons (c) and ephrin-B3 shRNA-transfected wild-type neurons (d). Scale bars in c,d, 5 μm. (e) The ratio of average GFP pixel intensities in dendritic shafts to the GFP intensities per pixel in puncta was used to measure the fraction of diffuse PSD-95-GFP in c,d (ANOVA, F(4,93) = 7.155, P values: wild type versus Efnb3<sup>−/−</sup>: 0.0105, wild type versus Efnb3<sup>−/−</sup> + S332D: 0.0233, Efnb3<sup>−/−</sup> versus Efnb3<sup>−/−</sup> + wt-eB3: 0.0285, Efnb3<sup>−/−</sup> versus Efnb3<sup>−/−</sup> + S332A: 0.0015, Efnb3<sup>−/−</sup> + wt-eB3 versus Efnb3<sup>−/−</sup> + S332D: 0.0487, Efnb3<sup>−/−</sup> + S332A versus Efnb3<sup>−/−</sup>, S332D, 0.0027; wild type (n = 22), Efnb3<sup>−/−</sup> (n = 31), Efnb3<sup>−/−</sup> + wt-eB3 (n = 18), Efnb3<sup>−/−</sup> + S332A (n = 9), Efnb3<sup>−/−</sup> (n = 32) (n = 18) neurons and ANOVA, F(2,60) = 18.45, wild type versus wild type + shRNA (n = 25 neurons): P < 0.0001, wild type + shRNA versus wild type + shRNA + rescue: P < 0.0001 (n = 16 neurons). (f) Representative images of DIV10 neurons transfected with PSD-95-GFP and either control (pSuper, n = 17 neurons), ephrin-B3 shRNA (n = 17 neurons) or ephrin-B3 shRNA + shRNA ephrin-B3 (rescue, n = 13 neurons) constructs at DIV0. Scale bar, 10 μm. Lower panels show high-resolution images of PSD-95-GFP puncta (arrowheads) from the section of a dendrite shown by the white square in upper panels. Scale bar, 3 μm. (g) Plot profiles showing the pattern of PSD-95-GFP localization along the section of dendritic shaft in f. (h-k) Quantification of PSD-95-GFP punctum density (h; F(2,44) = 6.322, *P = 0.0365, **P = 0.0043), average pixel intensities of GFP fluorescence in puncta (i; F(2,44) = 10.16, *P = 0.0127, ***P = 0.0002), average pixel intensities of GFP fluorescence in dendritic shafts; (j; F(2,44) = 8.496, *P = 0.0133, ***P = 0.0009) and average pixel intensities of total GFP fluorescence (puncta + shaft) per neuron (k; ANOVA, F(2,44) = 1.527, P = 0.2284) from experiments shown in f. Averages were obtained from at least three independent experiments. All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Tukey’s post hoc test.

was enriched in the dendritic shaft as compared with the levels found in spines (Fig. 3d,f, P < 0.05, ANOVA). To confirm the role of Ser332 in the synaptic localization of ephrin-B3 in vivo, biochemical fractionation was used to generate synaptic fractions from P21–23 mouse cortex<sup>22,29</sup>. Ephrin-B3 was enriched in PSD fractions together with PSD-95 and NMDA glutamate receptor subunits (Fig. 3e and Supplementary Fig. 5c). In contrast, phosphorylated ephrin-B3 recognized by the ephrin-B3 pSer322 antibody was significantly more abundant in Triton X-100–solubilized synaptic plasma membranes (Fig. 3e,g and Supplementary Fig. 5c, P < 0.05, t-test), indicating that phosphorylated ephrin-B3 is preferentially localized extrasynaptically. We observed similar enrichment of ephrin-B3 in crude synaptosomal and pure synaptosomal fractions prepared from P21 mouse brain (Supplementary Fig. 5d). Moreover, probing with antibodies against total ephrin-B3 and pSer332 ephrin-B3 demonstrated that phosphorylated ephrin-B3 was 80% less enriched in synapto- somes and 65% less enriched in crude synaptosomal fractions compared to nonsynaptic fractions (Supplementary Fig. 5d–f, P < 0.0001, ANOVA). These experiments indicate that phosphorylation of Ser332, a site that is required for ephrin-B3 to interact with PSD-95, reduces levels of ephrin-B3 at synaptic sites.

Phosphorylation on Ser332 regulates localization of PSD-95 To begin to test whether Ser332 might be phosphorylated and regulate the synaptic localization of ephrin-B3 under physiological conditions, sensory information was deprived from barrel cortex in one hemisphere of P21 male mice by whisker trimming. Whisker
trimming reduces neuronal activity in the deprived barrels. After 3 d of deprivation, the barrel cortices of deprived and undriven hemispheres were collected and synaptosomes were purified (Fig. 3h). Consistent with the model, in synaptosomes from deprived barrel cortex the level of ephrin-B3 was significantly increased (Fig. 3i,k and Supplementary Fig. 5g, P < 0.01, paired t-test) and the fraction of the total ephrin-B3 that was phosphorylated at pSer332 was significantly decreased (Fig. 3i,l and Supplementary Fig. 5g, P < 0.01, paired t-test). Levels of other synaptic proteins tested, including calcium/calmodulin-dependent kinase II (CaMKII), were unchanged (Fig. 3i). Thus, sensory deprivation leads to lower levels of phosphorylated ephrin-B3, likely driving the accumulation of ephrin-B3 at synapses.

Phosphorylation of Ser332 reduces the ability of ephrin-B3 to bind PSD-95. Therefore, we hypothesized that in deprived barrel cortex the ephrin-B3–PSD-95 interaction should be increased. Remarkably, whisker trimming led to a significant increase in the amount of PSD-95 that coimmunoprecipitated with ephrin-B3 from deprived synaptosomes (Fig. 3j, m and Supplementary Fig. 5h, P < 0.01, paired t-test). Overall, these results indicate that changes in sensory activity modulate ephrin-B3 phosphorylation in vivo, which regulates the localization of ephrin-B3 at synapses and its association with PSD-95.

Ephrin-B3 interacts with PSD-95 specifically at synaptic sites and appears to regulate the synaptic localization of PSD-95 in response to changes in sensory stimuli. To test whether this interaction is necessary for normal synaptic localization of PSD-95, we prepared synaptosomes from wild-type and Efnb3−/− mouse cortex either during periods of rapid synaptogenesis (P10) or when synaptogenesis is largely complete (P21)23,27. Consistent with previous work, the expression level of PSD-95 in brain and the synaptic expression levels of CamKIIα, GluN2A and GluN2B were unchanged in the Efnb3−/− animal31,32 (Supplementary Fig. 6a–d). Accordingly, Efnb3−/− cortex at both Postnatal day 10 (P10) and P21, levels of PSD-95 normalized to synaptic CamKIIα were significantly lower than control (Fig. 4a and Supplementary Fig. 6g; P = 0.0458, t = 3.298, d.f. = 3. P10 wild-type (n = 2 mice), P10 Efnb3−/− (n = 3 mice) and P = 0.016, t = 2.894, d.f. = 10, P21 wild-type (n = 6 mice), P21 Efnb3−/− (n = 6 mice), unpaired two-tailed Student's t-test). Similar results were found in PSD fractions prepared from P20–21 Efnb3−/− cortex (Supplementary Fig. 7a–d, P < 0.05, t-test). These results suggest that ephrin-B3 is necessary to maintain normal levels of PSD-95 at synapses.

