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Eph RECEPTORS AS A TARGET TO DEVELOP NOVEL THERAPIES TO CONTROL NEURODEGENERATION AND NEUROPATHIC DISEASES.

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ABSTRACT

Eph receptors and ephrins represent an important molecular system that enable reciprocal communications between different cell types in the developing and adult nervous system and play critical roles in axonal guidance, regulation of neuronal progenitor positioning, controlling synapse formation, and neuronal plasticity. Dysfunction of Ephs-ephrins system is involved in neurological disorders, including neurodegenerative diseases (AD, ALS) and pain.

Class B of Ephs and ephrins has been implicated in the induction and persistence of various types of pain, including chronic neuropathic, inflammatory and cancer pain, as well as in the physical dependence on opiates. Especially, the activation of spinal EphB1 receptor by ephrin B1-Fc is critical to the development of bone cancer pain, morphine tolerance in treating bone cancer pain and produced thermal hyperalgesia and mechanical allodynia in mice. Moreover, EphB1 receptor is significantly up-regulated in the spinal dorsal horn following an escalating morphine treatment whereas spinal administration of EphB1 receptor blocking reagent, EphB2-Fc prevents and reverses bone cancer pain in animal models.

Characterization of EphB1 molecular mechanisms involved in neuropathic pain and its interaction with other cell surface proteins also involved in these pathologies, will be useful to develop novel EphB1 antagonist, capable to modulate these signaling pathways. To this aim, I investigated any functional cross-talk between intracellular signaling pathways triggered by mu-opioid receptor (MOR) and EphB1 receptors in different cell models co-expressing the two receptors.

EphB1 agonist receptor (ephrinB1-Fc) or the MOR agonist morphine determine a time-dependent increase p42/44 phosphorylation only when these ligands are administered as single agents whereas their co-administration occluded p42/p44 MAPK activation. Such cross-talk, as well as EphB1 and MOR expression, are modified in neuronal cells subjected to differentiation or to exposure to the pro-inflammatory agent TNF-α; thus, suggesting a differential role played by the functional interaction between EphB1 and MOR depending on the physiological state of neuronal cells.

Some members of EphA receptors play a role in adult neuronal functions. The most abundantly Eph receptor expressed in the hippocampus and cortex is EphA4, which regulates neuronal plasticity that occur during learning and memory formation. However, aberrant EphA4 levels and excessive activity inhibit neuronal repair and promote neurodegenerative processes. Possible strategies to target EphA4 for pharmacological intervention include inhibiting ligand binding or kinase activity. I set-up a cell-based assay suitable to test peptide and small molecules in order to develop inhibitors with increased potency and improved pharmacological properties.

Efforts to characterize and optimize peptides and small molecules that target specific Eph receptors and ephrins, could provide useful leads for innovative pharmacological approaches to treat neurological diseases.

INTRODUCTION

1. Eph receptors and ephrins

Erythropoietin-producing hepatocellular (Eph) receptors are the largest family of receptor tyrosine kinases (RTKs), containing a unique ligand binding domain on the extracellular side and a structurally conserved tyrosine kinase domain on the intracellular side.

A distinctive feature of Ephs is their binding to cell surface-bound ligands, called ephrins. Their interaction thus relies on direct contact between neighbouring cells, whereby Ephs and ephrins transduce interdependent signals into each interacting cell.
Upon ephrin binding, the tyrosine kinase domain of Ephs is activated, initiating "foward" signaling in the receptor expressing cells. At the same time, signals are also induced in the ligand-expressing cells, referred to as "reverse" signaling.

Ephs are classified on the basis of sequence homology and binding preferences:in the human genome there are 9 EphA receptors(EphA1–A8, EphA10), which preferentially bind 5 glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands, and 5 EphB receptors(EphB1–EphB4, EphB6), whichpreferentially bind 3 transmembrane ephrin-B ligands (ephrins-B1–B3)[1].Ephs and ephrins interact promiscuously within each subclass, but cross-class interactions are known for EphA4, interacting with B-type ephrins [2,3], and ephrin-A5, activating EphB2 as well as EphAs [4].

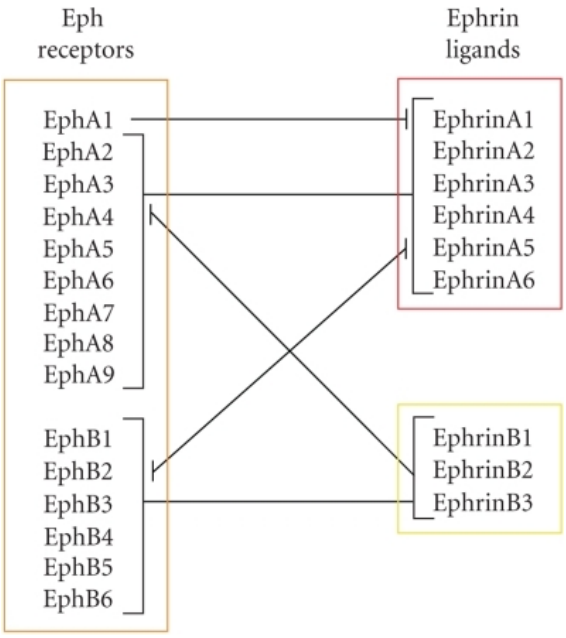


Figure 1: Major interactions of Eph receptors and ephrin ligands. Class A Eph receptors preferentially bind all A-type ephrins and class B Eph receptors bind all B-type ligands. However, there are some exceptions, as EphA1 primarily binds ephrinA1, EphA4 binds both, A- and B-type ligands, and ephrinA5 binds EphA receptors as well as EphB2 (Mosch B. et al, *J Oncol.* 2010) [5].

The interactions between Ephs and cell-bound ephrins are complex: a nucleating hetero-tetrameric complex between Ephs and ephrins on opposing cells initially triggers lateral extension of Eph/ephrin clusters via distinct ephrin/Eph and Eph/Eph interactions; only then association of signalling/adaptor proteins modulating focal adhesions as well asthe Ras-MAP kinase and PI3-kinase signalling circuits follows, where the overall size and composition of these clusters can determine the signaling outcome[6, 7, 8].

Eph receptors and ephrins engage in a multitude of activities. They typically mediate contact-dependent communication between cells of the same or different type to control cell morphology, adhesion, movement, proliferation, survival and differentiation [9].
Eph activitiesare also involved in specialized cellular functions such as synaptic plasticity, insulin secretion, bone remodeling and immune function [10].

1.1 The structural basis of Eph signalling

1.1.1 Eph and ephrin structures

The Eph-receptor extracellular domain is composed of the ligand-binding globular domain (LBD), a cysteine-rich domain (encompassing the sushi andepidermal growth factor (EGF)–like domain) and two fibronectin III (FNIII) repeats, which seems to be involved in receptor dimerization[6].
The cytoplasmic part of Eph receptors can be divided in several functional units; the SRC homology 2 (SH2) binding sites, the juxtamembrane region that contains two conserved tyrosine residues and regulates kinase activity, a typical tyrosine kinase domain, a sterile-alpha-motif (SAM) domain and a PDZ binding motif.
The solved structure of the SAM domain (~70 amino acids) indicates that it could form dimers and oligomers and be involved in signal trasduction [11, 12].
The PDZ-binding motif, located in the carboxy-terminal 4-5 amino-acid residues, contains a consensus binding sequence that includes a hydrophobic residue (usually valine or isoleucine) at the very carboxyl terminus (Figure 2).

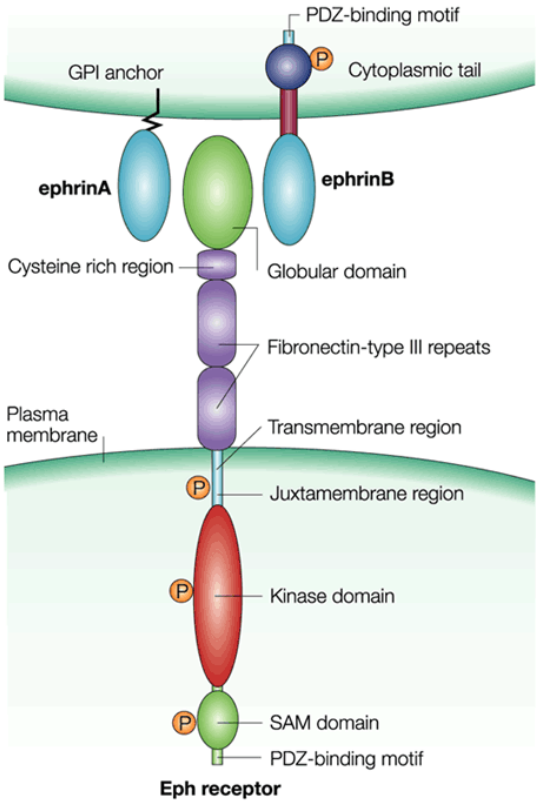


Figure 2: Structure of Eph receptors and ephrin ligands. PDZ: Postsynaptic density 95-Discs large-Zonula occludentes-1-protein, SAM: Sterile alpha motif, GPI: glycosylphosphatidylinositol (Kullander K. and Klein R. *Nat Rev Mol Cell Biol.* 2002) [13].

Both ephrin classes include a conserved Eph receptor-binding domain, which is connected to the plasma membrane by a linker segment whose length can be affected by alternative splicing. The ephrin-As are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, although they can also be released to activate EphA receptors at a distance [14, 15], whereas the ephrin-Bs contain a transmembrane segment and a short cytoplasmic region.

1.1.2 Eph-ephrin complex formation and activation

A defining characteristic of Ephs compared to other RTKs is thatonly membrane-bound or artificially clustered ligands can trigger receptor signaling. Prior to ephrin contact, Ephs are largely distributed across the plasma membrane and display minimal kinase activity; after activation Eph clusters appear rapidly at discrete spots on cell surface [4].
To induce downstream signaling, Ephreceptors need a high local density of ligands [16].
The first step in the initiation of Eph-mediated signaling is the recognition and binding of Eph receptors and ephrin ligands located on closely opposed cell surfaces.
The first cristal strycture of an Eph/ephrin complex [17, 18] indicated that the proteins form a tetrameric, ring-like assembly in which two receptors and two ligand molecules

interact via distinct interfaces. One of the Eph/ephrin interfaces is very extensive and is responsible for the high-affinity ligand-receptor dimerization. The second interface is smaller and is suggested to be responsible for the assembly of the dimers into functional tetrameric 2:2 complexes.

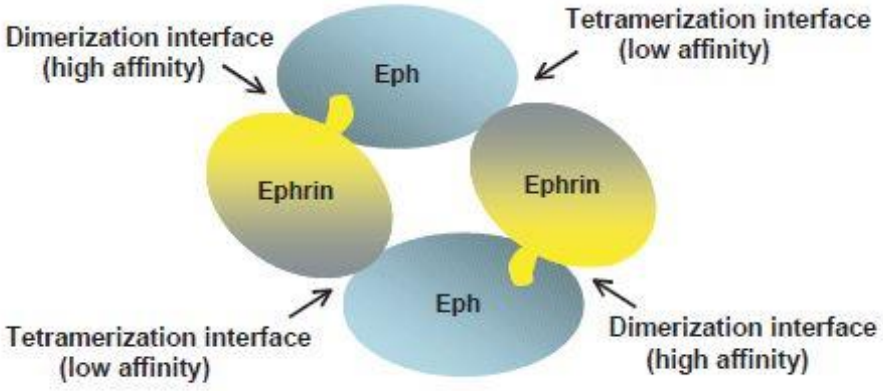


Figure 3: Dimerization and tetramerization interfaces of a ligand-receptor tetrameric complex (Murai KKand Pasquale EB.*J Cell Sci.* 2003) [19].

A-class ligand/receptor recognition seems to proceed though a "lock-and-key" type mechanism where the G-H loop of ephrinA1 is inserted in a hydrophobic channel on the surface of for example EphA2, while B-class receptor/ephrin recognition proceeds through an "induced-fit" mechanism. For example, the loops forming the side of the ephrin-binding channel of the EphB2 receptor rearrange upon ligand binding, thus requiring energy to generate the extensive interaction surface that is complementary to the ephrin G-H loop [20, 21, 22].
Eph/ephrin complexes can progressively aggregate into larger clusters, the size of which might depend on the densities of Eph receptors and ephrins on the cell surface [9].
The degree of clustering may not only affect signal strengthbut may also differentially regulate downstream pathways, thus leading to variable outcomes [9, 23].
Other regions of the Eph and ephrin proteins are also involved in the final positioning of bidirectional signaling complexes, including the extracellular cysteine-rich linker and the intacellular SAM domain of the receptors, and the C-terminal PDZ-domain binding sites found in mostEph receptor and the ephrin-B ligands.

Ligand binding serves to bring together two catalytically repressed kinase domains and to hold them in an orientation favoring phosphorylation intrans. One of the monomers consequently phosphorylates regulatory sequences on the other monomer, leading to the activation of its catalytic domain. The active kinase domains can then phosphorylate other molecules, including the kinase domains of neighboring receptors, andinitiatedownstream signaling cascades.

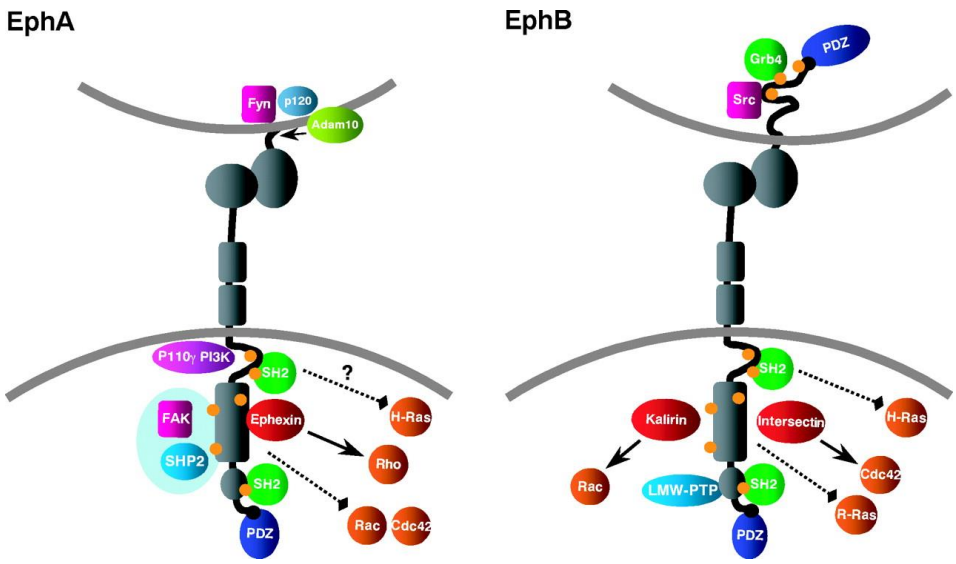


Figure 4:Forward and reverse signals communicated downstream of Eph-ephrin complexes. Some of the known EphA–ephrin-A orEphB–ephrin-B signaling pathways are highlighted. SH2 and PDZ refer to a number of identified proteins containing SH2 or PDZdomains. Grb4 is the only SH2-domain-containing protein known to bind to ephrins (Murai KKand Pasquale EB.*J Cell Sci.* 2003) [19].

1.1.3 Forward signaling

In the unphosphorylated form the Eph receptors are kept in an inactive state by the association of a helix from the juxtamembrane domain with the kinase domain [24]. As a consequence of ephrin binding, the Eph receptors cluster and the kinase domains come in close proximity, favoring trans-phoshorylation of the cytoplasmic domains which in turn releases the inhibitory interactions with the juxtamembrane domain and promotes kinase activity.
There are also differences in the kinase domains within the Eph receptor family. For example, the "gatekeeper" residue in the hinge region between the kinase domain lobes, which controls access to a hydrophobic pocket adjacent to the ATP binding site, is a threonine in most Eph receptors but a valine in EphA6 and an isoleucine in EphA7. Hence, EphA6 and EphA7 likely differ from the other Eph receptors in their sensitivity to kinase inhibitors and possibly substrate specificity [25].

Tyrosine phosphorylation creates binding sites for proteins that contain Src-homology 2 (SH2) domains, including non-receptor tyrosine kinases of the Src and Abl families and adaptors such as Nck and Crk, which are crucial for signal transduction [26, 27].

The interaction ofPDZ domain-containing proteins withthe carboxy-terminal tails of Eph receptors also contributes to signaling. Particularly important effectors are Rho and Ras family GTPases.
Eph receptors regulate actin dynamics through small GTPases of the Rho family (Rho, Rac and Cdc42),which impact cell shape, adhesion, and movement. Rho GTPases cycle between an active GTP-bound conformation and an inactive GDP-bound

conformation.The Eph receptors can influence these conversions by regulating both guanine nucleotide exchange factors (GEFs, which facilitate GDP to GTP exchange) and GTPase-activating proteins (GAPs, which promote GTP hydrolysis to GDP). Regulation of GEFs and GAPs by Eph receptors can involve constitutive or ephrin-induced association, tyrosine phosphorylation, or even ubiquitination and degradation [25].
Interestingly, whereas most other RTK families use these central regulators of cellular physiology to stimulate cell proliferation, survival, and forward movement, the Eph receptors can use them to inhibit cell growth and achieve cell repulsion. In neurons, Rho activation inhibits neurite outgrowth and promotes growth cone collapse and axon retraction[28, 29, 30].
The collapse or local retraction of neuronal growth cones and dendritic spines (the small protrusions on dendrites bearing excitatory synapses) are well-known repulsive effects of EphA receptors that depend on Rho family GTPases. Growth cone collapse involves RhoA activation, for example by the GEF Ephexin1, and Rac1 inactivation, for example by the GAP α2-Chimaerin [25].
Activation and inactivation of Rho family GTPases may occur with different spatial and/or temporal resolution to achieve growth cone collapse and regulate dendritic spines.
EphB receptor forward signaling can also promote synapse formation through ubiquitination and degradation of the Rho-GEF Ephexin5, which decreases RhoA activity without obvious effects on spine morphology [31].

Eph receptors also regulate the activities of small GTPases of the Ras family. The best-characterized member of this family, H-Ras, activates a MAP kinase cascade culminating in the phosphorylation and activation of the Erk1/Erk2 MAP kinases.
A common mechanism of Eph receptor-dependent Erk inhibition is through p120RasGAP, which inactivates H-Ras. Through p120RasGAP, the Eph receptors can also inhibit another Ras family GTPase, R-Ras, causing the reduced integrin activity that is important for retraction of cell processes and decreased malignancy [32].
In some cases, however, Eph receptors behave similarly to other RTKs and activate the Ras-Erk pathway. For example, in cultured mouse mesenchymal cells, ephrin-B1/EphB signaling activates Erk to promote proliferation and regulate immediate early gene transcription [25]. In P19 embryonal carcinoma cells and microvascular endothelial cells, ephrin-stimulated EphB1 recruits the adaptors Shc and Grb2 to activate H-Ras and increase cell migration [25]. Interestingly, the activation of EphB4 by ephrin-B2 in MCF7 breast cancer cells promotes Erk1/2 activation through an unusual pathway that seems to require the PP2A serine/threonine phosphatase [25].
Signaling by the Eph receptors, however, is not always consistent and can lead to divergent outcomes. The kinase inactive Eph receptors and alternatively spliced forms lacking the kinase domain can modulate signaling outcome by reducing signal strength in the clusters as well as by contributing distinctive signals. Other aspects of the cellular context and implementation of positive and negative feedback loops, further contribute to the diversity of Eph receptor activities.

1.1.4 Reverse signaling

Forward signaling is clearly an important mechanism used by Eph receptors to modify cell behavior. However, the Ephreceptors also can activate 'reverse' signaling through ephrin ligand binding[9, 27].
The B-type transmembrane ephrin ligands do not possess any intrinsic catalytic activity for signaling, but rely upon a scaffolding activity that recruits signaling molecules to transmit functional effects within the cell.
The cytoplasmic domain of ephrinB ligands contains five conserved tyrosine residues, which can be phosphorylated by Src family kinases upon Eph receptor engagement (Figure 5) [33, 34]. At least one SH2-domain-containing protein binds to tyrosine-phosphorylated ephrin-B1 [35]. The adaptor protein Grb4, which has three SH3 domains and an SH2 domain, could link ephrin-Bs to a vast signaling network that modifies cell morphology through reorganization of the actin cytoskeleton.Ephrin-B signaling through Grb4 controls axon pruning, synapse formation and dendritic spine morphogenesis in the developing mouse hippocampus [36, 37].
A mechanism that may serve to turn off phosphorylationdependent ephrin-B reverse signals involves the delayed recruitment of the phosphotyrosine phosphatase PTP-BL, which can dephosphorylate the ephrin-B cytoplasmic domainand inactivate Src family kinases. The interaction of PTB-BL with ephrin-B1 is mediated by the PDZ domain of the phosphatase, which binds the C-terminal PDZ-binding motif of ephrin-B molecules. PTP-BL can act as a negative regulator of ephrinB phosphorylation, through a switch mechanism that mediatinga shift from phosphotyrosine-dependent signaling to PDZ domain dependent signaling [34, 38].

For example, the adaptor PDZ-RGS3 connects ephrin-Bs to G-protein coupled receptors that control neuronal cell migration and neural progenitor self-renewal.The GTPase activating protein PDZ-RGS3, which catalyzes the hydrolysis of GTP to GDP in the Gα subunits of heterotrimeric G proteins, binds to the PDZ-binding motif of ephrin-B molecules. It also inhibits SDF1 (stromal cell derived factor 1)-mediated cerebellar granule cell chemotaxis through the CXCR4 G-protein coupled chemokine receptor.
During cerebellar development, activation of ephrin-B signaling by EphB receptors may attenuate granule cell attraction to SDF-1, which is expressed at the pial surface, and allow cells to migrate from the external to the internal granule cell layer [38].
Similar to its role in neurons, ephrin-B reverse signaling may also inhibit the migratory and invasive effects of the CXCR4 G protein-coupled chemokine receptor in cancer cells [9, 39].

Furthermore, ephrin-Bs can signal through their intracellular domain via non-PDZ or SH2 interactionsthatmodulate epithelial cell-cell junctions through the Par polarity complex and disrupt gap junctional communication [40].

Another protein is the Signal Transducer and Activator of Transcription 3 (STAT3) protein, and interaction between ephrin-Bs and STAT3 reveals that ephrin-Bs transduce signals from the cell surface to the nucleus. In the case of ephrin-B2, the interaction with STAT3 has been suggested to be important for orchestrating pericyte/endothelial cell assembly [41].

