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EphB Receptors Interact with NMDA Receptors and Regulate Excitatory Synapse Formation

Matthew B. Dalva,*§ Mari A. Takasu,*§ Michael Z. Lin,* Steven M. Shamah,* Linda Hu,* Nicholas W. Gale,† and Michael E. Greenberg*‡

*Division of Neuroscience, Children's Hospital, and the Department of Neurobiology
Harvard Medical School
Boston, Massachusetts 02115

†Regeneron Pharmaceuticals, Inc.
Tarrytown, New York 10591

Summary

EphB receptor tyrosine kinases are enriched at synapses, suggesting that these receptors play a role in synapse formation or function. We find that EphrinB binding to EphB induces a direct interaction of EphB with NMDA-type glutamate receptors. This interaction occurs at the cell surface and is mediated by the extracellular regions of the two receptors, but does not require the kinase activity of EphB. The kinase activity of EphB may be important for subsequent steps in synapse formation, as perturbation of EphB tyrosine kinase activity affects the number of synaptic specializations that form in cultured neurons. These findings indicate that EphrinB activation of EphB promotes an association of EphB with NMDA receptors that may be critical for synapse development or function.

Introduction

Synapse formation proceeds through a coordinated series of events that includes presynaptic clustering of neurotransmitter-filled vesicles, and postsynaptic clustering of neurotransmitter receptors (Vaughn, 1989; Sanes and Lichtman, 1999). At most excitatory synapses in the central nervous system (CNS), glutamate-containing vesicles are released and act via NMDA (N-methyl-D-aspartate), non-NMDA (AMPA [α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid] and kainate) and metabotropic-type glutamate neurotransmitter receptors that are concentrated at postsynaptic sites. AMPA-type glutamate receptors mediate fast excitatory transmission and are comprised of various combinations of four subunits termed GluR1–4. The NMDA receptor channel is comprised of NR1 subunits that form functional NMDA-sensitive channels and NR2 subunits (A-D) that modulate NR1 function (Hollmann and Heinemann, 1994). The NMDA receptor channel is calcium permeable and has been implicated in the initiation of synaptic plasticity, excitotoxicity, and neuronal plasticity (Choi, 1988; Bliss and Collingridge, 1993; Katz and Shatz, 1996).

While many of the components of the mature excitatory CNS synapse have been characterized, including proteins involved in synaptic vesicle trafficking and proteins of the postsynaptic density (Sudhof, 1995; Garner et al., 2000), the molecular events that initiate the process of synapse formation and maturation in the CNS have only begun to be elucidated. In contrast, formation of the neuromuscular junction (NMJ) has been characterized in detail (Sanes and Lichtman, 1999). At the NMJ, a receptor tyrosine kinase (RTK), MuSK, is critical for initiating synapse formation. MuSK colocalizes with acetylcholine receptors (AchRs) at mature NMJs (Valenzuela et al., 1995), and activation of the MuSK RTK by a heparin sulfate proteoglycan, agrin, leads to clustering of AchRs in myoblasts (Glass et al., 1996). However, agrin-deficient mice have no apparent defects in formation of glutamatergic or GABAergic synapses on CNS neurons (Li et al., 1999; Serpinskaya et al., 1999), suggesting that molecules other than agrin or MuSK mediate the formation of CNS synapses. Several molecules, including WNT-7a, and neuroligin (Hall et al., 2000; Scheiffele et al., 2000), have been implicated in regulation of the formation of presynaptic specializations in the CNS. However, the identification of regulators of the formation and maturation of postsynaptic specializations has proceeded more slowly.

In recent studies, EphB receptors were localized to synaptic sites in hippocampal neurons and at the postsynaptic density of CA1 pyramidal neurons, raising the possibility that the Eph receptor might play a role in CNS synapse formation or function (Torres et al., 1998; Buchert et al., 1999). We considered the possibility that EphB receptors might regulate the development of synaptic connections and postsynaptic specializations in the CNS. Eph RTKs form a large family of at least 14 receptors with eight ligands, termed ephrins. Both ephrins and Eph receptors are expressed throughout the developing and mature nervous system, and members of this family regulate axon guidance and target recognition (Flanagan and Vanderhaeghen, 1998).

Eph receptors are divided into two classes, EphA and EphB receptors, based on their ability to bind the ligands, ephrinAs or ephrinBs, respectively. EphrinAs associate with the plasma membrane via a glycosyl phosphatidylinositol (GPI) linkage, while the ephrinBs are transmembrane proteins (Flanagan and Vanderhaeghen, 1998). Ephrins must be clustered to bind and activate their cognate Eph receptors (Davis et al., 1994), and both ligands and receptors are cell-surface associated, leading to the proposal that ephrins and Eph receptors may regulate cell-to-cell signaling. Not only does ephrinB binding activate the EphB RTK, but, in addition, upon binding to EphB receptors, the cytoplasmic domains of the ephrinBs become tyrosine phosphorylated. This indicates that ephrinBs as well as the EphBs may serve as signaling molecules (Bruckner and Klein, 1998; Flanagan and Vanderhaeghen, 1998; Holland et al., 1998).

The observation that the EphB2 RTK and ephrinB1 localize at sites of excitatory synaptic connections where glutamate receptors are localized, led us to test whether Eph receptors might interact with glutamate

‡To whom correspondence should be addressed (e-mail: greenberg@a1.tch.harvard.edu).
§These authors contributed equally to this work.

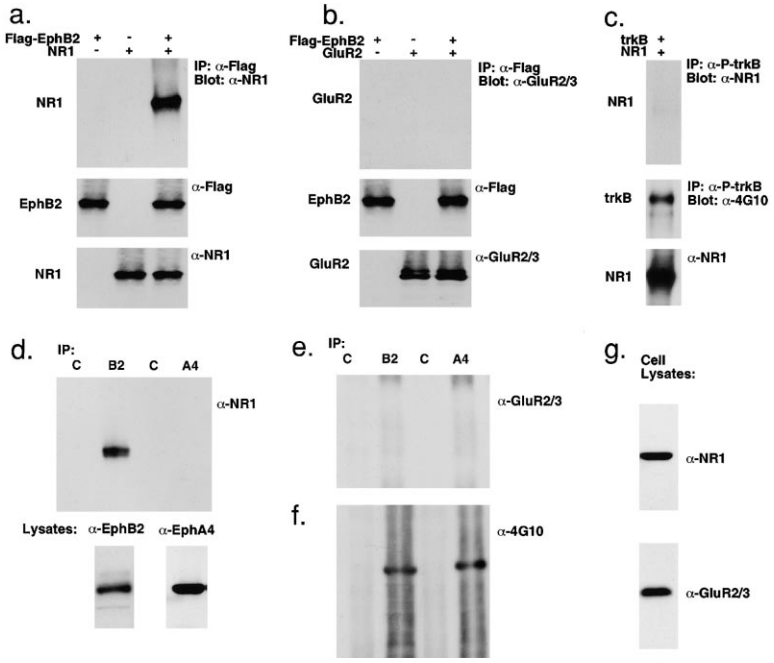

Figure 1. Coassociation of EphB2 Receptor and NR1 in Heterologous Cells and in Rat Cortex

(A) 293T cells were transfected with FLAG-EphB2, NR1, or FLAG-EphB2 and NR1. Cell lysates were immunoprecipitated with anti-FLAG antibody, and then immunoblotted with anti-NR1 antibodies. For all 293T experiments, the lower panels show Western blots from the same set of lysates used for the immunoprecipitation (A–D).

(B) 293T cell lysates transfected with FLAG-EphB2, GluR2, or FLAG-EphB2 and GluR2 were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-GluR2/3 antibodies. GluR2/3 Western blots of lysates used for immunoprecipitation were performed at the same time and indicate that the anti-GluR2/3 antibody effectively detects GluR2/3.

(C) 293T cell lysates expressing TrkB and NR1 were immunoprecipitated with a phospho-TrkB antibody, then immunoblotted with an anti-NR1 antibody. In the same experiments in which trkB and NR1 were found not to coimmunoprecipitate, EphB2 and NR1 were found to coimmunoprecipitate.