In wild-type brain slices, PSD-95-GFP was punctate and predominantly localized to dendritic spines adjacent to presynaptic specializations (Fig. 4c and Supplementary Fig. 8a,b). In Efnb3−/− neurons, by contrast, PSD-95-GFP fluorescence was found in a diffuse pattern throughout the dendritic arbor (Fig. 4c). The PSD-95-GFP fluorescence in the dendritic shaft likely represents freely migrating PSD-95–GFP puncta (Supplementary Fig. 6b). Ephrin-B3 appears to regulate the synaptic localization of PSD-95 in response to changes in sensory stimuli. To test whether this interaction is necessary for normal synaptic localization of PSD-95, we prepared organotypic slice cultures from wild-type and Efnb3−/− mouse cortices and conducted molecular replacement experiments (Fig. 4b). We hypothesized that synaptic localization of PSD-95 would be rescued by ephrin-B3 mutants that promote the interaction with PSD-95 (Fig. 4b). We generated ephrin-B3 constructs with point mutations in two putative coiled-coil domains (Fig. 4a). Synaptic localization of PSD-95 was rescued by expression of the eB3F–eB3 mutant (Fig. 4a, c, d). Similarly, synaptic localization of PSD-95 was rescued by expression of the eB3F–eB3 mutant (Fig. 4a, c, d). In contrast, in Efnb3−/− neurons, by contrast, PSD-95-GFP fluorescence was found in a diffuse pattern throughout the dendritic arbor (Fig. 4c). The PSD-95-GFP fluorescence in the dendritic shaft likely represents freely migrating PSD-95–GFP puncta (Supplementary Fig. 6b). The mechanism responsible for the synaptic localization of PSD-95 is complex and may involve multiple signaling cascades and regulatory proteins. To further investigate the role of ephrin-B3 in synaptic localization of PSD-95, we analyzed the behavior of ephrin-B3 mutants in vivo and in vitro.

Figure 5 Organization and localization of ephrin-B3 is independent of PSD-95. (a) Immunostaining of DIV10 neurons cotransfected with tdTomato, wild-type Flag-ephrin-B3 and either control (n = 30 neurons) or PSD-95 shRNA (n = 34 neurons) constructs at DIV0. Ephrin-B3 is localized to synapses (arrowheads: vGlut1–PSD-95–Flag, arrows: vGlut1–Flag colocalization) and displays punctate organization in both control and PSD-95 shRNA transfected neurons. Scale bar, 5 μm. (b,c) Localization of endogenous ephrin-B3 in DIV10 neurons transfected with GFP and either control (n = 10 neurons) or PSD-95 shRNA (n = 12 neurons) constructs. Scale bars, 5 μm (left) and 15 μm (right). (d–g) Quantification of synapse density (d; vGlut1/PSD-95 colocalization, P < 0.0001, t = 4.872, d.f. = 62), Flag-ephrin-B3 punctum density (e; P = 0.4130, t = 0.8241, d.f. = 52), fraction of synaptic ephrin-B3 (f; vGlut1–Flag colocalization, P = 0.1686, t = 1.393, d.f. = 62) and pattern of ephrin-B3 expression (g; ratio of Flag in shaft/Flag in puncta, P = 0.1101, t = 1.623, d.f. = 57). (h) Synapse density shown by vGlut1 immunostaining is significantly reduced in PSD-95 shRNA transfected neurons (c) compared to control (b). P = 0.0114, t = 2.785, d.f. = 20. (i) The fraction of synaptic ephrin-B3 (quantified as a percent of ephrin-B3 colocalization with vGlut1, arrowheads in b and c) is unchanged between control and PSD-95 shRNA transfected neurons (P = 0.6654, t = 0.4390, d.f. = 20). All data are represented as mean (box) and individual data points (dots). *P < 0.05, ***P < 0.001, unpaired (two-tailed) t-test.
Ephrin-B3 regulates PSD-95 mobility. (a) Schematic of FRAP experiments. (b) Representative FRAP images of DIV10 rat cortical neurons transfected with PSD-95-GFP. Recovery of bleached puncta (circle) was monitored for 1 h at 1-min intervals. Scale bar, 3 μm. (c) Recovery plots of 1-h FRAP experiments (n = 6 neurons, 3 independent experiments). Mean recovery curve ± s.e.m. (d) Cortical neurons transfected with PSD-95-GFP and either control or ephrin-B3 shRNA at DIV0. At DIV10 neurons were loaded with FM 4-64 (shown in high contrast) to mark presynaptic release sites. Representative FRAP images of synaptic (control, n = 25; shRNA, n = 16) and nonsynaptic (control, n = 15; shRNA, n = 11) PSD-95-GFP puncta from at least three independently transfected neurons. Scale bar, 2 μm. (e) Quantification of FRAP in d. Mean recovery curve; error bars show s.e.m. (K-S test: control versus syn., eB3 shRNA versus syn. ***P < 0.0001; control versus non-syn., eB3 shRNA versus non-syn., ****P < 0.0001; eB3 shRNA versus syn., eB3 shRNA versus non-syn., **P = 0.0002). (f) Mobile fractions of synaptic (syn.) and nonsynaptic (non-syn.) PSD-95-GFP puncta at early (5 min) and late (18 min) phases of FRAP in ephrin-B3 shRNA transfected neurons compared to the control (5 min, F(3,63) = 8.860, control syn. versus shRNA syn.: P < 0.0001, control syn. versus shRNA non-syn.: P = 0.0015, control non-syn. versus shRNA syn.: P = 0.0015, control non-syn. versus shRNA non-syn.: 0.0228; 18 min, F(3,63) = 8.883, control syn. versus shRNA syn.: P < 0.0001, control syn. versus shRNA non-syn.: P = 0.0089, control non-syn. versus shRNA syn.: P = 0.0002, control non-syn. versus shRNA non-syn.: 0.0196. Graphs (f) show the mean ± s.e.m. *P < 0.05 one way ANOVA with Fisher’s LSD post hoc.

diffusible or unbound PSD-95-GFP. Consistent with this, the ratio of PSD-95-GFP fluorescence per pixel in PSD-95-GFP puncta to the average intensity in puncta-free regions of the dendritic shaft was significantly higher in ephrin-B3 null neurons (Fig. 4e; P < 0.0001, ANOVA). Single-neuron rescue of ephrin-B3 expression caused the relocalization of PSD-95-GFP puncta from dendritic shafts to dendritic spines and rescued the diffuse dendritic PSD-95-GFP pattern (Fig. 4c,e, P < 0.0001, ANOVA), indicating that the change in PSD-95-GFP was due to the loss of ephrin-B3 expression. These findings were phenocopied with single-neuron knockdown and rescue of ephrin-B3 expression22 in wild-type brain slices (Fig. 4d,e; P < 0.0001, ANOVA) and cultured neurons (Fig. 4f-k). Knockdown of EphB2, another trans-synaptic organizing protein known to regulate synapse formation23, caused a significant (>40%; P < 0.0001) decrease in PSD-95-GFP puncta density, but did not alter the localization of PSD-95-GFP in the dendritic shaft (Supplementary Fig. 8c-g). Together these findings indicate that ephrin-B3 likely functions postsynaptically to regulate PSD-95-GFP localization cell autonomously.

