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# "TARGETING EphB1 RECEPTOR IN CELLULAR MODELS: ANALYSIS OF SIGNALING PATHWAYS AND CROSS-TALK WITH OPIOID RECEPTORS"


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# Abstract

The activation of EphB1 receptors by their membrane bound ligands ephrins in nociceptive neurons have been implicated in the onset and maintenance of different types of pain and in side effects produced as consequence of prolonged Mor activation. The use of EphB1 blocking reagents rescue opioid-induced analgesia suggesting that antagonists targeting EphB1 receptor could represent a novel class of analgesics.

A potential inverse relation between EphB1 and Mor receptors was displayed in a previous investigation by Lombardo Sara where showed that the co-administration of Mor and EphB1 agonists resulted in an occlusion of morphine-mediated p42/44 MAPK phosphorylation.

Therefore, the aim of the research was elucidated the signaling pathway and mechanism responsible for the functional crosstalk triggered by the co-administration of both ligands and at the same time developed and tested different EphB1 antagonist peptidomimetics.

As results: *ephrinB1/EphB1 activation through PI3K signaling pathways blunted p42/44 activation morphine-mediated and PKC-dependent in SHSY5Y native cells where in addition EphB1 signaling activation seems to be the responsible for blocking the capacity of the morphine to modulate adenylyl cyclase activity. *SHSY5Y cells induced to PMA differentiation produced up regulation of Mor expression and down regulation of EphB1 receptors allowing that morphine significantly activate p42/p44 MAPK even when ephrinB1 was co-administered. *Conversely, in PMA-differentiated SHSY5Y cells exposed to TNF-α, EphB1 expression was significantly up regulated and the morphine failed to increase p42/44 MAPK phosphorylation. * Moreover, were found two novel antagonist peptides capable to counteract ephrinB1-Fc-mediated activation of p42/p44 MAPK phosphorylation.

Concluding that when EphB1 is activated morphine no longer triggers PKC-dependent signaling events downstream of Mor activation, explaining the reasons why opioid analgesics are less effective in the treatment of different pain states. At this regards, novel EphB1 receptor antagonists may represent a novel strategy to reduce the negative impact of ephrin system.

# 1. Introduccion

## 1.1 Eph receptors and ephrin ligands

### 1.1.1 Functions and classification

Eph (erythropoyetin—producing hepatocellular carcinoma) receptors, the largest subfamily of receptor tyrosine kinases (RTKs), and their membrane bound ephrin ligands are cell surface molecules and important mediators of cell–cell communication, that play a fundamental role in cell behavior during embryogenesis and adult life [1–7]. Several studies have demonstrated that are involved in important functions as: cell shape, adhesion and repulsion, cell positioning and migration; tissue boundary formation during somatogenesis; development of the vascular system; tumor invasion and metastasis; immune function; hematopoiesis; blood clotting; stem cells development; tissue repair and maintenance; angiogenesis, control of tissue morphogenesis and patterning; cell sorting at compartmental boundaries and bone formation. [1,2,8,9] In nervous system are regulators of axon guidance during development; neural and synaptic plasticity; long term potentiation, synaptogenesis, dendritic filopodia motility, and neural crest cell [1,2,5,10,11]

Eph receptors, based on the structure and the ability to bind their membrane attached ephrin ligands, are divided into EphA and EphB receptors. In mammals, there are nine members of EphA class (A1-A8, A10) and five members of EphB class (B1-B4, B6) while Ephrins-As (A1-A5) are bound by a glycophosphatidylinositol (GPI) anchor, and ephrin-Bs (B1-B3) contain a short intracellular signaling domain [5,6,10,11]. Furthermore, Ephs and ephrins interact promiscuously within each subclass. Thus cross-class interactions known are EphA4, interacting with B-type ephrins and ephrin-A5 can activate EphB2 as well as EphAs [12].

### 1.1.2 Structure

The extracellular region of all Eph receptors consists in highly conserved N- terminal globular domain called ligand binding-domain which is necessary for ligand recognition and binding site that mediates receptor-ephrin interaction between cells [11,13,14].

The globular domain is followed by a unique cysteine-rich motif (Cys domain, comprising sushi and epidermal growth factor (EGF)-like motifs) and two fibronectin type III motifs, which appear to be involved in receptor–receptor dimerization interactions [13] and facilitate clustering of multiple Eph-ephrin complexes [11,15].

In the juxtamembrane region, there is a highly conserved motif containing two tyrosine residues, which are the major autophosphorylation sites involved in receptor signaling [13,15].

The tyrosine kinase domain has been proposed to serve as a binding site for activators of the small GTPases, which regulate cytoskeletal organization. A conserved region of 60–70 amino acids in the carboxyl-terminal tail of the Eph receptors forms a sterile alpha motif (SAM) domain, which has been implicated in receptor dimerization and mediates cell–cell initiated signal transduction [13,15].

Eph receptors also possess a post- synaptic density protein zona occludens (PDZ)-binding domain. This domain is important in the assembly of Eph or ephrin complexes, that allow interactions with other regulatory molecules (Figure 1), and in their localization to specific sites within the cell, such as membrane raft microdomains and synapses [13,15].

The EphrinB display an extracellular Eph receptor binding domain, a single-pass transmembrane region, a short intracellular domain with several sites for tyrosine and serine phosphorylation, and a C-terminal PDZ-binding motif [16] which is phosphorylated by Src family kinases upon ephrin stimulation by Eph receptors (Figure 1).

Ephrin-Bs domains include three tyrosine residues enabling the recruitment of Src Homology 2/3 (SH2/SH3) adaptor proteins, a PDZ binding domain enabling interaction with adaptor-proteins such as glutamate receptor interacting protein 1 (GRIP1), a D-domain for interaction with Erk/MAPK, and a Grb4 domain enabling interactions with the G protein-coupled receptor kinase-interacting protein 1 (GIT1) [17].

Ephrin-As, in contrast, lack a transmembrane domain and are attached to the cell membrane through a GPI-anchor. Therefore, ephrin-As rely on other receptors and mechanisms to transduce intracellular signals, for instance, p75 neurotrophin receptor (p75NTR) and the TrkB neurotrophin receptor tyrosine kinase have been involved in reverse signaling ephrin-A-mediated which is involved in axon guidance [16,18].

