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EphBs: an integral link between synaptic function and synaptopathies

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Abstract

Assembly and function of neuronal circuits rely on selective cell-cell interactions to control axon targeting, generate pre- and postsynaptic specialization, and recruit neurotransmitter receptors. In neurons, EphB receptor tyrosine kinases mediate excitatory synaptogenesis early in development, and then later coordinate synaptic function by controlling synaptic glutamate receptor localization and function. EphBs direct synapse formation and function to regulate cellular morphology through downstream signaling mechanisms and by interacting with glutamate receptors. In humans, defective EphB-dependent regulation of NMDA receptor (NMDAR) localization and function is associated with neurological disorders including neuropathic pain, anxiety disorders, and Alzheimer's disease (AD). Here, we propose that EphBs act as a central organizer of excitatory synapse formation and function, and as a key regulator of diseases linked to NMDAR dysfunction.

Keywords

Alzheimer's disease; Neuropathic pain; Synaptogenesis; NMDA receptors; Receptor tyrosine kinase; Ephrin

Introduction

Synapses enable information flow within the central nervous system. Early in development there is a period of new synapse addition followed by synapse maturation [1, 2]. Formation of excitatory synapses requires precise coordination between two contacting neurons to organize a presynaptic terminal capable of neurotransmitter release, and a postsynaptic specialization equipped with the proper neurotransmitter receptors [1-4]. Maturation of synapses involves the pruning of inappropriate connections, stabilization of pre- and postsynaptic components, and formation of appropriate morphological specializations. Finally, at the mature synapse proper function requires synaptic maintenance and plasticity [2]. The improper formation and function of synapses can have devastating consequences.

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Malfunctions in synaptic formation and maturation are implicated in a wide variety of neurodevelopmental disorders, including autism spectrum disorders (ASDs). Furthermore, alterations in synaptic function contribute to neuropathic pain, anxiety disorders, and AD[5-9].

Excitatory synaptic transmission is mediated by AMPA-type [10] and NMDA-type [11] glutamate receptors. AMPA receptors (AMPA) and NMDARs are directed to the cell surface and synaptic sites by both neuronal activity and intermolecular interactions. At synaptic sites, glutamate receptors are dynamically regulated and changes in the size and number of AMPARs and NMDARs at synaptic sites underlie the expression of synaptic plasticity [12-16]. Many of the molecular mechanisms governing glutamate receptor trafficking, retention, and maintenance at synaptic sites are well characterized. Especially important are associations of glutamate receptors with scaffolding proteins such as postsynaptic density protein 95 (PSD-95) and glutamate receptor-interacting protein 1 (GRIP1), and with synaptic adhesion molecules such as EphBs, neuroligins, and epidermal growth factor receptor family (ErbBs) [1, 3, 4]. Dynamic glutamate receptor trafficking is not only important for normal brain function, but deficits in trafficking are associated with diseases including AD, addiction and schizophrenia [11, 17].

Numerous cell signaling and adhesion molecules coordinate the differentiation, morphological changes, and organization of proteins required to generate a functional synapse [1, 3, 4]. One member of these multifunctional synaptogenic molecules are the EphBs [9, 18-20], part of the larger Eph (erythropoietin-producing hepatocellular carcinoma) family of receptor tyrosine kinases (RTKs). The Eph receptors are divided into two classes (A and B) by their ability to bind their membrane-attached ephrin ligands. In mammals there are nine members of the EphA class (A1-A8, A10) and five members of the EphB class (B1-B4, B6) [18]. Ephrin-As (A1-A5) are attached by a Glycophosphatidylinositol (GPI) anchor, whereas ephrin-Bs (B1-B3) contain a short intracellular signaling domain [21]. Typically, EphAs specifically bind ephrin-A ligands, and EphBs bind ephrin-B ligands. However, EphA4 has a high binding affinity for ephrin-B ligands and EphB2 binds ephrin-A5 [22, 23]. Generally, Ephs coordinate signaling events that occur between cells including: axon guidance, synaptogenesis, dendritic filopodia motility, neural crest cell and stem cell migration, angiogenesis, cell sorting at compartmental boundaries, bone formation, and synaptic plasticity [18, 20, 24, 25].

This review focuses on EphB/ephrin-B signaling mechanisms that control NMDAR function and localization. We highlight how EphB-dependent misregulation of NMDARs contributes to neurological diseases such as neuropathic pain, anxiety disorders, and AD.

EphBs and Synapse Formation

In vivo and *in vitro* there is a rapid phase of synapse addition early in neuronal development followed by a plateau phase, and later synaptic loss as contacts mature [26-28]. During the phase of rapid synaptogenesis, dendrites and axons have motile filopodia that appear to search for contacts. Different molecules control specific aspects of new synapse accumulation: Synaptic Cell Adhesion Molecule 1 (SynCAM1) restricts the number of filopodia at axonal growth cones [29], neuroligin stabilizes dendritic filopodia [30], and EphBs controls dendritic filopodia motility enabling synapse formation [28]. EphB-dependent synapse formation driven by filopodia motility requires ephrin-B binding, EphB kinase activity, and p21 activated kinase (PAK) [28], suggesting a model (Figure 1a-c) in which filopodia find appropriate target axons, and motility subsequently decreases leading to stabilized synaptic contacts. Next, through trans-synaptic interactions with ephrin-Bs,

EphBs initiate a program of pre- and postsynaptic maturation through extracellular protein-protein interactions and intracellular signaling.