(D–F) P1 cortical lysates were immunoprecipitated with anti-EphB2 (B2), anti-EphA4 (A4) or control (C) antibodies, then immunoblotted with anti-NR1 antibodies (D), with anti-GluR2/3 antibodies (E), or with anti-phospho-tyrosine antibodies (F).

(G) The same cortical lysates that were used for the immunoprecipitations in (D)–(F) were immunoblotted with anti-NR1 antibodies (upper) or anti-GluR2/3 antibodies (lower). The anti-NR1 antibodies and the anti-GluR2/3 antibodies detected the presence of NR1 and GluR2/3 in the lysates.

receptors. We find that ephrinB activation of EphBs leads to the recruitment of NMDA receptors to an EphB receptor complex and influences the number of pre- and postsynaptic specializations that form in cultured neurons. Strikingly, the interaction between EphB and NMDA receptors is mediated by the extracellular domains of these two proteins.

Results

Association of EphBs and NMDA Receptors

We investigated the possibility that glutamate receptors form a complex with EphB receptors. Expression vectors encoding either NMDA receptor subunit NR1 or AMPA receptor subunits GluR1–3 were cotransfected into 293T cells along with a vector encoding FLAG-tagged EphB2. We found that NR1 and EphB2 coimmunoprecipitate using antibodies against either FLAG or NR1 (Figure 1A and data not shown). NR1 also coimmunoprecipitates with EphB1, EphB3, or EphB4 (data not shown). These interactions appear to be specific, as GluR1, GluR2, and GluR3 failed to coprecipitate with EphB2 (Figure 1B; data not shown for GluR1), and NR1 failed to coprecipitate with other neuronally-expressed RTKs, EphA3, EphA5 or TrkB (Segal and Greenberg, 1996; data not shown; Figure 1C). These findings indicate that NMDA receptors and EphBs specifically interact with one another when expressed in 293T cells.

As EphB2 has previously been shown to be localized at synapses, we next asked if endogenous NMDA receptors and EphB2 interact. When EphB2 was immunoprecipitated from P1 brain lysates, and the immunoprecipitates probed with antibodies to either NR1, GluR1, or GluR2/3, we found that NR1, but not GluR1 or GluR2/3, coimmunoprecipitate with EphB2 (Figures 1D and 1E). However, if we immunoprecipitated EphA4, another class of Eph receptor that can also bind ephrinB, NR1 was not coimmunoprecipitated (Figure 1D), although both EphB2 and EphA4 were tyrosine phosphorylated in these brain lysates (Figure 1F). These results suggest that the ability of Eph receptors to interact with NR1 is specific to the EphB family of receptors, and demonstrate that NR1 and EphB receptors interact in neurons during the time that synaptic connections are forming (Vaughn, 1989).

To determine whether the interaction between NR1 and EphBs requires ephrinB binding to EphBs, we activated EphBs with either ephrinB1 or ephrinB2 (see Experimental Procedures; Davis et al., 1994). We found that NR1 coimmunoprecipitated with EphB2 only following activation of EphB2 with aggregated ephrinB1-Fc (Figure 2A) or ephrinB2-Fc (data not shown). Treatment of cultured neurons with unaggregated ephrinB ligands did not induce tyrosine phosphorylation of the EphB receptors and did not result in the coimmunoprecipitation of NR1 with EphBs (data not shown). The ephrinB-induced association of EphBs with NR1 was specific, as exposure of cortical neurons to ephrinB1 did not stimulate an interaction between EphBs and GluR1–3 (Figure 2B). The ephrinB1 treatment did not result in increased levels of NR1 or EphB expression, suggesting that the ephrin-induced interaction between NR1 and EphB was the result of an association between pre-existing proteins (Figure 2D). In addition, ephrin-induced association of

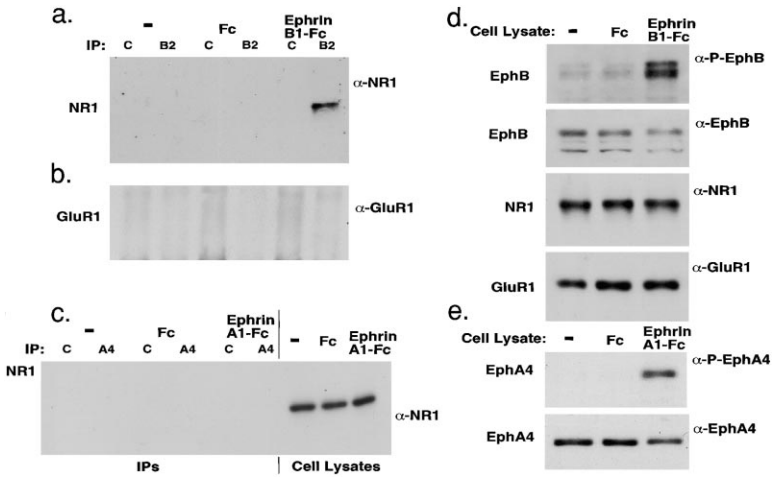

Figure 2. EphrinB1 Induces an Interaction of EphBs with NR1 in Cultured Cortical Neurons

(A and B) Cortical neurons at E17 + 1 DIV were stimulated with ephrinB1-Fc, Fc, or vehicle, lysed, and immunoprecipitated with anti-EphB2 (B2) or control (C) antibodies. The immunoprecipitates were divided into duplicate sets which were electrophoresed on one SDS polyacrylamide gel and transferred to a PVDF membrane. The membrane was then divided and immunoblotted with anti-NR1 antibodies (A), or with anti-GluR1 antibodies (B). The ability of the GluR1 antibody to recognize GluR1 is shown in the Western blots of the same lysates as those used for the immunoprecipitation in (D).

(C) Cortical neurons at E17 + 1 DIV were stimulated with ephrinA1-Fc, Fc, or vehicle, lysed and immunoprecipitated with anti-EphA4 (A4) or control (C) antibodies, then immunoblotted with anti-NR1 antibodies. On the same gel, cell lysates from each condition were run and immunoblotted with anti-NR1 antibodies. The anti-NR1 antibodies detected NR1 in the extracts, indicating the efficacy of this antibody and suggesting that the failure to detect NR1 in the EphA immunoprecipitates reflects a failure of EphA to interact with NR1.

(D) The same neuronal lysates as those used in (A) and (B) from ephrinB1-Fc, Fc, or vehicle-stimulated cells were immunoblotted with anti-phospho-EphB2, EphB2, NR1, or GluR1 antibodies.

(E) The same neuronal lysates that were used in (C) were immunoblotted with anti-phospho-EphA4 or EphA4 antibodies.

the Eph receptors and NR1 appears to be restricted to the EphB subfamily, as NR1 failed to coimmunoprecipitate with EphA4 when this receptor was activated with ephrinA1 (Gale et al., 1996; Figure 2C).

Ephrins and Eph receptors are not expressed at detectable levels in 293T cells (Nakamoto et al., 1996). Thus, the observation that, when overexpressed in 293T cells, EphB and NMDA receptors are capable of interacting in the absence of exogenously applied ephrin (Figure 1) may be explained by the presence of ephrins in the serum-containing media or may be due to high levels of EphB2 expression. In brain lysates, the EphB and NMDA receptor interaction may be mediated by endogenously expressed ephrins. In E18+1 DIV cultured neurons, endogenous ephrins are not present at high levels. Only with the addition of exogenous ephrins are EphBs activated, and only upon the addition of ephrinB does NR1 coimmunoprecipitate with EphB.