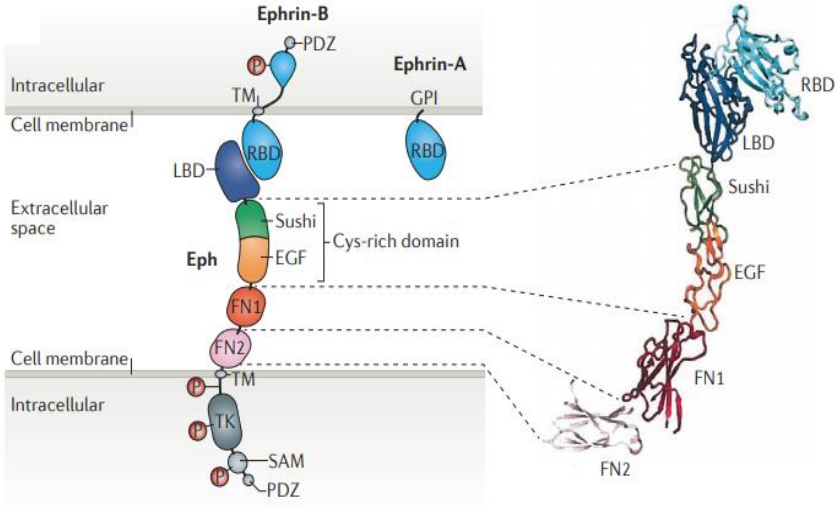


**Figure 1.** Structure of Eph receptors and ephrin ligands. Eph and ephrin signaling relies on specific functional domains; on the extracellular side, Eph receptor is composed by ligand binding domain (LBD) which binds to the receptor-binding domain (RBD) of ephrins, followed by a Cys-rich domain encompassing the sushi and epidermal grown factor (EGF)-like domains, and two fibronectin (FN) domains. The intracellular side of Eph receptor is composed of the transmembrane region (TM), the Tyr kinase domain (TK), the sterile alpha motif (SAM) and the PDZ domain. EphrinsA class is linked to the membrane via a glycosylphosphatidylinositol (GPI) linkage, whereas the B class has a transmembrane domain and an intracellular PDZ domain. Eph–ephrin interaction results in phosphorylation of Tyr residues (P) present in the juxtamembrane domain between the TK and TM domains, as well as in the TK and SAM domains. The juxtamembrane phosphorylation is required for TK function, which is crucial for many Eph signalling-induced biological responses [15].

## 1.1.3 EphB and ephrinB system

### 1.1.3.1 Formation and activation

In contrast, to other tyrosine kinases receptors where receptor dimerization is enough to trigger biological activity, Eph receptors need high local density of their ligands and the formation of clusters as pre-requisite to form Eph/ephrin signaling assemblies, which in turn induce a precise downstream signaling pathways and biological response [3,12,19].

The formation and activation of Eph/ephrin system is considered a multiple-step process where Eph and ephrin domains interact with each other. Prior to activation, the Eph receptors are loosely distributed on the cell surface and display minimal kinase activity, unless receptor expression levels are considerably elevated [3].

Upon cell–cell contact, ligands and receptors bind each other with 1:1 stoichiometry and nanomolar affinity [20]. Eph receptors use two separate interfaces for the assembly of signaling-competent clusters. The first step, in the initiation of Eph-mediated signaling, is the recognition and binding of Eph receptors and ligands located on closely opposed cell surfaces. This process, knowing as DIMERISATION INTERFACE; produce high-affinity dimers that are the predominant form of the complex. [2,20,21] However, is possible that residues of Eph receptor could produce lower-affinity binding, principally inEphrinB2, wich could lead to the association of two homodimers [16].

The second interface, called as CLUSTERING INTERFACE, is produced within the cys-rich domain of the Eph molecules and is responsible for the formation of the higher-order functional tetrameric 2:2 complexes, in which each receptor interacts with two ligands and each ligand with two receptors (Figure 2). For instante, Crystallographic studies of the EphB2– ephrinB2 complex reveal a tetrameric complex forming a ring-like structure. [2,12,20,21]. The existence of these two separate and independent interacting interfaces allows the Eph receptors to utilize the so-called 'seeding' mechanism for the assembly of clusters [3].

Finally, binding of ephrins to the extracellular part of Eph receptors not only activates their cytoplasmic tyrosine kinase domain, but also leads to the transduction of a reverse signal into the ephrin-bearing cell [3]

Importantly, the disruption of ephrin–ephrin homodimers and the Eph–ephrin tetramer formation also results in a repositioning of the B-ephrin transmembrane and cytoplasmic domains, converting them from an inactive to an active configuration [20]

Furthermore, is important point out that ligand-independent signaling can also occur if the receptor concentration is not enough, for example, on the surface of tumor cells.

Furthermore, Eph assemblies can be independent of ephrin binding for example, EphA4-ECD (ectodomain receptor) can bind FNIII-domain of a neighboring receptor molecule without the presence of ephrins [3]

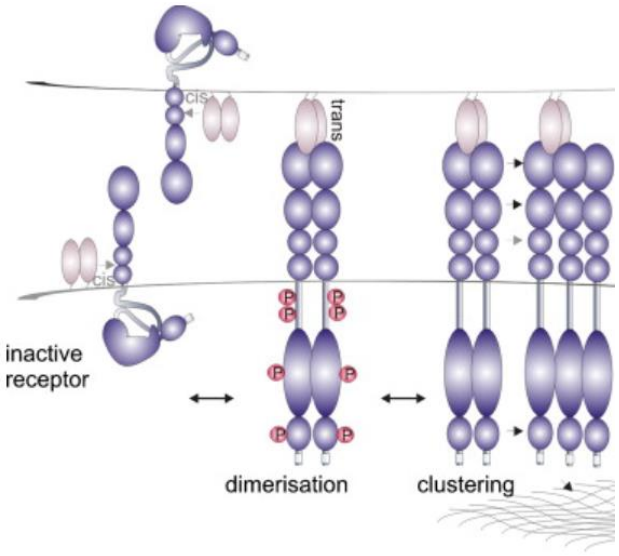


**Figure 2**. Eph receptor clustering and activation. The inactive Eph receptor present auto-inhibited conformation, released upon phosphorylation and activation. Ephrin binding to one receptor initiates dimerisation into an Eph/ephrin heterotetramer. Further clustering is facilitated by receptor–receptor interactions between multiple domains of adjacent Ephs, including co-clustering of A and B type Ephs [12].