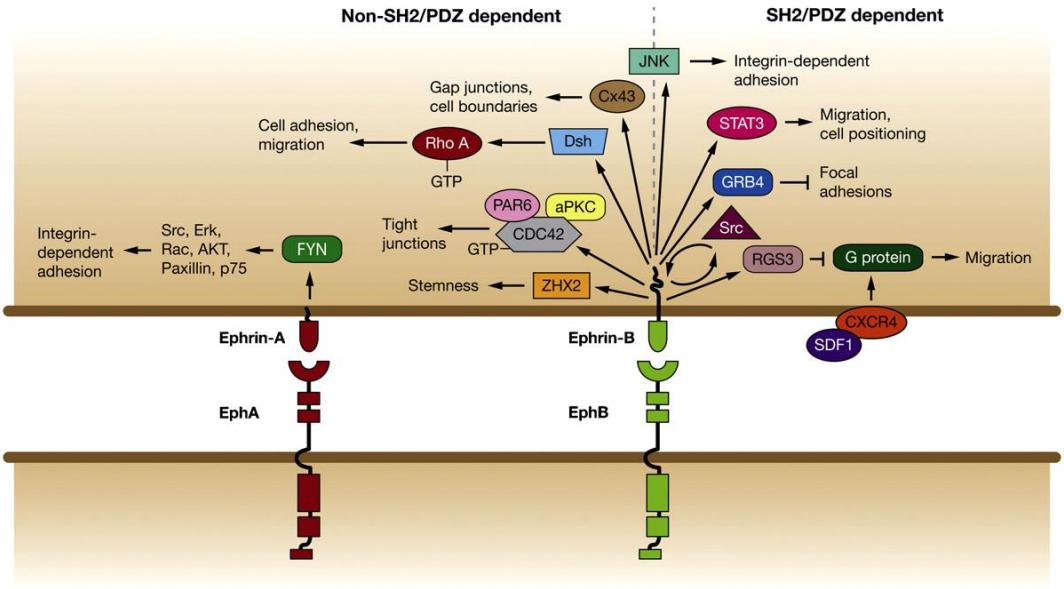


Figure 5. SH2/PDZ-dependent and -independent reverse signaling pathways of ephrin-A and ephrin-B. Ephrin-A signaling via Fyn affectsdownstream effectors regulating integrin-dependent cell adhesion. Ephrin-B reverse signaling pathways that are non-SH2/PDZ dependent are located to left of the dotted line and SH2/PDZdependent signaling is located to the right, such as regulation Src. (Daar IO. *Semin Cell Dev Biol.*2012) [40].

Ephrin-A ligands can also convey reverse signals that modify cell behavior.Like many GPI anchored proteins, the ephrin-A molecules are targeted to lipid rafts, where they presumably assemble into protein complexes that transduce intracellular signals.
Clustering of ephrin-A molecules with EphA-Fc fusion proteins recruits the Src family kinase Fyn to lipid rafts. This is accompanied by the redistribution of vinculin, activation of MAP kinase, tyrosine phosphorylation of a 120 Kd lipid raft protein, and increased cell substrate adhesion (Figure 5).
Ephrin-A5 can increase cell-substrate adhesion in fibroblasts and astrocytes by activating the Src family kinase Fyn and integrins, and seems able to also promote invasiveness [42]. Interestingly, activated Fyn can increase the surface amount of sphingomyelin clusters and hence inhibit the trafficking of ephrin-As from endosomes to the plasma membrane, which serves as a negative feedback loop for ephrin-A signaling.
Furthermore, the neurotrophin receptors TrkB and p75NTR, and the ADAM10 membrane metalloprotease, have also been found to associate with ephrin-As on the plasma membrane.Studies in neurons have implicated the p75 neurotrophin receptor and the

TrkB and Ret RTKs as transmembrane binding partners that enableephrin-A-dependent reverse signals involved in axon guidance and branching [25].
Cleavage of ephrin-As occurs when ADAM10 is present on the plasma membraneof opposing cells.This could serve a dual function. Ephrin-A cleavage from the cell surface allows Eph-receptor-bearing structures such as growth cones to change their response to ephrin-A molecules from adhesion to repulsion. In addition, the cleaved ligand is no longer able to transmit signals [19].

1.1.5 Signal termination

Eph/ephrin-mediated cell repulsion and disengagement require that the high-affinity interaction between Eph and ephrin proteins is terminated. Two mechanisms are known to disrupt this interaction: endocytosis and proteolytic cleavage.
Endocytosis can remove complete Eph/ephrin complexes from the surface to enable cell disengagement.Once ephrin-Bs bind to their cognate receptors, the internalization of the receptor-ligand complexes immediately occurrsenablingcell retraction. Interactions between EphB- and ephrinB-positive cells induce formation of intracellular vesicles that contain the full-length proteins in a complex. Previous studies have shown that EphB1 induces clustering and subsequent endocytosis of ephrin-B1, which is mediated by a clathrin-dependent pathway. The balance between forward and reverse endocytosis (either being endocytosed into the contacting cell or into the cell of residence for the ephrin ligand) depends on many cell types, receptor/ligand type, surface densities, oligomerization states, and activation of downstream signaling pathways [41].
In the case of proteolytic cleavage, there are membrane associated metalloproteases (e.g., presenilin, MMPs, ADAMs) that can cleave both ephrins and Eph receptors. The cleavage by these metalloproteases of both ephrin-A and ephrin-B proteins breakes the adhesion between cells.

2. Eph receptors and ephrins in cancer and neurological diseases

2.1 Eph-ephrin system in cancer

During the past two decades Eph receptors and eprins have been found deregulated in many cancer cell types and associated to tumorogenesis, tumor angiogenesis, invasion, metastasis and cancer stem cell function.

The upregulation through activated oncogenic pathways, the epigenetic silencing by promoter hypermethylation, chromosomal alterations and changes in mRNA stability are the main mechanisms of Eph-ephrin alteration expression. Surprisingly, the increased and decreased expression of these proteins have been linked to tumor progression, highlighting the complexity of Eph-ephrin signaling [43].

Moreover, ligand-induced Eph receptor signaling in tumor cells plays a role in tumor suppression, whereas ligand-independent Eph receptor signaling functions in tumor promotion. The apparent paradox in which the same Eph receptor or ephrin ligand acts as a tumor promoter or tumor suppressor in different tumors or even in the same tumor at different stages, is related to the fact that Eph receptors and ephrins can signal independently of each other through cross-talk with other signaling systems. For example, EphA2 has been found to enhance tumor cell proliferation and motility in cells overexpressing EGF receptor family members, an activity that likely contributes to tumorigenesis and metastatic progression in a mouse ErbB2 mammary adenocarcinoma model, promoting Erk and RhoA GTPase activity [9].

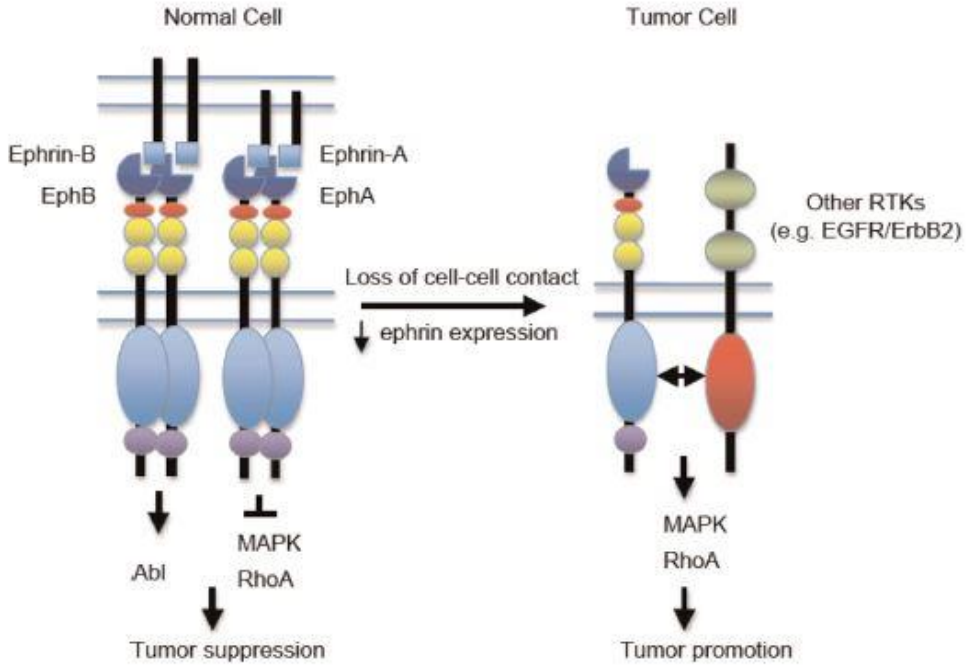


Figure 6: Eph receptor function in tumor promotion and tumor suppression. In normal cells, engagement of Eph receptors with ephrins on adjacent cells *in trans* induces receptor forward signaling, leading to inhibition of Ras/mitogen-activated protein kinase (MAPK) activity, or suppression of Crk

activation via Abl kinase activity, and tumor suppression. In tumor cells, disruption of cell-cell junctions inhibits Eph receptor interaction with endogenous ephrins *in trans*. In addition, Eph receptors are often upregulated whereas ephrins are downregulated. Crosstalk between Eph receptors and other receptor tyrosine kinases such as ErbB2 and epidermal growth factor receptor (EGFR) results in increased activity of the Ras-MAPK pathway and the RhoA GTPase, and enhanced tumor malignancy. (Vaught D et al, *Breast Cancer Research* 2008) [44].

Differences in Eph-ephrin signal outcome depend on multiple factors, including Ephs and/or ephrins involved, cellular context and disease stage.
EphA2 and EphB4 are the Eph receptors most extensively studied in breast cancer. Both receptors are widely expressed but poorly tyrosine phosphorylated, suggesting a low level of ephrin-dependent activation. Indeed, the levels of ephrin-B2, the preferred ligand for EphB4, are low in these cell lines, and high EphA2 expression also correlates with low ephrin-A expression. Intriguingly, even when ephrin-A1 is present, its ability to activate EphA2 may be impaired in breast cancer cells that lack E-cadherin. Loss of cell-cell adhesion in tumor cells impairs activation of Eph receptor by ephrins on adjacent cells. Thus, the oncogenic activity of EphA2 and EphB4 in human breast cancer cell lines, may be either independent of ephrin stimulation or manifest itself when ephrin stimulation is low.
Overexpression of EphA2 in a human mammary epithelial cell line, as well as in melanoma cells, has been shown to cause oncogenic transformation. EphA2 knockdown by RNA interference or with antisense oligonucleotides inhibits the tumorigenicity of several types of cancer cells, including a breast cancer cell line. Similarly, EphB4 knockdown inhibits breast cancer cell survival, migration, and invasion, and also tumor growth in a mouse xenograft model [9].
By contrast, high level of ephrin-dependent EphA2 and EphB4 forward signaling suppress tumorogenesis. Stimulation of EphA receptors with soluble ephrin-A1-Fc ligand reduces Erk phosphorylation in tumor cell lines, fibroblasts, and primary aortic endothelial cells, and suppresses growth of primary keratinocytes and prostate carcinoma cells. In addition to EphA2, EphB4 forward signaling also appears to inhibit tumor progression via Abl-Crk pathway impairing tumor cell growth and motility ofbreast cancer cells [45]. Furthermore, EphB receptor signaling is also able to suppress tumor expansion in colon cancer [44].
To add more complexity to EphB4 role in cancer cells, it has also been reported that in some cancers, such as melanoma, ephrin-B2–dependent EphB4 signaling enhances the migratory and invasive ability of the cells. These effects require EphB4 signaling and activation of the RhoA GTPase. In addition, signaling by another Eph receptor (EphB2) has been shown to promote the invasive ability of human glioma cells through phosphorylation of the R-Ras GTPase. Interestingly, however, the EphB2/R-Ras pathway inhibits glioma cell proliferation. Hence, the cellular context also seems to play an important role in determining the tumor-promoting or tumor-suppressing effects of Eph receptors in cancer [46].

Additionally, mutations that impair Eph receptor signaling ability or up-regulation of tyrosine phosphatases that dephosphorylate Eph receptors may also promote

tumorigenesis. For example, *EPHB2* mutations have been identified in human prostate, gastric, colorectal and melanoma tumors. Some of these mutations may impair kinase function, and some are accompanied by loss of heterozygosity, suggesting a tumor suppressor role for EphB2 forward signaling. Furthermore, a number of Eph receptors, particularly *EPHA3* and *EPHA5,* are frequently mutated in lung cancer. The mutations are typically scattered throughout the Eph domains, including the ephrin-binding domain and other extracellular regions. Elucidating the effects of the mutations will provide important insight into the functional roles of the Eph system in cancer [10].

Several Eph receptors and ephrins play an important role in tumour angiogenesis, critical for growth, survival, and malignant progression of tumors, by mediating communication of vascular cells with other vascular cells, as well as tumour cells. The main roles in tumor angiogenesis have so far been attributed to EphA2 forward signaling and ephrin-B2 reverse signaling based on a series of in vitro and in vivo experiments with mouse tumor models [9].
Interaction with ephrin-A1 ispresent in tumor endothelial cells as well as in other tumor cells and is responsible for activating endothelial EphA2 which promotes angiogenesis through PI3 kinase, Vav guanine nucleotide exchange factors and Rac1 signaling effectors. *In vitro* and *in vivo* data also indicate that EphA2 forward signaling can increase blood vessel permeability, perhaps in part through phosphorylation of claudins, in epithelial and in melanoma cell lines.
Interestingly, the upregulation of EphA2 and ephrin-A1observed in pancreatic tumors of mice treated with VEGF inhibitors suggests that EPHA2-dependent angiogenesis may contribute to the development of resistance to anti-VEGFtherapies, perhaps by promoting endothelial coverage by pericytes and smooth muscle cells.
EphB4 and ephrin-B2 are also involved in tumor angiogenesis. During the development, they are characteristically expressed in the endothelial cells of veins and arteries, respectively, and enable arterial-venous vessel segregation and vascular remodeling. Reverse signaling by ephrin-B2, and possibly other ephrin-Bs, in tumor endothelialcells, pericytes and smooth muscle cells likely depends on interaction with several EphB receptors expressed by vascular and/or tumor cells and has been shown to be important for blood vessel assembly, enlargement and decreased permeability both in cell culture and *invivo*. Ephrin-B2in the tumor endothelium may enhance the recruitment of bone marrow-derived endothelial progenitor cells that could participate in tumor vascularization, through a mechanism involving EphB4-dependent upregulation of selectin ligands. [10].

An emerging theme in cancer therapy is the growing relevance of targeting “cancer stem cells,” which are the cells that can repopulate the tumor and cause recurrence even when most of the tumor mass has been eliminated. Because Eph receptors are expressed in most adult stem cell niches and in many types of cancers, it has been long suspected that this family of RTKs may also be essential in regulating cancer stem-like cell (CSC) function.

Positive as well as negative effects on proliferation, apoptosis, and differentiation have been reported depending on the Eph/ephrin involved and the stem cell considered.
In human glioblastoma, tumor propagating cells with stem-like characteristic (TPCs) overexpress EphA2 receptor, suggesting that EphA2 activity in this contest is ligand indipendent signaling. Indeed, ephrinA1-Fc stimulation or EphA2 downregulation by siRNA, induces the differentiation and loss of tumor initiating capacity of TPCs.
In glioma and in prostate cancer cells EphA2 forms a reciprocal loop with AKT-mTOR1, one of the major oncogenic signaling pathways in cancer, promoting Akt oncogenic pathway in absence of its ligand.
Besides EphA2, also EphA3 has been expressed in more aggressive and indifferentiated mesenchymal glioblastoma multiforme (GMB) cells contributing to mantain tumor cells in a tumorogenic state [43]. Knockdown of EphA3 induced neural and glial cell differentiation. Thus, loss of either EphA2 or EphA3 was shown to be accompanied by an increase in ERK activity. As sustained MAPK signaling was reported to drive differentiation of neural progenitors, it is possible that EphA2 or EphA3 maintains CSCs in an undifferentiated state by inhibition of ERK activity [47].
The EphA2 receptor has also been implicated in playing a critical role in lung cancer CSCs. JNK signaling may be involved in EphA2-dependent CSCs self-renewal in lung cancer, as EphA2 regulates JNK and mTORC1 signaling in lung cancer cells.
Class B of Eph receptors have been shown to be expressed in intestinal stem cells. In colon cancer, constitutive Wnt signaling upregulates the expression of EphB2, EphB3, and EphB4 receptors. When EphB-expressing tumor cells reach the epithelium, they encounter normal cells expressing ephrin-Bs that restrict tumor cell expansion, resulting in *in situ* adenoma growth. As tumor development progresses, B class Eph RTK expression is silenced despite the persistence of Wnt signaling, which is concomitant with tumor invasion into surrounding tissues. Thus, loss of EphB expression at later stages of colon cancer, correlates with the transition from adenoma to malignant adenocarcinoma [47].

2.2 Eph receptors in nervous system

Eph receptors and ephrins are highly expressed in the developing nervous system, where theyregulate the spatial organization of cell populations, tissue patterning, axon guidance, and theformation of synaptic connections. Some members remain substantially expressedin the adult nervous system, where they control the structure and function of synapses and variousaspects of neural stem and progenitor cell biology. The Eph/ephrin system has alsobeen linked to neuropathologies ranging from inhibition of neural repair after traumatic injuryand stroke to neurodegenerative diseases and chronic neuropathic pain [48].

2.2.1 EphBs and ephrinBs in pain signaling

The EphB-ephrin-B system has been implicated in the induction and persistence of various types of pain, including chronic neuropathic pain caused by peripheral nerve injury, inflammatory pain, and cancer pain, as well as in the physical dependence on opiates. The mechanism underlying pain involves increased activation of post-synaptic EphB receptors, particularly EphB1, in neurons of the spinal cord by presynaptic ephrin-B ligands expressed in pain sensory neurons as well as hyperexcitability of the sensory neurons [48].
EphB1 is expressed in the post synaptic membrane of neurons in laminae I-III of the dorsal horns of the spinal cord and modulates the function of GluN2B-containing NMDA receptors.
Upregulation of EphB1 receptors in the spinal cord and its subsequent activation of forward signaling, leads to the recruitment of Src kinase, which phosphorylates GluN2B at tyrosine Y1472. NMDA receptor activation leads to large cellular influx of calcium ions and hyperalgesia in both inflammatory and neuropathic pain [49, 50].

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 [51].

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 maintenance, rather than the onset of thermal and mechanical hypersensitivity [52].

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 mediated by MMPs. Moreover, EphB1-ephrinB2 signaling contributes to chronic bone cancer pain by increasing glial release of pro-inflammatory cytokines in spinal cord, the same cytokines that are able to hamper opioid-mediated signaling; on the contrary, blocking EphB1 receptor signaling in spinal cord relieves chronic bone cancer pain and rescues opioid analgesic effect. Thus, EphB1 receptor may be a potential target for treating bonecancer pain and reducing opioid tolerance in treating bonecancer pain clinically [53].

Other studies demonstrate a significant contribution of ephrinB1 and ephrinB2 to spinal cord pain processing. For instance, chronic constriction injury of the sciatic nerve causes a time-dependant up-regulation of ephrinB1 expression in dorsal root ganglia (DRG) and spinal cord that corresponds to the development of thermal hyperalgesia [54]. 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. LPA induces ephrinB1 gene expression while an antisense oligodeoxynucleotide against ephrinB1 reduces LPA-induced thermal hyperalgesia and allodynia [55].

Finally, 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 [56]. Similarly, peripheral tissue damage causes an increase of 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 reduces thermal hyperalgesia and mechanical allodynia in the Seltzer model of neuropathic pain [57].

2.2.2 Ephs-ephrins in neurodegenerative diseases

The Eph/ephrin system has been implicated in several neurodegenerative diseases. An example is Alzheimer's disease (AD), that is characterized by cognitive decline associated with synaptic loss and network disfunction. Proteolysis of amyloid precursor protein by the presenilin/γ-secretase intramembrane protease complex is critical to generate the cytotoxic β amyloid (Aβ) peptides. The neurotoxicity of amyloid-β (Aβ) oligomers is mediated by interaction with synaptic receptors. Multiple forms of crosstalk have emerged between the presenilin/γ-secretase/Aβ system and Eph receptors and ephrins known to regulate cell communication at synapsis level. Only three Eph receptors have been implicated in Alzheimer's disease, in particular EphB2, EphA4 and EphA1.
Soluble Aβ oligomers bind to the extracellular fibronectine type III domains of EphB2 and cause EphB2 proteosomal degradation, leading to decreased NMDA receptor-mediated calcium current, reduction of long-term potentiation (LTP) and impairment of synaptic transmission. Importantly, EphB2 restoration can reverse the electophysiological deficit caused by the defective NMDA component and also improve the cognitive and behavioral functions in an Alzheimer's mouse model [58].

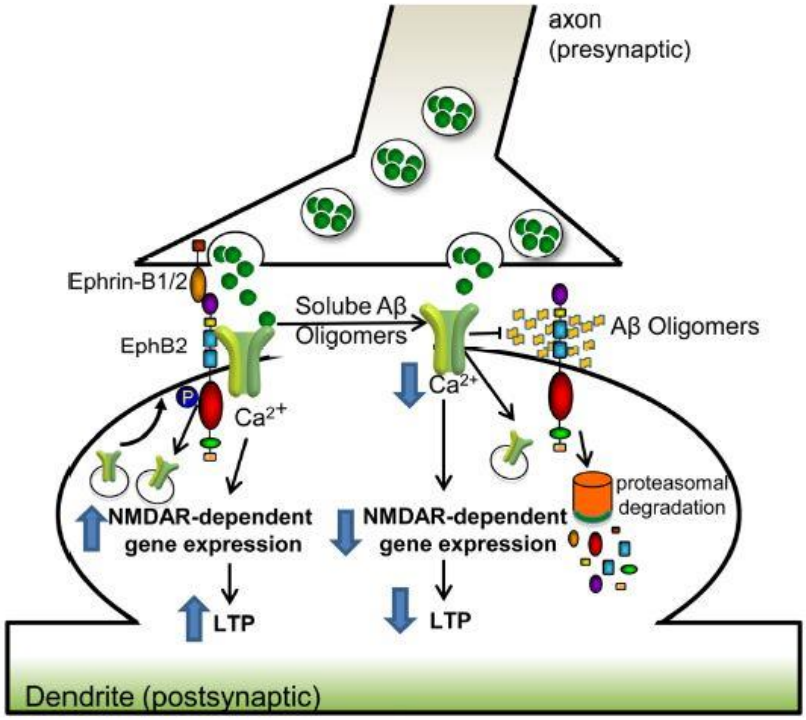


Figure 7: EphB2 in Alzheimer's disease. Soluble Aβ oligomers, which are overabundant in AD, bind directly to EphB2. The EphB2-Aβ interaction inhibits receptor activation and causes internalization and degradation of both EphBs and NMDARs. Degradation of EphB receptors inhibits their ability to retain NMDARs on the membrane. Fewer NMDARs on the cell surface leads to decreased calcium influx and none of the changes in gene transcription required for LTP (Sheffler-Collins SI and Dalva MB, *Trends Neurosci.* 2012)[59].