Coclustering of EphB2 and NR1 Receptors

We next asked whether ephrinB1 binding to EphBs induces EphB and NMDA receptors to form clusters on neurons as receptor clustering on the cell surface is an important aspect of synapse formation. Cultured neurons were plated at low density, treated with aggregated ephrinB1-Fc or control reagents, and the distribution of EphBs and NR1 was examined. At 1 DIV, we do not detect synaptic specializations and neurons are immature with short dendrites. Prior to ephrinB stimulation, approximately 25% of cells contained small clusters of EphB receptor staining with the majority of EphB staining distributed along the cell surface. However, after 5 min of exposure of neurons to ephrinB1, EphB was tyrosine phosphorylated and formed clusters along neurites and cell bodies (Figure 3A) in approximately 62% of neurons, whereas EphB clusters were observed in approximately 26% of neurons treated either with Fc alone or vehicle control (Figures 3B, 4A, and 4B). Interestingly, following ephrinB stimulation, we observe very few EphB-positive neurons without EphB clusters. In contrast to what we observed with ephrinB, exposure of cultured neurons to BDNF, the ligand for the TrkB RTK (Segal and Greenberg, 1996), failed to cluster TrkB, EphB, or NMDA receptors, although it did induce phosphorylation of TrkB (data not shown).

As it has previously been shown that EphB receptors colocalize with synaptic specializations (Torres et al., 1998; Buchert et al., 1999), we examined whether the ephrin-induced clustering of EphBs results in the coclustering of EphBs with subunits of glutamate receptors. Following one hour of treatment with ephrinB1-Fc, neurons displayed a significant increase in the number of NR1 clusters that colocalized with EphB2 clusters (39%; Figures 3B and 3D). In contrast, significantly fewer cells displayed coclusters of EphBs and NMDA receptors in the absence of exogenous ephrin (12%; Figure 3B). We failed to detect GluR1, GluR2/3 associated with EphB clusters in cultured neurons (data not shown). In addition, the presynaptic proteins SV-2 and synaptophysin did not cocluster with EphB and treatment with unaggregated ephrinB did not result in NR1 clustering. The anti-NR1 antibody staining was specifically competed with a GST-NR1 fusion protein but not with GST alone (data not shown), demonstrating staining specificity. The ephrin-induced clusters of EphBs and NR1 were localized to the cell surface of neurons by visualization of sequential planes of focus using confocal microscopy and by immunostaining of nonpermeabilized neurons (Figure 3C). The delay in the clustering and colocalization of NR1 and EphB suggests that an ephrinB initiated sequence of events may mediate the interaction and clustering of NR1 and EphBs. Alternatively, the delay might simply reflect a stochastic process where EphBs must encounter NR1 following ligand binding.

As functional NMDA-type receptor channels are predominantly comprised of NR1 and either NR2A or NR2B

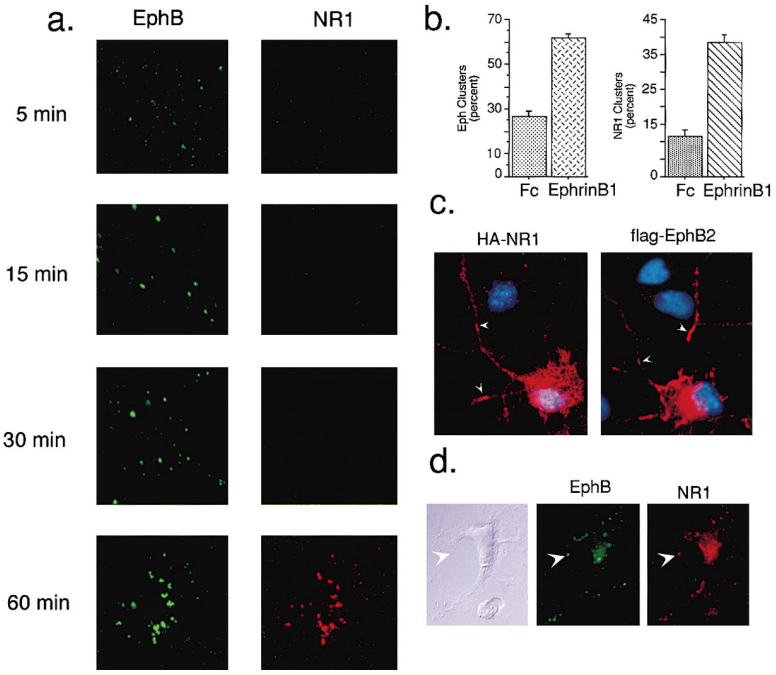

Figure 3. EphrinB1 Stimulation of Neurons Clusters EphBs and NR1 by 60 Minutes on the Cell Surface

(A) EphBs form clusters rapidly upon stimulation with ephrinB1-Fc, but NR1 forms clusters with a delayed time course. In green is anti-EphB staining. In red is anti-NR1 staining.
(B) Graphs of the percentage of cells containing clusters of EphBs or NR1 receptors following 60 min of ephrinB1 treatment.
(C) Immunostaining of cortical neurons transfected with either N-terminal-tagged HA-NR1 or FLAG-EphB2 expressed in nonpermeabilized cortical neurons stimulated for 1 hr with activated ephrinB2-Fc. In red: both HA-NR1 and flag-EphB2 cluster on the cell surface following ephrinB stimulation. Cell nuclei are stained with Hoechst in blue.
(D) Brightfield and immunofluorescent images of a neuron stimulated with ephrinB2-Fc for 1 hr. Arrows indicate examples of coclusters of EphBs (green) and NR1 (red). Scale bar ≈ 25 μm.

subunits (Hollmann and Heinemann, 1994), we examined whether NR2A and NR2B subunits also colocalized with EphB2 after ephrin treatment. Both NR2A and NR2B clustered and colocalized with EphB clusters following ephrinB1-Fc stimulation (Figures 4A and 4B). In 1 DIV cultured neurons, NR2A and NR2B also coimmunoprecipitate with EphB2 following ephrinB stimulation (data not shown).

Since NMDA and EphB receptors associate with a variety of cytoplasmic proteins that facilitate receptor signaling (Bruckner and Klein, 1998; O'Brien et al., 1998), we asked whether ephrinB1 treatment leads to the colocalization of some of these signaling molecules with the EphB and NMDA receptor clusters. Both CaMKII and Grb10, two signaling molecules that associate with NMDA or EphB receptors, respectively (Stein et al., 1996; Gardoni et al., 1998; Leonard et al., 1999) were found to colocalize with NR1 and EphB following ephrin stimulation (Figures 4C and 4D and data not shown). CamKII and Grb10 coimmunoprecipitated with EphB from P1 cortex, and following ephrinB2 stimulation of cultured neurons. However, when CamKII and EphB2 are expressed in 293T cells, CamKII does not coprecipitate with EphB2, suggesting that the interaction be-

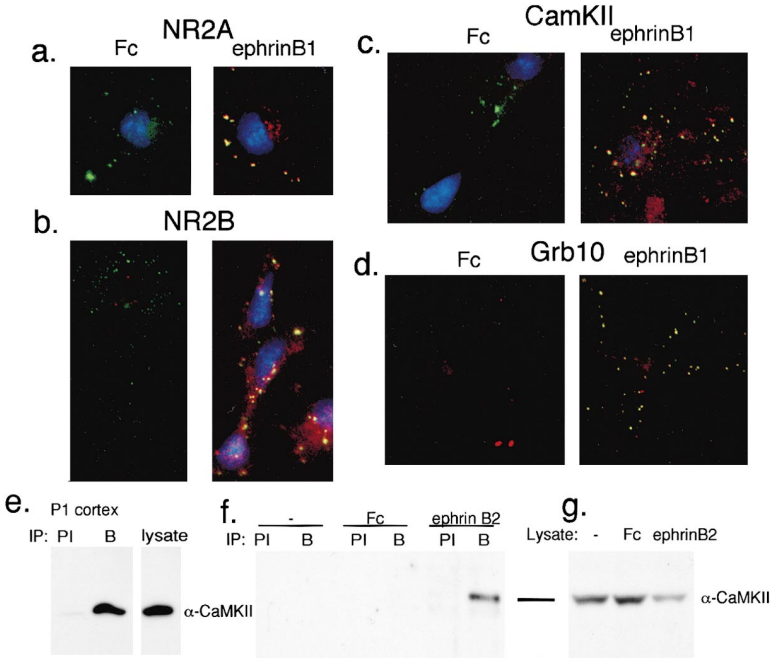

Figure 4. Association of NMDA Receptor Subunits and Signaling Molecules with Clusters of EphBs