### 1.1.3.2 Bidirectional signaling

The binding of ephrin molecules to Eph receptors induces conformational changes in both proteins [13], producing modifications of actin cytoskeleton and microtubular organization[22].

A distinguish feature of Eph-ephrin complexes is their ability to generate bidirectional signals that affect both the receptor-expressing and ephrin-expressing cells (Figure 3) [11]. Eph receptor "forward" signaling depends principally on the tyrosine kinase domain, which mediates autophosphorylation of the receptor as well as phosphorylation of other proteins and the associations of the receptor with various effector proteins [23].

Ephrin-B "reverse" signaling depends on tyrosine phosphorylation of the ephrin cytoplasmic region (mediated by Src family kinases and some receptor tyrosine kinases) and on associated proteins. For instance at the level of the nervous system, Eph/ephrin bidirectional signaling is important not only for the communication between neurons but also between neurons and glial cells [24]

Finally, Eph proteins and ephrins can simultaneously act as receptors and ligands, leading to bidirectional or parallel and antiparallel signaling (Figure 3), depending on the distribution of Ephs and ephrins between interacting cells, as well as the direction of signaling in single ephrin–Eph pairs. Ephrins can also induce signaling cascades independently of Eph proteins, a mode of signaling that seems to be evolutionarily conserved [15].

Futhermore, the interaction produced between Eph receptors and ephrin ligands in Eph receptors and ephrins co-expressed cells, is called CIS interaction in which ephrins can attenuate signaling of Eph receptors (Figure 2), for instance in nervous system Cis interaction allow the cell to precisely control the axonal trajectory [25,26]; whereas TRANS interaction (Figure 2) is produced between neigboring cells, facilitate forward signaling and, depending on the identity of Eph receptors and ephrins involved, serve as either repulsive or attractant cues for axonal guidance [25].

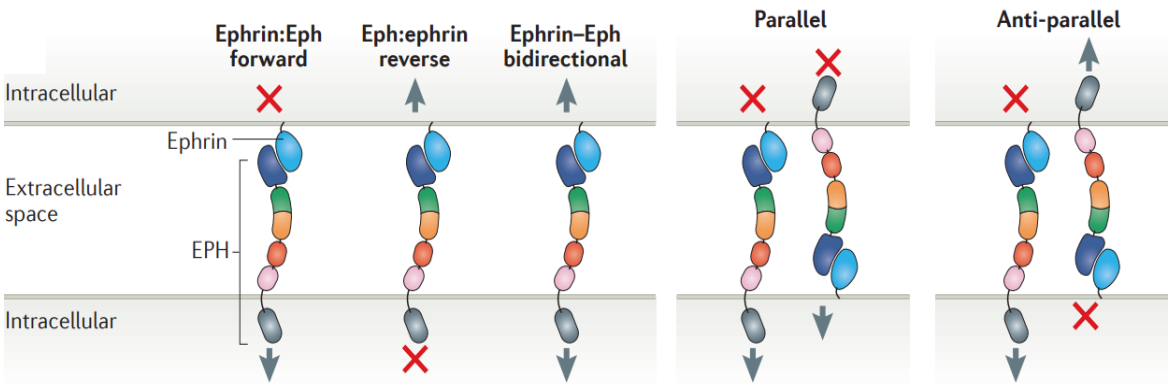


**Figure 3**. Eph–ephrin signaling**.** Forward signaling involves signal transduction from ephrins to Ephs; reverse signalling involves signalling from Ephs to ephrins; and bidirectional signaling involves the simultaneous activation of pathways downstream of ephrins and Ephs. Parallel signaling occurs when ephrins and Ephs are expressed on the same cell. Anti-parallel signalling is a special case of simultaneously occurring forward signalling, whereby ephrin–Eph signals are propagated in both directions [15].

#### 1.1.3.2.1 Forward signaling

The classic mode of forward signaling is from ephrins to Ephs and frequently results in cell retraction. This repulsive response is particularly important for axon guidance and sorting of Eph-expressing cells during development [15,27].

The signaling is initiated by autophosphorylation and Src family kinases-mediated phosphorylation of the intracellular tyrosine residues, resulting in the activation of the tyrosine kinase catalytic domain [16]. However the interactions also can be kinase independent, for example, EphB receptor in kinase independent mechanism plays essential roles in retina axon path finding [28]. Furthermore, Ephs can be negatively regulated by several protein tyrosine phosphatases and by ubiquitin ligase-mediated internalization and degradation [6].

Once the Eph receptors are phosphorylated, scaffold and adaptor proteins are activated. The adaptor proteins, containing Src-homology 2 (SH2) and Src-homology (SH3) domains, can bind and initiate phosphorylation of downstream substrates and allow protein–protein interactions, via SAM and PDZ-binding motifs, for contribute to signaling [16,29].

Forward signaling is involved in the activation of several cytoplasmic downstream signaling pathways such as Src family kinases, mitogen- activated protein kinase, p-21 activated kinase, chemokine pathways, heterotrimeric G-protein pathways, and integrin-mediated pathways (Figure 4) [1].

For each pathway to be activated, the recruitment of adaptor and scaffold proteins are necessary. Adaptor proteins involved in forward signaling are: Rho GTPase activating proteins (GAPs) (including RhoA, Cdc42, and Rac) which are involved in the regulation of the actin cytoskeleton and cell shape, movement, and adhesion [16], guanine nucleotide exchange factors (GEFs) (including ephexins, Vav2, Vav3 protein) [15]; ADAM10 family proteases [26]; non-catalytic regions of Tyr kinase adaptor protein 1 (Nck1) and Nck2 [29] and phosphoinositide 3-kinase (PI3K) (FIGURE 4) [15] which can be suppressed by forward signaling, blocking the activation of Akt - mTORC1 pathway [23].

GAPs and GEFs proteins are involved in Eph/ephrin mediated axon guidance events [26], among them α2-chimaerin, which is essential for axon guidance-dependent of EphA4 [11], and ephexin induce cytoskeletal collapse in growth cone regions exposed to ephrins. Principally, ephexin is essential for both normal axon outgrowth and ephrin-dependent axon repulsion in LMC neuron, modulating motor axon pathfinding at different stages [26].