Organization of presynaptic specializations

EphBs organize functional presynaptic specializations by binding specific ephrin-B ligands at sites of contact between dendrites and axons [31, 32]. The number of presynaptic specializations is decreased by knockdown either postsynaptic EphB2, presynaptic ephrin-B1, or presynaptic ephrin-B2 [31-33]. Ephrin-B1 and ephrin-B2 organize presynaptic terminals through interactions with presynaptic scaffolding molecules containing PSD-95, *Drosophila* discs large protein, and ZO-1 (PDZ)-binding domains. Specifically, ephrin-Bs recruit the adaptor protein syntenin-1 to new presynaptic sites and syntenin-1 is required in contacting axons for EphB-dependent formation of presynaptic specializations [32]. Syntenin-1 could then recruit synaptic vesicles by interacting with ELKS/RAB6-interacting/CAST family member (ERC2/CAST1) and Rab3-interacting molecule-1 (RIM1) [34]. Together, these results provide a link from postsynaptic EphB through presynaptic ephrin-B1/2 and syntenin-1 to the formation of functional presynaptic specializations (Figure 1d).

Regulation of postsynaptic specializations

EphBs regulate maturation of postsynaptic sites by inducing spine morphogenesis and recruiting neurotransmitter receptors (Table 1). Consistent with their importance in these functions, *EphB1*^{-/-}, *2*^{-/-}, *3*^{-/-} triple knockout (TKO) mice have reduced excitatory synapse density (~40% cortex, ~25% hippocampus) [31, 35]. The effects of EphBs on spine development appear similar in cortex and hippocampus. Spine density and postsynaptic density size are reduced in the hippocampus of EphB TKO animals [35]. Similarly, synapse and spine density are decreased in the cortex of EphB TKO, but not double knockout (DKO) animals [31]. In the cerebellum it appears that EphBs may act differently because EphB TKO mice have increased numbers of spines [36]. However, these may be so called “naked” spines that lack presynaptic specializations and are associated with defective synaptogenesis in the cerebellum [37]. More work will be needed to resolve the mechanisms mediating these differences in EphB function.

Changes in spine and synapse density in the cortex may result from decreases in dendritic filopodia motility [28]. Filopodia motility is impaired in cortical slices from EphB TKO, but not DKO animals [28]. In cultured hippocampal neurons, expression of any EphB is sufficient to rescue defects in synapse development [35], while re-expression of EphB2 in EphB TKO slices rescues deficits in synapse density and spine formation [31]. These findings suggest that any EphB is sufficient to initiate EphB-dependent synaptogenesis. Although EphBs may play redundant functions, individual EphBs can exert their effects on specific circuits or domains of neurons. The pattern of EphB expression in the hippocampus suggests localized function: EphB2 and EphB3 are expressed in CA1, and EphB2 and EphB1 are expressed in CA3 [35, 38, 39]. In the cortex, EphBs can preferentially affect dendritic protrusions in basal dendrites of pyramidal neurons [40]. Together, these findings suggest that in addition to regulating synapse formation, specific EphB family members may have selective functions in different brain regions.

EphBs control neuronal morphology and motility by modulation of the actin cytoskeleton. EphBs exert these effects through GTPases such as Rho, Rac1, and Cdc-42 [9, 18], by suppressing activity of the actin-severing protein cofilin [41, 42], and by the phosphorylation of the cell surface proteoglycan syndecan-2 [33]. A principle mechanism enabling EphBs to signal to the actin cytoskeleton is by interacting with guanine exchange factors (GEFs), which accelerate the exchange of GDP for GTP [9, 18]. EphBs interact directly with GEFs such as Rho/Rac1-GEF Kalirin-7[43], Rac1-GEF Tiam1[44, 45], and

with Rho-GEF intersectin-1 through the adaptor protein Numb [46, 47] recruiting these proteins to synapses and spines. In addition to their interaction with EphBs, intersectin-1 and Tiam1 can associate directly with NMDARs at synaptic sites to modulate receptor function [44-47]. By linking to Rac1 and Rho/Cdc-42 signaling pathways, EphBs can prevent depolymerization of the actin cytoskeleton. Preventing depolymerization drives synapses to mature and form mushroom shaped spines.

In addition to coupling with positive regulators of synapse development, the synaptogenic activities of EphBs are subjected to a specific negative regulatory pathway. EphB activity is inhibited by binding a RhoA-GEF called Ephexin5 [48]. Ephexin5's inhibition of EphB is released by ephrin-B activation of EphBs [48]. EphB activation causes phosphorylation of Ephexin5 on tyrosine-361 that enables the ubiquitin ligase E3A (Ube3A) to bind and ubiquitinate Ephexin5 [48]. Then ubiquitinated Ephexin5 undergoes proteasomal degradation, allowing EphBs to initiate synapse formation [48]. Notably, Ephexin5 knockout mice have increased synapse number [48]. Consistent with a negative regulatory function, overexpression of Ephexin5 reduces the number of excitatory synapses [48]. These findings link EphBs to human cognitive disorders: AS and ASDs. Mutations or deletions of Ube3A are a cause of AS and duplications in the 5q11-q13 locus, which includes Ube3A, are found in genetic forms of ASDs [6]. Whether these diseases result from a misregulation of Ube3A function during synapse maturation [49], or regulation of AMPAR trafficking [50] remains to be elucidated. Nonetheless, these findings place EphB signaling pathways in a key position to regulate diseases of cognitive dysfunction.