In all cases, green indicates EphB staining and blue is Hoechst nuclear stain. Yellow indicates colocalization of red and green immunostaining.
(A) In red is anti-NR2A antibody staining.
(B) In red is anti-NR2B antibody staining.
(C) In red is anti-CamKII antibody staining.
(D) In red is anti-Grb10 antibody staining.
(E) P1 cortical lysate immunoprecipitated with anti-EphB2 (B2) or control antibodies (PI) and immunoblotted with anti-CaMKII antibodies. Adjacent — P1 cortical lysates immunoblotted with anti-CaMKII antibodies.
(F) Cortical neurons at E18 + 1 DIV stimulated with ephrinB1, Fc, or vehicle, lysed and then immunoprecipitated with anti-EphB2 (B2) or control (PI) antibodies, then immunoblotted with anti-CamKII antibodies.
(G) Neuronal lysates from ephrinB1, Fc, or vehicle-stimulated cells immunoblotted with anti-CaMKII antibodies. Scale bar ≈ 25 μm.

tween these proteins requires components not present in 293T cells (Figure 4 and data not shown). Other proteins, such as PSD-95 and Nck, which have previously been shown to associate with either NMDA or EphB receptors (Bruckner and Klein, 1998; Garner et al., 2000), respectively, were not observed in the NMDA/EphB coclusters (data not shown), although this may reflect low expression levels of these proteins in 1 DIV cultured neurons.

Interaction Domain of the EphB2 Receptor

To characterize the nature of the association of EphB with NR1, we defined the domains of these proteins that are required for their interaction. Mutant receptor proteins were tested for their ability to interact in 293T cells by coprecipitation and in neurons by coclustering. Two EphB2 proteins with point mutations in conserved amino acids within the kinase domain that render the receptor kinase inactive (EphB2KD; Ullrich and Schlessinger, 1990) still coimmunoprecipitated NR1 from 293T cell lysates, suggesting that the kinase activity of EphB2 is not required for the interaction with NR1 (Figure 5B). Further, deletion of the cytoplasmic region of EphB2, including the SAM and C-terminal PDZ binding domains, failed to disrupt the interaction between EphB2 and NR1 (EphB2DI; Figure 5C; Holland et al., 1998). In neurons transfected with either wild-type FLAG-tagged EphB2 or EphB2DI, both proteins clustered and colocalized with endogenous NMDA receptors following stimulation with ephrinB1 (Figures 5I and 5K). By contrast, GluR1 did not cocluster with clusters of exogenously expressed EphB2 (data not shown). These results indicate that exogenously expressed EphB2 associates with NMDA receptors in a ligand-dependent manner, and that this interaction does not require the intracellular domain of EphB2.

The extracellular domain of EphB2 contains a cysteine rich domain, two fibronectin type III repeats, and a globular domain that is thought to be the site of ligand binding (Holland et al., 1998). We generated a chimeric receptor in which the extracellular domain of EphB2 was replaced with the extracellular domain of the fms RTK (Sherr et al., 1985). We found by Western blotting with anti-phospho-EphB2 antibodies that, when stimulated with the fms ligand CSF-1, the cytoplasmic domain of the chimeric receptor was tyrosine phosphorylated (data not shown; see Figure 5K). However, the chimeric receptor was unable to coimmunoprecipitate NR1, suggesting that the extracellular domain of EphB2 mediates the EphB2/NR1 interaction (Figure 5D). When neurons were transfected with fmsEphB2, stimulation with the fms RTK ligand (Sherr et al., 1985) induced the formation of tyrosine phosphorylated clusters of chimeric receptor, but these clusters did not colocalize with anti-NMDA receptor antibody staining (Figure 5J). To corroborate our findings with the chimeric receptor, we generated an additional EphB2 construct that lacks the EphB2 extracellular domain (TrExEphB2); this truncated EphB2 failed to coimmunoprecipitate NR1. Taken together, these results demonstrate that the extracellular domain of EphB2 is necessary and sufficient to mediate the interaction of EphB2 with NR1.

To examine whether the extracellular domain of NR1 is required for EphB2 and NR1 to interact, we generated a chimeric receptor that consists of the N-terminal extracellular domain and the first transmembrane domain of NR1 (ExTM_NR1). ExTM_NR1 was localized on the cell surface and, when coexpressed with EphB2, the two receptors coimmunoprecipitated in 293T cells (Figure 5F). ExTM_NR1 failed to coimmunoprecipitate with the chimeric fmsB2 construct, suggesting that the N-terminal portion of the NR1 interacts with the N-terminal portion of EphB2 (data not shown). In addition, we generated a chimeric NR1 that consisted of the N-terminal extracellular domain of GluR2 and the four transmembrane domains and C-terminal tail of NR1 (Chimeric NR1). The chimeric GluR2-NR1 was localized on the cell surface, but failed to coimmunoprecipitate with wild-type EphB2 (Figure 5G). These results demonstrate that the N-terminal extracellular domain of NR1 is both necessary and sufficient to mediate the interaction with EphB2 in 293T cells.

We next examined whether the interaction between the extracellular domains of NR1 and EphB2 is direct. To examine binding between the extracellular domains of EphB2 and NR1, we produced a fusion protein consisting of the extracellular domain of NR1 and a histidine tag (NR1-HIS) and bound the NR1-HIS to nickel agarose beads via the HIS tag. We found that only in the presence of ephrinB1-Fc did EphB2-Fc effectively bind to NR1-HIS (Figure 5H). These findings suggest that the extracellular domains of NR1, EphB2, and ephrinB1 form a ternary complex in vitro, although it remains possible that additional proteins participate in the interaction within neurons.

EphB2 Receptors and Synapse Development

As clustering of neurotransmitter receptors has been proposed as an initial step in the formation of synapses (Sanes and Lichtman, 1999) and EphB receptors are localized at sites of synaptic contact (Torres et al., 1998; Buchert et al., 1999), we investigated whether activation of EphB RTKs might affect the number of pre- and postsynaptic specializations that form in culture. We first examined whether ephrinB1 activation of EphB receptors affects the number of synaptic specializations that form in cultured neurons.

The time course of synapse formation was examined in neurons cultured for 1–10 DIV. The presence of synapses was documented using antibodies to visualize proteins that are selectively expressed pre- or postsynaptically (SV-2, synaptophysin, NR1, NR2A/B, PSD-95, and GluR2/3), and by the fluorescent vital indicator dye, FM1-43 , which labels presynaptic release sites (Ryan et al., 1993; Cochilla et al., 1999; Kavalali et al., 1999). Beginning at 7 DIV, we could detect small clusters of pre- and postsynaptic marker proteins that were adjacent to one another and displayed the morphology previously described for synapses that form in culture (data not shown; Craig et al., 1993; O'Brien et al., 1997; Rao et al., 1998; Kavalali et al., 1999). We also found by Western blotting that the expression levels of pre- and postsynaptic proteins increased substantially at 7 DIV (data not shown), indicating that functional synaptic connections become numerous in our culture system at around 7 DIV.