Amyloid-beta oligomers can serve as a ligand that aberrantly increase EphA4 kinase activity, which leads to c-Abl kinase activation in brain, causing neurodegeneration. EphA4 antagonists can prevent dendritic spine loss, the blockage of LTP induction and the apoptotic process caused by Aβoligomers [60]. In a recent study, a small molecule rhyncophylline, which binds EphA4 ligand binding pocket, has been identified as a novel EphA4 inhibitor. Rhyncophylline administration restores the impaired long-term potentiation a in transgenic mouse model of AD [61].

Finally, the failure of the processing of EphA4 by γ-secretase is involved in the pathogenesis of AD. The intracellular EphA4 fragment, which appear to be decreased in Alzherimers brain, promote the formation of the dendritic spine through activation of the Rac signaling pathway [62]. Thus, Eph/ephrin-based therapies against Alzheimer's disease could include restoring EphB2 expression and signaling, blocking EphA4-ephrin and EphA4-Aβ interaction, and increasing the EphA4 intracellular fragment.

EphA1 has also been linked to Alzheimer's disease, despite not being detectably expressed in the adult brain. Genome-wide association studies in patients with Alzheimer's disease have identified *EPHA1* as one of the 8 top loci frequently associated with late-onset Alzheimer's disease. Further studies are required to determine how *EPHA1* contributes to the risk of Alzheimer's disease and to determine whether it is a potential target for therapy [63, 64].

The Eph/ephrin system may also be involved in Parkinson's disease, a neurodegenerative disorder characterized by motor and cognitive symptoms. A soluble form of the ephrin-A1 ligand, ephrin-A1 Fc, promotes regeneration of the brain dopaminergic neurons that are lost in a rat Parkinson's model, and SNPs in several Eph receptors, including EphB1, have been associated with the disease [48].

Finally, EphA4 was recently identified as a modifier gene that can worsen the pathology of amyotrophic lateral sclerosis (ALS), a fatal disease involving progressive motor-neuron degeneration. Low EphA4 mRNA levels and EphA4 loss-of-function mutations in ALS patients were correlated with late disease onset and prolonged survival. Consistent with this, studies in ALS animal models suggest that decreasing EphA4 expression or pharmacological inhibition of EphA4-ephrin interaction could be of therapeutic benefit. [48].

These results collectively implicate ephrin–Eph signalling as an important target in therapies against cancer and neurological disorders in humans.

3. Cross-talk between Ephs-ephrins and other signaling pathways

The ability of Eph proteins to communicate with a variety of other cell surface proteins increases complexity of Eph-receptor–ephrin bidirectional signaling. The interactions between Eph/ephrin system and a number of cell surface receptors can behave as an agonist or an antagonist. This crosstalk may allow the Eph receptors and ephrins to broaden their repertoire of functions [19].

3.1 Ephs-ephrins regulate cell adhesion dynamics

3.1.1 Cadherins

The interaction of Eph receptors and ephrins with adhesion molecules such as E-cadherin could influence cell-cell attachment. Thereby, it is assumed that E-cadherin can influence the expression, cellular localization and the function of Eph receptors and vice versa.
One of the most prominent biological outcomes of Eph/ephrin signaling is the regulation of cell sorting, a process by which populations of cells physically segregate from each other to generate distinct tissues or compartments [65, 66]. The cellular and molecular mechanisms by which Eph/ephrin signaling control cell sorting behaviors are still not well characterized; however, because homotypic interactions via cadherins play an important role in cell sorting [67], it was postulated that these two pathways might cooperate to regulate cell adhesion and segregation.
In mammalian epithelial cells, it has been shown that EphA2 localizes to sites of cell–cell contact and that this subcellular localization is dependent on E-cadherin. Furthermore, in breast cancer cells, the inhibition of E-cadherin function reduces EphA2 phosphorylation and causes EphA2 restribution into membrane ruffles. Ectopic expression of E-cadherin in metastatic cells that lack endogenous E-cadherin, restores a normal pattern of EphA2 phosphorylation and localization, and lead to a decreased cell-extracellular matrix adhesion interplay [68]. Since the two proteins are expressed in overlapping patterns, loss of E-cadherin function may alter neoplastic cell growth and adhesion via effects on EphA2.
A direct role of E-cadherin in Eph/ephrin-induced cell sorting was reported by the Batlle group [69]. In the intestinal epithelium, EphB signaling controls the positioning of cell types along the crypt-villus axis. EphB activity suppresses also tumor progression in colorectal cancer (CRC). EphB receptors compartmentalize the expansion of CRC cells through a mechanism dependent on E-cadherin–mediated adhesion. This restricts the ability of EphB-positive tumor cells to invade or colonize ephrin-B–positive territories during tumor dissemination beyond the early stages.

To avoid repulsive interactions imposed by normal ephrin-B1-expressing intestinal cells at the onset of tumorigenesis, CRC cells could silence EphB expression.

Thus, de-regulated Eph-cadherin crosstalk might contribute to the tumorigenic potential of Eph/ephrin-positive cancer through alterations of cell-cell adhesion [70, 71].

3.1.2 Claudins

In contrast to cadherins, claudins have clearly been shown to directly interact with Eph/ephrin proteins in epithelial cells. Claudins are components of tight junctions located in the subapical region of the lateral membranes. Tight junctions serve as paracellular barriers restricting movements of molecules across epithelial barriers [72].
Direct interaction between EphA2 and claudin-4 extracellular domains lead to the phosphorylation of tyrosine residues of claudin-4, which reduces its integration in tight junctions, thus increasing paracellular permeability (Figure 8) [73].
Interestingly, claudin-4 also binds to ephrin-B1 and the interaction between these proteins occurs in *trans*, lending support to the notion that ephrins could have Eph-independent functions. The interaction between claudin-4 and ephrin-B1 involves their extracellular domain and may lead to tyrosine phosphorylation of ephrin-B1 that in turn affect intercellular adhesion (Figure 8) [74].

3.1.3 Integrins

Eph receptors can affect extracellular-matrix (ECM) adhesion and migration by modulating integrin signalling, although both positive and negative effects have been described.
While a number of reports show that Eph/ephrin signaling increases integrin-mediated cell adhesion [75, 76, 77, 78, 79, 80, 81], others demonstrate a counter-effect of Eph/ephrin on integrin mediated cell adhesion [82, 83, 84, 85, 86].
These outcomes are not linked to a specific class of Eph/ephrin pair, nor does it seems to be linked to either forward or reverse.
Opposite effects of Eph/ephrin signaling on integrin function may depends on distinct cellular contexts, since the above mentioned studies use different cell types (primary, transformed, or non-transformed cell lines) and different modes of expression of the proteins of interest (endogenous vs. ectopic).
The point of convergence between both pathways may involve cytoplasmic kinases (FAK, PI3K, MAPK) and/or small GTPases (Rac, Rho, Ras, Rap1). Only one study reports a direct interaction between an Eph receptor and an integrin [81].

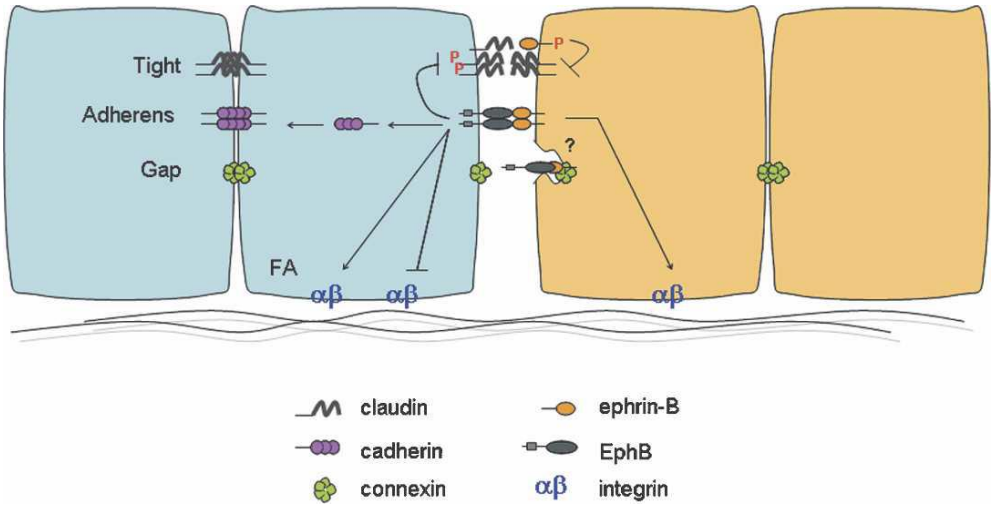


Figure 8. Regulation of adhesion proteins. Eph/ephrin signaling regulates cell–cell adhesion and cell–matrix adhesion by impinging on formation/stability of tight, adherens, and gap junctions, as well as on integrin function.
Activation of forward signaling induces the redistribution of E-cadherin to the cell surface while destabilizing claudins in Eph-expressing cells (blue), Interaction with claudins destabilizes tight junctions in ephrin-expressing cells (orange). Both forward and reverse signaling act on integrin-mediated adhesion. Together, these cascades participate in Eph/ephrin-induced cell sorting. (Arvanitis and Davy, *Genes Dev.* 2008) [39].

3.2 Cooperation with Ion Channels

Numerous molecular signals control different aspects of synapse development, including secreted factors that affect the competence of neurons to generate synapses, cell–cell adhesion proteins that locally drive the organization and maturation of synaptic specializations, and ligand or voltage-gated ion channels that respond to neuronal activity. There is a growing body of evidence for a tight cooperation between Eph/ephrins and ion channels in regulating excitatory neurotransmission and synaptic plasticity.

3.2.1 AMPA receptors

EphB receptors can regulate the surface localization of AMPA receptors (AMPARs) and are important for retention of AMPARs in the receptor recycling pool. In cultured neurons, EphB2 and AMPARs associate by each binding to the PDZ-adaptor proteins PICK1 and GRIP. Both PICK1 and GRIP bind to AMPARs acting in a opposite way: GRIP1 promote AMPA surface retention whereas PICK1 acts to remove AMPARs from the cell surface. This control of AMPAR trafficking requires ephrin-B activation, the PDZ-binding domain and kinase activation of EphB2 receptors [87].
EphB-dependent internalization of AMPARs likely relies on synaptojanin-1, a phosphatidylinositol 5'-phosphatase which is phosphorylated by EphB2, promoting activation of clathrin-mediated endocytotic mechanisms (Figure 9) [88].

3.2.2 NMDA receptors

More recently, EphB receptors have been shown to associate directly with NMDA receptors through their extracellular domains at synapses. This interaction is enhanced by the presence of ephrin-B acting in *trans.* Ephrin-B-induced activation of EphB receptors causes NMDA receptor clustering, potentially helping to initiate the development of the post-synaptic specialization. This interaction is functionally important because EphB2 activation enhances Src-mediated NMDA receptor phosphorylation, which results in increased glutamate-induced calcium influx through the NMDA receptor. The enhanced calcium influx through NMDAR also results in enhanced downstream transcription (Figure 9). This is consistent with the reduced NMDA-mediated currents in EphB2 knock out mice [59]. Thus, crosstalk between Eph and NMDA receptors could be important for early events during synaptogenesis and in modifying the physiological properties of synapses.
Eph-B/ephrin-B signaling positively regulates the activity of a number of ligand-gated ion channels, which underlies their role in regulating dendritic spine formation and synaptic plasticity.

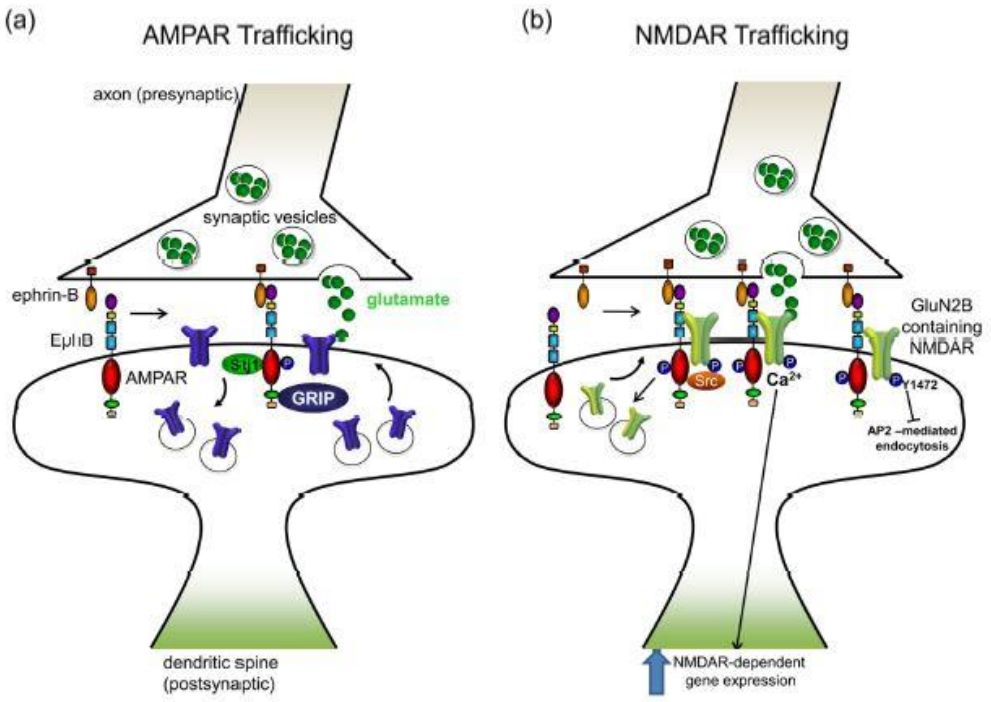


Figure 9. **EphBs regulate glutamate receptor trafficking**. (a) EphBs regulate AMPAR trafficking through a PDZ-dependent interaction with GRIP1, which promotes AMPAR insertion into the membrane from the recycling pool, and indirect interactions with synaptojanin-1 (Stj1), leading to AMPAR internalization. (b) After binding ephrin-B ligand, EphBs directly interact with NMDARs to regulate their synaptic surface localization and functions. Activation of EphBs promotes insertion of GluN2B-containing NMDARs into the

synaptic membrane of mature neurons, leading to an increase in calcium influx and in gene expression of c-fos (Sheffler-Collins SI and Dalva MB, *Trends Neurosci.* 2012) [59].

3.3 Eph-ephrin interaction with cell surface receptors

3.3.1 Cross-talk with other RTKs

It is highly likely that RTK signalling networks are necessarily interconnected to Ephs as they share prominent cytoplasmic signalling cascades, such as the MAPK or PI3K pathways [89]. Thus, it is not surprising that co-clustering and interdependent signalling between Ephs and other RTKs has been suggested.

Eph receptors interact in particular with erbB/EGFR family members: analysis of the EGFR interactome revealed amongst many other interaction partners, ligand-independent association of EphA2 and EphB4 [90, 91].
Interestingly, EGFR colocalises with EphA2 at cell-cell contacts [92] and adhesion-induced EphA2 expression is thought to be regulated by EGFR activation [93].
Human cancer cell lines, co-expressing both EGFR and EphA2, show that EphA2 expression level are correlated with cell adhesion. Adhesion-induced EphA2 expression is dependent upon activation of the epidermal growth factor receptor (EGFR), mitogen activated protein kinase kinase (MEK) and Src family kinases (SRC). This response is further enhanced by interactions with integrin ligands. The effect of ephrinA1-mediated reduction in cell viability by inhibiting EphA2 expression is overruled by activated EGFR in human cancer cells.
The functional cross-talk between the two receptor–ligand system could be important when targeting either receptor, highlighting the importance of considering multi-targeting drugs in cancer therapy.

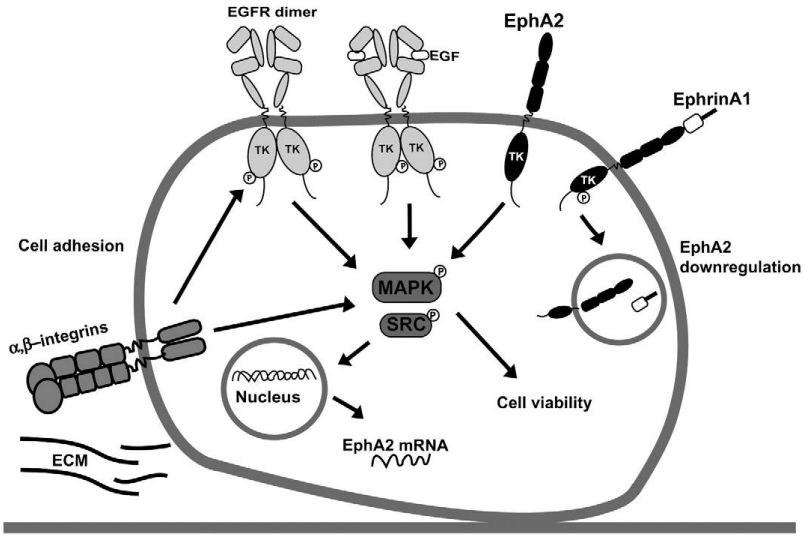


Figure 10. Regulatory mechanisms of adhesion-induced EphA2 expression in cancer cell lines co-expressing EGFR. Adhesion of cancer cells to the extracellular matrix induces the expression of EphA2 through induction of EGFR tyrosine phosphorylation and activation of MEK and SRC signaling pathways. In

turn, overexpression of EphA2 contributes to increased ERK activation and cell viability. EphrinA1 leads to EphA2 downregulation resulting in reduced ERK activity and cell viability. These effects are however abolished by activation of EGF-receptor ligand system favoring Ras/MAPK signaling and cell proliferation. Abbreviations: ECM: extracellular matrix, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, TK: tyrosine kinase, MAPK: mitogen activated protein kinase, P: tyrosine phosphorylation, and SRC: Src family of protein kinases (avian sarcoma virus oncogene homologue). (Larsen AB et al, *Cellular Signalling* 2010) [93].

Furthermore, association between EphA2 and ErbB2 promotes tumour progression of mammary tumours. EphA2 forms a complex with ErbB2 in human and murine breast carcinoma cells, resulting in enhanced activation of Ras-MAPK signaling and RhoA GTPase. Ras/MAPK contributes to cell proliferation, while activated Rho GTPase is required for tumor cell motility. EphA2 co-operates with ErbB2 to amplify its tumourigenic effects, conferring resistance to anti-ErbB2 therapy. Thus, EphA2 may be a novel target for tumors that are dependent upon ErbB receptor signaling [94].

Some studies have reported a direct agonistic interaction between FGFR and Eph/ephrin signaling pathways. Indeed, in mammalian cells EphA4 and FGFR could *trans*-phosphorylate each other and synergistically activate shared downstream signalling, such as potentiation of mitogen-activated protein kinase (MAPK) stimulation [95–97]. By contrast, activation of FGFR1 in EphB2-expressing cells inhibits repulsion and segregation responses to ephrinB1. This change in cell response is caused by inhibition of a positive feedback loop via transcriptional down-regulation of the Ras-ERK pathway while indirectly increasing the phosphorylation of unstimulated EphB2. These findings reveal a novel feedback loop that promotes high level EphB2 activation required for cell repulsion that is blocked by transcriptional targets of FGFR1 [98].
Antagonistic interaction between FGF and ephrin signaling pathways have also been elucidated. Injection of ephrin-B1 in both blastomeres of a two-cell stage *Xenopus* embryo resulted in blastomere dissociation at the mid-blastula stage, and the phenotype could be rescued by culturing the injected embryos in the presence of basic FGF [99]. Activated FGFR bound directly to ephrin-B1 in *cis* and induces its own phosphorylation on tyrosine, which in turn inhibits the ability of ephrin-B1 to induce blastomere dissociation in *Xenopus* embryos [100] (Figure 11).

Ephrins may also exhibit crosstalk with receptor tyrosine kinases. For example, the PDGF receptor phosphorylates the ephrin-B cytoplasmic domain [101]. Many types of central nervous system neurons express PDGF b receptors and respond to PDGF, including neurons of the cerebral cortex, which are known to express ephrin-B, suggesting a crosstalk between ephrin-signaling and the signaling cascade activated by tyrosine kinases.

Eph receptors and ephrin ligands have also been shown to contribute to embryonic vascular development [102]. In the study described by Ojima et al, [103], EphA2 stimulation by ephrinA1 in cultured bovine retinal endothelial cells inhibits VEGF-

induced VEGFR2 receptor phosphorylation and its downstream signaling cascades, including PKC (protein kinase C)-ERK (extracellular signal-regulated kinase) 1/2 and Akt. This inhibition results in the reduction of VEGF-induced angiogenic cell activity, including migration, tube formation, and cellular proliferation.
Moreover, inhibition of these receptor-associated signal cascades lead to a reduction of both VEGF-induced angiogenesis and vasopermeability in vivo. These findings suggest a novel therapeutic potential for EphA2/ephrinA1 in the treatment of neovascularization and vasopermeability abnormalities in diabetic retinopathy.

An example of a protein that may collaborate with EphB receptors during embryonic development is Ryk, an atypical RTK that contains a catalytically inactive tyrosine kinase domain.
Genetic studies in *Drosophila* and mouse have implicated Ryk in regulating axon guidance and craniofacial development, two developmental processes that involve cell migration [104]. Because these biological functions overlap with those attributed to Eph/ephrin signaling, a potential interaction has been sought between these pathways. In mouse, homozygous deletion of *Ryk* resulted in craniofacial defects similar to defects observed in *Eph-B2/Eph-B3*-deficient embryos. Intriguingly, Ryk-deficient mice have a cleft palate, which is similar to EphB2/EphB3 double-knockout mice and suggests that these proteins cooperate during palate formation [105].
In addition, Ryk associates with EphB2 and EphB3 receptors, by encouraging the recruitment of AF-6, a cell junction-associated PDZ-domain-containing protein, to Eph receptors; therefore, facilitating activation of downstream signaling events such as cell migration.
EphB2 and EphB3 may signal through Ryk by causing Ryk tyrosine phosphorylation. However, murine but not human Ryk is susceptible to this phosphorylation, indicating species-specific differences in the crosstalk between Eph receptors and Ryk [106].
Interaction between Ryk and Eph receptors is also involved in regulating the migration of neuroprogenitors. Indeed, overexpression of full-length Ryk, but not a mutant form that does not bind to EphB3, inhibits radial migration of cortical neuroprogenitors in the rat cortex. Conversely, a mutant form of Ryk lacking the kinase domain (including six out of nine tyrosines) had no effect on radial cell migration and still bound EphB3, suggesting that the function of Ryk in cortical cell migration is independent of tyrosine phosphorylation but correlates with EphB3 binding [107]. Taken together, these results suggest that Ryk and Eph receptors act as agonists in regulating cortical cell migration during development (Figure 11).