Role in synapse maturation

As neurons mature, synapse addition slows while synaptic function becomes more stable and reliable [26, 27]. EphBs also act as central organizers to coordinate these events. In the mature cerebral cortex and hippocampus, both immuno-electron microscopy and biochemical fractionation experiments indicate that EphBs are localized to the pre- and postsynaptic terminal suggesting that EphBs continue to play a role at mature synapses [51-53]. However, EphBs are not critical to maintain synapse density once synapses have formed [28]. Specifically, if EphB2 expression is reduced with short hairpin RNA (shRNA) knockdown at mature synapses (DIV14-21), there is no effect on synapse density [28]. Moreover, overexpression of EphB2 in neurons from TKO mice rescues synapse density early in development (DIV3) but fails to rescue synapse formation when expressed later (DIV10) [28]. These findings suggest that EphBs control synaptogenesis selectively during the rapid phase of synapse addition (DIV0-14) [28]. Interestingly, Ephexin5 expression is down-regulated as EphBs begin to function to control synaptogenesis (DIV7-8) [48, 54] suggesting a possible restrictive mechanism. After synapses begin to mature (>DIV14), EphBs are not required for synapse maintenance, but instead EphBs shift to control of synaptic function. While the causes of these changes in EphB function are unknown, the role of EphBs in the mature brain is the focus of the next two sections of this review.

EphB Trafficking and Regulation of AMPARs

Receptors undergo regulated delivery to the plasma membrane and removal from the cell surface [55]. For EphBs, receptor trafficking is critical to coordinate pre- and postsynaptic formation, and to regulate glutamate receptor function, while EphB receptor cleavage and internalization may mediate detachment of contacts and repulsion [21, 56-61]. Although the mechanisms controlling EphB delivery to the membrane are poorly understood, work from non-neuronal and neuronal systems demonstrate that EphB membrane localization is tightly regulated [21, 61, 62]. The key points of regulation are EphB receptor cleavage, internalization, and degradation [61]. Activated EphBs undergo proteolytic processing by A Disintegrin and Metalloproteinase 10 (ADAM10) and matrix metalloproteinases (MMPs)

[56, 57]. To enable events such as growth cone collapse, the EphB2 receptor can be endocytosed via clathrin-mediated mechanisms, ubiquitinated, and targeted for degradation in the proteasome [48, 55, 61, 62]. Interestingly, the entire EphB-ephrin-B receptor-ligand complex can be transendocytosed bidirectionally into the EphB or ephrin-B expressing cell [58, 60]. Although what differentiates among these various modes of receptor trafficking is poorly understood, receptor trafficking is clearly important for EphB dependent regulation of synaptic proteins.

EphBs can regulate both the surface localization and the function of AMPARs (Figure 2a). In cultured neurons, EphB2 and AMPARs associate by each binding to the PDZ-adaptor proteins Protein Interacting with C-Kinase 1 (PICK1) and GRIP1 [63, 64]. Both PICK1 and GRIP1 bind to AMPARs and are thought to act in opposition: GRIP1 promotes AMPAR surface retention, while PICK1 acts to remove AMPARs from the cell surface [12]. However, a link between PICK1, EphB2 and AMPAR trafficking has not been shown [65]. Instead, PICK1 appears to cluster EphB/ephrin-Bs at synaptic sites [64]. In neurons, GRIP1 appears to help localize both EphB2 and GluA2-containing AMPARs to the dendritic plasma membrane [66]. Consistent with a role for EphBs in control of AMPAR trafficking, EphBs are important for retention of AMPARs in the receptor recycling pool [31]. This control of AMPAR trafficking requires ephrin-B activation, the PDZ-binding domain, and kinase activation of EphB2 receptors [31]. EphB-dependent internalization of AMPARs likely relies on synaptojanin-1, which is phosphorylated by EphB2, promoting its activation [67]. Since GRIP1 and PICK1 preferentially interact with the GluA2 subunits, which regulate calcium influx through AMPARs [10, 12], these findings suggest that EphB receptors may regulate synaptic AMPAR subunit composition.

Physiological evidence demonstrates that EphBs modulate synaptic AMPAR function. The AMPAR-dependent component of miniature excitatory postsynaptic currents (mEPSC) in mature (DIV21-23) neurons is increased by EphB2 overexpression, and reduced by knockdown of EphB2 [53]. However, consistent with the different developmental functions of EphB2, knockdown of EphB2 in younger neurons has no effect on mEPSC amplitude [31]. The lack of a change in the level of GluA2 at cortical synapses in EphB TKO mice suggests that EphBs may regulate AMPAR receptor subunit composition rather than overall number [53]. However, additional work is required to demonstrate the impact of EphB-dependent regulation of AMPARs.

EphBs and NMDARs

EphB receptors regulate multiple facets of NMDAR surface localization, function, and downstream signaling. EphBs and NMDARs colocalize at synaptic sites, and mice lacking EphB2 have reduced levels of NMDARs at synapses in the hippocampus and cortex [31, 35, 68]. Upon ephrin-B binding, EphBs interact directly with NMDARs through their extracellular domains [68-71]. However, the specific region of the extracellular domains involved in this interaction for both EphB2 and the NMDAR remains to be identified. Ephrin-B activation of EphBs not only induces the EphB-NMDAR interaction, but also potentiates NMDAR function [68, 72]. Ephrin-B activation of the EphB-NMDAR interaction causes Src kinase-dependent phosphorylation of the GluN2B subunit of the NMDAR at tyrosine residues 1226, 1336 and 1472 [72]. Phosphorylation at tyrosine 1472 of GluN2B-containing NMDARs blocks binding of the clathrin adaptor protein 2 (AP2) complex, preventing clathrin-dependent endocytosis [73]. Thus, the EphB-NMDAR interaction can increase the surface retention of GluN2B-containing NMDARs.