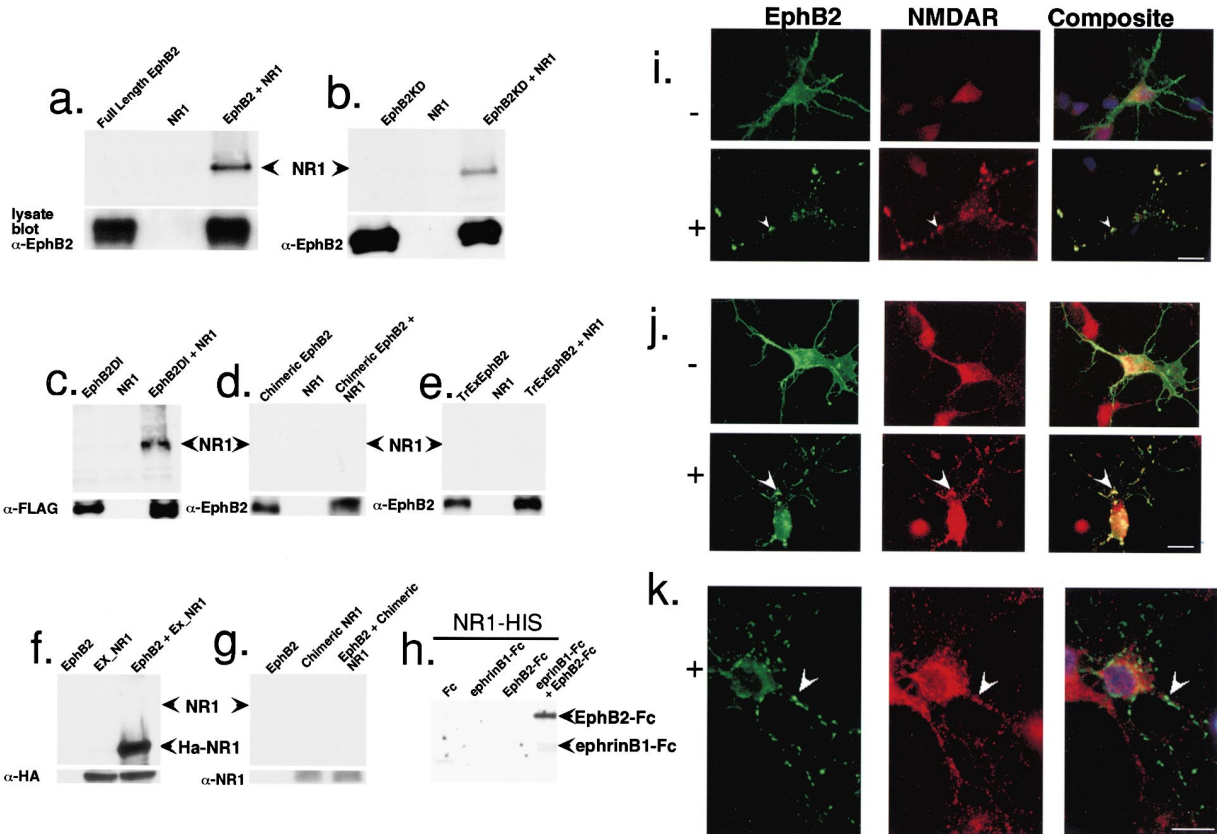

Figure 5. Immunoprecipitation and Immunostaining with EphB2 and NMDA Receptor Constructs

(A–G) 293T cells were transfected with an EphB2 construct, NR1 alone, or various NR1 and EphB2 constructs. All immunoblots are with anti-NR1 antibodies unless otherwise noted. In all cases where proteins were found not to coprecipitate, positive controls for immunoprecipitation, transfer, and antibody binding were included.

(A) 293T cells were transfected with wild-type FLAG-tagged EphB2 and lysates immunoprecipitated with anti-EphB2 antibody. (B) FLAG-EphB2 kinase inactive construct, EphB2KD, was coexpressed and immunoprecipitated with anti-EphB2 antibody. (C) FLAG-EphB2 receptor construct lacking the intracellular domain, EphB2DI, was coexpressed in 293T cells and lysates were immunoprecipitated with anti-FLAG antibodies. (D) Chimeric EphB2 construct comprised of the fms RTK extracellular domain and intracellular domain of EphB2 was introduced into 293T cells and lysates were immunoprecipitated with anti-EphB2 antibody. (E) An EphB2 construct lacking the extracellular domain, TrExEphB2, was expressed and lysates immunoprecipitated with anti-EphB2 antibodies. (F) A construct consisting of the N-terminal HA-tagged extracellular and first transmembrane domains of NR1 (ExTM_NR1) was coexpressed with FLAG-EphB2 in 293T cells and the lysates immunoprecipitated with anti-EphB2 and immunoblotted with anti-HA antibodies. (G) Chimeric GluR2-NR1 was coexpressed with FLAG-EphB2 in 293T cells and lysates immunoprecipitated with anti-EphB2. (H) In vitro binding assay. NR1-HIS binds EphB2-Fc only in the presence of ephrinB1-Fc. Anti-human IgG immunoreactivity shown.

(I–K) Cortical neurons were transfected with wild-type or mutant FLAG-tagged EphB2 constructs at E17 + 3 DIV and at 5 DIV, stimulated for one hour with EphrinB1-Fc or Fc alone, and stained with a mouse monoclonal anti-flag antibody (green). NMDA receptors were visualized with a polyclonal anti-NR2B antibody (red). Yellow in "composite" indicates colocalization of red and green staining and in blue is Hoechst stain. A plus sign indicates addition of ephrinB1-Fc for 60 min prior to fixation and a minus sign indicates addition of Fc 60 min prior to fixation. Scale bar = 25 μm. (I) Distribution of wild-type, exogenously introduced FLAG-EphB2 and endogenous NR2B after Fc treatment (−) and after stimulation with ephrinB1-Fc (+). White arrows indicate an example of endogenous NR2B clustering with clusters of exogenous EphB2 receptors. (J) As in (I), except exogenous FLAG-EphB2DI after Fc treatment (−), and ephrinB1-Fc (+) stimulation. Again, white arrow indicates an example of endogenous NR2B colocalized with EphB2DI. (K) The chimeric fms-EphB2 was expressed in neurons and recognized with an anti-phospho-EphB2 antibody (green), and NMDA receptors were visualized using a mouse monoclonal anti-NR1 antibody (red). A plus sign indicates addition of aggregated CSF-Fc, the ligand for Fms RTK. Note: The chimeric EphB2 is capable of clustering, is localized to the plasma membrane, and is specifically phosphorylated following ligand stimulation. White arrows indicate an example of a failure of clustered chimeric EphB2 to colocalize with NR1. Scale bar = 25 μm.

To determine whether ephrinB1 activation of EphB receptors affects the number of postsynaptic sites, we determined the density of NMDA receptor positive synapses on single neurons that had been treated with ephrinB1-Fc, Fc, vehicle, or BDNF. The experiments were conducted and analyzed in a blind fashion with respect to the experimental condition and were scored by two different investigators. We found that treatment from 4–7 DIV with BDNF, Fc, or vehicle did not change the number of NMDA receptor–containing postsynaptic specializations that form (Figure 6B). However, treatment with aggregated ephrinB1-Fc resulted in a significant increase in the number of NMDA receptor–containing specializations (Figure 6B). These findings suggest that long-term ephrinB1 treatment leads to an increase in the number of postsynaptic specializations