Next, using a molecular replacement strategy in ephrin-B3 null brain slices, we tested the necessity and sufficiency of Ser332 for the localization of PSD-95 in cortical neurons. Using bioistics, ephrin-B3 expression was rescued by expressing either S332D or S332A ephrin-B3 constructs in cortical neurons of Ephb3−/− brain slices. Remarkably, non-phosphorylatable S332A ephrin-B3, but not the phosphomimetic S332D ephrin-B3 mutant, rescued the pattern of PSD-95-GFP localization to resemble that of control neurons (Fig. 4c,e; P < 0.0001, one-way ANOVA). These data are consistent with our structure–function studies (Fig. 2) and suggest that phosphorylation of Ser332 of ephrin-B3 acts to control localization or stabilization of PSD-95 at synaptic sites.

Ephrin-B3 functions upstream of PSD-95

Our results are consistent with a model in which ephrin-B3 functions upstream of PSD-95 to retain PSD-95 at synapses. We next tested whether PSD-95 is required to organize ephrin-B3 clusters at synapses. We transfected neurons at DIV0 either with wild-type Flag-eB3 and PSD-95 shRNA or with PSD-95 shRNA alone, and processed them for immunocytochemistry at DIV10 (Fig. 5a-c). Knockdown of endogenous PSD-95 decreased PSD-95 expression (Supplementary Fig. 9a,b) and, as expected, caused a significant reduction in synapse density (Fig. 5a,d and Supplementary Fig. 9c,d; P < 0.0001, t-test) and the density of vGlut1+ puncta (Fig. 5b,c,h; P = 0.0114, t-test) compared to controls. However, unlike the effects of ephrin-B3 knockdown on PSD-95-GFP, PSD-95 knockdown had no effect on the puncta density of either Flag-eB3 or endogenous ephrin-B3 found at synaptic sites (Fig. 5e,f,i). Thus, ephrin-B3 is still able to localize to sites of cell-cell contact marked by presynaptic vGlut1, even when PSD-95 expression is markedly reduced. Moreover, Flag-ephrin-B3 remained punctate and did not become diffuse following knockdown of PSD-95 (Fig. 5g). Thus, PSD-95 is not required to organize ephrin-B3 or localize ephrin-B3 to synaptic sites, indicating that ephrin-B3 likely functions upstream of PSD-95.

Ephrin-B3 regulates PSD-95 stability at synapses

To test whether ephrin-B3 might control the stability of PSD-95 at synapses, we performed fluorescence recovery after photobleaching (FRAP) experiments (Fig. 6a). The baseline rate of PSD-95-GFP recovery 1 h after photobleaching was approximately 25% of the pre-bleach value (Fig. 6b,c and Supplementary Video 1, n = 6 neurons). Consistent with previous reports10,11,34, recovery of PSD-95-GFP fluorescence reached a plateau by about 20 min. Therefore, in...
subsequent experiments the recovery of PSD-95-GFP fluorescence was followed once every 20 s for 20 min (Fig. 6a)\(^3\).

We next determined the rate of turnover of PSD-95-GFP at functional synapses. Cultured cortical neurons transfected with PSD-95-GFP were loaded with FM4-64 dye to mark presynaptic release sites\(^{35}\) and FRAP was determined for PSD-95-GFP puncta (Fig. 6d). Twenty minutes after bleaching, fluorescence recovery of PSD-95-GFP was similar for synaptic and nonsynaptic PSD-95 puncta (20% of initial value, Fig. 6d–f; \(P = 0.8085\), Kolmogorov-Smirnov (K-S) nonparametric test).

Next we asked whether knockdown of ephrin-B3 expression might change the stability of PSD-95-GFP. Knockdown of ephrin-B3 resulted in significantly higher levels of PSD-95-GFP fluorescence recovery at both synaptic (\(P < 0.0001\), K-S test) and nonsynaptic sites (Fig. 6d,e) and Supplementary Video 2, \(P < 0.0001\), K-S test). GFP intensity in ephrin-B3 shRNA transfected neurons recovered ~30% in the first 5 min of imaging and remained significantly higher than the control for the remainder of the 20 min of imaging (Fig. 6f, \(P < 0.0001\), ANOVA), indicating that ephrin-B3 knockdown resulted in a significantly higher mobile pool of PSD-95-GFP at both synaptic and nonsynaptic sites. Notably, synaptic puncta recovered faster than nonsynaptic puncta after ephrin-B3 knockdown (Fig. 6d,e, \(P = 0.0002\), K-S test). However, there was no difference in the mean fraction of recovery between synaptic and nonsynaptic PSD-95-GFP puncta (Fig. 6f, \(P = 0.5\) at 5 min, \(P = 0.2\) at 18 min, ANOVA). Overall, these findings suggest that ephrin-B3 controls the mobility of a significant fraction of PSD-95 by regulating the rate of turnover or stabilization of PSD-95 at synaptic sites.

**Ser332 of ephrin-B3 is required for the stability of PSD-95**

Ephrin-B3 functions upstream of PSD-95 and regulates PSD-95 stability at synapses. We next sought to examine whether stabilization of PSD-95 by ephrin-B3 is regulated through the same domains that regulate PSD-95–ephrin-B3 interaction (Fig. 2) using FRAP combined with a molecular replacement strategy. Knockdown of ephrin-B3 significantly enhanced the recovery of bleached PSD-95-GFP (Fig. 7a,b and Supplementary Video 3, \(P < 0.0001\), K-S test), resulting in an increased pool of mobile PSD-95-GFP (Fig. 7c,d, \(P < 0.01\) (5 min), \(P < 0.05\) (18 min), ANOVA). Transfection of an shRNA-resistant wild-type Flag-eB3 construct rescued PSD-95-GFP recovery (Fig. 7a,b, \(P = 0.5185\), K-S test) and the mobile fraction of PSD-95-GFP (Fig. 7c,d) back to control levels, indicating that increased PSD-95-GFP mobility is due to ephrin-B3 knockdown.