In addition, EphB2 can increase calcium influx through GluN2B-containing NMDARs by decreasing calcium-dependent inactivation rates [53, 72 and Nolt, 2011]. Notably, EphB2

does not appear to act similarly on calcium inactivation of GluN2A-containing NMDARs [53]. The enhanced calcium influx through the NMDAR also results in enhanced downstream gene transcription [72]. In EphB TKO mice there is a reduction in GluN2B at synaptic sites in cerebral cortex and cultured hippocampal neurons [31, 35]. At the mature synapse, knockdown of EphB2 causes a reduction in mEPSC amplitude and NMDAR-dependent decay time, while overexpression of EphB2 causes an increase [53]. This study specifically implicated the trafficking of GluN2B-containing NMDARs to synaptic sites after ephrin-B activation in the mature brain [53]. A recent report suggests that EphBs may also interact with and regulate the function of nicotinic acetylcholine receptors (nAChRs) [74], suggesting the possibility that EphBs play a larger role in the regulation of neurotransmitter receptor function than previously appreciated. Regardless, these data support a model (Figure 2b) whereby EphBs are not required for synapse maintenance, but are instead critical regulators of NMDAR localization, function, and signaling in the mature brain.

The extracellular domain-mediated EphB-NMDAR interaction has also been implicated in NMDAR-dependent forms of hippocampal synaptic plasticity, particularly for EphB2. *EphB2*^{-/-} null mice have reduced synaptic NMDAR, but not AMPAR currents, reduced long-term potentiation (LTP), and long-term depression (LTD) [38, 69]. Importantly, these effects are rescued by transgenic expression of a truncated EphB2 receptor lacking its kinase domain [69]. These data suggest that EphB-dependent regulation of synaptic plasticity requires the extracellular domain-mediated interaction between EphB and NMDARs. Consistent with these deficits in synaptic plasticity, *EphB2*^{-/-} mice perform poorly in behavioral learning tasks such as the Morris water maze [69]. Together, these data imply that the EphB-NMDAR interaction is required for proper synaptic function, synaptic plasticity, and behavior.

EphB-NMDAR Interaction in Disease

Evidence suggests a synaptic origin for many diseases of neuronal development and the aging brain. Proper NMDAR synaptic localization and function appear to be linked to these synaptopathies. By direct interaction and functional modulation of the NMDAR, EphBs and ephrin-Bs appear to be key synaptic regulators implicated in many of these diseases. We will discuss three examples of EphB/ephrin-B signaling in disease: neuropathic pain, anxiety disorders and AD.

Neuropathic pain and hyperalgesia

Control of NMDAR function by EphBs plays a critical role in the enhancement of pain. In the spinal cord and periphery, neuropathic pain (chronic pain caused by injury) and hyperalgesia (increased sensitivity to painful stimuli) are linked to EphB1/ephrin-B signaling through modulation of NMDAR function. Remarkably, in four different models of pain - thermal hyperalgesia, spontaneous, crush, and mechanical allodynia - EphB1 functions similarly (Figure 3a-d). Despite differences in models, neurons, and brain regions mediating pain, downstream signaling mechanisms are well conserved. Therefore, EphB-dependent modulation of NMDAR function may be an important target for the control of pain.

Induction of neuropathic pain in the spinal cord is mediated by dysregulation of excitatory glutamatergic synapses between the axons from sensory neurons in the dorsal root ganglia (DRG) and second-order neurons in the dorsal horn (DH) [75]. At the DRG-DH synapses, induction of neuropathic pain depends on NMDAR function [75]. Specifically, NMDAR dependent LTP-like changes in synaptic strength are suggested as the cellular mechanism for pain amplification and hyperexcitability of the DRG-DH circuit [75].

Ephrin-B1, ephrin-B2, and EphB1 expression levels are upregulated after injury in the DRG and spinal cord [76-78]. Indeed, intrathecal injection of the extracellular domain of EphB1/B2-Fc fused to the fragment crystallizable (Fc) region of human IgG [70, 77, 79], or shRNA against ephrin-B2 [78], prevents EphB/ephrin-B signaling, and decreases hyperalgesia and mechanical allodynia suggesting a role for EphB signaling in regulation of pain after injury. Moreover, injection of ephrin-B2-Fc to activate EphBs induces pain [70, 77, 79, 80].

EphB-dependent modulation of NMDAR function appears to drive EphBs' function in pain responses (Figure 3b-d). Similar to the cerebral cortex, injury induced upregulation of EphB1 and ephrin-B expression appears to directly modulate the function of GluN2B-containing NMDARs. EphB-dependent hyperalgesia depends upon tyrosine phosphorylation of GluN2B in the spinal cord [81], while blocking NMDARs and Src kinase activity is sufficient to prevent hyperalgesia [70, 77, 79]. Src kinase exerts its effects by phosphorylating GluN2B at Y1472 [68, 70, 72]. EphB-dependent regulation of NMDAR function can induce changes in synaptic strength thought to underlie induction and maintenance of hyperalgesia. In the spinal cord, EphB activation lowers the threshold for LTP induction and increases phosphorylation of GluN2B-containing NMDARs [77, 79]. Moreover, inhibition of EphB signaling blocks pain-induced activity-dependent gene transcription of the immediate early gene *c-Fos* [77, 79]. These findings suggest that EphB/ephrin-B signaling is critical to suppressing the injury-induced hyperexcitability of the DRG-DH circuit.