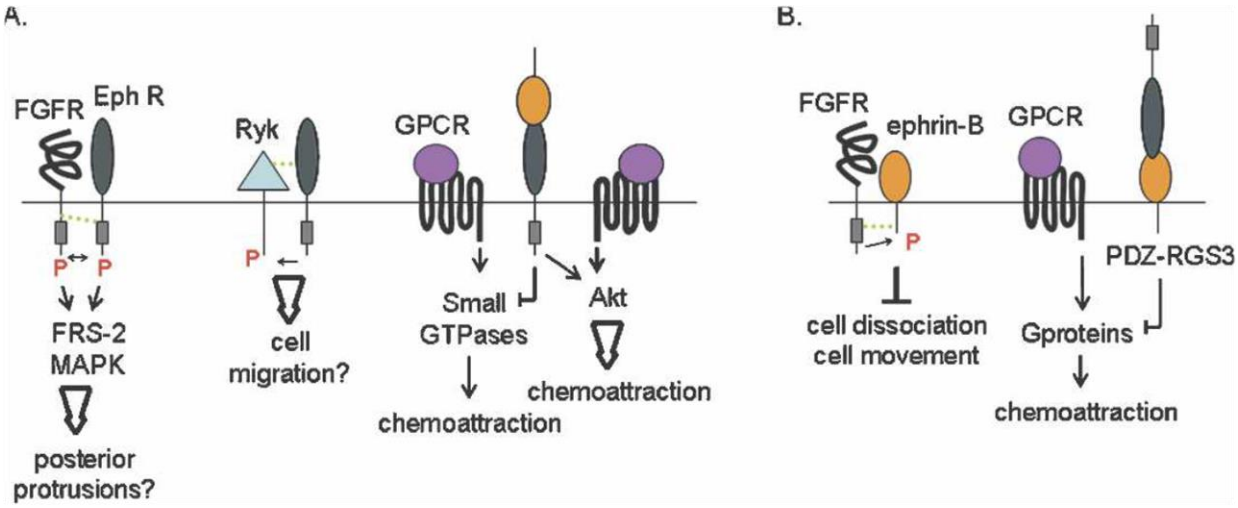


Figure 11. Interactions with cell surface receptors. (*A*) Eph receptors interact with FGFR, Ryk, and chemokine receptors. Direct interactions are indicated by dashed green lines. Arrows represent agonistic interaction, while blunted lines indicate antagonistic regulation of downstream effectors or biological processes. Tyrosine phosphorylation events are shown in red. (*B*) Ephrins interact with FGFR and chemokine receptors. (Arvanitis and Davy, *Genes Dev.* 2008) [39].

3.3.2 Cross-talk with GPCRs

There is considerable interest in the crosstalk between Ephs and the receptor CXCR4 for the chemokine SDF-1, which has been proposed to control endothelial cell movement in cooperation with EphB2 and EphB4. Stimulation of the EphB2 and EphB4 signaling cascades enhances SDF-1-induced chemotaxis in endothelial cells, and both pathways synergizes to activate/phosphorylate AKT regulating endothelial movement and morphogenesis of blood vessels [108].
Unlike this study, which demonstrates an agonistic relationship between Eph/ephrin and CXCR4/SDF-1 signaling, activation of ephrin-B reverse signaling inhibits SDF-1-induced chemotaxis of cerebellar granule cells. The mechanistic basis for this inhibition was partly elucidated by the identification of PDZ-RGS3, a protein that binds the cytoplasmic domain of ephrin-Bs and is able to inactivate G-protein signaling via its GAP activity (Figure 11). Similar results were obtained in T cells, as it was shown that activation of EphA receptors inhibits SDF-1-induced chemotaxis by altering the balance of small GTPases activity in these cells (Figure 11). Down-regulation of chemokine induced migration by Eph/ephrin signaling was also shown to be important to regulate trophoblast movement involved in arteriole remodeling during human placentation.
Although the data so far point to a cross-talk at the level of downstream effectors, it would be interesting to test for direct interactions between these proteins, especially in light of the fact that both pathways localize in lipid rafts [39].

Further insight into the crosstalk between Ephs, other RTKs and other types of transmembrane receptors will be essential for a better understanding of Eph function and

its potential application in the development of combination therapies, particularly for cancer and neurological disorders [109].

4. Strategies to target Eph receptors for pharmacological intervention

The altered expression and functional involvement of Eph receptors and ephrins in many diseases offers the opportunity for therapeutic strategies based on modulating the activities of these relevant family members. Areas of attention include cancer, inflammation, stem cell biology, nerve injury and degeneration, neoangiogenesis and tissue remodelling.
Different agents can be used to increase or inhibit the activities of a single Eph receptor or ephrin or multiple family members, and for targeted delivery of drugs and imaging agents in different disease conditions [48].

4.1 Molecules that Can Be Used to Interfere with Eph-Ephrin Signaling

4.1.1 Soluble EPHs and ephrins as part of fusion proteins

The activation of Eph receptors by ephrin ligands relies on direct contact between cells that express Ephs and ephrins to induce signalling. Preventing receptor-ligand interactions may be useful to inhibit Eph/ephrin function. A large number of molecules can be used for this purpose.
Recombinant Eph/ephrin extracellular domains (ECDs) are widely used as soluble surrogates for their membrane-bound counterparts to activate as well as inhibit forward signaling, reverse signaling, or both. These ECDs bind with high affinity and can have a long in vivo half-life, particularly when coupled to an Fc domain or albumin.
The dimeric EphA2 ectodomain fused to Fc (which inhibits EphA forward signaling but promotes reverse signaling) and the monomeric soluble EphB4 ectodomain (which inhibits both forward and reverse signaling) can both reduce tumor growth in mouse cancer models, at least in part by inhibiting tumor angiogenesis [27].
Scehnet et al. used the extracellular domain of EphB4 fused with human serum albumin to block ephrinB2 in Kaposi sarcoma cells in vitro, resulting in the inhibition of migration and invasion of Kaposi sarcoma cells in response to various growth factors [110].
On the basis of these and other preclinical studies that have also demonstrated the therapeutic effects of an EphB4–human serum albumin (HSA) fusion protein in colon, lung, breast, glioma, melanoma and prostate tumours [110, 111], soluble EphB4–HSA has entered Phase I clinical trials in patients with refractory or metastatic solid tumours.
Furthermore, in some systems, ephrin Fc proteins seem to function as Eph receptor inhibitors unless they are oligomerized with anti-Fc antibodies, perhaps because monomeric or dimeric ephrins are weaker activators than the endogenous, membrane-bound ephrins they displace. In contrast, Eph Fc proteins can promote ephrin reverse signaling. Eph/ephrin Fc proteins can also compete with their endogenous counterparts

and reduce their signaling ability. EphA2 Fc and EphA3 Fc, for example, can function as anticancer agents in mouse models by inhibiting EphA2 forward signaling in the tumor vasculature [112, 113]. Applications of ephrin ECDs also include attachment of ephrin-A1 Fc or the ephrin-B2 ECD to biomimetic hydrogels for therapeutic angiogenesis [114, 115] and of ephrin-A1 Fc to albumin microspheres to inhibit cancer cell growth and migration [116].

4.1.2 Monoclonal antibodies

Antibodies are particularly suitable for modulating the Eph/ephrin system, given their high binding affinity and specificity coupled with their long in vivo half-life. Both activating and inhibitory monoclonal antibodies recognizing Eph/ephrin ECDs have been developed for applications against cancer and angiogenesis, with particular focus on EphA2, EphA3, EphB4, and ephrin-B2.

Agonistic antibodies have been used to suppress tumour growth in mouse models. These agonists are activators of Eph-ephrin signalling that stimulate Eph forward signalling and could be used to negatively regulate tumour cell growth and to induce the degradation of Eph receptors in cancer cells. Coffman et al. targeted EphA2 on cancer cells using agonistic antibodies that simulate the effect of ligand binding, showing a decrease in tumour growth *in vivo* through protein degradation [117].

EphA2 was the first Eph receptor to be considered for therapeutic antibody development. From a panel of agonistic monoclonal antibodies (agonistic mAbs), the humanized version of mAb B233 with improved FcγRIII binding capacity (3F2-3M) suggested promising antibody-dependent cell-mediated cytotoxicity (ADCC)-mediated antitumour effects in ovarian, lung and breast cancer xenografts.

Treatment with 3F2-3M mAbs restored drug sensitivity in trastuzumab-resistant tumour cell lines by targeting the previously reported oncogenic ERBB2–EPHA2 crosstalk, and in combination with trastuzumab, there was synergistic inhibition of trastuzumab-resistant tumour growth *in vivo* [63].

The prominent overexpression of EPHA3 in a range of solid and haematological cancers, including glioblastoma-initiating cells and leukaemic stem cells, and its lack of expression on normal haematopoietic cells or their progenitors prompted development of the agonistic anti-EPHA3 mAb IIIA4 as an anticancer therapeutic. The IIIA4 mAb, which alone or in synergy with ephrin A5, triggers sustained EPHA3 activation, tumour cell contraction and apoptosis, effectively targets EPHA3+ tumour xenografts without binding to non-tumour mouse tissues [118].

Therefore, Eph agonist antibodies may also be useful in cancer treatment in combination with chemotherapy since they can also enhance the effects of established chemotherapeutic drugs such as tamoxifen, paclitaxel, and docetaxel. Additionally, EphB4 and ephrin-B2 inhibitory antibodies have shown efficacy in mouse tumor xenografts as antiangiogenic/anticancer agents, in some cases when combined with anti-VEGF therapy [48].

4.1.3 Peptides that bind to Eph receptors and inhibit ephrin binding

Another main approach to directly target the ephrin-binding pocket of the Eph receptors can be accomplished with peptides or chemical compounds.
Peptides have proven especially suitable for occupying the broad and shallow ephrin-binding pocket of the Eph receptors with high affinity and selectivity, and can function as antagonists as well as agonists (Figure 12 B, C) [119].

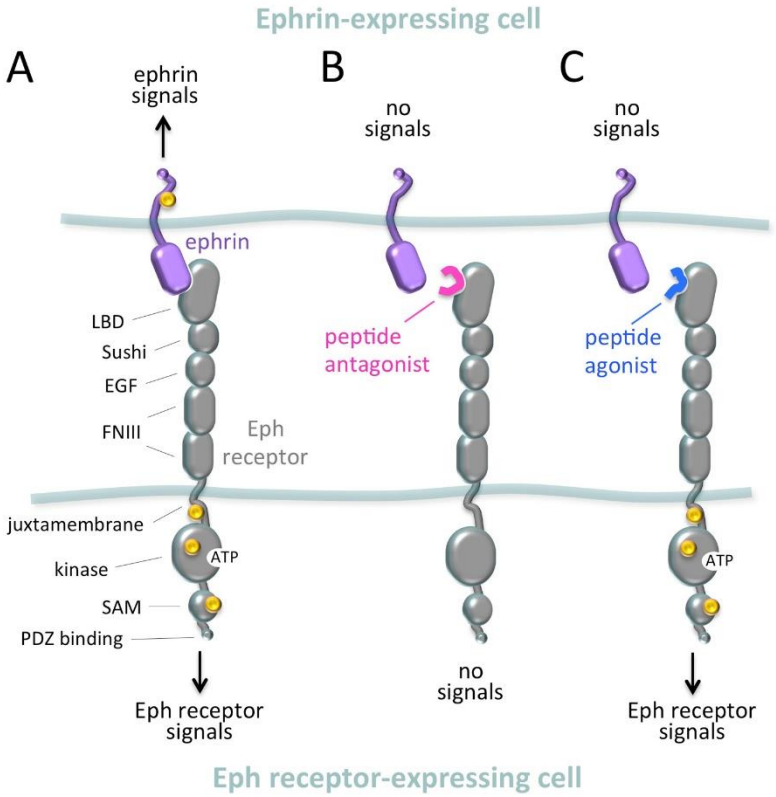


Figure 12. Effects of peptides on Eph receptor-ephrin bidirectional signaling
(A) Bidirectional signaling of an Eph receptor-ephrin complex where an Eph receptor-expressing cell comes near an ephrin-expressing cell. Signaling typically involves clustering of multiple Eph receptor-ephrin complexes, even though only one EphB receptor and one ephrin-B are shown for simplicity. (B) A peptide antagonist inhibits signaling by both the Eph receptor and the ephrin. (C) A peptide agonist activates Eph receptor signaling and inhibits ephrin signaling (Ryedl SJ and Pasquale EB, *Curr Drug Targets.* 2015) [119].

A series of dodecapeptides that can selectively target the ephrin-binding pocket of individual Eph receptors, or subset of receptors, were identified by phage display [120]. Additional evidence that some of the peptides bind to the ephrin-binding pocket includes NMR chemical shift perturbations that suggest an interaction of the peptides with residues of the ephrin-binding pocket [121, 122] and mutations of residues in the ephrin-binding pocket that affected peptide binding [121]. However, the most direct evidence comes from several X-ray crystal structures of peptide-Eph receptor complexes.

To date, the crystal structures of 4 peptides in complex with the EphA4, EphB2 or EphB4 LBDs have been solved, revealing that peptides can bind to the ephrin-binding pocket in a variety of orientations [123-125]. Moreover, the ability of several peptides to target only a single Eph receptor, despite the promiscuity in the binding of the ephrins to Eph receptors, suggests that the ephrin-binding pockets do have unique features that can be exploited by peptides to achieve strict selectivity.
In addition, peptide dimerization or oligomerization can also drastically increase binding affinity through the increased avidity of multivalent binding [126].

EphA2
The YSA and SWL dodecapeptides identified in phage display screens exhibit strict selectivity for EphA2 among the Eph receptors, compete with each other for binding and inhibit ephrin binding.
YSA, SWL and derivative peptides are agonists that can promote EphA2 tyrosine phosphorylation (indicative of activation) and downstream signaling (including suppression of major oncogenic pathways such as RAS-ERK, AKT-mTORC1 and integrin-dependent pathways) as well as cause EphA2 degradation [127, 128].
The agonistic effects of the peptides are similar to those of the natural ephrin-A ligands but weaker, which may at least in part stem from the lower binding affinity of the peptides. The unmodified YSA and SWL peptides have low micromolar antagonistic potency (<15 μM), which can be substantially improved up to ~1 μM or less by C-terminal addition of lysine, biotin or other moieties attached through linkers. Furthermore, dimerization of SWL with a 6-carbon linker was shown to yield a bivalent peptide with >10 fold increased potency (0.3 μM) due to its simultaneously binding to 2 EphA2 LBDs [126]. Thus, this modifications to increase potency or the identification and improvement of new scaffolds will be needed to achieve more robust EphA2 activation triggered by peptide agonists.
Such higher affinity derivatives would also be more amenable to further development into therapeutic leads. These agents would then be useful to promote EphA2 activation, which is low in many tumors consistent with the fact that ephrin-induced EphA2 signaling can suppress tumorigenesis [27] On the other hand, EphA2 activation in endothelial cells is an important factor in pathological forms of angiogenesis. Thus antagonistic peptides, if they could be developed, could be useful to inhibit angiogenesis.

EphA4
A phage display screen to identify dodecapeptides binding to the EphA4 extracellular region identified 3 peptides (KYL, VTM and APY) that bind to the EphA4 LBD with low micromolar to submicromolar affinity and compete with each other for binding [121, 129]. There are common, as well as distinctive features, in the interaction of the 3 peptides with the ephrin-binding pocket of EphA4. In addition, several EphA4 mutations that disrupt ephrin-A5 binding do not similarly affect the binding of the peptides, suggesting substantial differences in the residues utilized for binding by the peptides and a natural

ephrin ligand. This is in agreement with the strict selectivity of these peptides for EphA4, which is in contrast to the receptor binding promiscuity of ephrin-A5.

Unlike KYL and VTM, which are linear, APY has a cyclic structure that results from a disulfide bond between C4 and C12 [121, 125]. APY has been crystallized in complex with the EphA4 LBD, illustrating the excellent fit of the peptide within the ephrin-binding pocket and the unique positioning of the surrounding DE, GH and JK loops of EphA4 in the complex. Information from the crystal structure also suggested modifications for improving the binding affinity of APY [125]. One was amidation of the C terminus of APY, which resulted in an additional intrapeptide hydrogen bond. Another was to introduce a methylene spacer in the backbone of APY by replacing Gly8 with βAla in the tight β-turn at the apex of the circular portion of APY. These modifications resulted in the peptide APY-βAla8.am, which exhibits a binding affinity of 30 nM and retains strict selectivity for EphA4.

The KYL, VTM, APY and APY-βAla8.am peptides are antagonists that can inhibit ephrin-induced EphA4 activation in *in vitro* biochemical assays, in cultured cells, and in mouse hippocampal slices [121, 125, 129]. Although APY-βAla8.am with its nanomolar binding affinity is the most potent of the EphA4 peptide antagonists, it was only recently developed and, therefore, so far most studies have used KYL as a research tool and to validate EphA4 as a potential drug target.
KYL was used in cell culture and in ex vivo models to implicate EphA4 in various biological processes as well as in rodent preclinical studies demonstrating the role of EphA4 in neuroprotection and neural repair.
Supporting a role for EphA4-ephrin interaction in axon guidance and inhibition of nerve regeneration after injury, KYL and very recently APY-βAla8.am were shown to inhibit EphA4-dependent growth cone collapse in retinal explants and/or cultures of cortical neurons [125, 130].
Importantly, KYL has also been used to corroborate the role of EphA4 signaling in neurodegenerative processes. Two studies have shown that blockage of the EphA4 LBD by KYL can inhibit EphA4 activation by amyloid-β oligomers, which are believed to play an important role in the synaptic dysfunction and cognitive impairment characteristic of Alzheimer's disease [60, 61].
Furthermore, intracerebral infusion of KYL was shown to restore normal synaptic plasticity in a transgenic mouse model of Alzheimer's disease [61]. In a rat model of spinal cord injury, KYL administered intrathecally enhanced the sprouting of injured axons as well as recovery of limb function [130], suggesting potential medical applications to promote nerve repair after injury by inhibiting ephrin-induced EphA4 signaling. Moreover, intracerebral infusion of KYL significantly delayed disease onset and increased survival in a rat model of amyotrophic lateral sclerosis (ALS), a lethal neurodegenerative disease characterized by progressive loss of motor neurons and for which therapy options are nearly absent [131].

In other applications outside the nervous system, the KYL peptide has been used in cell culture experiments to demonstrate the importance of ephrin-induced EphA4 activation in limiting integrin-mediated T-cell adhesion to endothelial cells, suggesting a role for EphA4 in regulating T-cell trafficking in vivo [132].
Finally, KYL was used in a co-culture model to demonstrate that interaction of EphA4 upregulated in breast cancer stem cells with ephrins expressed in a monocyte cell line elicits juxtacrine signals that induce secretion of cytokines sustaining the stem cell state [133]. This helped define EphA4 as a key receptor that mediates the interplay of breast cancer stem cells with monocytes and macrophages serving as niche cells that support breast cancer malignancy.

Measurement of peptide antagonistic activity after incubation in cell culture conditioned medium revealed that the KYL and APY peptides have a half-life of ~ 10 hours while VTM is stable for several days. However, all 3 peptides are rapidly degraded in plasma, with half-lives < 1 hour, which will have to be improved in derivatives to be used *in vivo* [121].

EphB2
Phage display screens identified SNEW as a dodecameric peptide that selectively binds to EphB2 with moderate affinity (KD = 6 μM) and inhibits EphB2-ephrin-B2 interaction in ELISAs with an IC50 value of 15 μM [120, 124].
Molecular dynamics simulations of SNEW in complex with EphB2 suggested that the first 4 residues of the SNEW peptide fit optimally in the ephrin-binding pocket, consistent with the crystal structure of the SNEW-EphB2 LBD complex, whereas C-terminal modifications could improve binding affinity.
Notably, 8 of the 13 peptides identified by panning on EphB2 also bound to EphB1, suggest a close similarity between the ephrin-binding pockets of the two receptors.

EphB1
Of the EphB1 receptor-targeting peptides, EWLS is a selective EphB1 antagonist that inhibits ephrin-B2 binding in ELISAs with an IC50 value of ~10 μM and also competes for EphB1 binding with the other 4 peptides identified by panning on EphB1 [120].

EphB4
For EphB4, phage display screens identified ~15 dodecameric peptides that preferentially bind to this receptor compared to the other EphB receptors [120]. Among a number of peptides that were chemically synthesized, TNYL was the best inhibitor of ephrin-B2 binding to EphB4, even though its potency was only 50-150 μM for the biotinylated and non-biotinylated versions, respectively.
The C-terminal extension of TNYL by addition of the RAW motif yielded TNYL-RAW, a 15 amino acid-long peptide that exhibits a dramatically increased potency compared to TNYL (by 4 orders of magnitude, with an IC50 value of 15 nM and a KD value of 2-3 nM for the binding of TNYL-RAW to mouse EphB4). The crystal structure of TNYL-RAW bound to the EphB4 LBD shows an extensive network of interactions between the peptide and residues

in the ephrin-binding pocket, since it binds in a different configuration compared to the ephrin-B2 G-H loop but interacts with some of the same EphB4 residues, including L48, L95 and T147. Importantly, these residues are not conserved in other Eph receptors, most likely contributing to the selectivity of TNYL-RAW for EphB4.
Stability studies revealed that TNYL-RAW has a very short half-life in cell culture medium and in plasma, suggesting high susceptibility to proteolytic degradation and clearance from blood circulation. Various strategies have been successfully used to inhibit peptide degradation and rapid blood clearance, including N-terminal modifications, conjugation to a 40 kDa branched polyethylene glycol (PEG) polymer or to nanoparticles, fusion to the Fc portion of an antibody, and complexation of the biotinylated peptide with streptavidin [134].
Furthermore, the cyclic cTNYL-RAW exhibits greatly increased stability in mouse plasma, presumably because the cyclic conformation inhibits peptide degradation by aminopeptidases as well as cleavage between R13 and A14 by trypsin-like proteases.

