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REVIEW

EphB and ephrinB in pain signaling

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The Ephrin type B receptors (EphB) and their ephrinB ligands are involved in distinct facets of cell physiology and pathophysiology. EphB-ephrinB signaling mediates synapse formation and plasticity by controlling the insertion, localization and function of glutamate receptors in synaptic membranes. Whereas, EphB-ephrinB signaling at the excitatory glutamatergic synapses in the dorsal horns of the spinal cord, has been implicated in the pathophysiology of pain. Here, the key evidence that support the participation of EphB-ephrinB signaling in pain processes are highlighted. Then, a possible role for the pseudokinase EphB6 in the EphB-ephrinB pain signaling complex is considered. These pathways are currently being intensely studied to exploit selective therapeutic targets for pain relief.

Keywords: EphB; ephrinB; receptor; pain

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Eph receptors and ephrin ligands

Members of the erythropoietin-producing hepatocellular carcinoma receptor family (Eph) are cell surface receptors that comprise approximately 25% of known receptor tyrosine kinases [1]. Eph are categorized into two subclasses, designated EphA and EphB, defined by their receptor-ligand binding preferences, membrane anchorage and sequence homology. At present sixteen Eph have been revealed, including ten type A Eph designated EphA1 to EphA10 and six type B Eph designated EphB1 to EphB6. The two subclasses exhibit related overall structure and characteristics, but differ in their recognition motifs. Although there is extensive structural information available on the extra-and intra-cellular regions of Eph in isolation, no three-dimensional protein structure is currently available for an entire receptor. The extracellular Eph moiety has an ephrin-binding globular domain, a cysteine-rich area with an epidermal growth factor-like motif and consecutive fibronectin type III regions. The cytoplasmic moiety has a short membrane region with a number of residues that are conserved among Eph, a tyrosine kinase domain, a sterile alpha-motif, and a density protein/disc/large zona occludens domain [2, 3, 4]. Eph are stimulated by Eph receptor interacting proteins (ephrins) ligands on adjacent cells mainly through *trans* interactions, thus limiting signaling to cell-to-cell interactions. Predominantly, EphA subclass members interact with five different A type ephrins (ephrinA1-A5), whereas EphB subclass members interact with three different B type ephrins (ephrinB1-B3) [3]. The EphrinA ligand is attached to the cell membrane surface by a glycosyl-phosphatidyl-inositol linkage. The EphrinB

ligand has a transmembrane motif followed by a short cytoplasmic domain with conserved tyrosine phosphorylation sites. Receptor-ligand interactions appear to be promiscuous, since EphA4 also binds ephrinB2 and ephrinB3 [5] and EphB2 also binds ephrinA5 [6]. However, efforts to identify Eph-ephrin specific interactions are ongoing and the roles of many interactions still remain unclear. Interestingly, Eph-ephrin interactions mediate bi-directional signaling. Eph forward signaling includes phosphorylation, autophosphorylation, and other effector protein interactions, while ephrin reverse signaling entails Src or receptor kinase tyrosine phosphorylation of the ephrin cytoplasmic domain [1,2]. Eph and ephrins are expressed in various tissues and organs where they mediate embryonic development processes, as well as physiological and pathophysiological conditions at maturity that include axon guidance, angiogenesis, cell migration, and cancer [2,3]. In nervous system development, EphB receptors and ephrinB ligands participate in synapse and neural circuit establishment [7, 8, 9] and control dendritic filopodia motility facilitating synapse formation, when dynamic filopodia processes are numerous [10]. In the adult nervous system, EphB-ephrinB signaling mediates neural plasticity at glutamaterigic synapses, by interacting directly with the extracellular domains of N-methyl-D-aspartic acid (NMDA) receptors to regulate their surface localization, function and downstream signaling [11, 12,13,14,15].