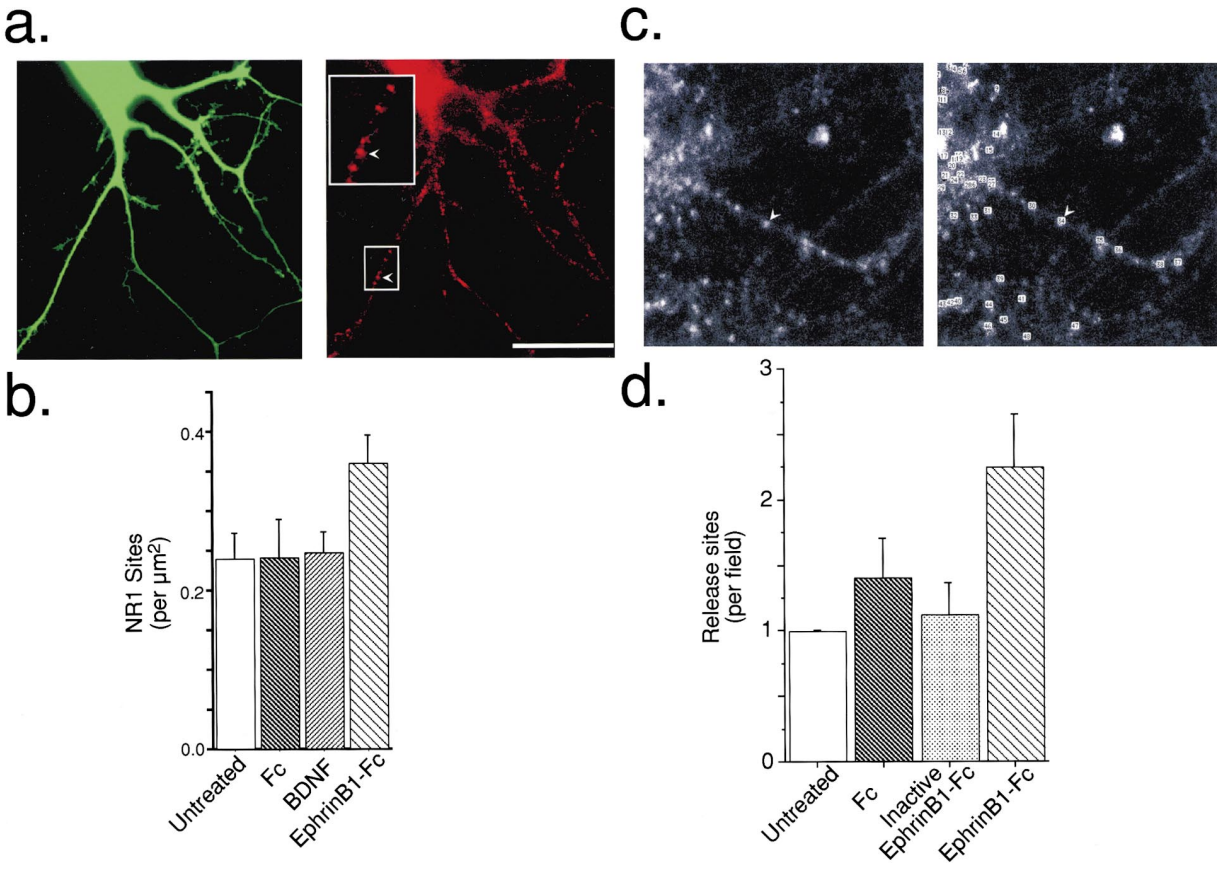

Figure 6. Effects of Long-term Stimulation of EphBs on Pre- and Postsynaptic Specializations

(A) GFP was introduced into neurons at 4 DIV, and at 7 DIV cells were labeled by immunostaining with a rat polyclonal anti-GFP antibody (green). Endogenous NMDA receptors were visualized with a mouse monoclonal anti-NR1 antibody (red). The arrow indicates an example of one of many NR1 postsynaptic sites. Scale bar = 25μm.

(B) Graph of the density of NR1 postsynaptic sites (per μm^2) on neurons cultured for 7 days. Cells were either left untreated, treated with Fc, BDNF, or ephrinB1-Fc. The density of NMDA receptor synaptic clusters was determined within cells labeled with GFP. EphrinB1-Fc treatment caused a significant increase in the density of NMDA receptor postsynaptic sites ($p < 0.05$; $n = 5$ cells in each condition).

(C) Subtracted image of FM1-43-labeled release sites and a computer generated count of these sites. White spots are sites of FM1-43 labeling. Sites of labeling that are counted are marked with small numbered boxes in the right panel. Note that large, nonspecific white regions are not counted. The white arrow in the left panel indicates an example of a discrete site of FM1-43 labeling and the white arrow in the right panel indicates that it was counted.

(D) Graph of normalized number of presynaptic release sites labeled by FM1-43. Neurons were either left untreated, treated with Fc, inactive ephrinB1-Fc, or activated ephrinB1-Fc. Only treatment with activated ephrinB1-Fc resulted in a significant increase in the number of presynaptic sites ($p < 0.01$).

in cultured neurons, raising the possibility that ephrinBs and EphBs might play a broader role in the maturation of synapses than simply the initial clustering of NMDA receptors.

To test whether EphB activation also affects the number of presynaptic release sites, and not simply the density of NR1 clusters, we employed the lipophilic dye FM1-43 to determine the number of functional presynaptic release sites that form in culture in the presence or absence of exogenously added ephrinB (Figure 6C). Four days of treatment with ephrinB1-Fc or Fc did not affect cell survival (data not shown), nor did we note any changes in neurite extension or distribution of cells on the culture dish. When comparing the normalized value of untreated cultures, Fc-treated neurons had 1.41 ± 0.29 release sites per field, while ephrinB1-Fc-treated cells had 2.25 ± 0.40 release sites per field (Figure 6D; $p < 0.01$ vehicle and $p < 0.05$ Fc; $n = 6$). This indicates that ephrinB1 treatment of cultured neurons causes a statistically significant increase in the number of functional presynaptic release sites. In contrast, treatment of cells with unaggregated ephrinB1, which does not result in tyrosine phosphorylation of the EphB receptor, did not significantly change the number of synaptic release sites (Figure 6D; 1.12 release sites per field; $n = 6$). These results demonstrate that ephrinB treatment leads to an increase in the number of pre- and postsynaptic specializations that form in cultured neurons in a manner that is dependent on the activation of EphB. The differences that we observe in the magnitude of the effects of ephrinB treatment on pre- and postsynaptic specializations may be due to differences in the rates of maturation of these specializations (Friedman et al., 2000).

To test further whether EphBs contribute to synapse development in culture, we inhibited EphB signaling and assessed the effect on synapse formation. At least four distinct EphBs are expressed in our neuronal cultures, and at least three of these receptors interact with NR1 (data not shown). Therefore, we employed dominant interfering forms of EphB that are capable of blocking signaling by multiple EphB family members. We used two different EphB2 mutants (EphB2DI or EphB2KD) that block the tyrosine phosphorylation of multiple EphB family members (EphB1–4) when overexpressed in 293T cells or in neurons following ligand stimulation (data not shown). When EphB2DI or EphB2KD were coexpressed with either TrkB or PDGFR in 293T cells, they failed to block the tyrosine phosphorylation of either TrkB or PDGFR, indicating that these dominant interfering EphB constructs can specifically block Eph signaling.

We transfected cultured cortical neurons at E18+4 DIV with each of the mutant EphB2 constructs (EphB2DI or EphB2KD) and assessed the effect on postsynaptic NR1 clusters containing specializations. Cells expressing EphB2KD or EphB2DI had significantly fewer postsynaptic specializations relative to control (EphB2KD = 0.26 ± 0.02 per μm^2, $p < 0.02$; EphB2DI = 0.28 ± 0.02 per μm^2, $p < 0.04$; Control = 0.36 ± 0.03 per μm^2; $n = 30$ each condition, Figures 7A and 7B). Neurons transfected with the wild-type EphB2 display a significant increase in the density of postsynaptic specializations relative to control (0.47 ± 0.04 per μm^2, $n = 30$, $p < 0.006$, Figures 7A and 7B), consistent with our findings that exposure of cultured neurons to ephrinB leads to an increase in the number of postsynaptic specializations. When the effect of wild-type EphB2 expression on the number of postsynaptic sites was compared with that of the dominant inhibitory EphB2 constructs, a highly significant decrease in the number of postsynaptic specializations that form was observed. These findings indicate that perturbations of EphB signaling affect the number of NR1 positive postsynaptic specializations that form in cultured neurons. In contrast, neurons transfected with constructs expressing trkB or a kinase-dead trkB (trkBKD) appeared not to affect the number of NR1 containing specializations, suggesting that the effects on the number of NR1 sites that result from modulation of EphB2 signaling are specific (Figure 7C). Taken together, our results suggest that while the cytoplasmic domain and the kinase activity of the EphB2 RTK are not required for the initial interaction of EphB and NMDA receptors, the cytoplasmic domain and in particular the kinase activity of EphBs may be important for subsequent events that contribute to the number of NMDA receptor-containing postsynaptic specializations.