Furthermore, GTPases protein specifically H-Ras family protein activates a MAP kinase cascade culminating in the phosphorylation and activation of the Erk1/Erk2 MAP kinases (Figure 4). This pathway Ras-MAP kinase is important in cell migration neurite outgrowth and axon guidance [30], for instance, in cultured mouse mesenchymal cells, ephrin-B1-EphB signaling activates Erk to promote proliferation and regulate immediate early gene transcription as well as increase cell migration in P19 embryonal carcinoma cells and microvascular endothelial cells by the recruitment of the adaptors Shc and Grb2 to activate H-Ras in ephrinB- EphB1signaling [23].

Vav family GEFs and ADAM10 family proteases are other kind of adaptor protein which have been proposed to mediate later events in the ephrin-Eph signaling cascade, enhancing endocytosis and proteolysis, which are essential for efficient cell detachment and a complete guidance response and receptor activation [7,26]

EphB1, EphB2, EphA2, EphA3 and EphA4 receptors are associated with Nck family protein which has been implicated in the organization of actin cytoskeleton, cell movement, and axon guidance due to SH2 and SH3 domain. For example, SH2 domain of Nck can bind with the first tyrosine residue localized in the juxtamembrane region of EphB1, while its SH3 domains bind proline-rich motifs on downstream target proteins, one of this is paxillin protein [29].

Cask-interactive proteins (Caskins) is a scaffold protein expressed in neurons and involved in the regulation of synaptic function. EphB1 receptor can form a complex with Caskin1 through the adaptor protein Nck participating in the regulation of actin cytoskeleton [29].

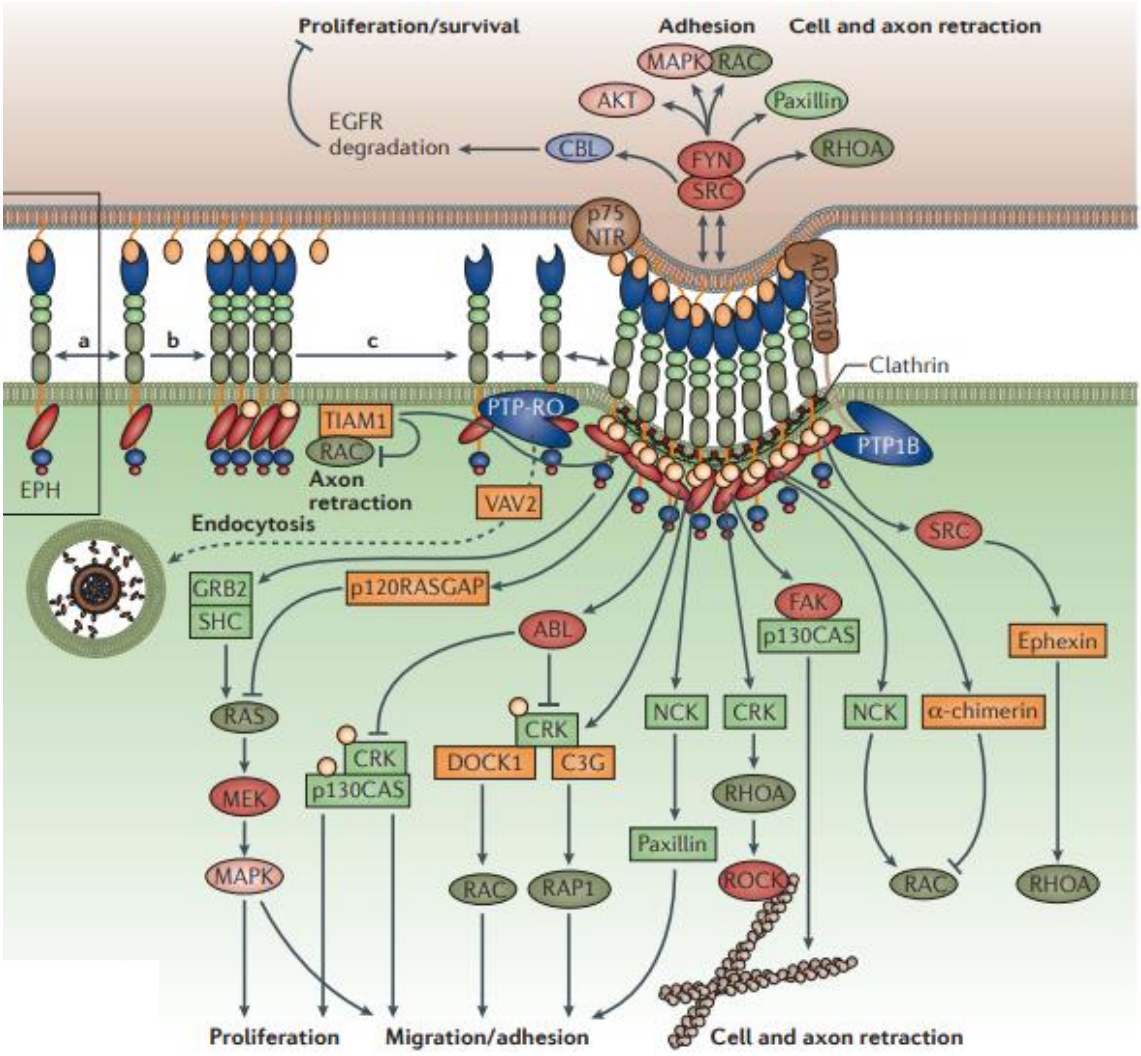


**Figure 4**. Downstream molecules activated in forward signaling (ephrin-Eph) and reverse signaling (Eph-ephrin)[31].

#### 1.1.3.2.2 Reverse signaling

Ephrin reverse signaling, which generally promotes adhesion [27], is also activated following interaction with Eph receptors (Figure 4).

The ephrin-A proteins are localized in lipid rafts, a specific microdomain of the plasma membrane [28], due to the lack an enzymatic domain [23]. The lipid raft provides a platform which allows ephrin-A to be constitutively associated and transduce reverse signals by interacting with other similarly localized molecules [28], for example Ephrin A signaling can be initiated when the glycosyl phosphatidylinositol–linked is associated with transmembrane partners, such as the p75NTR neurotrophin receptor, TrkB and

Ret receptor tyrosine kinases [32] as well as the Src-family kinase Fyn and Rho family small GTPases that lead to tyrosine phosphorylation of raft membrane proteins, which eventually brings about cytoskeletal rearrangement [28].