The ERK-binding domain and Ser332 in ephrin-B3 are required for the interaction between ephrin-B3 and PSD-95. To test whether the ephrin-B3 binding to PSD-95 might regulate PSD-95 mobility, we conducted molecular replacement studies. Transfection of neurons with PSD-95-GFP, shRNA targeting ephrin-B3 and an shRNA resistant Flag-eB3-L293A D-domain mutant failed to rescue either the diffusely distributed PSD-95-GFP (Supplementary Fig. 10a–c, \(P < 0.05\), ANOVA), the increased turnover (Fig. 7a,e and Supplementary Video 3, \(P < 0.0001\), K-S test), or elevated mobile fractions of PSD-95-GFP (Fig. 7c,d, \(P < 0.05\) (5 min), \(P < 0.01\) (18 min), ANOVA). Indeed, the recovery of PSD-95-GFP fluorescence in neurons transfected with Flag-eB3-L293A and ephrin-B3 shRNA was significantly higher than in neurons transfected with ephrin-B3 shRNA only (Fig. 7e, \(P < 0.047\), K-S test), suggesting that the L293A mutation may act as a dominant negative and suppress activity of endogenous ephrin-B3. Overall, these findings indicate that the ERK binding domain of ephrin-B3 is required for the stability of PSD-95 at synaptic sites.

To test whether phosphorylation of ephrin-B3 at Ser332 is necessary for the control of PSD-95 stability, we rescued ephrin-B3 expression with Flag-eB3-S332D. Consistent with Ser332 phosphorylation acting negatively, neurons transfected with the phosphomimetic Flag-eB3-S332D rescue construct had significantly higher PSD-95-GFP fluorescence intensity in dendritic shafts than control neurons (Supplementary Fig. 10a–c, \(P < 0.001\), ANOVA) and enhanced recovery of photobleached PSD-95-GFP puncta (Fig. 7a,f...
Figure 8 MAPK negatively regulates PSD-95 mobility. (a) Representative FRAP images of DIV10 control (n = 12) neurons cotransfected with PSD-95-GFP and pSuper constructs at DIV0 before and after treatment with U0126. Circles indicate PSD-95-GFP puncta that were photobleached. Scale bar, 3 µm. (b) Average FRAP traces from control neurons (K-S test, P < 0.0001). (c,d) PSD-95-GFP mobile fraction in control neurons before and after treatment with U0126 at early (5 min, P = 0.0821; t = 1.490, d.f. = 11) and late (18 min, P = 0.0203; t = 2.319, d.f. = 11) phases of FRAP. (e) Representative FRAP images of DIV10 neurons transfected with PSD-95-GFP and ephrin-B3 shRNA (n = 14) before and after treatment with U0126. Scale bar, 3 µm. (f) Average FRAP traces (K-S test, ***P < 0.0001; dotted lines represent control traces from b). (g,h) PSD-95-GFP mobile fraction in ephrin-B3 shRNA–transfected neurons before and after treatment with U0126 at 5 min (P = 0.0216; t = 2.240, d.f. = 13) and 18 min (P = 0.0329; t = 2.009, d.f. = 13) of recovery. Data are represented as paired observations (before and after U0126 treatment) of all neurons tested in control and ephrin-B3 shRNA conditions. *P < 0.05, two-tailed Student’s t-test.

and Supplementary Video 3, P < 0.0001, K-S test). Neurons transfected with shRNA-resistant Flag-eB3-S332D also had significantly higher levels of mobile PSD-95-GFP than controls (Fig. 7c,d, P < 0.05 (5 min), P < 0.01 (18 min), ANOVA). The transfection of the non-phosphorylatable Flag-ephrin-B3 mutant (Flag-eB3-S332A) that can interact with PSD-95 rescued the distribution (Supplementary Fig. 10a–c), recovery (Fig. 7a,g, P = 0.1196, K-S test) and mobile fractions of PSD-95 to control levels (Fig. 7c,d and Supplementary Video 3, P < 0.01, ANOVA). Thus, the Ser332 residue in ephrin-B3 negatively regulates the ephrin-B3 interaction with PSD-95 and is both necessary and sufficient to control PSD-95 stability.

MAP kinases are negative regulators of PSD-95 stability

Ephrin-B3 binding to ERK reduces ERK activity and prevents ERK translocation to the nucleus22, while PSD-95 stability requires an intact D-domain in ephrin-B3. Additionally, ERK-dependent phosphorylation of Ser332 negatively regulates the ephrin-B3–PSD-95 interaction and PSD-95-GFP mobility. Therefore, we tested whether ERK activity may negatively regulate ephrin-B3-dependent PSD-95-GFP turnover.

To examine the role of ERK in ephrin-B3-dependent regulation of PSD-95-GFP turnover, we blocked the upstream ERK activator MEK using U0126 (ref. 28) and asked whether this might rescue PSD-95-GFP mobility to normal levels in ephrin-B3 shRNA–transfected neurons (Fig. 8). FRAP experiments were conducted in single neurons transfected with PSD-95-GFP and either control or ephrin-B3 shRNA constructs before and after MEK inhibition. First, a single PSD-95-GFP punctum was bleached and its recovery determined. Then ERK activity was blocked with U0126 for 30 min, after which the mobility of a second PSD-95-GFP punctum in the same neuron was determined with FRAP.

Consistent with ERK playing a negative regulatory role, blockade of the MAPK pathway in wild-type neurons significantly decreased the recovery of PSD-95-GFP (Fig. 8a,b, P < 0.0001, K-S test). Interestingly, inhibition of ERK did not change PSD-95-GFP mobility at early time points during recovery (Fig. 8c), but led to a significant reduction in the mobile pool of PSD-95-GFP at later time points (Fig. 8d, P < 0.05, paired Student’s t-test), suggesting that ERK signaling might be particularly important in later phases of PSD-95 stabilization. Inhibition of ERK activity was also sufficient to restore PSD-95-GFP turnover in a neuron after knockdown of endogenous ephrin-B3, consistent with the idea that ERK is a negative regulator of PSD-95 turnover (Fig. 8e–h, P < 0.0001, K-S test, P < 0.05 paired Student’s t-test). Overall, our data are consistent with a model whereby ephrin-B3 controls the turnover rate of PSD-95 through its interaction with PSD-95 and through inhibition of ERK signaling.

DISCUSSION

The precise localization and scaffolding activity of PSD-95 is essential for normal synaptic function and behavior1,13. Yet how this intracellular synaptic organizer is localized and maintained at the correct sites of cell-cell contact has remained poorly understood. In the present study we show that the synaptic localization and stability of PSD-95 in cortical neurons depends on a direct interaction with the trans-synaptic organizing protein ephrin-B3. Ephrin-B3 appears to control the synaptic localization and stability of PSD-95 in a neuron after knockdown of endogenous ephrin-B3, consistent with the idea that ERK is a negative regulator of PSD-95 turnover. Overall, our data are consistent with a model whereby ephrin-B3 controls the turnover rate of PSD-95 through its interaction with PSD-95 and through inhibition of ERK signaling.
of ephrin-B3 on Ser332. Taken together, our findings provide the first example of a trans-synaptic negative regulatory signaling mechanism that stabilizes a key synaptic organizer, PSD-95, at synapses.