Discussion

EphB/NR1 Interaction

As more examples of molecules that regulate aspects of synapse formation or maturation are described, it is becoming clear that there may be a variety of factors that regulate the process of synapse development (Hall et al., 2000; Scheiffele et al., 2000). EphB receptors are localized at the postsynaptic membrane and associate with molecules such as PICK1 and AF6 that are established structural components of the synapse (Torres et al., 1998; Buchert et al., 1999). In addition, EphB receptors interact with a host of intracellular effector molecules including Src, Nck, and Grb2 (Bruckner and Klein, 1998; Yu and Salter, 1999), although the role for these molecules in synapse development is not yet clear. Here, we show that ephrinBs induce the association and clustering of EphBs with NMDA receptors. This interaction is mediated by the extracellular domains of both EphB2 and NR1 and is likely to be direct. In in vitro binding experiments, the extracellular domains of recombinant NR1 and EphB2 interact with one another in a complex with the extracellular domain of ephrinB1. These results suggest that, in neurons, the binding of ephrinB to EphB2 may lead to the direct recruitment of NR1 and its associated subunits (NR2A-B) to the EphB complex. The binding of ephrinB to EphB also results in the recruitment of other proteins, including CaMKII and Grb10, to the EphB/NMDA receptor complex. Taken together with previous findings, our results indicate that EphB receptors are linked to structural and signaling molecules at the synapse that may enable an EphB receptor–driven signal to contribute to the development or function of synapses.

The observation that EphB and NMDA receptors interact raises the intriguing possibility that cross-talk exists between these receptors to elicit changes in the functional properties of these proteins. One possibility is that the association of EphB and NMDA receptors could lead to changes in NMDA receptor function, perhaps via the phosphorylation of NMDA receptor subunits. The EphB RTK may phosphorylate the NMDA receptor either directly or via an associated kinase. Members of the Src family of tyrosine kinases, Src and Fyn, are good candidates to mediate EphB-dependent tyrosine phosphorylation of the NMDA receptor, as both of these Src family members bind Eph receptors and have been shown to regulate NMDA receptor function (Bruckner and Klein, 1998; Yu and Salter, 1999). CaMKII is another good candidate, as we have shown it is recruited to the EphB/NMDA receptor complex, and CaMKII has been shown to phosphorylate the NMDA receptor (Omkumar et al., 1996). Phosphorylation of the NMDA receptor may alter its channel conductance and NMDA receptor phosphorylation may underlie aspects of LTP (Malinow et al., 1988; Swope et al., 1999; Yu and Salter, 1999). Thus, the assembly of the EphB and NMDA receptor complex may lead to changes in the channel properties of the NMDA receptor that could play an important role in synapse development or plasticity. This conclusion is supported by our observations that ephrin stimulation of EphBs enhances the formation of both pre- and postsynaptic specializations, and that blocking the ability of EphB to signal via their receptor tyrosine kinase activity suppresses the number of postsynaptic specializations.

Synapse Development

Many issues remain to be clarified regarding the mechanism by which EphBs promote synapse development. These include characterization of the signaling pathways that trigger the clustering of the EphB and NMDA receptors and induce synapse formation, and the clarification of whether the interaction between EphBs and

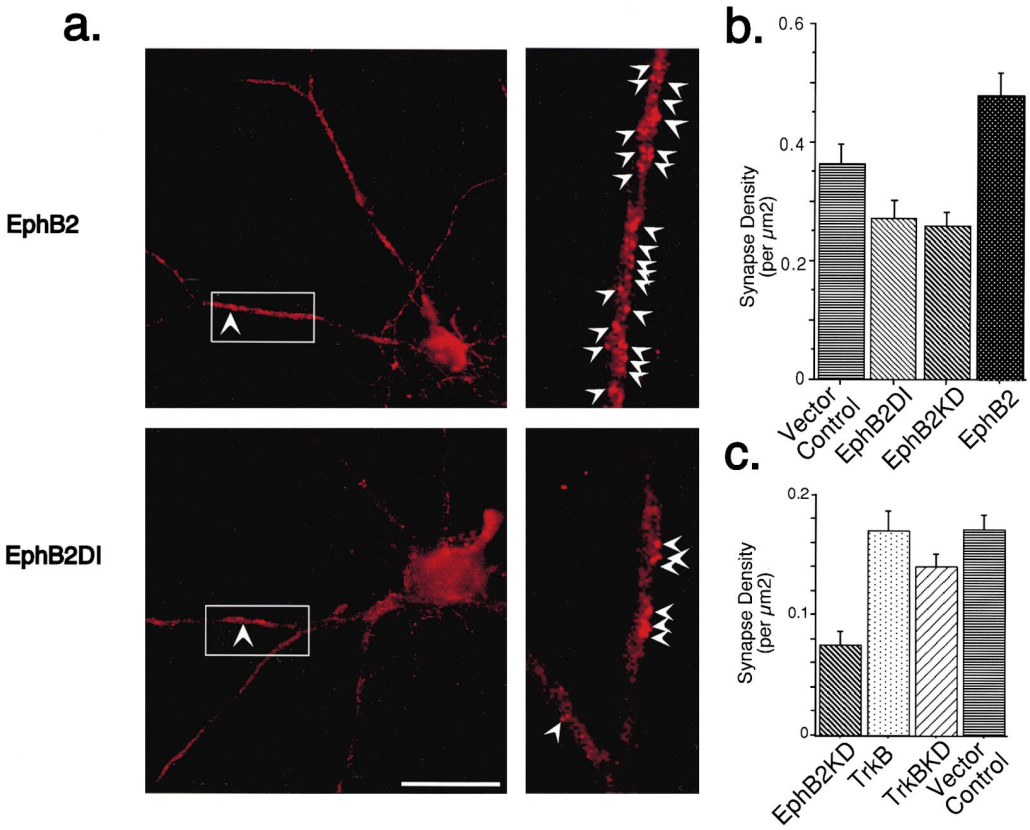

Figure 7. Effects of Overexpressing EphB2 Constructs on NMDA Receptor Postsynaptic Sites

Cultures of cortical neurons were transfected at 4 DIV and processed for immunocytochemistry at 7 DIV. Transfected cells were identified by GFP staining and sites were counted as in Figure 6.

(A) Mouse monoclonal anti-NR1 antibody staining of neurons transfected with either wild-type EphB2 or EphB2DI and GFP. Arrows indicate examples of NMDA receptor staining counted as clusters. Insert on right is a 4.5× magnified view of boxed regions. Scale bar = 25 μm.

(B) Graph of four independent experiments of the density of NR1 sites on the proximal dendrites of a GFP- labeled neuron cotransfected with various EphB2 constructs. Overexpression of EphB2DI or EphB2KD results in a significant decrease ($p < 0.04$) and overexpression of wild-type EphB2 results in a significant increase in the density of postsynaptic NMDA receptors ($p < 0.006$).

(C) Graph of two independent experiments of the density of NR1 sites in GFP-labeled neurons cotransfected with EphB2KD, trkB, trkBKD, or vector control. Overexpression of trkB ($n = 9$ cells) or trkBKD ($n = 17$ cells) did not lead to a significant change in the density of NR1 sites.

NR1 might play a role in synaptic plasticity. Mice lacking EphB family members have already been generated using gene disruption strategies and have no reported synapse formation defect (Henkemeyer et al., 1996; Orioli et al., 1996; Birgbauer et al., 2000). However, given that numerous EphB receptor family members can interact with NR1, these results are not surprising. In our own preliminary studies of mice lacking both EphB2 and EphB3 (Orioli et al., 1996), we find that EphB receptor family members that are still expressed in these mice are capable of associating with NMDA receptors following ligand stimulation (M. B. D, M. E. G., I. Grundwald, and R. Klein, unpublished data). Thus, in the future, to address the role of EphB in vivo, it will be necessary to employ strategies designed to disrupt the function of multiple members of the EphB family during development. In addition, several studies have found that early during development, EphBs localized on axons, while ephrinB localized on targets (Flanagan and Vanderhaeghen, 1998). However, in adult hippocampus, EphB has been shown to be postsynaptic (Buchert et al., 1999). It will be important to undertake a detailed examination of the localization of EphB and ephrins during synapse formation.