Futhermore, Ephrin-As to interact with the EphA or in some cases with EphB2 receptors, can interact in cis mode with other co-expressed membrane-associated molecules. This action interactions may help in the transduction of reverse signals.

Integrins are the principal molecules which interact with ephrin-A in cis mode. Integrins are transmembrane molecules with many subtypes that mediate adhesion through interactions with the extracellular matrix. Some studies have seen that β1-integrin can interact with ephrin-A5 in the lipid raft and help mediate reverse signaling driven cell adhesion upon engagement with EphA receptor. This interaction is sufficient to sustain neurite outgrowth in retinal ganglion neurons [28].

Ephrin-Bs structurally have an extracellular domain, a transmembrane domain and a cytoplasmic domain which enables these molecules to interact not only with membrane exposed proteins but also with intracellular proteins, which allow them to transduce distinct reverse signals [28]. In addition ephrinBs can transduce reverse signaling in a tyrosine phosphorylation-dependent or -independent [33]manner, as well as PDZ-dependent manner [23].

Reserve signaling have been studied in many biological processes including cell adhesion, migration, and gene expressions [7]. For example, the phosphorylation of EphrinB ligands provides a docking site for the SH2 domain-containing adaptor protein Grb4 [6,31], this complex is used as signal transducer and activator of transcription STAT3, and subsequent activation of Jak-2-dependent phosphorylation that lead the migration to the nucleus, where regulates a variety of target genes [16]. Furthermore, EphrinB–Grb4 complex results in the activation of focal adhesion kinase (FAK) catalytic activity and recruitment of the G-protein-coupled receptor kinase interacting protein (GIT) which is involved in cytoskeleton regulation [16]. Another important complex formed in reverse EphrinB signaling is Grb4–Pak1–Dock180 wich is implicated in control axon pathfinding [15].

Moreover EphrinB1 activation affects cell adhesion and migration *in vitro* and *in vivo* by the interaction with CNK1 scafoold protein. CNK1 - ephrinB1 complex can promote cell migration through RhoA and JNK activity. This finding has demonstrated that the overexpression of EphrinB1 increases cell motility in cancer cell lines, however, CNK1 depletion by siRNA, abrogates ephrinB1-mediated cell migration and JNK activation. [33]

Finally, Ephrin-Bs can also interact in cis mode with numerous membranes expressed proteins such as fibroblast growth factor (FGF) receptor, adhesion molecules integrins, claudins, and connexin playing a principal role in cell migration and cell-cell adhesion. For instance, FGF modulates ephrinB1 signaling to regulate the positioning of retinal progenitor cells within the definitive eye field [28].

### 1.1.4 Internalization and proteolytic cleavage

Eph receptor–ephrin binding can also lead to endocytosis, proteolytic cleavage, or both, generating intracellular Eph/ephrin fragments with distinctive signaling abilities and often leading to proteosomal or lysosomal degradation and signal termination [32]**.** For the attenuation, termination and remotion of Eph/ephrin, the complex formed from the cell surface can be subjected to endocytosis of vesicles containing plasma membrane fragments derived from both cells proteolytic cleavage, by disintegrin, metalloproteinase and γ -secretase [1,7,23], and tyrosine phosphatase activity [1].

In the endocytosis, Eph/ephrin complexes can be internalized into either the Eph receptor- or the ephrin-expressing cells through the formation of vesicles containing plasma membrane fragments derived from both cells [23]. The internalization of the receptor-ligand complexes immediately occurred during cell retraction. An implication of this unusual mechanism is that the two cells exchange Eph receptors or ephrins and possibly their associated proteins, which may continue to signal from intracellular compartments [11]. For instance, the interactions between EphB1-ephrinB1 induce formation of intracellular vesicles that contain the full-length proteins in a complex; the subsequent endocytosis is mediated by a clathrin-dependent pathway [7,16]. The direction of endocytosis depends on the cell type, for example, glial cells are effective at engulfing EphB2, but not ephrin-Bs from neurons [15].

Eph–ephrin complexes produced by trans-endocytosis can convert adhesive interactions into cell repulsion by activating metalloproteases, such as ADAM (A Disintegrin And Metalloproteases) family members. EphB receptors also interact with ADAM10 and E-cadherin. these bindings to ephrin-Bs in trans interaction mode provokes shedding of E-cadherin by ADAM10 preferentially in the ephrin-B-expressing cells [23]. Other proteins activated in trans-endocytosis are Rac-1 and ubiquitin ligase Cbl proteins; the firt one can removal adhesive complexes from cell–cell contact sites allowing cell separation and repulsive effects whereas the second one can interact with several Eph receptors promoting their ubiquitination [23].

The major proteins involved proteolityc cleavage of ephrin-Eph signaling are ADAMS; these proteins produce cell-cell repulsion that is important for neuronal axon guidance and also for establishment of the arterial and venous vascular networks [34]. ADAM10 specifically cleaves the A-class ephrins allowing termination of EphA signaling [3]. When ADAM10 cleave ephrin from the opposing cell, only receptor-bound ligand is cleaved, breaking the molecular tethers between the opposing cell surfaces, allowing internalization of the EphA3/ ephrin-A5 complexes into the Eph-expressing cell. ADAM13 is also reported to cleave ephrin-B1 and B2 ligands, and other substrates, including fibronectin (FN) and Cadherin-11 [34]

EphBs and ephrin-Bs have also been reported to undergo cleavage by MMPs (Matrix-metalloproteases), regulating other MMP functions such as degradation of extracellular matrix facilitating cell migration and invasion. In gastric carcinoma cells, silencing of EphA2 expression inhibits cell proliferation, invasion and expression of MMP 9 in vitro and in vivo [34].

On the other hands, Ephrin-B ligands can also undergo metalloprotease/γ-secretase processing following binding to EphB receptors [23]. Moreover, EphB2 receptor can also be processed by γ-secretase, via both ligand dependent and independent pathways. During ligand induced signaling, while the ephrin is sequentially processed by metalloprotease and γ-secretase, the receptor ectodomain cleavage occurs in endosomes, leads to receptor degradation, and is metalloprotease-independent [34]

Finally, PTPs (Protein tyrosine phosphatases) can also regulate Eph/ephrin signaling and might be important for controlling the formation or dissolution of the receptor/ligand clusters [3]

## 1.2 EphB/EphrinB system in SNC

Eph receptor and its membrane anchored ligand ephrin are considered key players in earlier and mature nervous system [1,4,8], both EphA and B receptors as well as ephrins A and B are expressed in the pre-synaptic and post-synaptic neurons as well as in adult olfactory bulb, hippocampus, cerebellum [35] and cortex [24]. Further their interactions lead to the bidirectional signaling cascades triggering several signaling pathways [10,36] allowing not only communication between neurons but also between neurons and glial cells [24,35] specifically with astrocytes [18].