Evidence for EphB/ephrinB signaling in pain

The initial evidence that linked EphB-NMDA receptor interactions with excitatory synapse formation has led to several studies that support the participation of EphB-ephrinB signaling in inflammatory, neuropathic and cancer pain processing [1,11]. EphB1 is expressed in the post synaptic membrane of neurons in laminae I-III of the dorsal horns of the spinal cord. EphB-Fc fragment neutralizing receptor bodies and ephrinB-Fc stabilized activators are standard in experimental protocols of the murine pain models use to explore the *in vivo* involvement of EphB-ephrinB signaling in pain. Intrathecal infusion of EprinB2-Fc, which bind and activate EphB receptors and EphB1-Fc, which contend with EphB receptors for ephrinB binding, cause and inhibit NR2B phosphorylation, respectively [16]. Tissue damage induces ephrinB subclass redistribution at the presynaptic membranes of the central terminals of primary sensor neurons. This initiates a series of events that begin with EphB activation, followed by Src kinase mediated signaling and culminate in NMDA receptor activation and hyperalgesia [17, 18, 19]. Upregulated EphB1 expression modulates the function of GluN2B-containing NMDA receptors and depends upon tyrosine phosphorylation of GluN2B in the spinal cord. Src kinase exerts its effects by phosphorylating GluN2B at Y1472. NMDA receptor activation leads to large cellular influx of calcium ions and hyperalgesia in both inflammatory and neuropathic pain [15, 18, 19]. EphB1 is also involved in the development of long-term potentiation at synapses of C-fibers in the superficial dorsal horn neurons of the spinal cord [17, 19, 20]. Long-term potentiation of synaptic transmission has emerged as an important contributor to pain pathology. Long-term potentiation is influenced by alterations in the numbers, activity and properties of glutamate receptors and voltage-gated Ca^{2+}channels. EphB activation in the spinal cord reduces the long-term potentiation induction threshold and increases the phosphorylation of GluN2B-containing NMDA receptors [17]. Interestingly, EphB1 forward but not reverse signaling is crucial for bone cancer pain development. Blocking EphB1 forward signaling with EphB2-Fc prevented and alleviated pain behaviors, related c-fos induction and astrocyte activation. EphB2-Fc merger with endogenous ephrinB ligands result in EphB1 substitution and cleavage [21]. One of the major critiques of the aforementioned studies is that the EphB-Fc and ephrinB-Fc chimeras used to disrupt EphB-ephrinB interactions are not specific for a given EphB receptor or ephrinB ligand, respectively. However, recent EphB1 knockout mice ($EphB1^{-/-}$) experiments conducted with different murine inflammation and neuropathic pain models, have confirmed the significance of forward EphB1 signaling as a contributor to both inflammatory and neuropathic pain processing. In EphB1 knockout mice, NR2B phosphorylation, microglia stimulation and c-fos induction were abridged. Of particular note, in long term pain models both wild type and EphB1 knockout mice developed mechanical and thermal hyperalgesia, but recovery was more rapid in EphB1 knockout mice. Thus, in some pain models functional EphB1 appears essential for the preservation but not the commencement of thermal and mechanical hypersensitivity [22].

Other studies demonstrate the significant contributions of ephrinB1 and ephrinB2 to spinal cord pain processing. For instance, chronic constriction injury of the sciatic nerve caused a time-dependant up-regulation of ephrinB1 expression in dorsal root ganglia (DRG) and spinal cords that corresponded to the development of thermal hyperalgesia [23]. Comparable results have been reported with other neuropathic pain models. The lysophosphatidic acid (LPA) neuropathic pain model involves intrathecal injections of LPA at the lumbar 5-6 levels to induce nerve injury. Gene expression profiling of dorsal root ganglia harvested from experimental animals indicated that LPA-induced ephrinB1 gene expression while antisense oligodeoxynucleotide ephrinB1 treatments reduced LPA-induced thermal hyperalgesia and allodynia [24]. In

contrast, ephrinB1 expression was significantly decreased in DRG in the Walker 256 carcinoma cell model of cancer pain [25]. EphrinB2 is expressed on peptidergic neurons in laminae I and II of the dorsal horn, on afferent C-fibers but not on nonpeptidergic IB4 fibers. EphrinB2 protein expression increased in DRG neurons in a time-dependent manner in a murine neuropathic pain model that employed crushing the left L5 spinal nerve [26]. Similarly, peripheral tissue damage caused increased ephrinB2 expression in presynaptic membranes of the dorsal horn of the spinal cord, while deletion of ephrinB2 in Nav1.8 positive nociceptive neurons attenuated mechanical hyperalgesia induced by Complete Freund's adjuvant and significantly reduced thermal hyperalgesia and mechanical allodynia in the Seltzer model of neuropathic pain [27]. Visceral inflammation evokes hyperexcitability in nociceptive neurons and is associated with increased voltage-gated sodium channel Nav 1.8 current density and protein expression [28].