With respect to targeting EphB receptors, the EphB2-binding peptide SNEW and the EphB4-binding peptide TNYL-RAW can inhibit the ephrin-induced tyrosine phosphorylation of their target EphB receptor as well as tyrosine phosphorylation of ephrin-B ligands, which is mediated by kinases such as SRC [120, 134].
Given their selectivity, the SNEW and TNYL-RAW peptides have been used as tools in studies to implicate EphB2, EphB4 or both receptors in various biological processes. SNEW and TNYL-RAW can block human umbilical vein endothelial (HUVE) cell retraction caused by ephrin-induced EphB2 activation [120] and ephrin-induced EphB4 activation respectively [135], indicating the ability of the peptides to counteract the cell shape changes and anti-migratory effects mediated by the EphB2 and EphB4 receptors.
Both SNEW and TNYL-RAW have also been used in *in vitro* experiments demonstrating the importance of EphB receptor-ephrin-B2 signaling in the angiogenic responses of endothelial cells and their supporting vascular mural cells [134]. Finally, EphB2 and EphB4 can promote tumorigenesis by interacting with ephrin-B ligands [46], opening the possibility of using antagonist peptides for cancer therapy.

Peptides can also have some disadvantages, including their potentially poor pharmacokinetic parameters and oral bioavailability. Additional properties are needed for in vivo use of peptides, including high resistance to plasma proteases and persistence in the blood circulation. N-terminal modifications to prevent digestion by aminopeptidases present in the blood, inclusion of unnatural amino acids, and cyclization have been successfully used to obtain more metabolically stable Eph receptor-targeting peptides. In addition, PEGylation or inclusion into nanoparticles can prevent rapid clearance through the kidneys and the reticuloendothelial system, prolonging peptide lifetime in the circulation.

4.1.4 Kinase inhibitors

Small molecules bind the ATP-binding pocket in the Eph kinase domain. Advantages of small-molecule kinase inhibitors are their extensive track record as drugs; potential for oral bioavailability; and, in many cases, ease of synthesis.
A particularly promising inhibitor is NVP-BHG712, an EphB4-specific inhibitor developed by Novartis, which has good pharmacokinetic properties. It inhibits EphB4 phosphorylation in tissues after oral administration and vascular endothelial growth factor (VEGF)-driven angiogenesis *in vivo*.
However, most kinase inhibitors exhibit poor selectivity and target multiple kinases. Interestingly Src, Abl and platelet-derived growth factor (PDGF) and EGF receptors have a threonine gatekeeper residue. The gatekeeper residue, that controls access of inhibitors to a deep hydrophobic pocket adjacent to the ATP binding site, is also a threonine in most Eph receptors. This is consistent with the selectivity profile observed for many of the kinase inhibitors targeting Eph receptors. For example, dasatinib and nilotinib were first identified as Src and Abl inhibitors but also potently target Eph receptors [136].
Surprisingly dasatinib, a multi-targeted kinase inhibitor already used in the treatment of chronic myelogenous leukemia and under clinical evaluation to treat solid tumors, potently inhibits EphA2 and other Eph receptors besides its primary targets Abl and Src. Interestingly, EphA2 has also been identified as a biomarker for dasatinib sensitivity of cancer cells [27].

4.1.5 Interfering RNA

An additional approach for therapies directed against intracellular targets is the regulation of the gene expression using small interfering RNA or antisense oligodeoxynucleotides.
These agents are highly selective and downregulate Eph/ephrin expression, but their in vivo delivery can be inefficient. The most promising results were obtained with *EPHA2* siRNA administered to tumors using neutral liposomes; this agent inhibits tumor growth and metastasis in mouse models of ovarian cancer, particularly when combined with delivery of siRNA silencing focal adhesion kinase (FAK) or with paclitaxel chemotherapy [27].
Targeted knockdown of EphB4 expression by siRNA and antisense oligodeoxynucleotides led to poor survival of breast cancer cells and increased apoptosis. Furthermore, antisense oligodeoxynucleotides-mediated EphB4 knockdown resulted in the suppression of tumour growth in a murine tumour xenograft model [137].

4.2 Targeting Molecules for Delivery of Therapeutics and Imaging Agents

Besides modulating Eph receptor/ephrin function, antibodies and peptides can also serve to deliver conjugated agents for imaging and therapy by using various nanoparticles to fight tumors and other diseases expressing target Eph receptors.

Several chemotherapeutic drugs and toxins conjugated to Eph antibodies or to an ephrin, which cause receptor-mediated drug internalization, appear promising according to initial studies.

EPHA2- or EPHB2-targeting antibodies coupled to derivatives of the peptide drug auristatin, which disrupts microtubule dynamics, inhibit the growth of several cancers in rodent models. For example, 1C1 EphA2 agonistic antibody–drug conjugate with the microtubule inhibitor auristatin, was reported to convincingly block tumor xenograft growth, reduce metastasis and improve survival rates in ovarian and endometrial carcinoma xenografts without any obvious adverse effects.

Likewise, cytotoxic ephrin A1–drug conjugates actually in preclinical development, include ephrin A1–exotoxin A, which effectively killed EPHA2+ glioblastoma, breast and prostate carcinoma cell lines. Moreover, transfection of human mesenchymal stem cells with a soluble ephrin A1–*Pseudomonas* exotoxin fusion, induces glioblastoma killing and reduction in tumour volume after intratumoural injection of the engineered mesenchymal stem cells [63].

Additionally, another way to achieve tumor selective delivery of chemotherapeutic drugs is their conjugation to peptides targeting cell surface receptors that are highly expressed in tumors but poorly expressed in most normal tissues, such as EphA2 and EphB4.

EphA2-specific YSA agonistic peptide and its improved derivatives conjugated to paclitaxel enhance the anti-tumor effects of the chemotherapeutic drug paclitaxel in a PC3 prostate cancer mouse xenograft model and decrease vascularization in a mouse syngeneic renal cancer model without overt signs of toxicity.

The YSA peptide fused to the homodimeric p19 siRNA-binding protein or conjugated to the outer shell of hydrogel nanoparticles has also been successfully used to deliver functional siRNAs inside EphA2-positive ovarian cancer cells in culture, leading to siRNA-mediated gene knockdown [119].

Peptides have several favorable features as conjugated targeting agents compared to antibodies, including ease of synthesis, low immunogenicity and toxicity and a small size that enables more efficient tissue penetration. In addition, peptides can not only escort drugs to target tissues but also help make them more soluble and bioavailable.

Antibodies, ephrins and peptides can also be used to deliver imaging agents for diagnostic purposes. Promising results have been obtained in animal models by using an EPHA2 antibody labeled with ^{64}Cu through the chelating agent 1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid (DOTA) for radioimmunoPET imaging and an EPHA3 antibody coupled to ^{111}Indium for gamma camera imaging [138, 139].

Ephrin-As conjugated to a radioisotope can also target tumor cells overexpressing EphA receptors, although rapid clearance from the circulation will have to be overcome for in vivo use, particularly against solid tumors [139]. Additionally, ephrin-A1-targeted, gold-coated nanoshells were used for in vitro photothermal ablation of cancer cells [140].
With regard to the EphB/ephrin-B system, the high-affinity EphB4-targeting peptide TNYL-RAW have been used to deliver imaging agents and therapeutic nanoparticles to mouse tumor xenografts.
The ^{64}Cu-DOTA-TNYL-RAW peptide was successfully used to image EphB4-positive PC3 prostate cancer and CT26 colon cancer cells in mouse tumor xenografts by small animal PET-CT [119]. Another version of the peptide, Cy5.5-TNYL-RAWK-^{64}Cu-DOTA, labeled with the near infrared dye Cy5.5 at the N terminus and with 64Cu-DOTA attached to an added C-terminal lysine, was developed for dual modality microPET-CT and near-infrared fluorescence optical imaging of orthotopic glioblastoma xenograft mouse models [141]. This derivative also retained high EphB4 binding affinity. When systemically administered in mice with intracranial tumors derived from EphB4-expressing U251 cells, Cy5.5-TNYL-RAWK-^{64}Cu-DOTA labeled both the tumor cells and the tumor vasculature. This could represent a way to monitor tumors by imaging their blood vessels through EphB4 targeting.
The cyclic version of the peptide, cTNYL-RAW, was conjugated through a PEG linker to hollow gold nanospheres, which absorb in the near-infrared region and have strong photothermal conduction. These nanospheres were additionally loaded with the chemotherapeutic drug doxorubicin. The peptide selectively targeted the nanospheres to several EphB4-positive cancer cells in culture and in mouse tumor xenografts after intravenous injection [142].
Furthermore, TNYL-RAW peptide-conjugated polymeric micellar nanoparticles labelled with the near-infrared dye indocyanine 7 (Cy7) and with ^{111}In allow multimodal whole-body imaging of tumour-bearing mice by single-photon emission computed tomography and near-infrared microscopy, and this technique holds great promise for the development of non-invasive strategies for visualizing tumour lesions [63].

4.3 Vaccine-based immunotherapy

Owing to the notable overexpression of EPHs in tumours, Eph receptors represent possible targets for anticancer vaccines. EPHA2, EPHA3 and an EPHB6 isoform have been identified as sources of tumor-associated peptide antigens that are recognized by cancer-specific cytotoxic T cells [27].
In an experimental approach, EphA2-derived peptides that induce specific, tumour-reactive CD8+ or CD4+ T cell responses might be able to serve as agents for immunotherapy of renal cell carcinoma [137]. Moreover, EPHB6 peptides induce cytotoxic T cells in peripheral samples from human leukocyte antigen A2-positive (HLA-A2+) glioma patients and EPHA2 peptide-pulsed dendritic cell vaccines induce natural

killer, CD4+ and CD8+ T cells and inhibit growth and metastasis in mouse EPHA2+ tumour models.

Yamaguchi et al. investigated the effectiveness of vaccination dendritic cells (DCs) loaded with EphA2-derived peptides (Eph-DCs) in a murine colon cancer model, demonstrating that immunization with Eph-DCs suppressed MC38 tumour (with EphA2 overexpression) growth compared with the control group, and in contrast, Eph-DC vaccination had no influence on BL6 tumour (without EphA2 expression) growth [143].

Additionally, a bispecific single-chain antibody that simultaneously binds both EPHA2 and the T-cell receptor/CD3 complex causes T-cell-mediated destruction of EPHA2-positive tumor cells *in vitro* and decreases tumor growth *in vivo* [144], enhancing the antitumor efficacy of the antibodies in xenograft models.

Interestingly, agonists and drugs that stimulate Eph receptor degradation may inhibit tumor growth at least in part by enhancing the presentation of Eph-derived peptides that can be recognized by effector T-cells. Vaccination with Eph-derived epitopes also shows promise as a strategy to elicit tumor rejection [27].

5. AIM OF THE RESEARCH

Eph receptors, and their membrane-bound ligands, the ephrins, are expressed in the nervous system, where they modulate different processes, such as neurogenesis, neuronal migration, axon guidance, synaptogenesis, and they significantly contribute to contact-dependent neuron-glia communication [145, 146].

Eph receptor signaling typically starts through cell-cell contact and is then transmitted bidirectionally. The receptor-expressing cell and the ligand-expressing cell transduce a signal known as forward signal and reverse signal, respectively. The Eph-ephrin system modulates many biological activities during embryogenesis, the developement of the central nervous system as well as in the adult. Alterations of the Eph-ephrin system have been linked to neuropathologies ranging from inhibition of neural repair after traumatic injury and stroke to neurodegenerative diseases and chronic neuropathic pain, which nowadays have an important social impact but still no effective therapies.

The EphB-ephrinB system has been recently implicated in the onset and maintenance of different types of pain: expression of both EphB and ephrin-Bs is increased in different animal models of neuropathic pain [54]. Indeed, intrathecal injection of ephrin-B1-Fc determines dose- and time-dependent hyperalgesia through activation of PI3K and p42/p44 MAPK [147,148], whereas intrathecal administration of EphB1-Fc reduces formalin-induced inflammation and chronic-constrictive injury-induced neuropathic pain behaviors in mice [148]. Furthermore, ephrin-B-EphB receptor signaling contributes to bone cancer pain via pro-inflammatory cytokines, whereas the blockade of spinal EphB1 receptor activation relieves pain and rescues the opioid-mediated analgesia that is lost in a mouse model of cancer-related chronic pain [149, 53]. Hence, the EphB-ephrin-B system is emerging as a potentially new target, which could be exploited for designing new therapies for the treatment of these diseases.

Adding to the complexity of Eph-receptor–ephrin bidirectional signaling is the ability of Eph proteins to communicate with a variety of other cell surface proteins. This cross-talk may allow the Eph receptors and ephrins to broaden their repertoire of functions. A large body of evidence show cross-communication between G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) which activate overlapping signaling patways [151]. Activation of RTKs by GPCRs ligands is called transactivation and is an important pathway that links GPCRs to the extracellular signal-regulated kinase (ERK) signaling [152]. Reciprocally, RTKs utilize GPCR signaling molecules to transduce signals, and even RTK ligands can transactivate GPCRs. Thus, RTKs and GPCRs can form a platform in which protein signalling components specific for each receptor are shared to produce an integrated response upon engagement of ligands [152].

There is considerable interest in the crosstalk between EphB receptors and GPCRs. Mu opioid receptors (MOR), belonging to the GPCR super-family, are expressed both in central and peripheral nervous system, where they can be activated by both endogenous

and exogenous opioids, thus eliciting a significant analgesia through different signaling events, including adenylyl cyclase inhibition and activation of p42/p44 MAPK phosphorylation [153].

Morphine and related MOR agonists are among the most effective and widely used pain killers, but their clinical use is limited by relevant side effects as respiratory depression, tolerance, and addiction [154]. Furthermore, opiates are poorly effective in different types of chronic pain accompanied by sustained glial activation and altered neuron-glia cross-talk, including neuropathic pain [155].

All the above mentioned findings highlight an opposite effect determined by EphB1 and MOR through their effects on p42/p44 MAPK activation (hyperalgesia vs analgesia) and suggest a potential inverse relation between the activation of the EphB1-ephrin-B system and the effects elicited by MOR agonists. However, any cross-talk between EphB1 and MOR, and its potential influence on the reduced analgesic effects of opioids in different chronic pain states, has been so far poorly investigated.

Therefore, the aim of the research has been to investigate the functional cross-talk between intracellular signaling pathways triggered by EphB1 and MOR receptors in different cell models co-expressing the two receptors.

First, we characterized EphB1 and MOR receptor-mediated signaling in HEK293 cells expressing recombinant EphB1 and MOR, and evaluated the activation profile of intracellular p42/44 MAPK proteins. Subsequently, the investigation of any perturbation of opioid-mediated signaling (receptor expression, Src, PKC and MAPK activation) by the concomitant activation of the EphB1 receptor by its soluble agonist (ephrin-B1 Fc), was conducted in the human neuroblastoma SH-SY5Y cell line, which endogenously expresses both receptors, before and after differentiation, in presence or absence of pro-inflammatory stimuli.

Investigating the role of EphB1 signaling and linking its cross-talk with the MOR receptor to the activation of signaling pathways related to the onset and manteinance of chronic pain, will help understand the molecular processes contributing to the ineffectiveness of opioid analgesics.

Characterization of the contribution of EphB1 to the molecular mechanisms involved in neuropathic pain and validating the EphB1 receptor as a new druggable target will be useful to guide development of novel peptidomimetic EphB1 antagonists, capable to block or at least reduce the intracellular signaling induced by ephrin-B-induced EphB1 activation.

Studies conducted in the laboratory of Dr. Pasquale have identified a number of peptides that bind to Eph receptors and inhibit ephrin binding by using phage display approaches [120]. Most of the peptides are antagonists, but the peptides targeting EphA2 are agonists that activate receptor signaling similarly to the natural ephrin ligands. Interestingly, some of the identified peptides are highly specific and bind to only one Eph receptor family member, conversely the natural ephrin ligands promiscuously bind to multiple Eph

receptors. Thus, Eph receptor-targeting peptides represent valuable pharmacological tools to study the functional importance of specific Eph receptors in tumors and the nervous system and could be used as leads to develop therapies against cancer and neurological disorders.

During my period of research at the Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, under the supervision of Dr. Pasquale, a major specialist in the field of the Eph-ephrin system, I worked on a research project focused on the development and characterization of peptide antagonists and kinase inhibitors targeting Eph4, a receptor that plays a role in neurodegenerative diseases and cancer.

EphA4 is a cell surface receptor tyrosine kinase preferentially expressed in neurons. In the normal brain, EphA4 activity can control the dynamic reorganization of neuronal connection and the changes in synaptic transmission that occur during learning and memory formation. However, aberrant levels of EphA4 and its excessive activity inhibits neuronal repair and promotes neurodegenerative processes such as those occurring in amyotrophic lateral sclerosis (ALS) and Alzheimer's disease [60,61,156].

Possible strategies to target EphA4 for pharmacological intervention include inhibiting ligand binding or kinase activity. In order to identify EphA4 inhibitors with good pharmacological properties and in vivo bioavailability, I set up a cell-based assay suitable to test peptides and small molecules and used it to evaluate several peptides and kinase inhibitors targeting EphA4.

6. MATERIALS AND METHODS

6.1. Reagents

p-CMV6-entry vector and p-CMV6-EphB1 plasmids were purchased from OriGene Technologies (Rockville, USA), pcDNA3.1^{+}-OPRM 1 plasmid was obtained from the cDNA Resource Center (www.cdna.org).
Ephrin-B1 Fc (473-EB-200), ephrin-A5 Fc (374-EA-200) and recombinant human TNFα (210-TA) were from R&D System (Minneapolis, MN), whereas human Fc (ICN55911) was from MP Biomedical (Santa Ana, CA); Morphine hydro-chloride was from Boehringer Ingelheim limited (Bracknell, UK).
Gö6976, PP2 and PMA (Phorbol 12-myristate 13-acetate) were purchased from Sigma-Aldrich (Milan, Italy) and dissolved in dimethylsulphoxide (DMSO); the final concentration of DMSO was less than 0.1% and did not cause any significant effect on the activities tested in this study. Naloxone was from Tocris (Bristol, UK).
Anti-EphB1, Anti phospho-p42/44, total p42/44 (diluted 1:1000) were from Cell Signaling Technologies (EuroClone, Pero, Italy). Anti β-actin (diluted 1:5000) was from Abcam (Cambridge, United Kingdom).
Anti-Phospho-EphA3 (Tyr779) mAb (diluited 1:4000) was from Cell Signaling Technologies, anti-αEphA4 Exp 953 (170 µg/ml) Rabbit pAb (used at a final concentration of ~1 ug/ml) was produced by Dr. Pasquale Laboratory (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA) [157], anti-β-tubulin (T0198) (diluited 1:25000) was from Sigma-Aldrich.
All other reagents were of analytical grade or the highest purity available, purchased from Sigma-Aldrich.

6.2. Cell cultures

Human embryonic kidney HEK293 cells were obtained from European Collection of Cell Culture (Salisbury, UK). Cells were grown in Eagle's Minimal Essential Medium (EMEM) (Lonza Group Ltd, Basel, Switzerland) supplemented with 2 mM L-Glutamine (Lonza), 1x non-essential aminoacids (Life Technologies, Monza, Italy) and 1x antibiotic-antimycotic solution (Life Technologies) (defined cell culture medium), containing 10 % fetal bovine serum (Thermo Scientific) and cultured at 37°C in a humidified atmosphere of 5% CO_2.
Human neuroblastoma SH-SY5Y cells (European Collection of Cell Culture, Salisbury, UK) were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 1% L-glutamine (Lonza), 1x non-essential amino acids, and 1x antibiotic-antimycotic solution and cultured at 37°C in a humidified atmosphere of 5% CO_2.

HEK293 AD cell line, which is a derivative of the HEK 293 cell line with increased adherence, stably expressing human EphA4, were grown in Dulbecco's Modified Eagle's Medium (DMEM; Corning, Tewksbury, MA) supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 1x non-essential amino acids, and 1x antibiotic-antimycotic solution + 1mg/ml G418.

6.3. Cell transfection and treatments

HEK293 were plated in 6-well plates and at 70–80% confluence were transiently co-transfected with 1.5 ug/well of p-CMV6-EphB1 plasmid and 1.5 ug/well pcDNA3.1^{+}-OPRM1 plasmid using the Polyethylenimine branched Transfection Reagent (PEI) (Sigma, Steinheim, Germany), according to the manufacturer's protocol and treatments were started 48 h later.
HEK293 cells, tansfected to express human EphB1 and MOR, and SH-SY5Y cells (both native and differentiated), endogenously expressing the two receptors, were treated with EphrinB1-Fc (1µg/ml) or Morphine (1µM) or both ligands, or left untreated. For differentiation, SH-SY5Y cells were exposed to PMA (16 nM; 5 days); during the last 48 h of differentiation cells were exposed to TNFα (10 ng/ml; 48 h) or to vehicle.

To activate EphA4 in EphA4-HEK293 AD cells, the cells were stimulated for 15 min with 0.4 µg/ml ephrin-A5 Fc or Fc. In addition, some wells were pre-treated for 1h with Kinase inhibitor 1, Kinase inhibitor 2 and Dasatinib or vehicle control, before the addition of 0.4 µg/ml ephrin-A5 Fc for 15 min.
In some experiments, EphA4-HEK293 AD cells were pre-treated/incubated for 30 min with different concentrations of APY-d10, APY-d11 or vehicle control, and then stimulated for 10 min with 0.5 µg/ml ephrin-A5 Fc.

6.4. Saturation binding assay

Radioligand binding assays were performed as previously described [158]. EphB1-MOR-transfected HEK293 and SH-SY5Y cell membranes were prepared by homogenizing cells in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, and 1 mM benzamidine, with a Polytron homogenizer. After centrifugation (1000 g for 10 min at 4°C), supernatants were centrifuged (18000 x g for 30 min at 4°C) and the pellet suspended in 50 mM Tris–HCl buffer, pH 7.4, containing 5 mM $MgCl_2$. Protein concentration was determined by the BCA assay kit (Thermo Fisher). For saturation binding experiments, cell membranes (40 µg/assay tube) were incubated in 100 mM Tris–HCl, pH 7.4, containing 0.3% bovine serum albumin with increasing concentrations of [^{3}H]-DAMGO (0.1-10 nM in EphB1-MOR-HEK293 and 0.1-50 nM in SH-SY5Y) (PerkinElmer Life Sciences). Non-specific binding was determined in the presence of

Naloxone (Tocris) (50 μM). After 90 min incubation at 25°C, bound ligand was isolated by rapid filtration on Whatman GF/B filters (Schleicher & Schuell, Dassel, Germany). Filters were washed with 20 mL of ice-cold 50 mM Tris–HCl buffer, pH 7.4, and left in scintillation fluid overnight before counting. Data were fitted by non-linear least-square regression and the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) was used to calculate receptor density (B_{max}) and ligand affinity (K_d). Data are expressed as fmol of [^{3}H]-DAMGO bound and normalized to cell homogenate protein content.