In the earlier nervous system Eph and ephrin are higher expressed, while in mature adult system are expressed at lower levels, but can be up-regulated after neural injury on different cell types as astrocytes, neurons and oligodendrocytes [13].

During the last decade, several studies have demonstrated that the activation of Eph receptors in a kinase-independent or dependent manner are involved in different process at nervous system level, for instance, in axon guidance [13], early excitatory synaptogenesis [8] and then later coordinate synaptic function principally synapsis plasticity [36] during learning, memory and in response to injuries [31].

Since several studies have demonstrated that EphB receptors and ephrinB ligands possess a relevant role in earlier and mature nervous system, this chapter will be focused in the involvement of EphB/ephrinB system in SNC.

### 1.2.1 EphB/EphrinB system in early SNC

The capacity of Ephs and ephrins to act in communications processes of over short distances, such as cell–cell contact, [3,27] and in adhesive and repulsive responses between interacting cells [6] have suggested that this system is involved in nervous system development.

Both forward and reverse signaling of EphB receptors and ephrinB ligands have been involved in the establishment of neuronal connectivity guiding axons to the appropriate targets (axon pathfinding), neuronal cell migration [3,27], regulation of synaptic connections [11,27], neurogenesis [15], proliferation and

migration of neural progenitors [15], topographic maps, spine morphogenesis [17], and regulation of neural tube closure [15]

Furthermore, in the developing of nervous system, repulsive interactions between Eph receptors and their ligands are required in diverse areas, including anterior commissure formation, spinal cord, motor neuron and neural crest cell migration [13], allowing the interactions between pre-synaptic terminals and postsynaptic sites [6].

In synaptogenesis [18,28] and dendritic spine morphogenesis [17,24,35], the three class of EphB receptors are essential in the formation of up to 40% of excitatory synapses in the developing hippocampus and cortex [35,37]. Principally, EphB2 has been considered as indispensable in the maturation of the pre- and postsynaptic sides of excitatory synapses, in fact, EphB2 activation is sufficient to promote the assembly of presynaptic structures even in non-neuronal cells [11]. Moreover, Lai and collaborators observed that in triple knockout mice (TKO) (lacking EphB1/B2/B3) fewer synapses were formed producing a failiture in the formation of dendritic spines in the hippocampus [10], suggesting that ephrin-B/EphB signaling promotes spine formation and maturation [13]

EphB2 receptor in kinase activity manner can interact, clusterize and endocytositade others receptors which in turn are important in excitatory synapsis, such as NMDA and AMPA neurotransmitter receptors[11]. Likewise, ephrin B by PDZ-binding domain can interact with post-synaptic PDZ domain-containing proteins [10] allowing bind syntenin-1 in synaptogenesis [17] and growth-factor-receptor-bound protein 4 (Grb4) in cytoskeletal remodeling. [7]

EphB receptors and ephrinB ligands are expressed at synaptic terminals of hippocampal, cortical neurons, and presynaptic astrocyte [18]. During synapse formation in cortical and hippocampal neurons, forward signaling of presynaptic ephrinB2 trigger activation of postsynaptic EphB2 receptors which directly interacts with NMDA receptor, activating $Ca^{2+}$ influx and gene expression promoting dendritic spine morphogenesis [10,11,15,18]

Forward signaling ephrinB1/EphB also triggers the activation of Rho family GTPases among them Cdc 42, Rac1, Kalirin (Kalirin-5 and Kalirin-7), intersectin and Tiam [11]. Kalirin and Tiam promote the exchange of

GDP for GTP and thereby activate Rac1 activity which promotes actin cytoskeleton rearrangement (FIGURE 5) [10]. The activation of Rac1 also acts on PAK (p21 activated kinase) promoting spine formation, maturation and synapse formation (Figure 5) [10,24,38] ,as well as, the ubiquitination and degradation of Rho-GEF Ephexin5 promotes synapse formation, however, the reduction of RhoA activity do not show effects on spine morphology [23] indicating a negative regulation in excitatory synapse [7].

Furthermore, EphB forward signaling can phosphorylate focal adhesion kinase FAK [24] and heparin sulphate proteoglycan syndecan-2 [38], which interacts with the synaptic PDZ domain protein caskin and promotes spine maturation [24,25]. Specifically, EphB1 receptor can form a complex with Caskin1, through the adaptor protein Nck by its tyrosine 594, to bind SH2 domain, while its SH3 domains may interact with paxillin and Nck-interacting kinase (NIK) participating in the regulation of actin cytoskeleton [29]. In addition, EphB1 receptor has been considered as axonal adhesion molecule due to in forward and reverse signaling can regulate cell adhesion by controlling integrin β1 activation in oligodendrocytes (Figure 5) which have a positive effect on the ability of the oligodendrocytes to extend myelin sheets [39].

Reverse signaling has also been involved on axon pruning, synapse formation and dendritic spine morphogenesis in the developing mouse hippocampus through the phosphorylation of adaptor protein Grb4 [23,40] as well as in morphological and functional maturation of developing retinotectal synapses in the Xenopus optic tectum due to the activation of ephrin-B by postsynaptic EphB2 receptor [11].

Presynaptic ephrin B1 and ephrinB2 with postsynaptic EphB2 are also necessary in synaptogenesis, in fact reverse signaling allow the formation of normal synapsis due to the interaction with syntenin-1 [17] whereas, Ephrin-B3 interact with GRIP1, PICK1 and syntenin-1 proteins regulating the synapse and spine formation in the hippocampus and cortex (Figure 5) [17].