**Regulation of synaptic localization of PSD-95**

PSD-95 stabilizes proteins at the PSD that underlie synaptic function, plasticity and development. During experience-dependent plasticity, PSD-95 appears to promote the delivery of AMPA receptors to synaptic sites, and changes in sensory-driven neuronal activity alter the stability of PSD-95 at synaptic sites. However, the removal of PSD-95 binding partners, such as NMDA receptors and metabotropic glutamate receptors, has little impact on the mobility or synaptic localization of PSD-95 (ref. 10), nor does the stability of PSD-95 at synapses appear to rely on an intact actin cytoskeleton. Moreover, post-translational modifications of PSD-95, such as palmitoylation and phosphorylation, affect PSD-95 stability by regulating its membrane incorporation but are not sufficient to explain the synaptic stability and localization of PSD-95 (refs. 13–16). Indeed, it has been difficult to identify a molecule whose loss causes destabilization of PSD-95 at synapses. Our results identify a new interacting protein that localizes and stabilizes PSD-95 to synapses, regulates PSD-95 mobility and likely links changes in PSD-95 synaptic localization to neuronal activity. The ephrin-B3–PSD-95 interaction occurs selectively at synapses, is direct and specific for ephrin-B3 and is negatively regulated by the activity-driven MAPK-dependent phosphorylation of ephrin-B3 on Ser332. The observation that ephrin-B3 forms synaptically localized puncta after knockdown of PSD-95 indicates that ephrin-B3 acts upstream of PSD-95 to regulate PSD-95 turnover and stability, and suggests that ephrin-B3 may affect the organization of the synaptic scaffold as a whole by anchoring PSD-95 to synaptic sites.

Consistent with this model, PSD-95 knockout mice and mice lacking ephrin-B3 have no apparent decrease in synapse number, but they display a reduced AMPA receptor/NMDA receptor excitatory postsynaptic current ratio in the hippocampus, while Ephb3−/− mice have defects in the synaptic localization of PSD-95. Given the similarities between the phenotypes of these knockouts, it is perhaps surprising that the ephrin-B3–PSD-95 interaction was not identified earlier. Unlike many interactions, binding of ephrin-B3 to PSD-95 is negatively regulated by phosphorylation and occurs only at synaptic sites in vivo. Thus, identification of this interaction was challenging in experiments that used whole-cell extracts not enriched for synaptic components, which contain high levels of phosphorylated ephrin-B3, or in experiments that were enriched for phosphorylated synaptic proteins. Indeed, we were unable to detect the coimmunoprecipitation of ephrin-B3 with PSD-95 in whole-brain lysates. While mice lacking ephrin-B3 have reduced levels of synaptic PSD-95, additional proteins likely aid in organizing PSD-95 because some PSD-95 clusters form in the absence of ephrin-B3 (refs. 34,39,40).

**Negative regulation of PSD-95 stability by MAPK**

At many excitatory synapses, activation of postsynaptic NMDA-type glutamate receptors by different patterns of synaptic activity induce increases or decreases in the strength of synaptic transmission. Both the formation of new synapses and synaptic plasticity are likely to rely on modifications of PSDs and presynaptic terminals. PSD-95 appears to be an important element that links changes in experience to changes in glutamate receptor number and synaptic strength. Here we demonstrate that the activity-induced MAPK–ERK pathway, known to be important to LTP and learning, is linked to ephrin-B3 signaling and the control of PSD-95 stability. The functional significance of this interaction is indicated by the observation that sensory deprivation resulted in reduced levels of ephrin-B3 Ser332 phosphorylation and increased association of PSD-95 in synaptic fractions. Strikingly, the results of blocking ERK activity indicate that the MAPK pathway is a negative regulator of PSD-95 stability in neurons, and, as such, activation of MAPKs might act to mobilize PSD-95. This negative regulatory function of ERK and MAPKs in controlling PSD-95 stability is consistent with the high degree of PSD-95 mobility observed during LTP-induced structural plasticity. By enabling enhanced PSD-95 mobility, ERK activation could allow PSDs to undergo repositioning in spines and promote addition of PSD-95 to re-establish the steady-state composition of the PSD. Consistent with this, loss of ephrin-B3 has been shown to disrupt various forms of plasticity associated with LTP and LTD. Thus, phosphorylation of ephrin-B3 at Ser332 functions as an activity-regulated molecular switch controlling the stability and localization of PSD-95.

**Implications for synapse density and stability**

Overexpression of either PSD-95 or ephrin-B3 alone results in increased synapse density, while knockdown of either PSD-95 or ephrin-B3 expression reduces synapse density. Interestingly, Ephb3−/− mice have reduced density of dendritic spines but exhibit normal density of synapses in cortex. Thus, ephrin-B3 does not function as a classic synaptogenic molecule that is required for synapse assembly. Instead, relative levels of ephrin-B3 between neurons regulate overall synapse density in individual neurons, consistent with a transcellular competition that may govern synapse density in the developing cortex. Because accumulation of PSD-95 at PSDs has been shown to be essential for regulating the stability of synaptic structures, (but see ref. 45), one attractive model suggested by our findings is that ephrin-B3 might regulate synapse density by controlling the stabilization or maintenance of synapses by direct stabilization of PSD-95 at synaptic sites or by negative regulation of ERK signaling. By enabling signaling between a trans-synaptic organizer and a scaffolding protein, ephrin-B3–PSD-95 interaction could influence how neurons decide whether to accept or reject potential synaptic contacts. Consistent with this model, increased ERK signaling causes the breakdown of fixed, mature structures and emergence of de novo filopodia. Indeed, we observed that synapse density was reduced when ephrin-B3 was knocked down and was rescued by ephrin-B3 mutants that promote interaction with and stabilization of PSD-95 at synapses. Thus, ephrin-B3 may serve to integrate both positive synaptogenic signaling driven by PSD-95 and negative synaptic loss signaling driven by ERK signaling.