Taken together, these findings suggest an EphB-ephrinB signaling complex that necessitates interaction between pre-synaptic ephrinB1 and/or ephrinB2 in Nav1.8 positive neurons with post-synaptic EphB1 in dorsal horn neurons, to mediate forward signaling NMDA receptor regulation of pain sensation. What is less clear is whether other members of the EphB and ephrinB families are involved in this complex and whether EphB-ephrinB reverse signaling occurs between neurons in the dorsal horn of the spinal cord, under normal physiological conditions.

Possible involvement of EphB6 in pain

Several proteins possess kinase-like domains that lack at least one of the accepted catalytic residues. These proteins are predicted to be catalytically inoperative and are accordingly categorized as pseudokinases [29]. However, pseudokinases are crucial participants in the regulation of various cellular pathways. Indeed, pseudokinases are able to catalyze phosphotransfers similar to other receptor tyrosine kinases and also act as 'scaffolds' to control downstream signaling through regulated protein-protein interactions [30]. Therefore, the versatility of receptor tyrosine kinase signaling in cellular processes may be partly defined by the influence of the kinase-null participants. Both Eph receptor subclasses possess pseudokinases and these receptors appear to have key regulatory functions in modulating the Eph-Ephrin signaling complex. Messenger RNA of the EphA subclass pseudokinase EphA10 is present in the testis [31] and EphB10 protein has subsequently been used as a breast cancer marker [32], but details of its molecular role in cellular processes remain elusive. EphB6 is the distinctive member in the EphB subclass, in that its kinase domain contains several alterations in the Eph conserved amino acids and consequently has been predicted to lack catalytic activity [33]. We propose that EphB6 is an integral payer of EphB-ephrinB pain signaling. We have recently reported the first data in our efforts to elucidate a role for EphB6 in inflammatory pain processing, at the spinal cord. Colitis, induced with the administration of 4% (wt/vol) DSS in the drinking water of mice, significantly decreased EphB6 protein expression levels in neurons of the lower thoracic superficial layers in the dorsal horns of the spinal cord, the location of neurons that receive the majority of nociceptive information from the colon, via the primary afferents [34]. An interesting untested hypothesis is that Eph-ephrin signaling magnitude and direction is mediated by the balance between kinase versus pseudokinase protein expression levels. Nonetheless, the explicit molecular mechanism of EphB6 involvement, if any, in spinal cord pain processing and/or transmission is still largely unclear.

The greater part of the EphB6 literature is cancer research based. EphB6 loss or mutations have been reported in neuroblastomas, leukemia and breast and lung cancers. Several of these reports document EphB6 phosphotransfer activities and heterodimer formations with other EphB receptors, analogous to classical receptor tyrosine kinases signaling mechanisms [35, 36, 37]. For example, EphB6 can activate the tyrosine kinase ZAP-70, a protein localized adjacent to the surface membranes of T cells and natural killer cells, when bound to ephrins [38]. The mechanism involved is unknown, and it is not clear whether the pseudokinase domain is involved in the phosphorylation. When Myc-tagged human EphB6 is transiently expressed in COS-7 cells and exposed to ephrinB1 it undergoes inducible tyrosine phosphorylation [33]. In addition, EphB6 protein expression induction in cell lines can alter cell adhesion and migration fates and the differential responses are associated with Src kinase-dependent phosphorylation of the cytoplasmic domain of EphB6 [39]. EphB1 overexpression in difference cell lines have resulted in augmented EphB6 phosphorylation that involved the functional catalytic activity of EphB1. Indeed, EphB1 not only transphosphorylated EphB6, but together they also formed a stable hetero-complex [33]. Consistent with these findings, ErbB3 the pseudokinase member of the EGF receptor family forms a heterodimeric complex with the active kinase ErbB2 when bund to neuregulin. The interaction of the ErbB3 kinase null domain with the ErbB2 functional tyrosine kinase domain causes the autophosphorylation and activation of the ErbB2 tyrosine kinase domain [40]. This and other evidence suggest that the lack of evolutionary pressure to conserve phosphoryl transfer activity because of the presence of multiple ErbB genes being coupled by allosteric mechanisms of activation rather than