6.5. Western blotting assay

To detect EphB1 and β-actin, EphB1-MOR-HEK293, native and differentiated SH-SY5Y cells were scraped in cold phosphate-buffered saline, pelleted, and resuspended in 100 μL of T-PER Tissue Protein Extraction Reagent (Pierce) plus protease inhibitors composed of 0,5 mg/mL Benzamidine, 2 μg/mL Aprotinin, 2 μg/mL Leupeptin, 2 mM phenylmethlysulfonyl fluoride, and a cocktail of phosphatase inhibitors 100x (Sigma). After 10 min of gently agitation at 4 °C, samples were centrifuged at 10,000 g for 10 minutes to collect supernatants. Protein concentration was measured by BCA assay (Pierce).
Briefly, HEK-293- MOR-EphB1 cells and SHSY5Y cells (native and differentiated) were plated into 6-well plates until a confluence of 70-80% was reached; then, the cells were serum-starved for 16-18 h and subsequently exposed to different treatments in cell culture medium alone. To detect p42/44 MAPK phosphorylation, cells were scraped off and pelleted after 15 min of exposure. Cell were washed in PBS and lysed in MAPK Lysis Buffer (50mM Tris-Cl, 300mM NaCl, 1mM EDTA, 1mM Na_3VO_4, 1mM NaF, and 10% glycerol, 1% Triton X-100 1% protease and phosphatase inhibitors cocktail). The homogenates were sonicated for 10 seconds (speed 4) and then centrifugates at 17000 g for 25 minutes at 4°C. The supernatants were collected and protein concentration was measured by BCA assay (Pierce). Proteins (15 μg to assay ERK1/2 or 50 μg to assay EphB1 and β-actin) were separated by SDS-PAGE on 12% or 10% (w/v) acrylamide/bisacrylamide gels, respectively, and electrotransfered onto nitrocellulose membranes. Membranes were incubated in TBS-T (20 mmol/L Tris–HCl, pH 7.5, 137 mmol/L NaCl, 0.1% (v/v) Tween 20) containing 5% (w/v) bovine serum albumin for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody, rinsed with TBS-T, and incubated with peroxidase-conjugated anti-rabbit secondary antibodies, peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA) at 25°C for 1.5 h and the blots were developed with Clarity™ Western ECL substrate (Bio-Rad Laboratories, Segrate, Milan, Italy). Blot images were digitally acquired by LAS3000 Imager (Fujifilm Corporation, Stamford, CT, USA) and protein expression semi-quantitatively analyzed using AIDA software (Raytest Isotopenmessgeraete GmbH, Mannheim, Germany).
After treatments, HEK293 AD stably expressing human EphA4 were washed in PBS and

then lysed in Sample buffer 2X. Cell lysates were analyzed by immunoblotting with the following antibodies: anti-Phospho-EphA3 (Tyr779) Rabbit mAb (Cell Signaling) diluted 1:4000, anti-αEphA4 Exp 953 (170 μg/ml) Rabbit pAb (Pasquale Lab) diluted ~1 ug/ml, anti-β-tubulin Mouse (Sigma-Aldrich) diluited 1:25000. Incubation with primary antibodies was followed by incubation with anti-rabbit, anti-mouse secondary antibodies conjugated to HRP (anti-rabbit and anti-mouse from EMD Millipore, Billerica, MA) diluited 1:5000 to 1:10000. Immunoblots were developed with ECL chemiluminescence HRP detection reagent (GE Healthcare). Signal intensity of immunoblot bands was quantified by using the histogram function of Photoshop.

6.6. Total RNA preparation and real-time RT-PCR analysis

SH-SY5Y cells were maintained in cell culture medium containing 10% fetal bovine serum, and, after treatments, were collected from tissue culture flasks, centrifuged (500 g for 5 min) and rinsed with phosphate-buffered saline. Total cellular RNA was extracted with Tri-reagent® (Sigma-Aldrich) and digested with Rnase-free Dnase (Thermo Fisher Scientific) for 15 min at 25°C according to the manufacturer's instructions. A 2-μg sample was reverse-transcribed using the High Capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturer's instructions. Real-time PCR was employed for relative quantification of human EPHB1 and human MOPr transcripts using the StepOne Instrument (Life Technologies) and the GoTaq® qPCR master mix (Promega, Madison, Wisconsin, USA). This 'hot start' reaction mix contains Taq DNA polymerase, dNTP mix, and the fluorescent dye SYBR Green I for real-time detection of double-stranded DNA. Reactions were set up in 10 μL including 100 ng of target DNA. To amplify human EphB1 cDNA, a sense primer (5′-GACTGACGATGATTACAAGTCAGAGC-3′) and an antisense primer (5′-AGATGGCCACCAAGGACACA -3′) were used at 0.25 μM final concentration for producing a 101-bp fragment (1953–2053 bp; GenBank Accession nº. NM_004441.4). To amplify the human hMOPr cDNA, a sense primer (5′-CTGGGTCAACTTGTCCCACT-3′) and an antisense primer (5′-TGGAGTAGAGGGCCATGATC-3′) were used at 0.25 μM final concentration for producing a 146-bp fragment (327–472 bp; GenBank Accession nº. NM_000914). As a control, a 169-bp fragment of the human L19 ribosomal protein gene was amplified with a sense primer (5'-CTAGTGTCCTCCGCTGTGG-3') and an antisense primer (5'-AAGGTGTTTTTCCGGCATC-3') at 0.25 μM final concentration, producing a fragment (62–230 bp; GenBank Accession no. BC062709). Human MOPr cDNA amplification was performed as follows: 95°C for 10 min followed by 40 cycles of 95°C for 5 s, 62°C for 20 s, and 68°C for 10 s. Human EPHB1 and L19 amplifications were carried out as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. After that, the temperature was lowered to 60°C for 30 s and the specificity of the reaction was verified by analysis of the melting curve once the appropriate double-stranded DNA melting temperature had been reached.

Relative expression of RT-PCR products was determined using the $\Delta\Delta C_T$ method [159];

where C_T is the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and $\Delta\Delta C_T$ = [C_T gene of interest (unknown sample) – C_T L19 (unknown sample)] - [C_T gene of interest (calibrator sample) - C_T L19 (calibrator sample)]. One of the control samples was chosen as the calibrator sample and used in each PCR. Each sample was run in triplicate and the mean C_T was used in the $\Delta\Delta C_T$ equation. L19 was chosen for normalization because this gene showed consistent expression relative to other housekeeping genes among the treatment groups in our experiments.

6.7. Statistical analysis

All data are presented as mean ± SEM for the number of experiments indicated and were analyzed by one-way ANOVA followed by Newman–Keuls test. The GraphPad Prism, version 5.0 (GraphPad Software, Inc.) was used, and P values <0.05 were considered significant.

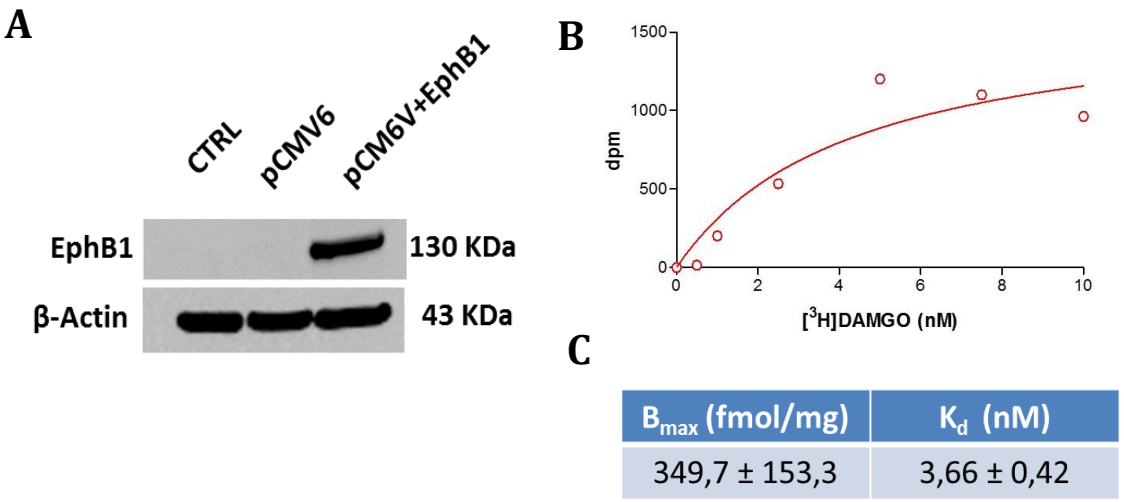
7. RESULTS

7.1 Evaluation of any cross-talk between EphB1 and MOR in trasfected HEK293 cell lines.

The human embryonic kidney cell line HEK293 has been extensively used as an expression tool for recombinant proteins, since its biochemical machinery is capable of carrying out most of the post-translational folding and processing required to generate functional and mature protein from a wide spectrum of both mammalian and non-mammalian nucleic acid [160]. This cell line has also been used to study a variety of questions in neurobiology. The fidelity of HEK cells express exogenous receptors make this cells amenable to many kinds of transfection procedures, permitting the expression of proteins for many purposes. Indeed, this cell line is useful as a transient transfection tool for the evaluation of pharmacological properties of receptors and for the study of their signals transduction.
In our study, we used HEK293 cell lines, as a transient expression system for EphB1 tyrosine kinase receptor and mu-opioid receptor (MOR), belonging to GPCR family.
After the administration of ephrinB1-Fc, ligand of EphB1, or morphine, known ligand of MOR, I evaluated the phosphorylation levels of p42/44 MAPK proteins at different times. Because both ephrin-B1 Fc-activated EphB1 and morphine-activated MOR lead an increase in p42/44 phosphorylation levels, p42/44 MAPK represents a convergence point for evaluating the interaction between EphB1 and MOR when activated at the same time by co-administrating both ligands.

7.1.1. EphB1 and MOR are expressed in transfected HEK-293 cells

In this experiment, we employed the human embryonic kidney cell line HEK293 transiently co-transfected with 1.5 ug/well of p-CMV6-EphB1 plasmid and 1.5 ug/well pcDNA3.1^{+}-OPRM1 by using the Polyethylenimine branched Transfection Reagent (PEI). 48 hours post-transfection, the cell lisate was analyzed by immunoblotting to evaluate EphB1 expression by using anti-EphB1 antibody (Figure 1A). Since there are no available antibodies recognizing Mu opioid receptors (MORs), saturation receptor-binding assays carried out using [^{3}H]-DAMGO, as described under Materials and Methods, ascertained that MOR receptors were expressed in EphB1-MOR-HEK293 cell membranes (B_{max}= 349.7 $\pm$ 153.3 fmol/mg protein; K_D= 3.66 $\pm$ 0.42) (Figure 2B,C).



C

B_{max} (fmol/mg)	K_d (nM)
349,7 ± 153,3	3,66 ± 0,42

Figure 1: Western blot analysis of EphB1 receptor expression levels (A), saturation binding of [^{3}H]-DAMGO to MOR (B) and B_{max} and K_d values relative to MOR expression (C), in HEK-EphB1-MOR cells. β-actin was used as a loading control.

7.1.2. EphrinB1-Fc and morphine determine a time-dependent activation of p42/p44 MAPK phosphorylation in HEK-EphB1-MOR cells.

To examine the time course of p42/44 MAPK activation in response to EphB1 or to MOR receptor stimulation, transiently transfected HEK 293 cells were exposed to either ephrin-B1 Fc or morphine at different times, and phosphorylated p42/44 MAPK were determined by western blot.

EphB1-MOR-expressing HEK 293 cells, starved for 16-18 hours, were exposed to ephrinB1-Fc for either 0, 15, 30, and 60 min, or morphine for 0, 5, 15, 30, 60 and 120 min. Cell lysates were analyzed by immunoblotting using anti-P-p42/44 and anti-p42/44 antibodies.

Ephrin B1-Fc induced a time-dependent p42/44 phosphorylation that peaked at 15 minutes of exposure (~2-fold over basal; Figure 2A). Morphine-induced p42/44 MAPK phosphorylation peaked at 15 minutes of exposure as well (Figure 2B).

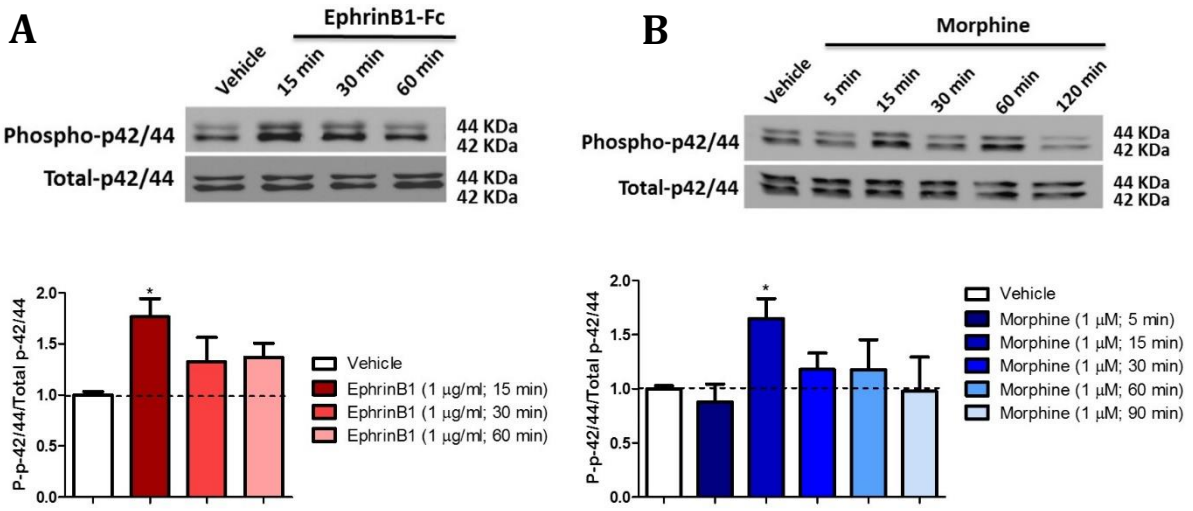


Figure 2: Western blot analysis of p42/44 MAPK phosphorylation levels in HEK-EphB1-MOR cells treated with vehicle, EphrinB1-Fc (1µg/ml; 0-60 min), or morphine (1µM; 0-120 min). * = $p<0.05$ vs Vehicle (values are the mean ± SEM; n=8); Total-p42/44 was used as a control.

7.1.3. EphrinB1-Fc and morphine co-administration to HEK-EphB1-MOR cells occludes p42/p44 MAPK phosphorylation

To evaluate the involvement of the p42/44 MAPK pathway in the EphB1-mediated signaling by concomitant activation of MOR-mediated signaling, phosphorylation levels of p42/44 in EphB1-MOR-HEK293 exposed to both ephrin-B1 Fc (1µg/ml) and morphine (1 µM) are examined by immunoblotting.

EphB1-MOR-expressing HEK 293 cells, starved for 16-18 hours, were exposed to ephrinB1-Fc (1µg/ml) or morphine (1 µM) alone, or with both ligands for 15 min.

Cells were lysed with MAPK lysis buffer, as described in material and methods, and protein extracted; 15 µg of proteins from the total extract were separated by SDS-PAGE at 12% of polyacrylamide for detection of p42/44 MAPK and examined by immunoblotting by using anti-P-p42/44 and anti-total p42/44 antibodies (Figure 3).

Morphine and ephrin-B1 Fc significantly increase p42/44 MAPK phosphorylation when administered as a single agents. On the other hand, their coadministration occludes p42/p44 MAPK activation.

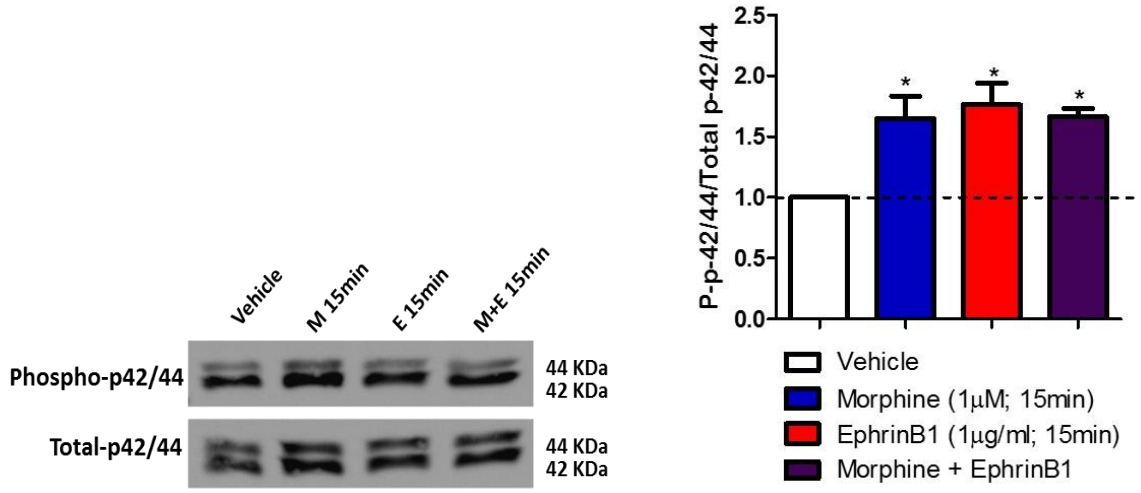


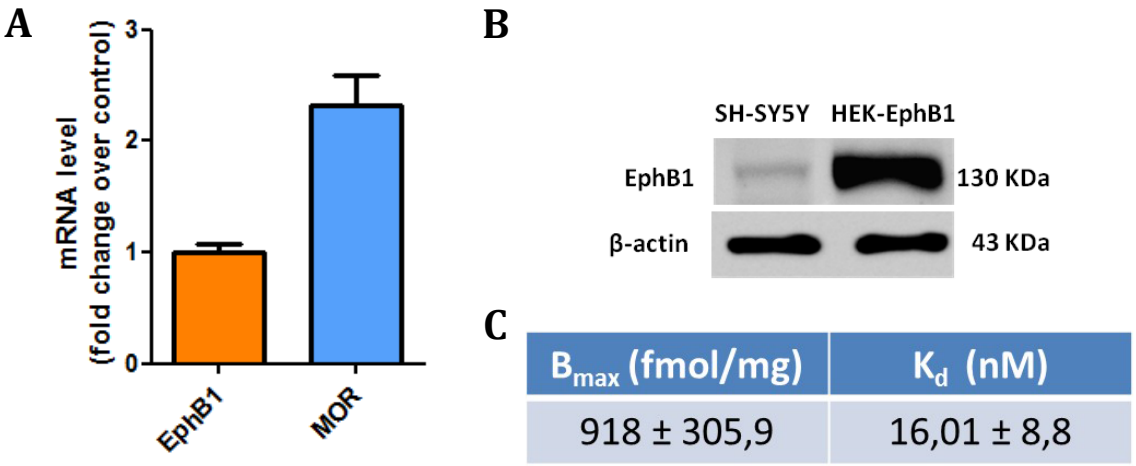
Figure 3: Western blot analysis of p42/44 MAPK phosphorylation levels in HEK-EphB1-MOR cells treated with vehicle, EphrinB1-Fc (1μg/ml; 15min), morphine (1μM; 15 min) or co-treated with both EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min). * = $p<0.05$ vs vehicle; (values are the mean ± SEM; n=4).

7.2 Evaluation of any cross-talk between EphB1 and MOR receptors in human neuroblastoma cell line SH-SY5Y.

7.2.1 EphB1 and MOR are endogenously expressed in SH-SY5Y cells

In this experiment, I have utilized the human neuroblastoma cell line SH-SY5Y that constitutively expresses EphB1 and MOR, reproducing a neuronal-like phenotype. To evaluate REST and MOR mRNA levels, I developed a real-time PCR technique by amplifying a cDNA sequence of 101bp of EphB1 mRNA and a cDNA sequence of 146bp of MOR mRNA, as previously described. The protein content of EphB1 and MOR was analyzed by western blot and saturation binding assay, respectively.

EphB1 and MOR are endogenously expressed in SH-SY5Y neuroblastoma cell line, as confirmed by evaluating their mRNA levels; EphB1 mRNA levels are lower in comparison to MOR mRNA levels (Figure 4A). EphB1 protein content, detected by western blot, shows endogenous levels lower in comparison to EphB1-transfected HEK293 cells (Figure 4B) whereas MOR are expressed in SH-SY5Y cell membranes (B_{max}= 918 ± 305.9 fmol/mg protein; K_D= 16.01 ± 8.8) (Figure 4 C).



B_{max} (fmol/mg)	K_d (nM)
918 ± 305,9	16,01 ± 8,8

Figure 4: Real-time PCR of EphB1 and MOR mRNA levels in SH-SY5Y cells (A); western blot analysis of EphB1 receptor expression levels in SH-SY5Yand in HEK293 cells transfected with cDNA coding for this receptor (B); B_{max} and K_d values relative to MOR expression (C) in SH-SY5Y.

7.2.2 EphrinB1-Fc and morphine show a time-dependent activation of p42/p44 MAPK phosphorylation in SH-SY5Y cells

To examine the time course of p42/44 MAPK activation in response to EphB1- receptor or to mu-opioid receptor stimulation, SH-SY5Y cells were exposed to either ephrin-B1 Fc or morphine at different times, and phosphorylated p42/44 MAPK were determined by western blot.

SH-SY5Y cells were starved for 16-18 hours prior to ephrin-B1 Fc or morphine exposure for 5, 15 and 30 min. Cell lysates were analyzed by immunoblotting using anti-p-p42/44 and anti-p42/44 antibodies.

Ephrin-B1 Fc, as well as morphine, induce a time-dependent p42/44 phosphorylation that peacks at 15 minutes of exposure (Figure 5A, B).