EphB-dependent neuropathic hyperalgesia appears to be mediated specifically by EphB1. EphB1 is expressed at high levels in the spinal cord, and *EphB1*^{-/-} and *EphB1*^{+/-} mice have significantly reduced thermal hyperalgesia and pain-induced hyperexcitability of DH neurons [82, 83]. Similar results are observed when ephrin-B2 is deleted conditionally from nociceptive sensory neurons [84]. Consistent with the importance of EphB1 in control of pain sensitivity, *EphB1*^{-/-} mice show defects in enhancement of pain after morphine-induced withdrawal [82]. EphB1 appears to be the only EphB receptor required for ephrin-B1 induced hyperalgesia because treatment with ephrin-B1-Fc has no effect on pain sensitivity in *EphB1*^{-/-} or *EphB1*^{+/-} mice [82]. These changes in hyperalgesia in *EphB1*^{-/-} mice are linked to NMDAR-dependent increases in phosphorylation of Ca²⁺/Calmodulin-Dependent Protein Kinase II (p-CaMKII), Extracellular Signal-Regulated Kinase (p-ERK) and cAMP Responsive Element Binding Protein (p-CREB) and the induction of *c-fos* expression [82, 83]. Interestingly, EphB1 upregulation after injury appears to be dependent on MMP-2/9, although the specific signaling mechanisms are unknown [85].

The EphB-dependent modulation of NMDAR function is also critically important in pain sensitivity outside of the spinal cord in the periphery. As in the spinal cord, in the peripheral nervous system injection of ephrin-B1-Fc, which activates EphBs, induces hyperalgesia dependent on NMDARs [86-88]. This leads to increased *c-fos* expression and activation of NMDAR-dependent phosphorylation of two pathways: Mitogen-Activated Protein Kinases (MAPKs) (P38 Regulated/Activated Kinase (p-p38), pERK and Jun N-terminal kinase (pJNK)) [86, 87] and Phosphoinositide 3-kinase (PI3K), Protein Kinase B (Akt), and ERK [88]. Together, these results suggest that in the periphery a similar mechanism for hyperalgesia occurs with EphB1 upregulation, NMDAR-dependent phosphorylation of targets required for synaptic plasticity, and changes in gene transcription.

Similar to the central and peripheral pain mechanisms, the EphB-dependent modulation of NMDAR function is implicated in cancer-induced pain for both bone and pancreatic cancer. In models of cancer-induced pain, expression of ephrin-B1 and EphB1 are upregulated [85, 89, 90]. Here, the EphB-NMDAR interaction is also specifically implicated because blocking EphB1 signaling alleviates mechanical allodynia [85]. The mechanism for this

alleviation is decreased activation of GluN2B-containing NMDARs, which reduces the phosphorylation of downstream targets pSrc (Tyr418), pERK1/2, pCaMKII, and pCREB, and gene transcription of *c-fos* [85]. The changes in EphB and ephrin-B1 expression in cancer-induced pain are likely to be downstream of the inflammatory cytokines Interleukin-1 beta (IL-1 β), Interleukin 6 (IL-6) and Tumor necrosis factor-alpha (TNF- α) [89]. Consistent with this, inhibiting the Cholecystokinin B receptor (CCK2)/gastrin receptor with a drug (Z-360) reduces IL-1 β levels, prevents upregulation of ephrin-B1 expression, and reduces GluN2B phosphorylation [90]. The observation that blocking EphB1 activation alleviates morphine tolerance in models of bone cancer pain [85] suggests that modulating EphB signaling could provide an avenue for treating chronic pain. Thus, EphB-dependent regulation of NMDAR function is linked to both the induction and expression of chronic pain.

Anxiety

Emerging evidence links both the regulation of EphB receptor cleavage and EphB-dependent regulation of NMDAR function to anxiety (Figure 4a). Severe or sustained stress can result in changes to synaptic architecture and function mirroring those found after induction of synaptic plasticity, and lead to behavioral changes associated with fear and anxiety disorders [91]. The pathogenic plasticity involved in these changes requires NMDAR activation and increases neuronal activity in the hippocampus and amygdala [91]. Antagonists of NMDAR receptors can produce anxiolytic effects in animal models of anxiety like the elevated plus maze [92]. Several lines of evidence suggest that changes in the extracellular matrix mediated by proteolysis promote an anxiety response [93-96]. The serine protease neuropsin (also known as kalikrein-related peptidase 8) is highly expressed in the hippocampus and amygdala [97]. Furthermore, genetic variations in human neuropsin are associated with bipolar disorder and cognitive functions [98]. Neuropsin-deficient mice have defects in spatial working memory, impaired ability in the Morris water maze assay, and reduced long-lasting LTP [97, 99-101]. In the amygdala, EphB2 and neuropsin colocalize and neuropsin expression is upregulated after stress [71, 98]. Furthermore, neuropsin upregulation and colocalization with EphBs in the amygdala results in cleavage of the EphB2 ectodomain but increases EphB2 expression [71, 98]. Neuropsin-dependent cleavage of EphB2 increases the dynamics of the EphB-NMDAR interaction, likely explaining the changes in NMDAR currents observed in neuropsin null mice [71]. Furthermore, injection of function blocking antibodies to EphB2 or neuropsin can prevent behaviorally stress-induced anxiety in the elevated plus maze task [71]. Together, these data suggest that targeting neuropsin-dependent cleavage of EphB2 is a potential strategy for treating stress-related and anxiety disorders.

In models of stress, somewhat unexpectedly EphBs appear to increase the amount of NMDAR currents at the synapse after neuropsin-dependent cleavage [71]. One potential explanation for the molecular mechanism of these findings comes from the studies on γ -secretase cleavage of EphB2 [57, 102]. Intriguingly, after MMP-dependent cleavage of the EphB2 extracellular domain, the γ -secretase releases a kinase-active fragment in non-neuronal cells [102]. Expression of a soluble kinase active intracellular fragment was shown to phosphorylate GluN2B subunits of NMDARs, resulting in increased NMDAR surface localization [102]. Whether a similar mechanism plays a role in neurons of the amygdala remains unknown. However, these non-neuronal experiments raise the possibility that EphBs may regulate NMDAR function both through direct interactions and by indirectly phosphorylating the NMDAR.