**Implications for plasticity**

Homeostatic scaling of synaptic strength appears to rely on the bidirectional adjustments in synaptic AMPA receptor abundance driven by changes in the availability of MAGUKs such as PSD-95 and SAP-102. However, the molecular mechanisms driving activity-regulated changes in PSD-95 stability at synaptic sites have been more difficult to determine. The activity-regulated modulation of ephrin-B3–PSD-95 interaction provides an attractive molecular mechanism for regulating synaptic strength. Consistent with this model, sensory deprivation that results in homeostatic scaling-up of deprived inputs reduced ephrin-B3 phosphorylation and enhanced the ephrin-B3–PSD-95 interaction. This mechanism could drive more PSD-95 to a subset of synapses, perhaps only those with reduced levels of neuronal activity. Consistent with the importance of ephrins in these events, in the Drosophila ephrin homologues are required for...
glutamate receptor–dependent homeostatic plasticity at the neuromuscular junction\(^49\). In the future, it will be important to determine how the ephrin-B3–PSD-95 interaction might be linked to abnormal changes in synapse numbers and morphology associated with disease states such as intellectual disability and autism spectrum disorder\(^50\).

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

M.H. and M.B.D. designed the project. Biochemistry, live imaging and sensory deprivation were performed by M.H. Immunocytochemistry and imaging was performed by M.H., N.T.H., N.L.X. and S.J.L.M. Organotypic slice culture was performed by M.H. and N.T.H. Immunohistochemistry was performed by N.T.H. M.H., N.T.H., N.L.X. and S.J.L.M. analyzed the data. The manuscript was written by M.H. and M.B.D.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All animal studies were performed according to the Institutional Animal Care and Use Committee guidelines at Thomas Jefferson University. Wild-type and ephrin-B3 null littermates were generated and genotyped as described previously22,23. E17–18 timed pregnant rats and wild-type CD-1 male mice used for whisker trimming were purchased from Charles River Laboratories Inc. (Wilmington, MA).

Sensory deprivation. CD-1 P21 wild-type males were anesthetized with 4% isoflurane using isoflurane vaporizer (VSS, Rockmart, GA). All facial vibrissae on the right side were trimmed, leaving the left side untouched. Trimming was done every 24 h for 3 d while maintaining the animals in standard (un-enriched) conditions. After 72 h of sensory deprivation, animals were sacrificed, barrel cortices from deprived and undeprived (control) hemispheres were rapidly dissected and flash frozen in liquid nitrogen. The tissue was used for synaptosomal preparation and biochemical analysis.

Cortical neuronal and organotypic slice cultures. Dissociated cortical neurons were prepared from embryonic day 17–18 (E17–18) rat cerebral cortex as described previously22,23 and cultured in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), glutamine (Sigma, St. Louis, MO) and penicillin-streptomycin (Sigma, St. Louis, MO) and laminin (BD Biosciences). Neurons were plated at 150,000 per well in 24-well plates for transfection experiments and were maintained in a humidified 37 °C incubator with 5% CO2. For organotypic cortical brain slices, 300-µm slices were made from postnatal day 4 (P4) to P6 wild-type or Efnb3−/− mice using a vibratome (Warner Instruments, Hamden, CT). Slices were cultured in medium containing 50 ml Neurobasal with B27 supplement, 25 ml of Hank’s balanced salt solution (HBSS, Invitrogen), 25 ml of heat-inactivated horse serum (Invitrogen), 0.65 g of dextrose (Sigma), 1 ml of 1 M HEPES, pH 7.0 (Invitrogen) and 1 ml of penicillin-streptomycin, at pH 7.2. Efnb3−/− mice were generated and outcrossed into the CD1 background by M. Henkemeier (University of Texas Southwestern Medical Center, Dallas, TX)25.

Depolarization of neurons with KCl. DIV14 rat cortical neurons were treated with TTX (1 µM, Tocris) for 4 h at 37 °C to inhibit action potentials and bring the overall levels of neuronal activity to baseline. To block ERK activation, some wells were treated for 4 h with either U0126 (10 µM, Sigma) or PD98059 (100 µM, Sigma) MEK inhibitors28 along with TTX. After 4 h, neuronal activity was elevated by depolarizing neurons with 55 mM KCl for 1 h at 37 °C (ref. 53) in the presence of TTX and either the presence or absence of MEK inhibitors. After 1 h, neurons were immediately lysed with boiling 6× sample buffer, separated by SDS-PAGE and processed for western blot analysis.

cDNA and shRNA constructs. PSD-95-GFP, all Flag-tagged ephrin-B3 constructs and ephrin-B3 shRNA constructs used for transfections and biochemical experiments have been previously generated and characterized in the Dalva laboratory22,23,27,29. For neuronal transfections, we cloned wild-type and mutant ephrin-B3 cDNAs into a pENTR4 plasmid that contains the neuron Xpress epitope, cat. no. 34-3600), anti-ephrin-B3 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), anti-ephrin-B2 (1:500, R&D Systems, Minneapolis, MN, cat. no. AF473), anti-glutamate transporter 1 (anti-vGlut1; 1:5,000, Millipore, cat. no. AB5905). Antibodies and reagents. For the generation of a phospho-specific Ser332 (pSer332) antibody that recognizes phosphorylated ephrin-B3 Ser332, three rabbits were subcutaneously injected (Covance, Princeton, NJ) with custom-synthesized KLH-conjugated pSer332 peptide (IVQDGPPQ-pS-PPNIYYKV, EZBiolab, Inc., Carmel, IN). The antibody was affinity-purified from rabbit sera using the pSer332 peptide and tested for specificity in biochemical and imaging preparations (Supplementary Fig. 2). In all subsequent experiments, pSer332 rabbit antiserum 1176 was used at 1:4,000 for western blotting and rabbit antiserum 1178 was used at 1:2,000 for immunocytochemistry and immunohistochemistry.

Primary antibodies. mouse monoclonal anti-PSD-95 clone K28/43 (1:1,000, Neuromab, UC Davis, Davis, CA, clone 28/43), anti-PSD-95 (1:2,000, Thermo Fisher Scientific, Pittsburgh, PA, cat. no. MAI-046), anti-M2 Flag (1:1,000, Sigma, Saint Louis, MO, cat. no. F3165), anti-pan-ephrin-B (1:1,000, Thermo Fisher Scientific, cat. no. 37-8100), anti-glycerolaldehyde-3-phosphate dehydrogenase (GAPDH, clone 6C5, 1:500, Millipore, Temecula, CA, cat. no. AB5874), anti-GluN2B clone N39/36 (1:300, Neuromab); rabbit polyclonal anti-CaMKII (1:1,000, Cell Signaling, Danvers, MA, cat. no. 3357) anti-Flag (1:1,000, Sigma, cat. no. F7425), anti-ephrin-B3 (1:100, Thermo Fisher Scientific, extracellular epitope, cat. no. 34-3600), anti-ephrin-B3 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, intracellular epitope, cat. no. sc20724), anti-GluN2A (1:1,000, Millipore, cat. no. 07-632), rabbit monoclonal anti-phospho-p44/p42 MAPK (Erk1/2, 2029, and 204; 1:1,000, Cell Signaling, cat. no. 4695), goat anti-p44/p42 MAPK (Erk1/2; 1:1,000), Cell Signaling, cat. no. 4377; goat polyclonal anti-ephrin-B1 (1:500, R&D Systems, Minneapolis, MN, cat. no. AF473), anti-ephrin-B2 (1:500, R&D Systems, cat. no. AF496); guinea pig polyclonal anti-vesicular glucose transporter 1 (anti-vGlut1; 1:5,000, Millipore, cat. no. AB5905).