Other important process, in earlier nervous system, in which ephrinB/EphB system has been involved is axon guidance. EphrinB and EphB have the capacity of guide long-distance axonal growth cones. Different studies in mice have demonstrated that the ubiquitous replacement of the intracellular domain of EphB1 and EphB2 by β-galactosidase, mirrors the phenotype of loss of function of the gene encoding ephrin-B1 in the intervening tissues, such as the ventral telencephalon, indicating that the establishment of neural

connections between the thalamus and the cortex depends on forward signaling [15]. Furthermore, EphB1 expression is sufficient for axon repulsion and for the ipsilateral trajectory of retinal axons. [15]

Finally, is important to review the involvement of EphB receptor and ephrinB ligands in other processes in early nervous system. For example, in neuronal migration the lack of ephrinB ligands, in Cajal–Retzius cells, can elicit defects in this process [15] as well as in neurogenesis EphB1 and ephrin-B3 play an important role in migration of neural progenitors in the hippocampus. In fact the lack of EphB1 significantly reduces the number of neural progenitors in the hippocampus, prevents migration and organization of neural progenitors, and affects other aspects of neurogenesis such as polarity, cell positioning and proliferation [35,41]. Furthermore, EphB receptor can activate β- catenin, independently of Wnt signal, leading to the upregulation of proneural gene expression, allowing Ephs to influence transcriptional control of cell fate [27]. During embryogenesis ephrinBs allowing cell division and cellular differentiation by the alternatively interaction with growth factor receptors, such as the fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDFGR), epidermal growth factor receptor (EGFR), and the TIE2 receptor [7].

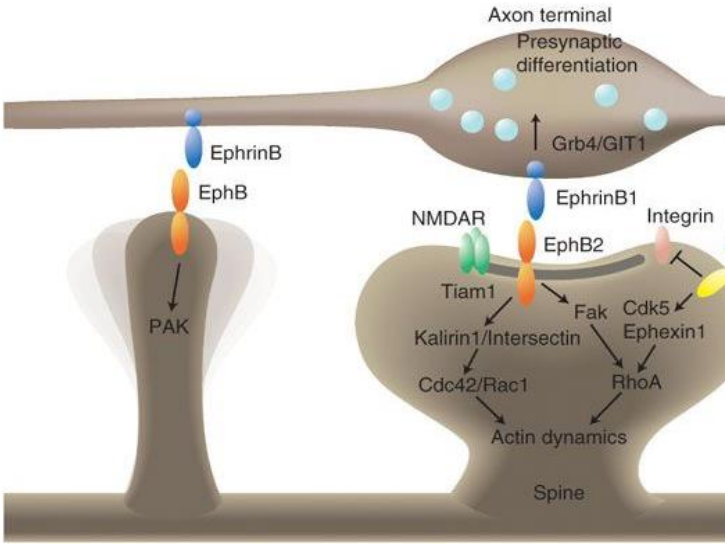


**Figure 5.** EphB and ephrin-B modulate spine and synapse formation. EphB forward signaling via PAK promotes filopodial motility. EphB activation by ephrinB-Fc recruits the Rac1 GEF Tiam1 to EphB complexes containing NMDARs. EphBs also modulate the activity of the Rho family GTPases by activating the Rho-GEFs kalirin and intersectin, and signal via FAK to activate RhoA. EphB1 forward signaling involves recruitment of Cdk5 and activation of ephexin1 and RhoA, which regulates actin reorganization. EphrinB reverse signaling may lead to presynaptic differentiation, possibly involving the Grb4/GIT1 protein complex [5].

### 1.2.2 EphB/EphrinB system in adult SNC

EphB and ephrinB persist in presynaptic and postsynaptic sites in the adult brain principally in regions where neuronal circuits continue to be remodeled in response to environmental changes [11] such as hippocampus and cerebral cortex [31], specifically, in pyramidal neurons [8,18], primary sensory neurons, spinal dorsal horn neurons [42] and astrocytes [36].

The principal roles of EphB and ephrinB in the adult brain are the regulation of synaptic transmission and morphology, which in turn also are controlled by synaptic glutamate receptor [8,43], synaptic plasticity [31,36] and in response to nerve injury [31].

Forward signaling has a crucial role in synapse formation and synaptic plasticity in the hippocampus, in fact, changes in synaptic transmission and neuronal morphology are involved in the modulation of learning [11,31] and in memory formation [36].

Long-term potentiation (LTP), long-term depression (LTD) and depotentiation are phenomena underlaying synaptic plasticityinvolved in EphB/ephrinB system [31].

Thus, forward signaling between ephrinB3 [31] and EphB1 [42], or EphB2 receptors [13] has been involved in long-term potentiation (LTP). The activation of EphB2 can in turn trigger the modulation of NMDA-receptor-mediated calcium influx via a Src- family kinase pathway having direct impact on synaptic transmission [17,30], in fact, in the mature brain EphBs are required for normal levels of synaptic NMDA receptors [37].

In addition, the activation of mediators as RAC1 guanine exchange factor, T lymphoma invasion metastasis-inducing protein 1 (TIAM1) [31], Grb4, Pick1, syntenin [27], MAP kinase and Src family kinases [25] is indispensable for the formation of dendritic spines and establishment of functional synaptic plasticity [27,35].

On the other hand, the structure of ephrinsB play an important role in synaptic plasticity [27] since only PDZ domain-binding site of ephrin-B2 is required in LTP, LTD, depotentiation [11].

Reverse signaling has also been involved in memory formation due to its importance in synaptic transmission, plasticity and neuronal morphogenesis [24,36]. The activation of ephrinB signaling, by EphB2-

Fc in Xenopus retinotectal system, leads to an enhanced transmission by an early increase in the presynaptic transmitter release and a delayed postsynaptic glutamate responses [44]. Other studies in cultured hippocampal neurons have demostrated that the activation of ephrinB by EphB2-Fc promotes spine maturation leading to higher proportion of spines with larger spine heads [10]. On the contraty has been also observed that mutation in ephrinB1, either due to the lack the cytoplasmic domain or for mutation of the six intracellular tyrosine residues, leads to formation of immature filopodia indicating that reverse signaling and tyrosine phosphorylation are fundamental in neuronal processes [10,28]. Furthermore, upon the activation of ephrinB the activation of downstream pathways such as G-protein-coupled receptor kinase-interacting protein 1 (GIT 1) can regulate synapse formation by the activation of Grb4, in fact, ephrinB/Grb4/ GIT1 complex is necessary in spine morphogenesis and synapse formation [10].

## 1.3 EphB and EphrinB are involved several pathological processes

Despite that EphA and B receptors have been studied in cancer, inflammation and in neurological deseases, during these chapter we will focus only in the role of EphB receptors and ephrinB ligands in these pathologies.