Alzheimer's disease

AD is a progressive neurodegenerative disorder characterized by declarative memory defects and dementia [103]. Among the many pathological changes seen in patient brains are loss of excitatory synapses and increased neuronal death [5, 104]. A reduction in synapse density in the cortex and hippocampus is found during the onset of AD and correlates with the level of cognitive impairment [5, 104]. EphB and NMDAR subunit expression are both reduced early in AD progression [42, 105, 106]. Furthermore, there are reduced levels of GluN2 subunit phosphorylation in postmortem brain samples from AD patients [106]. Thus, EphB-dependent modulation of NMDAR function appears to play a role in the pathogenesis of AD.

Building on these observations, studies have explored whether these effects on NMDAR expression are linked to prominent disease mechanisms such as amyloid- β (A β) [107]. In AD patients, the level of A β in the brain correlates with disease onset and progression [107]. In mouse models and in cell culture, the presence of A β can cause NMDAR endocytosis, reduced surface expression of NMDARs, and reduced NMDAR currents [108]. Also, in mouse models of AD, the A β -dependent reduction in EphB2 expression levels occurs prior to detectable behavioral impairments [42]. A β -dependent reductions in GluN2B-containing NMDARs have also been observed [108].

Recently, a link has emerged between EphB2, A β , and AD through NMDAR phosphorylation and regulation at the plasma membrane (Figure 4b). Consistent with experiments demonstrating reduced EphB2 expression levels in AD [42], A β binds to the extracellular FnIII domains of EphB2, causing receptor internalization and degradation [109]. The functional consequence of the removal of EphB2 from the cell surface by A β appears to be reduced NMDAR surface expression [109]. Furthermore, EphB2 depletion by knockdown or knockout phenocopies A β -dependent reduction in NMDAR surface localization [53, 109]. Similarly, targeted knockdown of EphB2 in the dentate gyrus causes deficits in LTP and NMDAR (but not AMPAR) currents [109]. These findings are consistent with previous observations on the effects on LTP in mice lacking EphB2 and on synaptic function following the knockdown of EphB2 in cortical neurons [38, 53, 68, 69]. The effects of targeted knockdown of EphB2 in the dentate gyrus are mirrored in a mouse model of AD that overexpresses human amyloid precursor protein (hAPP) [109]. Not only did knockdown of EphB2 cause defects in LTP, but viral overexpression of EphB2 in the dentate gyrus of hAPP overexpressing mice rescued LTP and NMDAR current deficits to wild-type levels [109]. Remarkably, targeted overexpression of EphB2 via lentiviral infection in the dentate gyrus was able to rescue hippocampal-dependent cognitive deficits in the Morris water maze in these mice [109]. These effects were seen despite only a relatively low percentage of neurons in the dentate gyrus being infected, suggesting that functional modulation of synapses for only a subset of neurons in a network can have significant effects on behavior. In summary, increasing neuronal EphB2 levels in hAPP mice reversed cognitive and behavioral defects associated with AD, while EphB2 knockdown appears to mimic many of the defects in NMDAR function in these mice.

Conclusions and Future Directions

EphBs regulate excitatory synapse development and function at mammalian synapses by controlling dendritic morphology and excitatory neurotransmitter receptor content. Dysfunction of EphB-dependent control of synaptic NMDAR function and surface localization appears to be involved in neurological disorders including AD and pain. In each of these diseases, the role of EphBs is linked to direct extracellular interaction between EphB and NMDARs. However, the exact domain mediating this interaction is unknown (see Box 1). In anxiety, EphB-dependent regulation of NMDAR function is also important, but

may be regulated by a different mechanism governed by the release of the EphB intracellular domain. However, in these three cases, EphB-dependent regulation of NMDAR function and surface localization has profound consequences. Given the potential role EphB-NMDAR in human disease, understanding the exact cellular mechanisms will likely be of therapeutic significance.

In addition to EphBs, a number of other regulators of synapse development have developmental shifts in function. Focal adhesion kinase [41, 110], SynCAM [111], Synaptic Adhesion-like Molecule 1/2 (SALM1/2)[112, 113], and neuroligin/neurexin [114, 115] all regulate synaptic function in mature neurons, while controlling synaptogenesis during development. These findings suggest that synaptic organizing proteins play dual functions, first to generate synapses and later to control their function. The dual functions of these proteins could explain some of their complex linkages to disease. We propose that there may be sufficient redundancy in synaptogenic factors that most synapses can still form normally after malfunction of any one of these organizers. Once the circuitry begins to mature, however, each of these proteins is required for proper mature synaptic function and breakdowns in these molecules might then manifest as neuronal or cognitive dysfunction. Thus, greater understanding of these synaptic organizing proteins is needed in three broad areas: 1) to investigate their functions in organizing synapses, 2) to appreciate how the activity of these molecules changes during development, and 3) to determine their different roles in controlling synaptic function. Exploring these areas will be critical to discerning brain function and treating synaptopathies.