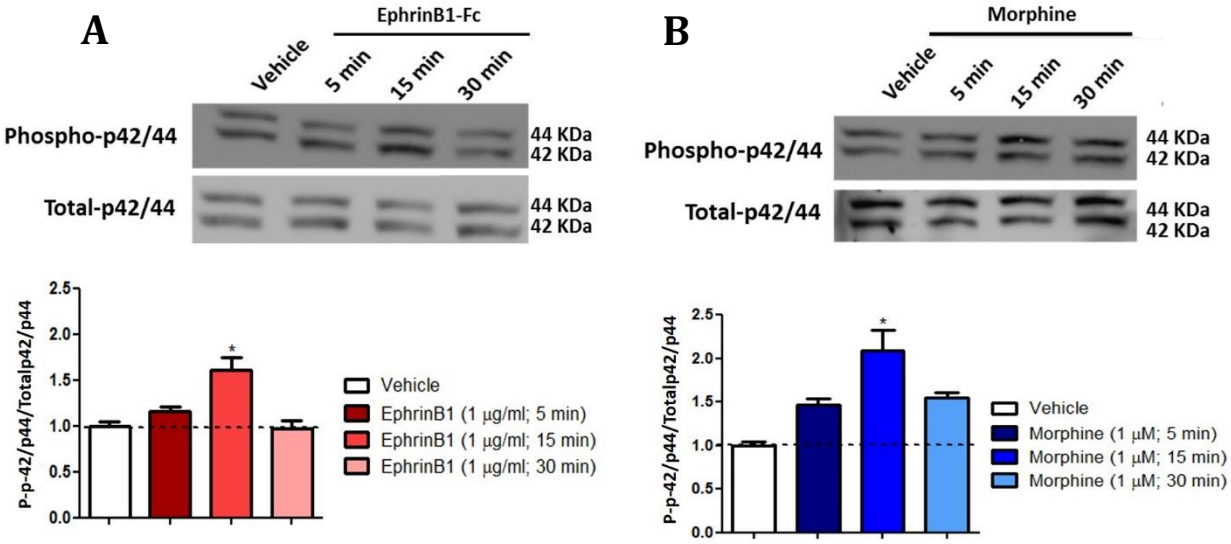


Figure 5: Western blot analysis of p42/44 MAPK phosphorylation levels in SH-SY5Y cells treated with vehicle, EphrinB1-Fc (1µg/ml; 5-30 min), or morphine (1µM; 5-30 min). * = p<0.05 vs Vehicle; (values are the mean ± SEM; n=12).

7.2.3. EphrinB1-Fc and morphine co-administration to SH-SY5Y cells occludes p42/p44 MAPK phosphorylation

To further explore the involvement of the p42/44 MAPK pathway in EphB1-mediated signaling by concomitant activation of MOR-mediated signaling, phosphorylation levels of p42/44 in SH-SY5Y exposed to both ephrin-B1 Fc (1µg/ml) and morphine (1 µM) were evaluated by western blot.

SH-SY5Y cells, starved for 16-18 hours, were exposed to ephrinB1-Fc (1µg/ml) or morphine (1 µM) alone or in presence of both ligands for 15 min.

Cells were lysed in MAPK lysis buffer and protein extracted; 15 ug of proteins from the total extract were separated by SDS-PAGE at 12% of polyacrylamide for detection of p42/44 MAPK and examinated by immunoblotting by using anti-P-p42/44 and anti-total p42/44 antibodies (Figure 6).

Morphine by interacting with MOR triggers the phosphorylation of p42/44 MAPK after 15 minutes of exposure; the same result is observed for EphB1 binding to ephrin-B1 Fc. However, the co-administration of ephrin-B1-Fc and morphine don't exhibit a synergistic effect caused by EphB1- and MOR-activated signaling pathways on p42/44 MAPK phosphorylation.

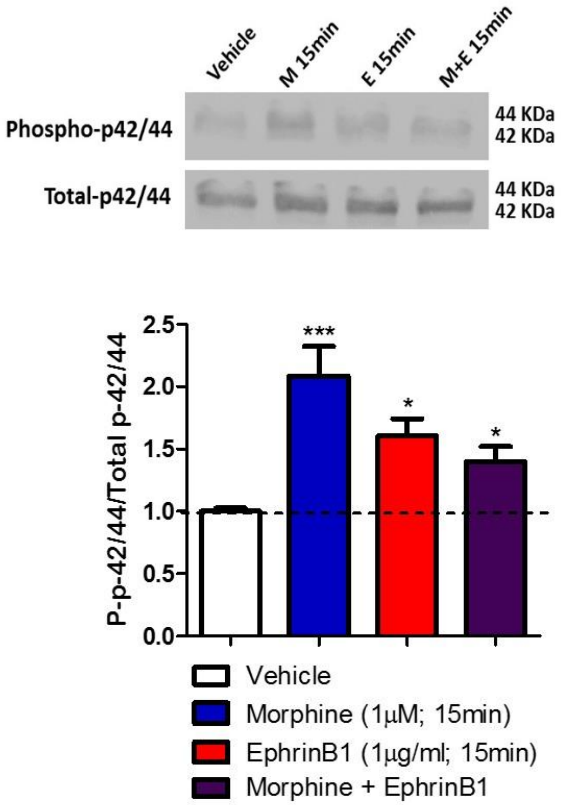


Figure 6: Western blot analysis of p42/44 MAPK phosphorylation levels in SH-SY5Y cells treated with vehicle, EphrinB1-Fc (1μg/ml; 15min), morphine (1μM; 15 min) or co-treated with both EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min). *** = $p<0.001$ vs vehicle * = $p<0.05$ vs vehicle; (values are the mean ± SEM; n=4).

7.2.4. p42/p44 MAPK activation by ephrinB1-Fc is PKC- and Src-independent

To elucidate any contribution of Src and PKC in mediating p42/44-dependent signaling pathways after activation of EphB1 with ephrinB1-Fc, phosphorylated p42/44 were examined by western blot analysis. SH-SY5Y were pre-treated for 30 min with Gö6976 (5 μM), a PKC inhibitor which only inhibits classical isoforms of this enzyme families, or PP2 (5 μM), a selective inhibitor of Src-family tyrosine kinases, administered before ephrin-B1 Fc stimulation. Using an antibody that recognize dually phosphorylated active form of p42/44, I ascertained that phosphorylation of p42/44 was maximally elevated by ephrin B1-Fc (1 μg/ml) 15 min after treatment. EphB1-mediated p42/44 MAPK activation was not abrogated by the PKC inhibitor Gö6976 and the Src inhibitor PP2. This suggests that p42/p44 MAPK activation by ephrinB1-Fc is PKC- and Src-independent.

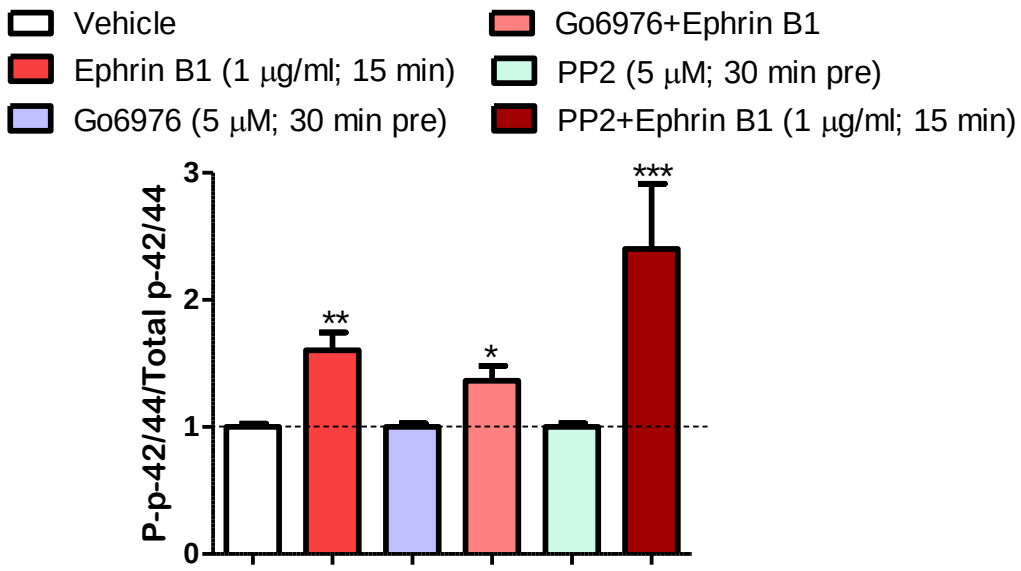


Figure 7: Western blot analysis of p42/44 MAPK phosphorylation levels in SY-SY5Y cells treated with vehicle or EphrinB1-Fc (1μg/ml; 15 min), with or without Gö6976 (5 mM) or PP2 (5 mM) 30 min prior to ephrinB1-Fc. **=p<0.01 vs vehicle; *=p<0.05 vs Go6976; ***=p<0.001 vs PP2; values are the mean ± SEM of three independent experiments.

7.2.5. p42/p44 MAPK activation by morphine is PKC-dependent and Src-independent

To elucidate any contribution of Src and PKC in mediating p42/44-dependent signaling pathways after activation of MOR by morphine, phosphorylated p42/44 were examined by western blot analysis. SH-SY5Y were pre-treated for 30 min with Gö6976 (5 μM), a PKC inhibitor which only inhibits classical isoforms, or PP2 (5 μM), a selective inhibitor of Src-family tyrosine kinases, administered before mophine exposure. Using an antibody that recognizes dually phosphorylated active form of p42/44, we ascertained that phosphorylation of p42/44 was maximally elevated by morphine (1 μM) 15 min after treatment. MOR-mediated p42/44 MAPK activation was abrogated by the PKC inhibitor Gö6976, but not by the Src inhibitor PP2. This experiment, therefore, suggests that PKC contributes in mediating p42/p44 MAPK activation by morphine, whereas Src is not involved in this pathway.

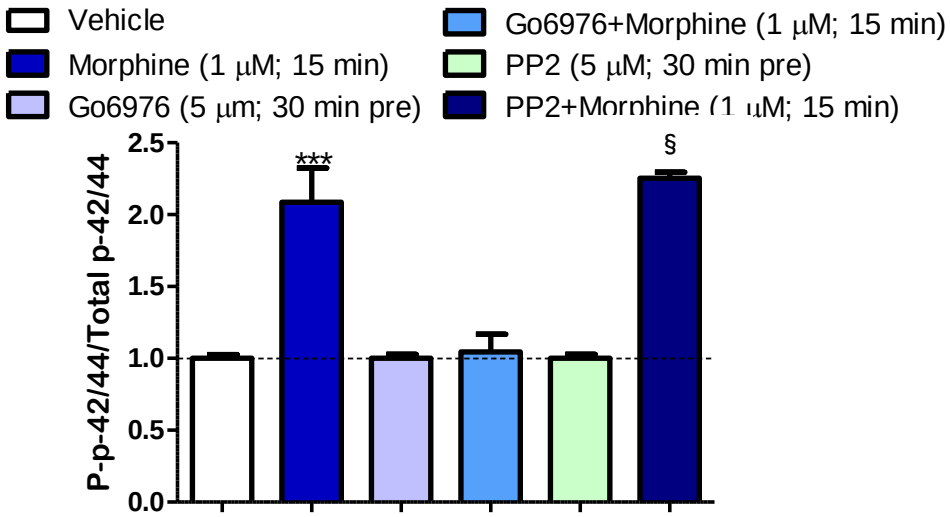


Figure 8: Western blot analysis of p42/44 MAPK phosphorylation levels in SY-SY5Y cells treated with vehicle or morphine (1μM; 15 min), with or without Gö6976 (5 mM) or PP2 (5 mM) 30 min prior to morphine. ***=p<0.001 vs vehicle; §=p<0.001 vs PP2; values are the mean ± SEM of three independent experiments.

7.3 Evaluation of any cross-talk between EphB1 and MOR in a PMA-differentiated SH-SY5Y cells

Neuroblastoma cell lines are a suitable model for investigating the mechanisms that induce neuronal differentiation, after exposure to phorbol esters which block cell growth and induce neurite outgrowth of neuroblastoma cell line SH-SY5Y [161].
First, I evaluated any transcriptional change in EphB1 and MOR transcripts, and also of their protein contents, after exposure to phorbol 12-myristate 13-acetate (PMA) for 5 days. Then, Iexplored anyinvolvement of the p42/44 MAPK pathway in the EphB1-mediated signaling by concomitant activation of MOR-mediated signaling in PMA-differentiated SH-SY5Y, exposed to both ephrin-B1 Fc (1μg/ml) and morphine (1 μM).

7.3.1 EphB1 and MOR expression is altered in PMA-differentiated SH-SY5Y cells

To evaluate any change in EphB1 and MOR mRNA levels in PMA-differentiated SH-SY5Y cells, we developed a real-time PCR technique by amplifying a cDNA sequence of 101bp of REST mRNA and a cDNA sequence of 146bp of MOR mRNA.
To induce cell differentiation, SH-SY5Y cells were exposed to PMA (16 nM) for 5 days or to the vehicle alone. Then, the cells were collected and total cellular RNA was extracted; a 2-μg sample was reverse-transcribed and the real-time PCR was employed for relative quantification of human EPHB1 and human MOPr transcript. The protein content of EphB1 and MOR was analyzed by western blot and saturation binding assay, respectively.

Exposure of SH-SY5Y cells to PMA (16nM) for 5 days induced a significant (P<0.001) decrease in EphB1 mRNA levels in comparison to control cells (Figure 9A). An opposite effect was observed in MOR mRNA levels, which induced a significant (P<0.001) increase in comparison to control cells, as expected [162] (Figure 9 B).

The levels of the corresponding EphB1 and MOR proteins in total cell extracts were evaluated by western blotting or saturation binding assay, respectively, (Figure 9 C and D). They follow an expression pattern similar to mRNA levels.

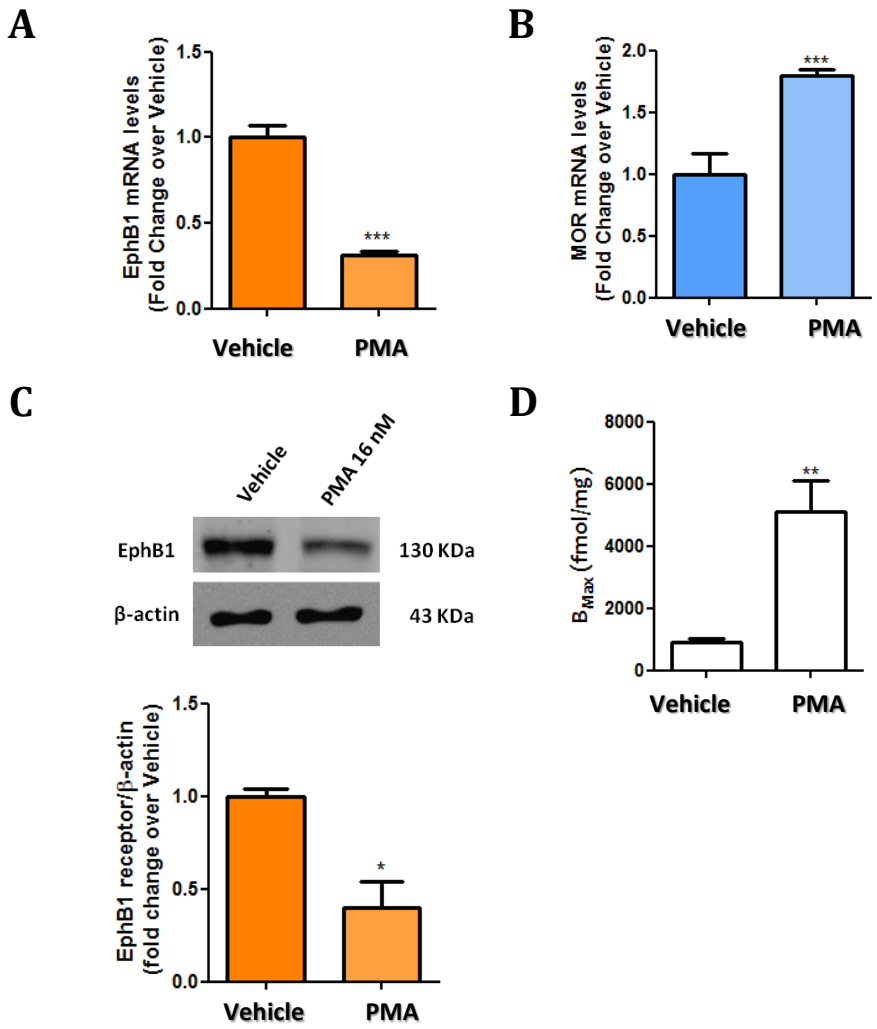


Figure 9: PMA down-regulates EphB1 mRNA levels while up-regulates MOR mRNA levels (A and B) in SH-SY5Y cells. Exposure to PMA (16nM) for 5 days decreases REST protein levels (C) while increases MOR protein levels (D) in comparison to control cells.

Real-time PCR of EphB1 receptor (A) and MOR (C) mRNA levels, western blot analysis of EphB1 receptor expression levels (B) and B_{max} values relative to MOR expression (D) in SH-SY5Y cells exposed to vehicle or PMA (16 nM; 5 d). *** = p<0.001, ** = p<0.01, * = p< 0.05 vs vehicle; (values are the mean ± SEM; n=6).

7.3.2 EphrinB1-Fc administration in PMA-differentiated SH-SY5Y cells no longer occludes morphine-mediated phosphorylation of p42/p44 MAPK

To further explore the involvement of the p42/44 MAPK pathway in the EphB1-mediated cell signaling by a concomitant activation of MOR-mediated signaling, phosphorylation levels of p42/44 in PMA-differentiated SH-SY5Y exposed to both ephrin-B1 Fc (1µg/ml) and morphine (1 µM) were examined by western blot.
SH-SY5Y cells, starved for 16-18 hours, were exposed to ephrinB1-Fc (1µg/ml) or morphine (1 µM) alone, or with both ligands for 15 min. Cells were lysed in MAPK lysis buffer and protein were extracted; 15 ug of protein from the total extract were separated by SDS-PAGE at 12% of polyacrylamide for detection of p42/44 MAPK and examined by immunoblotting by using anti-P-p42/44 and anti-total p42/44 antibodies (Figure 10).
Morphine interacting with MOR triggers the activation of p42/44 MAPK after 15 minutes of exposure, the same result is observed for EphB1 interacting with ephrin-B1 Fc. Surprisingly, the co-administration of ephrin-B1-Fc and morphine no longer occludes p42/p44 MAPK activation.

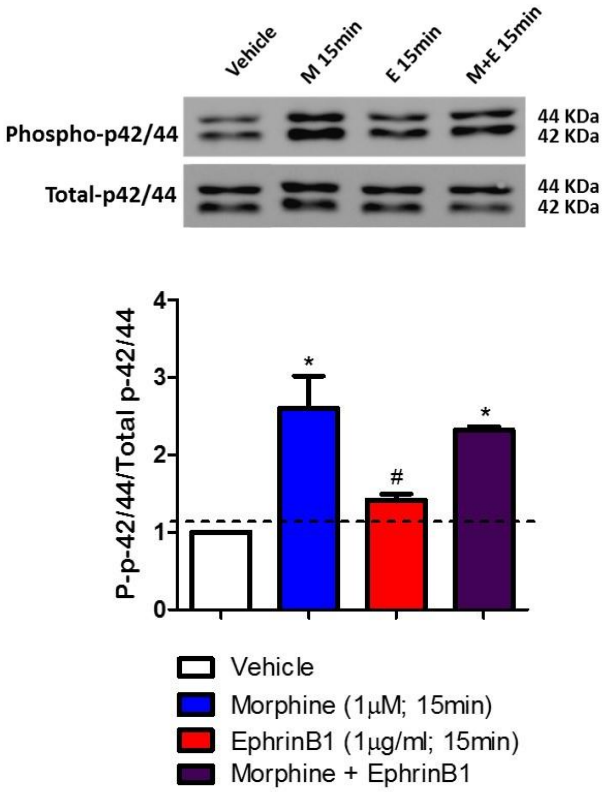


Figure 10: Western blot analysis of p42/44 MAPK phosphorylation levels in PMA-differentiated SH-SY5Y human neuroblastoma cells treated with vehicle, EphrinB1-Fc (1µg/ml; 15min), morphine (1µM; 15 min) or co-treated with both EphrinB1-Fc (1µg/ml; 15min) and morphine (1µM; 15 min). * = $p<0.05$ vs vehicle and ephrinB1-Fc; #=$p<0.05$ vs Morphine+EphrinB1; (values are the mean ± SEM; n=4).

7.4 Evaluation of any cross-talk between EphB1 and MOR in PMA-differentiated SH-SY5Y cells exposed to a pro-inflammatory stimulus

To evaluate transcriptional changes of EphB1 and MOR in PMA-differentiated SH-SY5Y after exposure to pro-inflammatori stimuli, by mimicking the neuroinflammatory response that contributes to different neuropathic pain states, a real-time PCR technique was developed.

To this aim, cells were exposed to PMA (16 nM) for 5 days or to vehicle; during the last 48 h of differentiation cells were exposed to TNFα (10 ng/ml; 48 h) or to the vehicle alone. Then, cells were collected and total cellular RNA was extracted; 2-µg samples were reverse-transcribed and real-time PCR was employed for the relative quantification of human EPHB1 and human MOPr transcripts. The protein content of EphB1 and MOR was also analyzed by western blot and saturation binding assay, respectively.

Exposure of PMA-differentiated SH-SY5Y cells to TNFα (10 ng/mL) for 48 hours induced a significant (P<0.001) increase in EphB1 mRNA levels in comparison to control cells (Figure 11A). In the same way, MOR mRNA levels were significantly (P<0.001) elevated in comparison to control cells (Figure 11 B).

The levels of the corresponding EphB1 proteins in total extracts were evaluated by western blotting (Figure 9 C; D) and followed a pattern similar to mRNA expression.