Secondary antibodies. Highly preadsorbed anti-mouse DyLight 488 (cat. no. 115-485-003), anti-rabbit DyLight 488 (cat. no. 711-485-152) and
anti-guinea pig Alexa-647 (cat. no. 706-605-148) were obtained from Jackson ImmunoResearch (West Grove, PA) and used at 1:500. Anti-mouse (cat. no. 610-151-121) and anti-rabbit (cat. no. 611-151-122) Atto 425-conjugated secondary antibodies were obtained from Rockland, Inc. (Gilbertsville, PA) and used at 1:250. Horseradish peroxidase-conjugated secondary antibodies (anti-mouse, cat. no. 715-035-151; anti-rabbit, cat. no. 111-035-144) were obtained from Jackson ImmunoResearch and used at 1:20,000.

Immunocytochemistry and immunohistochemistry. Dissociated cortical neurones were fixed at indicated times in 4% paraformaldehyde (PFA), 2% sucrose in PBS for 8 min at room temperature. Neurons were then washed three times in PBS, blocked and permeabilized for 2 h at room temperature in 1% ovalbumin, 0.2% gelatin from cold-water fish in PBS containing 0.01% saponin. Neurons were then stained for 2 h at room temperature with indicated primary antibodies, washed three times in PBS and then immunostained with corresponding secondary antibodies for 45 min at room temperature. After washing three times in PBS, coverslips were mounted with MOWIOL and used either for the conventional confocal or stimulated emission depletion (STED) imaging.

For immunohistochemistry, P10–16 mice were perfused transcardially with PBS followed by 4% PFA. The brains were removed and postfixed overnight in 4% PFA at 4 °C. 30-µm sections were then made using a VT-1000S vibratome, permeabilized for 25 min at room temperature in PBS containing 0.5% saponin, then blocked overnight at 4 °C in PBS containing 0.1% saponin, 10% horse serum and 1% BSA. Sections were then incubated overnight at 4 °C in blocking solution containing the indicated primary antibodies, after which they were washed three times for 10 min in PBS and incubated for 2 h at room temperature with blocking solution containing the indicated secondary antibodies. Sections were then washed three times for 10 min PBS and mounted with AquaMount mounting media.

Organotypic slice immunohistochemistry. Biolistically transfected slices were fixed by submersion in 4% PFA plus 0.1% glutaraldehyde for 30 min at room temperature. The fixed slices were removed from the culture plate membranes and washed three times for 10 min in PBS, then submerged in 30% sucrose/PBS overnight at 4 °C. All further steps were carried out with the sections free-floating. The slices were then re-sectioned at 30–50 µm using a sliding microtome, permeabilized for 25 min at room temperature in PBS containing 0.5% Triton X-100 (except for ephrin-B3 staining, for which we used 0.5% saponin), then blocked overnight at 4 °C in PBS containing 10% horse serum, 1% BSA and 0.2% Triton X-100 (except for ephrin-B3 staining, for which we used 0.1% saponin). For primary immunostaining, rabbit anti-GFP (cat. no. A6455, Thermo Fisher Scientific) and guinea pig anti-vGlut1 antibodies were both used at 1:2,500, and sections were incubated overnight at 4 °C. The sections were then washed three times for 10 min in PBS and incubated with the indicated secondary antibodies for 2 h at room temperature. The sections were washed three times for 10 min in PBS and mounted with Aquamount mounting medium.

Postsynaptic density and synaptosomes. Postsynaptic densities were prepared by biochemical fractionation of cortical hemispheres from wild-type and Ephb3−/− littersmates as described previously29. Synaptosomes were prepared from P10 and P21 wild-type and Ephb3−/− mice as previously described22. Briefly, brains were homogenized on ice in 0.32 M sucrose, 4 mM HEPEs, pH 7.4, containing protease inhibitor cocktail (Sigma) and 1 mM PMSE. After removing the nuclear fraction by centrifugation at 1,000g for 10 min, and sections were incubated overnight at 4 °C. The sections were then washed three times for 10 min in PBS and incubated with the indicated secondary antibodies for 2 h at room temperature. The sections were washed three times for 10 min in PBS and mounted with Aquamount mounting medium.

Immunoprecipitation. Synaptosomal pellets were resuspended and homogenized in lysis buffer (50 mM Tris, 140 mM NaCl, 0.5% NP40, pH 7.4) containing general protease inhibitor cocktail (Sigma) and 1 mM PMSE and phosphatase inhibitors (2 mM EGTA, Sigma), 50 mM NaF (Sigma), 1 mM NaN3VO4 (Sigma), 10 mM Na2MoO4 (Sigma) and 0.1 mM ammonium molybdate (Sigma). HEK 293T cells were lysed in 50 mM Tris, 140 mM NaCl, 10 mM NaF, 1 mM NaN3VO4, 1% NP40, pH 7.4. Insoluble material was removed by centrifuging at 10,000g for 20 min at 4 °C and the remaining supernatant was immunoprecipitated with the indicated antibodies for 4 h at 4 °C. Then 20 µl of protein G beads (blocked in 1% BSA in lysis buffer) were added to the supernatant and incubated for 1 h more at 4 °C. Beads were washed three times with lysis buffer and once with Tris-buffered saline containing 10 mM NaF, 1 mM NaN3VO4 with protease inhibitor cocktail (Sigma) and 1 mM PMSE. Proteins were subsequently eluted from the beads by boiling in 4x SDS sample buffer for 5 min. Eluted proteins were separated on SDS-PAGE gels, transferred to PVDF membrane (Millipore), blocked with 5% milk, labeled with the indicated primary and HRP-conjugated secondary antibodies, and visualized with Western Lightning Plus-ECL (PerkinElmer, Waltham, MA). In HEK 293T cell coimmunoprecipitation experiments, we performed densitometric analysis to determine the level of coimmunoprecipitated protein. To account for the differences in protein expression we normalized the amount of coimmunoprecipitated proteins to the amount of each construct present in the lysate in each of six experiments conducted.

Pull-down assay. Cloned intracellular ephrin-B3 fragments were used to generate Flag-tagged ephrin-B3 cytotoxic domains using the cell-free HeLa in vitro translation system (Invitrogen). Cytosolic ephrin-B3 domains were purified by incubating with Flag antibody–bound protein-G beads for 1 h at 4 °C in binding buffer (50 mM Tris-HCl, 140 mM NaCl and 0.5% NP40, pH 7.5) including protease inhibitor cocktail (Sigma) and 1 mM PMSE. After washing three times with binding buffer, 160 ng of recombinant PSD-95-GST (Novus Biologicals, Littleton, CO) was subsequently added to the mixture and incubated 1 h more at 4 °C with gentle agitation. Beads were then washed three times in binding buffer and bound complexes were eluted by boiling in 4x SDS protein sample buffer. We measured the amount of PSD-95-GST in pull-down experiments and normalized to it baseline levels of PSD-95-GST found in no-DNA controls.