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Glossary

Allodynia	Pain caused by an innocuous stimulus
Amyloid precursor protein (APP)	Neuronal integral membrane protein concentrated at synaptic sites. Proteolysis of APP first extracellular by Beta-secretase 1 (BACE1) then subsequently γ -secretase generates the 40-42 amino acid β -amyloid (A β) found in amyloid plaques of AD patients
Crush pain model	Under deep anesthesia a spinal nerve distal to the dorsal root ganglions (DRG) is crushed. Thermal hyperalgesia or mechanical allodynia are then tested
DH neuron	Integration of DRG inputs occurs in deep layers of spinal dorsal horn (DH) neurons. Output is carried to projection sites in the brain
DRG neuron	Nociceptive afferents carrying noxious stimuli (heat, noxious cold, pressure, or chemicals) have glutamatergic synapses onto relay neurons in the dorsal root ganglion (DRG)
GTPase	Small monomeric G-proteins which bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP) to stimulate downstream effectors

Guanine Nucleotide Exchange Factor (GEF)	A protein that activates small monomeric GTPase activity by accelerating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP)
Hyperalgesia	Increased sensitivity to pain
Hyperexcitability of DRG neurons	Characterized by lower current threshold for action potentials, an increase in spontaneous activity, and repetitive discharge
Intraplantar injection	Injection into the plantar surface of the paw
Intrathecal injection	Injection into the arachnoid membrane of the spinal cord
Mechanical Allodynia model	The plantar surface of each hind paw pricked with a sharp, cylindrical probe. Incidence of foot withdraw is measured
Miniature synaptic current	The postsynaptic current evoked by a single vesicle (or quanta) of neurotransmitter
Neuropathic pain	Chronic pain caused by injury to the central or peripheral nervous system
Receptor tyrosine kinase (RTK)	Cell-surface localized single-subunit transmembrane protein with intracellular catalytic activity to autophosphorylate and then phosphorylate tyrosine residues on signaling substrates
RNA Interference (RNAi)	A method using small hairpin RNAs (shRNAs) to bind the mRNA encoding a protein of interest to suppress its expression
Spontaneous pain model	Formalin is subcutaneously injected into a hind paw. Amount of time licking, biting, and flinching on the injected paw is assessed
Synaptic cell adhesion molecules	Pairs of molecules that interact across adjacent cells to stabilize the initial contacts between axon and dendrite to form a synapse. They also regulate the function of existing synapses through protein-protein interactions and intracellular signaling cascades
Thermal hyperalgesia pain model	Animals are placed in a testing box with a temperature-controlled floor. A heat source is then focused on the hind paw flushed to the floor and foot withdraw latency is measured

Box 1**Outstanding Questions**

- Which domains within EphBs and NMDARs mediate their interaction?
- How do EphBs differentially regulate downstream GEF signaling pathways?
- Does Ephexin-5 signaling contribute to ASDs?
- How does EphB regulate the function of GluN2B-containing NMDARs?
- Do EphBs regulate AMPARs at mature synapses?
- How is the activity of EphBs switched from synaptogenesis to synaptic maintenance?
- Are there pharmacological agents that can block the EphB-NMDAR interaction for neuropathic pain?
- What is the specific domain within A β that controls its binding to EphB2?
- Are there pharmacological agents that can potentiate the EphB-NMDAR interaction to enhance cognition in neurological disorders such as AD?

(a) EphB domain structure

(c) (d)

Figure 1. EphBs regulate excitatory synapse development

(a) EphB receptors contain the following main domains: Globular ligand-binding domain (LBD), Cysteine (Cys) Rich domain, Fibronectin type III repeat (FnIII) domains, receptor tyrosine kinase (RTK) domain, Sterile Alpha Motif (SAM) domain and PDZ-binding domain [21]. (b) Early in neuronal development (ie. in hippocampal or cortical rat primary neurons at DIV0-10), EphB receptors direct formation of excitatory synapses by regulating motility of filopodia via p21 activated kinase (PAK) and RTK activity [31]. (c) During the rapid phase of synapse addition (DIV7-14), EphBs interact *in trans* with ephrin-B1 or ephrin-B2 expressed on axons of adjacent cells. This EphB/ephrin-B interaction activates EphB kinase activity, which removes inhibition of synapse formation by the specific negative regulator Ephexin-5[48]. EphB activation phosphorylates Ephexin-5, inhibiting RhoA-GTPase activity and promoting ubiquitination and proteasomal degradation of Ephexin-5 by the E3 ligase Ube3A[48]. To promote synapse maturation, EphB kinase activation recruits GEFs to hydrolyze GDP into GTP, activating Rho-GTPases that enable synapse formation through PAK[28, 43]. (d) Postsynaptically, EphBs directly cluster NMDARs through an extracellular interaction[68-71], and cluster AMPARs via a PDZ-domain-dependent interaction with GRIP1[31, 67]. Furthermore, EphBs modulate the change in morphology of the actin cytoskeleton into mature mushroom-shaped dendritic spines. Presynaptically, EphBs direct presynaptic differentiation by clustering ephrin-B1 and ephrin-B2 at presynaptic terminals[31-33]. The EphB/ephrin-B1/2 interaction recruits the adaptor protein syntenin-1 to these signaling complexes through the PDZ-binding domain of ephrin-Bs[32]. Syntenin-1 enables EphBs to recruit the machinery required for neurotransmitter release to presynaptic specializations[34].

Figure 2. EphBs regulate glutamate receptor trafficking and function

(a) EphBs regulate AMPAR trafficking through a PDZ-dependent interaction with GRIP1 and indirect interactions with synaptojanin-1 (Stj1), a phosphatidylinositol 5'-phosphatase[67]. If an EphB receptor is interacting with GRIP1, kinase activation by ephrin-Bs promotes AMPAR insertion into the membrane from the recycling pool[31]. Alternatively, EphB kinase activation by ephrin-Bs can also promote AMPAR internalization by phosphorylation of Stj1, which activates clathrin-mediated endocytotic mechanisms[67]. (b) After binding ephrin-B ligand, EphBs directly interact with NMDARs to regulate their synaptic surface localization and function. Activation of EphBs promotes insertion of GluN2B-containing NMDARs into the synaptic membrane of mature neurons[53]. After activation, EphBs recruit Src kinase to phosphorylate GluN2B-containing NMDARs at Y1472 [72] blocking binding of the AP2 complex and clathrin-mediated endocytosis[73]. Functionally, EphB activation decreases calcium-dependent desensitization, mEPSC amplitude, and decay time of GluN2B-containing NMDAR[53]. This increased calcium influx through NMDARs also leads to EphB driven increases in NMDAR-dependent gene expression of *c-fos*[72].