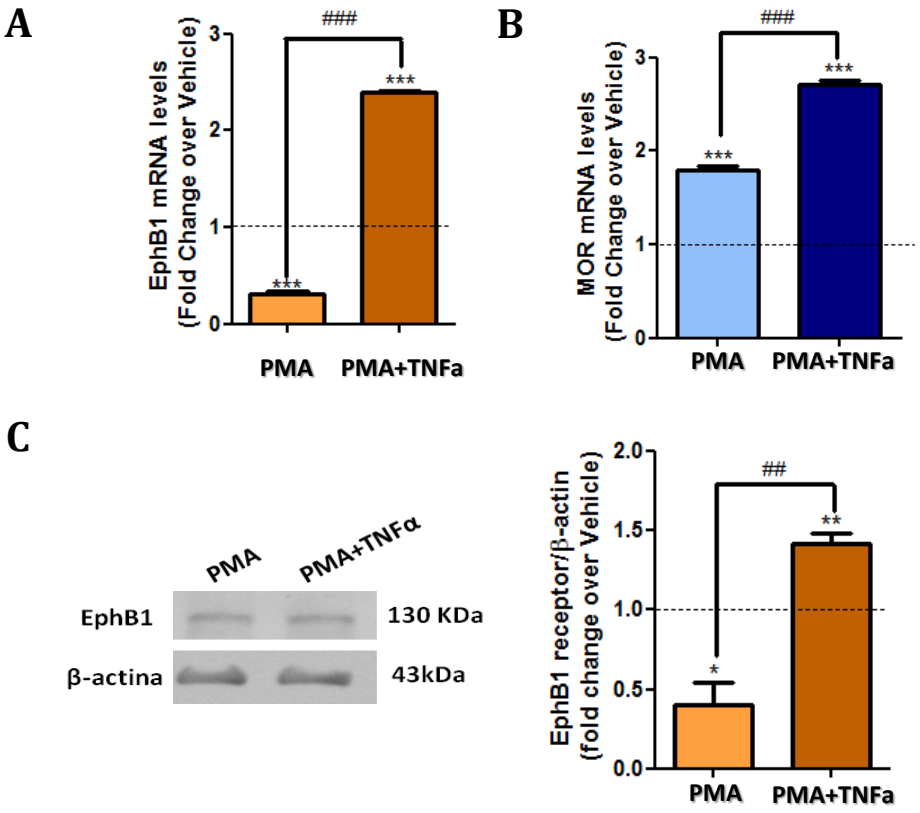


Figure 11: Real-time PCR of EphB1 receptor (A) and MOR (B) mRNA levels and western blot analysis of EphB1 receptor expression levels (C) in differentiated SH-SY5Y human neuroblastoma cells exposed to PMA (16 nM; 5 D) or PMA+TNF-a (10 ng/ml; 48 h). *** = p<0.001, ** = p<0.01, * = p< 0.05 vs undifferentiated cells treated with vehicle (dashed line); ## = p< 0.01, ### = p<0.001 vs PMA; values are the mean ± SEM of three independent experiments.

7.5. Targeting EphA4 for pharmacological intervention

7.5.1 APY-d3 derivatives can inhibit as well as activate the EphA4 receptor

To evaluate the potency of a multivalent derivative of the APY-d3 peptide, APY-d10, for inhibiton of ephrin-A5 Fc induced EphA4 tyrosine phosphorylation in comparison with the corresponding APY-d3 peptide [163], I performed a cell-based assay by using HEK293 cells transfected to stably express EphA4.
The cells were pre-incubated for 20 min with various concentrations of APY-d3 or APY-d10 and then treated with ephrin-A5 Fc at 0.5 µg/mL for an additional 15 min to activate EphA4 (Figure 1). Similar to the APY-d3 peptide, the derivative APY-d10 does not activate EphA4 tyrosine phosphorylation on its own and inhibits EphA4 phosphorylation induced by ephrin-A5 Fc stimulation. However, the APY-d10 peptide is a much more potent antagonist, as would be expected due to its much higher potency in ELISA assays measuring inhibition of ephrin-A5 binding to EphA4.

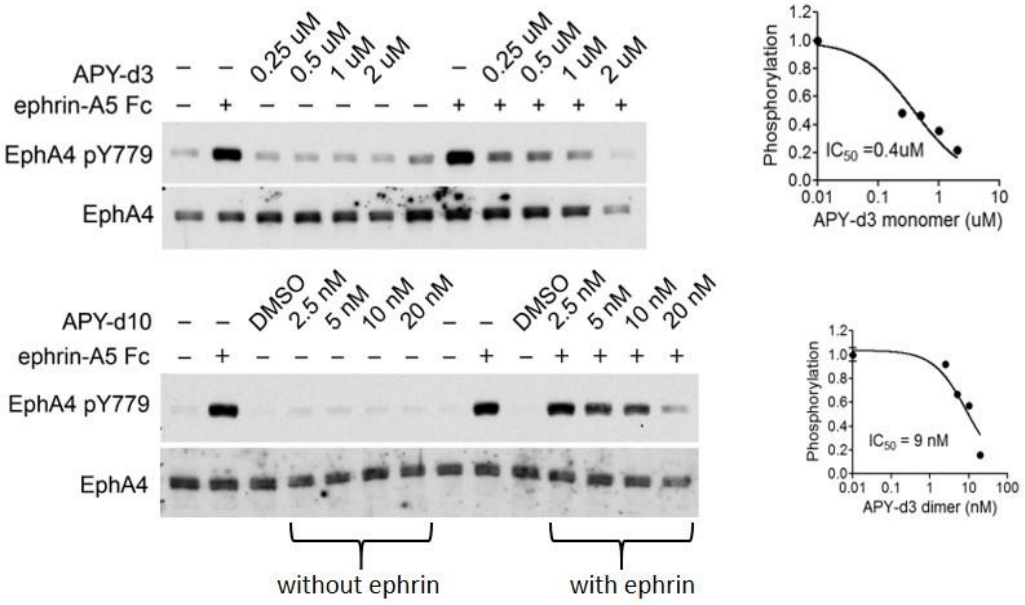


Figure 12. The APY-d10 peptide has much higher potency than the APY-d3 peptide, from which it is derived. Both peptides antagonize EphA4 tyrosine phosphorylation in a dose dependent manner, indicating inhibition of kinase activity after stimulation of HEK293 cells stably expressing EphA4 with the ephrin-A5 Fc ligand.

I also analysed the effect of another multivalent version of APY-d3, APY-d11, which also has increased potency in ELISA assays.
HEK293 cells stably expressing EphA4 were incubated for 20 min with various concentration of APY-d11 and then for 10 min with 0.5 µg/mL ephrin-A5 Fc to monitor the possible antagonistic activity of the peptide (right part of Figure 2). Alternatively, the cells were incubated for 30 min with only APY-d11 to monitor the possible agonistic activity of the peptide (left part of Figure 2). Ephrin-A5 Fc at 0.5 µg/mL for 10 min was used as a positive control for EphA4 activation.
Interestingly, these studies revealed that APY-d11 can efficiently activate EphA4 at low nanomolar concentrations (Figure 2) and thus might be used as a potent EphA4 agonist in cells that express multiple EphA receptors. In contrast, ephrin-A ligands promiscuously activate not only EphA4 but also other EphA receptors. Furthermore, APY-d3 peptide is more of 100-fold selective for EphA4, over other Eph receptors [163], and also the derivatives APY-d10 and APY-d11 have been show to be selective (as data not shown).

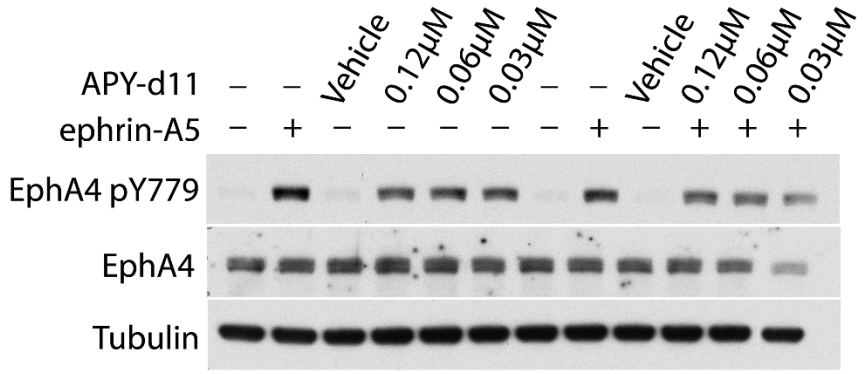


Figure 13. The APY-d11 peptide acts as an agonist of the EphA4 receptor. The peptide activates EphA4 tyrosine phosphorylation at low nanomolar concentrations, indicating that it stimulates EphA4 kinase activity (left portion of the blot). EphA4 phosphorylation induced by APY-d11 is similar to that induced by ephrin-A5 Fc, used as a positive control. In the right portion of the blot, EphA4 remains tyrosine phosphorylated in the presence of APY-d11 together with ephrin-A5 Fc, as expected if APY-d11 can inhibit ephrin binding but also activate EphA4. Blots for EphA4 and tubulin were used to verify the amount of protein in the lanes.

7.5.2 Characterization of Kinase Inhibitors targeting EphA4

Small molecule are well suited to target with high potency the ATP-binding pocket in the EphA4 kinase domain, but their selectivity is often low [164]. The EphA4 kinase domain has high sequence homology with non-receptor tyrosine kinases, such as SRC and ABL, and thus shares high binding affinity for some of the same kinase inhibitors, including Dasatinib. Interestingly, the crystal structure of the EphA4 kinase domain in complex with Dasatinib suggests that specific EphA4 inhibition should be achievable by exploiting an unusually large hydrofobic “pocket” in the ATP-binding site of EphA4 [165].
I set up a cell-based functional assay using the HEK293 cells stably expressing EphA4 to evaluate inhibition of EphA4 tyrosine phosphorylation by kinase inhibitors and used it to

compare the potency of Kinase Inhibitor 1, Kinase Inhibitor 2 and Dasatinib. The inhibitors were pre-incubated with the cells at different concentrations for 1 hour before addition of ephrin-A5 Fc for 15 min. The results revealed that Dasatinib is active at low nanomolar concentrations, as expected, while both Kinase Inhibitor 1 and Kinase Inhibitor 2 are active at low micromolar concentrations.

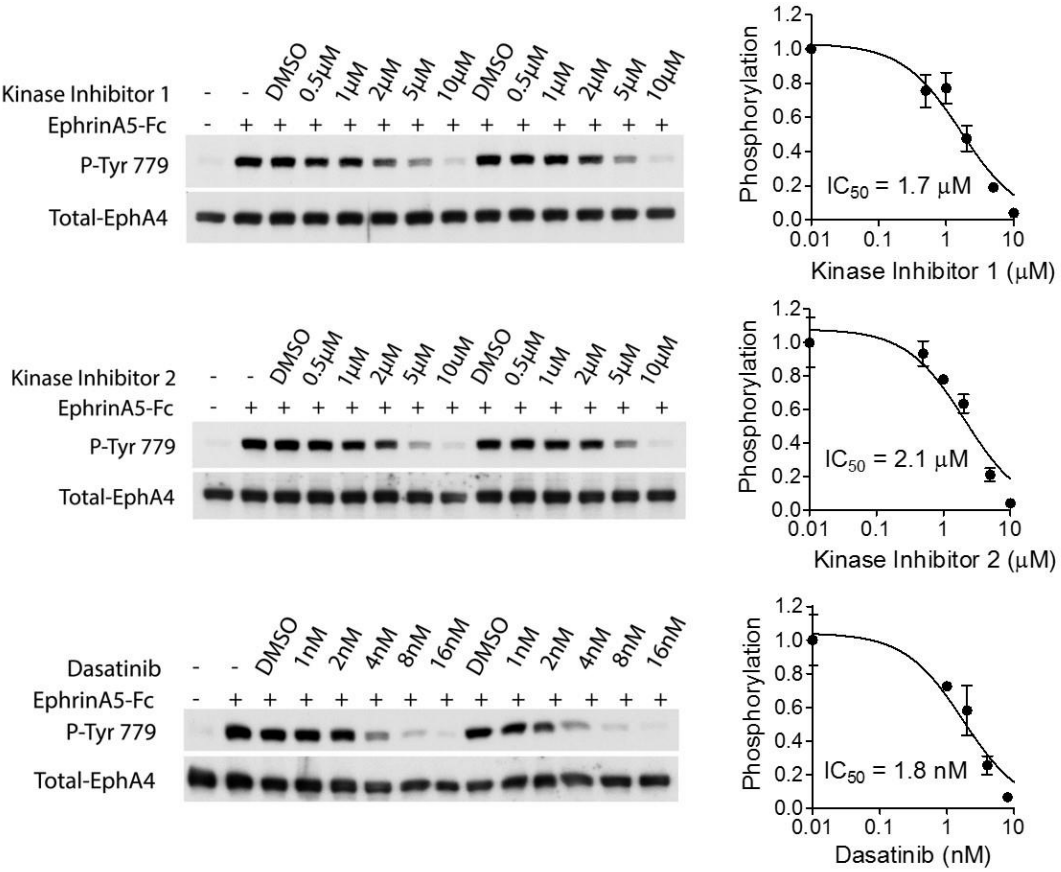


Fig. 14. Kinase Inhibitor 1 and Kinase Inhibitor 2 inhibit EphA4 tyrosine phosphorylation at micromolar concentrations while Dasatinib has nanomolar potency. Lysates from HEK293-EphA4 cells stimulated with ephrin-A5 Fc in the presence of different concentrations of the inhibitors were probed by immunoblotting using antibodies recognizing phoshorylated tyrosine 779 in the activation loop of EphA4 and EphA4 antibodies. The graphs show quantification of the bands and the calculated IC_{50} values for inhibition of EphA4 tyrosine phosphorylation.

8. DISCUSSION

Eph receptors and ephrins represent an important molecular system that enables reciprocal communication between different cells in the developing and adult nervous system and plays critical roles in axonal guidance, regulation of neuronal progenitor positioning, controlling synapse formation, and neuronal plasticity [145].
Disregulation of the Eph-ephrin signaling system is involved in pathological conditions related to pain and neurodegeneration.
Class B of Ephs and ephrins has been implicated in the induction and persistence of various types of pain, including chronic neuropathic, inflammatory and cancer pain, as well as in the physical dependence on opiates [48].
Despite these findings, the role played by EphB-ephrinB in neurophatic/cronic pain states remains unclear, due to different biological functions associated with individual Eph receptors and ephrin ligands, including any cross-talk with other cell surface proteins also contributing in these pathologies.
EphB1 receptor is expressed by nociceptive neurons in the spinal cord whereas ephrinBs are expressed by astrocyte and dorsal root ganglia (DRG), thus playing an important role in contact depend-neuron glia communication [146]. EphB1 receptor contributes to chronic pain states, partecipates to neuropathic pain in rat models [54] and to the development of opioid physical dependence [166]. Moreover, EphB-ephrin signaling contributes to chronic bone cancer pain by increasing glial release of pro-inflammatory cytokines in the spinal cord; the same cytokines able to hamper opioid mediated signaling; by contrast, spinal administration of an EphB1 receptor blocking reagent EphB2-Fc prevents and reverses bone cancer pain in animal models and rescues opioid analgesic effect [53].
However, the contribution of EphB1 signaling and its cross-communication with other receptor systems has been so far poorly investigated.
To this aim, I investigated any functional cross-talk between intracellular signaling pathways triggered by mu-opioid receptor (MOR) and EphB1 receptors in different cell models co-expressing both receptors.
I found that EphB1 agonist receptor (ephrinB1-Fc) or the MOR agonist morphine, administered as single agents to HEK-EphB1-MOR and to native SH-SY5Y cells, determine a time-dependent increase p42/44 phosphorylation (Figure 15 A) whereas their co-administration occludes p42/p44 MAPK activation (Figure 15 B).
Such cross-talk, as well as EphB1 and MOR expression, was modified in neuronal cells subjected to in vitro differentiation or to exposure to the pro-inflammatory agent TNF-α (Figure 15 C,D). Thus, suggesting a differential role played by the functional interaction between EphB1 and MOR depending on the physiological state of neuronal cells.

In conclusion, ephrinB1 interacting with EphB1 receptor is likely to prevent the contemporary activation of MOR-mediated intracellular signaling; experiments ongoing will deeply elucidate this ipothesis.
Further studies are needed to better charactherize the downstream proteins recruited by EphB1 and MOR activated by their respective ligands in differentiated human neuroblastoma SH-SY5Y cell line in presence or absence of pro-inflammatory stimuli.
To validate EphB1 as a new druggable targets in neuropatic pain condition could be useful to develop novel EphB1 antagonists to treat painful neuropathies.

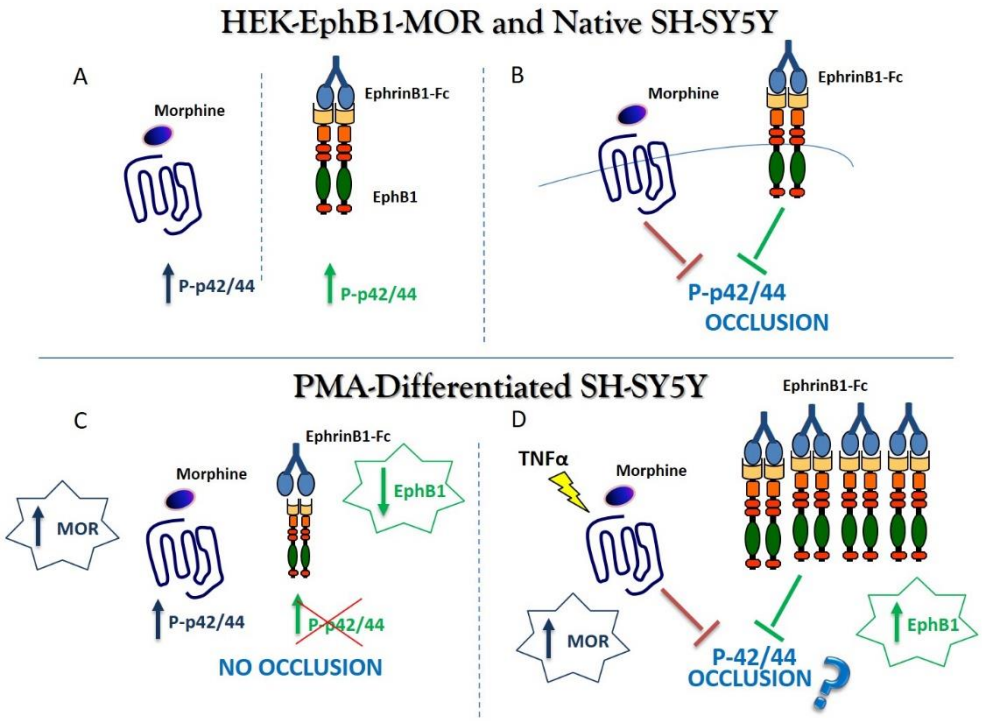


Figure 15: EphB1 may rapresent a novel target to develop effective therapeutics to treat painful neuropathies.
EphrinB1-Fc and morphine, administered as single agents to HEK-EphB1-MOR and to native SH-SY5Y cells, significantly increase p42/p44 MAPK phopshorylation (A); their co-administration occludes p42/p44 MAPK activation (B). In PMA-differentiated SH-SY5Y cells EphB1 is down-regulated and MOR up-regulated (C); under these conditions p42/p44 MAPK are activated by morphine both when administered alone and when co-administered with EphrinB1-Fc (C). In PMA-differentiated SH-SY5Y cells, a prolonged exposure to TNFα moderately up-regulates MOR and dramatically up-regulates EphB1 receptor expression (D), possibly restoring the occlusion of p42/p44 MAPK activation (D).

EphA4, a member of the Eph family of tyrosine kinase receptors, is predominantly expressed in the nervous system. In the adult, EphA4 is enriched in the hippocampus and cortex, two brain structures critical for learning and memory [129]. EphA4 signaling can be activated by all ephrin ligands, including the five GPI-linked ephrin-As and the three transmembrane ephrin-Bs. Through activation by the cell surface-ancored ephrin ligands, EphA4 plays important physiological roles in axon guidance during development, as well as in the structural remodeling of synapses in the adult brain [48].

However, increased EphA4 expression or disregulation of EphA4 kinase activity can hinder regeneration in the injured nervous systems as well as promote neurotoxicity and neurodegeneration. Indeed, EphA4 was identified as a gene promoting disease pathogenesis in amyotrophic lateral sclerosis (ASL), and studies in animal models and patients have shown that reduced EphA4 activity delays disease onset and slows disease progression [156]. Recent reports also suggest the possible involvement of EphA4 in the pathogenesis of other neurological disorders, such as Alzheimer's desease, where Aβ-oligomers can serve as ligands for EphA4 and aberrantly cause its excessive activation, leading to impaired sinaptic plasticity, loss of synaptic structure and neuronal death [60, 61]. Thus, EphA4 is a promising target for promoting neuronal repair after injury and to counteracting neurodegenerative processes.

The two main approaches that could be used to inhibit the detrimental effects of EphA4 activity in the nervous system involve the blockade of either its interaction with ephrin ligands or downstream kinase activity. Since the EphA4 ligand-binding pocket is quite broad and flexible, peptides are particular well suited to target it with high potency in order to block the deleterious effect of Aβ-oligomers. Indeed, an EphA4 antagonistic peptide (KYL, developed in the Prof. Pasquale lab) was used to block the binding of Aβ-oligomers to EphA4 in neuronal cultures, a rat ALS model and a mouse Alzheimer's model [60, 61, 156]. However, the KYL peptide has low potency.

I set up a cell-based assay suitable to test peptides and small molecules in order to develop in the future EphA4 inhibitors with increased potency and improved pharmacological properties. These studies have led to the characterization of a derivative of the APY-d3 peptide, APY-d10, as a much more potent antagonist in comparison with the corresponding APY-d3, with a relevant potency in the nanomolar range. Furthermore, I developed another APY-d3 derivative, APY-d11, which shows opposite effects, acting as EphA4 agonist. APY-d11 efficiently activates EphA4 at low nanomolar concentrations and thus might be used as specific, potent and selective EphA4 activator in cells that express multiple EphA receptors, overcoming the receptor binding promiscuity shown by ephrin ligands. Future studies will better characterize the agonist properties of APY-d11, to verify that the peptide activates the same downstream signaling pathways as the ephrin-As.

I also used our cell-based assay to test the potency of three kinase inhibitors against EphA4. One of the compounds analized is Dasatinib, a kinase inhibitor approved by the FDA for the treatment of chronic myelogenous leukemia (CMV). Dasatinib binds the EphA4 kinase domain [165] and is active at low nanomolar concentrations, as expected, while Kinase Inhibitor 1 and Kinase Inhibitor 2 are active at low micromolar concentration.

Small molecule antagonist targeting the Eph receptor ligand-binding domain so far displays a modest binding affinity in the micromolar range. On the other hand, small molecules that block Eph receptor forward signaling by targeting the ATP-binding pocket in the receptor kinase domain, have low selectivity.

Peptides that inhibit both Eph forward and ephrin reverse signals by disruption of Eph receptor-ephrin interaction, even if they exhibit a short half-life in cell culture or when

systemically administered *in vivo*, mainly due to proteolytic degradation and clearance by the kidney and reticuloendothelial system.
Efforts to characterize and optimize peptides and small molecules that target specific Eph receptors and ephrins, could provide useful novel leads with improved drug-like profiles for innovative pharmacological approaches to treat neurological diseases.

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