Imaging and analysis. Cortical neuronal cultures were imaged by confocal scanning microscopy with a Leica TCS SP5 and Leica TCS STED CW confocal microscope (Leica Microsystems, Mannheim, Germany). For conventional confocal microscopy, images were acquired with a 63x oil immersion objective (Leica) at 1.7–1.9x zoom and were z-projections of 4–10 images taken at 0.3-µm step intervals. For STED imaging, images were acquired with a 100x oil immersion objective (Leica) with 5–10x zoom to obtain 15–30 nm pixel sizes and were single optical sections.

Imaging of fixed brain sections and re-sectioned organotypic brain slices was conducted with a Leica TCS SP5 CW confocal microscope. Images were acquired with a 63x oil immersion objective. For analysis of pSer332 staining, stacks of images were taken at 0.3–0.5 µm intervals and analyzed in ImageJ with experimenter blinded to condition. For analysis of PSD-95-GFP/vGlut1 co-localization, images from at least two independent experiments per condition were taken at 0.25 µm intervals and analyzed in ImageJ with the experimenter blinded to condition.

Fluorescence recovery after photobleaching experiments were conducted with a Leica TCS SP2 confocal scanning microscope (Leica). Cultured neurones on coverslips were removed from the culture dish, placed in an imaging chamber with artificial CSF (ACSF) and imaged using a 40× water immersion lens (Leica) at 1.9x zoom with 488 nm laser at 25% power. For bleaching, selected puncta were zoomed 32× and bleached with 5 scans of a 488-nm laser at 100% intensity. Images were single optical sections acquired every 20 s for 20 min or every 60 s for 1 h. For FRAP of synaptic and nonsynaptic PSD-95-GFP puncta, neurones were loaded with FM4-64 dye for 15 min using spontaneous loading as described22 and then washed for 15 min with three 5-min washes of ACSF before imaging with a Leica TCS SP5 confocal microscope. For these experiments, bleaching of PSD-95-GFP puncta colocalized with and not colocalized with FM4-64 puncta was performed by the Leica bleach points algorithm associated with a Leica TCS SP5 confocal microscope using 50-ms pulses of a 488-nm laser line set at 100% power.

For brain slices, cultured cortical slices were removed from the culture dish, placed into ACSF and imaged on a two-photon Leica TCS SP2 confocal microscope equipped with a MatTai-series mode-locked titanium:sapphire laser tuned to 930 nm, pumped by a 12.5 W solid-state CW 532-nm laser source (Spectra Physics, Fremont, CA). Images are z-projections of 20–60 images taken at 0.5-µm intervals with HCX IRAPO L 25×/0.95 W lens. Images were analyzed in NIH ImageJ using built-in plug-ins and custom-made macros.

Fluorescence recovery after photobleaching analysis. GFP intensity of bleached puncta and four randomly selected unbleached puncta was quantified for each time point by selecting the regions of interest (ROIs) around selected puncta in every image in the time series. Four unbleached puncta served as a control for the
bleaching that occurred during image acquisition, and the recovery of bleached GFP puncta was normalized to the average intensity of unbleached puncta at each time point. The kinetics of bleached puncta recovery (represented as GFP intensity) were then determined by calculating the ratio of GFP fluorescence change over time to the GFP fluorescence before photobleaching using the equation \( \frac{F - F_0}{F_{pre}} \), where \( F \) is the GFP fluorescence measured at different time intervals, \( F_0 \) is the GFP fluorescence immediately after photobleaching at \( t = 0 \) s and \( F_{pre} \) is the GFP fluorescence intensity before photobleaching.

**Puncta coclustering analysis.** Analyses were performed blind to condition. Puncta coclusters were analyzed using automated ImageJ custom-built macros, as described previously\(^{22,23,51}\). Briefly, each channel was thresholded with the same value across experimental conditions and converted to a binary image. Puncta were defined as continuous pixels of 0.5–7.5 \( \mu m \) along at least 50 \( \mu m \) of dendrite and coclustering as >1 pixel of overlap between the channels.

**Puncta and dendritic shaft fluorescence analysis.** For the cultured neurons transfected with PSD-95-GFP or tdTomato and Flag-tagged ephrin-B3 constructs and for the wild-type and Ephb\(^{3−/−}\) neurons in organotypic cortical slices transfected with tdTomato/PSD-95-GFP construct, average puncta pixel intensity per cell was determined by thresholding GFP or Flag intensities in puncta along at least 50 \( \mu m \) of tdTomato labeled dendrite. Thresholded puncta intensities were then subtracted from the whole cell, leaving behind dendritic segments without puncta. Subtracted dendrites were re-thresholded to new values and the regions of interests (ROIs) were drawn around dendritic segments that calculating shaft fluorescence values between subtracted puncta. Calculating the ratios of average shaft to average puncta intensities on a per-cell basis was used to quantify relative amounts of PSD-95-GFP and Flag-tagged ephrin-B3 in different subcellular compartments.

**STED puncta analysis in dendritic spines.** Super-resolution analysis was performed on DIV21 neurons transfected with tdTomato, ephrin-B3 shRNA and Flag-tagged ephrin-B3 Ser332 mutant constructs or in wild-type neurons transfected with tdTomato only. Dendritic spine localization of ephrin-B3 and PSD-95 was performed on a per-spine basis. Spines were identified individually by thresholding tdTomato and drawing ROIs around the spine head. Spine localization of ephrin-B3 puncta and PSD-95 was determined by examining colocalization between thresholded PSD-95, ephrin-B3 or Flag immunostaining and tdTomato in the selected regions. Synaptic localization was measured as colocalization with vGlut1 clusters, which were imaged in conventional resolution (~250 nm). Analysis was performed blind to transfection condition. Puncta counted were restricted by size as described above.

**Statistical analyses.** Data are expressed as means ± s.e.m. Statistical significance of differences among groups was determined by one-way analysis of variance followed by the post hoc test described in individual figure legends, or with a \( t \)-test. Distribution of the data was assumed to be normal, but this was not formally tested. No statistical methods were used to determine sample sizes, but our sample sizes are similar to those reported in previous publications\(^{22,23,27,51}\). The nonparametric Kolmogorov-Smirnov test was used to determine statistical significance of PSD-95-GFP puncta recovery for the FRAP experiments. Probability values of less than 5% were considered statistically significant. Unless stated otherwise, statistical measures were conducted on a per-cell basis, collected from a minimum of three independent experiments.

A Supplementary Methods Checklist is available.

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