Figure 3. EphBs and neuropathic pain

(a) Under physiological pain conditions, ephrin-B and EphB1 expression remains low and GluN2B-containing NMDAR signaling remains normal[75]. (b) For pathological central pain, both ephrin-Bs and EphB1 are upregulated[76-78]. Activation of EphBs leads to recruitment and activation of Src, insertion of NMDARs into the membrane, GluN2B phosphorylation at Y1472, and increased calcium influx leading to *c-fos* gene transcription[70, 77, 79, 81]. (c) Pathological peripheral pain shares common mechanisms with central pain. However, three parallel signaling pathways have been well characterized leading to gene transcription of *c-fos* and CRE[86-88]. First, Src phosphorylates CamKII, which phosphorylates CREB causing nuclear translocation and Cyclic AMP Response Element (CRE) gene transcription[87]. Second, PI3K is phosphorylated, which phosphorylates Akt, which phosphorylates ERK, which translocates to the nucleus to activate *c-fos* gene transcription[88]. Finally, JNK gets phosphorylated and activated, which phosphorylates p-38 and converges to activate ERK[86]. (d) Pathological cancer-induced pain shares a remarkable number of the same mechanisms with pathological central and peripheral pain[85, 89, 90]. Unique to cancer-induced pain is that src kinase directly phosphorylates ERK to activate gene transcription of *c-fos*[85]. Additionally, inflammatory cytokines TNFα, IL-6, and IL-1β are upregulated leading to hyperalgesia, and hyperexcitability of nerve afferents[89, 90].

(a)

Anxiety

Figure 4. EphBs and neurological disorders

(a) Physiological EphB signaling affects both synaptic plasticity and synapse maturation. Ephrin-B activation of EphBs promotes the direct EphB- NMDAR interaction[68]. This interaction modulates NMDAR function by increasing calcium influx, inserting new GluN2B-containing NMDARs into the membrane, and activating calcium-dependent gene transcription required for LTP[53, 72]. In animal models of anxiety, there is a stress-induced upregulation of neuropsin [71, 97, 100]. At the membrane, neuropsin cleaves the EphB receptor ectodomain, releasing the intracellular domain into the cytosol[71, 98]. This intracellular kinase may be active and able to phosphorylate downstream intracellular targets including NMDARs[102]. Cleavage of the EphB ectodomain dissociates the EphB-NMDAR interaction, leading to NMDAR internalization and activation of FK506 Binding Protein-51 (Fkbp51) gene transcription in the nucleus[71]. (b) In AD, there is an overabundance of soluble Aβ oligomers, which appear to bind directly to EphB2[109]. The EphB2-Aβ interaction inhibits receptor activation and causes internalization and degradation of both EphBs and NMDARs[109]. Degradation of EphB receptors inhibits their ability to retain NMDARs on the membrane potentially through the EphB-NMDAR interaction[109]. Fewer NMDARs on the cell surface leads to decreased calcium influx and none of the changes in gene transcription required for LTP[15].

Table 1
EphB signaling pathways: evidence for regulatory roles at synapses during development and in the mature nervous system

Manipulation	Description	Functional effects at the synapse	Refs
<i>EphB2</i>			
↓EphB2	siRNA knockdown in developing cultured cortical neurons (before DIV10)	-↓ Functional synapses	[31]
	siRNA knockdown in mature cultured cortical neurons (after DIV14)	-No effect on synaptic density -↓ NMDAR/AMPA-dependent component of mEPSCs	[28, 53]
↑EphB2	Overexpression of EphB2 in mature (DIV21-23) cortical neurons	-↑NMDAR/AMPA-dependent component of mEPSCs	[53]
EphB2 ^{-/-}	KO mouse lacking EphB2	-↓NMDAR currents - no change in AMPAR currents	[38, 69]
		-↓ LTP	
		-↓ LTD	
EphB1 ^{-/-} /2 ^{-/-} /3 ^{-/-}	Triple KO mouse lacking all three neuronally expressed EphBs	-↓ excitatory synapse density, filopodial motility, spine number, and synaptic GluN2B-containing NMDARs	[28, 31, 35, 53]
↑EphB2 in EphB1 ^{-/-} /2 ^{-/-} /3 ^{-/-}	Expression of EphB2 in cultured cortical neurons derived from triple KO mice (before 10 DIV)	- Rescues defects in synapse development	[28]
↑EphB2 in EphB1 ^{-/-} /2 ^{-/-} /3 ^{-/-}	Expression of EphB2 in cultured cortical neurons derived from triple KO mice (after 14 DIV)	-No effect on defects in synapse development	[28]
<i>Ephrins</i>			
↓ephrin-B1	shRNA knockdown in developing cultured cortical neurons (DIV10-21)	-↓ presynaptic specializations	[32]
↓ephrin-B2	shRNA knockdown in developing cultured cortical neurons (DIV21)	-↓ presynaptic specializations	[33]
<i>Other regulatory molecules</i>			
Ephexin 5 ^{-/-}	KO mouse lacking Ephexin 5	-↑ synapse number in the hippocampus	[48]