Eph receptors and ephrins have well-known functions in the establishment of segmental boundaries as well as in mediating repulsive axon guidance cues (Flanagan and Vanderhaeghen, 1998). The findings reported here suggest that in addition to their previously described roles, EphB and ephrinB1/B2 may regulate initial steps in the development of synaptic connections. Eph receptors and ephrins may function not simply as a stop or boundary signal, but also as a signal initiating further differentiation. For EphBs and ephrinBs, this differentiation process appears to play a role in the formation of synaptic contacts. The ephrinB/EphB interaction then may contribute to the recruitment of proteins that form the macromolecular complexes that constitute a functional synapse. Our study suggests that one of the first steps in this process may be recruitment of the NMDA receptor to the nascent postsynaptic specialization (Rao et al., 1998). As the EphB/NMDA receptor complex forms at postsynaptic sites, a signal may be transmitted to the nascent presynaptic site. Given the capacity of the

ephrinB/EphB complex for reciprocal signaling (Holland et al., 1996; Bruckner et al., 1997), it is possible that as the EphB/NMDA receptor complex forms, a signal is sent from EphB to ephrinB or other proteins that could play a critical role in the maturation of presynaptic components. Alternatively, ephrinB may function in concert with other factors such as wnts or neuroligins that then promote the maturation of pre- and postsynaptic specializations (Hall et al., 2000; Scheiffele et al., 2000). Further analysis will be required to assess the various roles that ephrinBs and EphBs play in orchestrating the formation of functional synapses in vitro and in vivo.

Experimental Procedures

Cell Culture and Transfection

Cortical and hippocampal neurons were made from E17–E19 animals using previously described methods (Xia et al., 1996). Cells were plated at 25,000–50,000 cells per well of a 24-well plate for the coclustering assays and long-term ephrinB1 stimulation, at 100–125,000 per well for transfection, and at 10×10^6 cells per 100 mm dish for biochemical studies. 293T cells and neurons were transfected using the calcium phosphate precipitation method (Xia et al., 1996).

Activation of Ligands

Activation of the Eph and chimeric receptors was achieved with clustered multimeric ligands. EphrinA1-Fc, ephrinB1-Fc, ephrinB2-Fc, and Fc were provided by Regeneron Pharmaceuticals. CSF-Fc was generated from sequences encoding human CSF-1 (amino acids1–197) amplified by PCR and cloned in place of the ephrinA1 sequence in pJFE14-ephrinA1-Fc (Regeneron). Ligands were multimerized using 50 ng/ml anti-human Fc (Jackson Labs) and used at 500 ng/ml to stimulate neurons.

Immunoprecipitation and Western Analysis

For EphB2 interaction assays, cells were lysed in standard NP-40 or RIPA lysis buffers (cultured 293Ts or neurons) or buffer HO (brain lysates; Buchert et al., 1999). Lysates were incubated with primary antibody and bound to protein G Sepharose beads, separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted. The following antibodies were purchased: anti-FLAG M2 (Eastman-Kodak), anti-NR1 (Pharmingen), anti-NR2A (Affinity Bioreagents), anti-NR2B (Affinity Bioreagents), anti-GluR1 (Chemicon), anti-GluR2/3 (Chemicon), and anti-phophotyrosine4G10 (UBI). Anti-phospho-TrkB was a gift from Dr. R. Segal. Phospho-Eph antibodies were generated against a phosphopeptide of the sequence CMKI pYIDPFTpYEDPNE for phospho-EphB2, and CVRTpYVDPFTpY EDPNQ. A GST-EphA4 fusion protein containing mouse EphB2 amino acids 888–963 and a GST-EphA4 fusion protein containing mouse EphA4 amino acids 909–986 were used to prepare antibodies against EphB2 and EphA4.

In Vitro Binding Assay

NR1-HIS was transfected into 293T cells. Opti-Mem (Gibco) supernatants were collected for six days. ProBond Resin nickel beads (Invitrogen) were washed 3 times in PBS and then incubated with NR1-HIS supernatant overnight at 4°C. The NR1-HIS beads were washed 3 times in binding solution (pH 7.8 binding buffer (Invitrogen) + 150 mM imidazole (Sigma) + 0.01% NP-40). After overnight incubation with either Fc (4μg), ephrinB1-Fc (2μg + 2μg Fc), EphB2-Fc (2μg + 2μg Fc), or ephrinB1-Fc and EphB2-Fc (2μg each), beads were washed 3 times in the binding solution and 2 times in pH 7.8 binding buffer alone. After washing, SDS sample buffer was added and proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-human IgG to recognize the Fc domain of the Fc tagged proteins.

Immunostaining

Cells were stimulated for 0 to 60 min with clustered, multimeric ligands and fixed for 8 min with 4% paraformaldehyde and 4% sucrose in PBS. For the clustering assay, neurons were stained with ephrinB1-Fc (Regeneron Pharmaceuticals), anti-EphB2, anti-phospho-EphB2, anti-Nck (Transduction Laboratories), anti-PSD95 (UBI) antibodies. Secondary anti-Cy2 (1:250) and anti-Cy3 (1:250) were applied for 1–2 hr at room temperature and nuclei were stained with Hoechst and coverslips mounted with Aqua-Mount (Lerner #13800).

Imaging

Images were acquired using a Noran OZ Confocal System on a Nikon Diaphot 200 Inverted Microscope with either a 40× Nikon oil objective or 20× Leitz air objective. Some images were acquired with OpenLab (Intervision) on a Nikon Diaphot 300 inverted microscope with a 60× objective.

FM1-43 Imaging

At 7 DIV, coverslips containing neurons were removed from the culture medium and placed into an artificial cerebrospinal fluid (ACSF; 145 mM NaCl, 5 mM KCl, 3.5 mM $CaCl_2$, 1.6 mM $MgCl_2$, and 10 mM HEPES) on an unheated stage of the confocal microscope and neurons were then stained for 1 min in a solution of ASCF containing 55 mM KCl and the vital dye FM1-43 (1 μM) to label sites of transmitter release (Ryan et al., 1993; Cochilla et al., 1999). Stained cells were washed for 5 min and then destained by depolarizing with ASCF containing 55 mM potassium to release dye trapped in synaptic vesicles. During the wash and following depolarization, images were acquired. Synaptic release sites were identified by subtracting images of FM1-43 stained cells following the first wash from those following depolarization (Figure 6A). We determined the sites where the staining changed and counted these discrete spots using the Macintosh program NIHImage. These discrete spots have previously been shown to be sites of neurotransmitter release (Ryan et al., 1993; Cochilla et al., 1999).

GFP/NR1 Imaging

Cells were stained with anti-GFP antibodies and anti-NR1 antibodies and stained with secondary Cy2 and Cy3, respectively. Images were acquired on a confocal microscope with constant settings in each of 5 independent experiments.

Analysis

FM1-43 Experiments

For each coverslip, we imaged two different locations and averaged the number of release sites. The number of release sites per field were counted using NIHImage to identify and count sites that met brightness and size criteria. Brightness criteria were set so no grayscale value less than 35 was ever accepted, and to maximize the number of sites counted. The size criteria were set to accept sites between 0.08–0.25 μm^2. Results were then normalized to the vehicle control and averaged over six independent experiments.

GFP/NR1 Experiments

Using the GFP image as a mask, all other NR1 labeling was removed by subtraction. Regions of the proximal dendrites were selected for analysis in a blind fashion. Approximately 200 μm^2 of dendrite were selected for each cell analyzed. The number of NR1 synaptic clusters that met brightness and size criteria were counted by NIHImage on a Macintosh computer program. Brightness criteria were set so that no grayscale value less than 80 was accepted, and to maximize the number of sites counted. The size criteria were set to accept sites between 0.04–0.25 μm^2. The density of synaptic connections was calculated and the statistical analysis was done with StatView.

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