phosphorylation, may be the reason for the loss of catalytic activity of ErbB3 [41]. Comparable to ErbB, Eph appear to exert their effects through allosteric mechanisms of activation by forming homodimers and heterodimers. The capacity of EphB subclass members to form homodimers or heterodimers within their own class and with subclass A, is in the early stages of being characterized. One model of Eph-ephrin interaction portray a basic tetrameric Eph-ephrin complex formation, where each Eph receptor interacts with two ephrin ligands and each ephrin ligand with two Eph receptors [42]. X-ray crystallographic studies have revealed two dissimilar ephrin-binding sites on opposite sides of the EphB2 extracellular region. While biophysical solution studies indicate that the larger interface EphB-ephrinB binding site mediates the initial high affinity association between EphB2 and ephrinB2 and the smaller interface site directs further assembly of two EphB2-ephrinB2 heterodimers into an activated circular tetramer [43]. Nevertheless, there is no general consensus on the precise stoichiometry of Eph-ephrin signaling complexes *in vivo.*

EphB6 interacts with a number of proteins in mammalian cells including aldolase A, dynactin and clusterin [44] and these proteins may promote its ability to form homodimers or heterodimers. EphB6 is capable of forming heterodimers with both EphB1 and EphB2 in mammalian cells [33, 45] and undergoes trans-phosphorylation when heteromerized to EphB1 [33]. Recently, EphB6 in conjunction with testosterone has been shown to regulate vascular smooth muscle contractility and modulate blood pressure. Three weeks after castration, EphB6 KO *(EphB6$^{-/-}$)* mice exhibited augmented mean arterial, systolic and diastolic blood pressure and when compared to castrated wild-type control mice. These findings combined with complimentary *in vitro* experiments indicate that increased blood pressure manifestations, in castration EphB6 KO mice, were due to the absence of reverse signaling from EphB6 to ephrinB ligands, mediated by the scaffold protein, glutamate receptor interacting protein 1 [46]. An analogous mechanism may exist in the dorsal horns of the spinal cord, where the reduction in spinal cord EphB6 receptor expression in the postsynaptic membrane in spinal horn neurons with DSS inflammation, seen in our resent study [34], was triggered by the increase in ephrinB1 and/or ephrinB2 protein expression on axons of primary sensory DRG neurons at the glutamatergic synapses in spinal dorsal horn superficial laminae, with the development of hyperalgesia in murine pain models [21, 22, 23,27]. We propose an untested basic model for this mechanistic scenario. Under normal physiological conditions, two EphB6 interact with two ephrinB1 or ephrinB2 on adjacent neurons to dampen and stabilize sensory neuron excitability, in the dorsal horns of the spinal cord. This signal would be transmitted reversely through the ephrinB ligand via an unknown ephrinB-associating protein. Peripheral tissue damage detected by nociceptive primary sensory neurons then lead to a shift in the predominant heterotetramers in the synaptic membranes, from the stable reverse signaling EphB6-ephrinB1 or EphB6-ephrinB2, to the forward signaling EphB1-ephrinB1 or EphB1-ephrinB2, required for the maintenance of thermal and mechanical hypersensitivity.

Conclusions

The mechanisms of EphB-ephrinB signaling in spinal cord pain processing are in the early stages of being resolved. Deciphering a role for EphB6 in EphB-ephrinB signaling may be fundamental to unraveling the fine details of this signaling complex. Our finding that colitis alters EphB6 expression in the dorsal horns of the spinal cord should direct attention to this EphB subclass member, which is often neglected beyond its role in cancer. Understand the intricate pain signaling mechanisms of EphB-ephrinB signaling pathways in sensory neurons, should facilitate effective new therapeutic pain relief strategies, to either reinforce or disrupt EphB-ephrinB associated adaptor/scaffolding or downstream signaling protein interactions. Undoubtedly, the coming decades will see the emergence of novel pain relief modes based on such strategies.

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Conflicting interests

The author declares that he has no conflict of interest.

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