### 1.3.1 EphB/EphrinB system in cancer

Type A and B of receptors and ligands normally act as tumor suppressors, however, in tumor cell arepotentially converted into oncogenic proteins [3] being involved in a wide variety of human cancers [45]. They can affect the growth, migration, and invasion of cancer cells *in vitro* as well as tumor growth, invasiveness, angiogenesis, and metastasis *in vivo* [36].

Depending on the activated signaling pathway have been involved in the differents tumorigenic process [11] for example, Eph forward signaling promotes cell segregation and is considered as tumor suppressors since can inhibit oncogenic signaling pathways, such as the HRAS-Erk, PI3 kinase-Akt and Abl-Crk pathways [23,46], in colorectal, breast, prostate, and skin cancer cells both *in vitro* and *in vivo* [11], whereas reverse signaling is often tumor promoting, driving neoangiogenesis and invasion [31]

Abnormal expression of EphB1 receptor has been detected in metastatic gastric carcinoma, colorectal cancer, ovary serous carcinoma and renal cell carcinoma [47–49] ,as well as, in different brain tumors, principally the up-regulation of EphB1expression in medulloblastomas whereas the down-regultion in gliomas [45,47,50].

Interestingly, depending on the cellular context and whether EphB1 receptor forward signaling is dependent or indenpendent of ephrins ligands, EphB1 can act as tumor suppressor or tumor promoter [47].

Alterations in the expression of EphB1 have been involved in 1.49% of the cases of glioma [35]. In glioblastoma multiforme (GBM) patients with higher EphB1 expression levels showed longer survival rates [47]. Therefore, the overexpression and ligand-dependent EphB1 signaling can inhibit cell migration and invasion upon ephrin-B2 ligand stimulation [35] which is considered as a negative regulator for glioma cell motility and invasion that in turns is a positive predictor for glioma patient survival (Figure 6) [47].

Forward signaling ephrinB2/EphB1 activates several adaptor proteins that promotes cell migration, among them EphB1 recruits adaptor protein Grb2, p52$^{Shc}$ and Src whereby can activate MAPK/ERK regulating events involved in cell motility [51], as well as, can induce tyrosine phosphorylation of paxillin and form complex with Grb2, Grb7, integrins, Nck, paxillin, and FAK proteins in a c-Src-dependent manner [35]

In medulloblastoma EphB1 expression have an important role in radiation resistance and in cell migration. EphB1 knockdown Daoy cells have demonstrated that the lack of EphB1 receptor reduces cell growth, viability and migration, as well as, the expression of important cell cycle regulators as cyclinE, besides increases the percentage of cells in G1 phase and enhancements radiation sensitivity not only in culture cells but also in a genetically engineered mouse medulloblastoma model. Therefore EphB1 has been considered as therapeutic target [45].

Furthermore, EphB1can interact with other tyrosine kinases receptors as epidermal growth factor receptor (EGFR), contributing to the metastatic behavior of medulloblastoma cells as well as interacts with β1-integrin producing cell migration and chemotaxis via stimulation of Src activity [45,51,52]

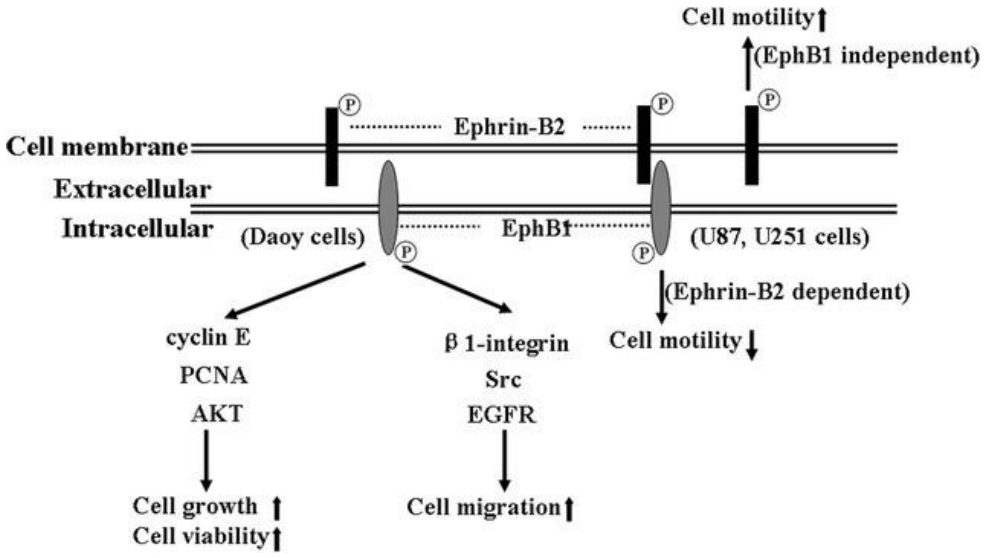


**Figure 6**. EphB1/ephrins signaling in brain tumors. EphB1 upon the stimulation of ephrinB2 activate three signaling pathways. In U87 and U251 cell lines, forward signaling ephrinB2/EphB1 supressess cell motility. Whereas, EphB1 expression in meduloblastoma cell line DAOY promotes cell migration [35].

### 1.3.2 EphB/EphrinB system in inflammation

Eph/ephrin interaction has been involved in the movement of inflammatory cells to the site of infection or tissue injury [1] as well as in the disruption of endothelial–epithelial barriers and adhesion of leukocytes to endothelial cells [31].

Principally, EphB/ephrin B system has been involved in the regulation of T cell maturation and proinflammatory gene expression [23,53]. For instance, an increase in ephrin-B1/EphB1 expression in peripheral blood lymphocytes has observed in patients with rheumatoid arthritis, as well as, EphB1/2 and ephrin-B1/2 are up-regulated in the intestinal cells of patients with Crohn's disease [1].

Furthermore, in animal models of rheumatoid arthritis it has observed that the activation of EphB1 receptor by EphrinB1–Fc fusion protein results in an increase of production of TNF-α from lymphocytes and stimulation of the release of interleukin-6 from synoviocytes, as well as, an increase in the number of peripheral blood lymphocytes migration into the joint enhancing lymphocyte migration [1,31].

On the other hands, in intestinal epithelial cells the stimulation of ephrin-B1 or B2, by EphB1-Fc, induced pro-inflammatory genes such as cyclooxygenase-2 and monocyte chemotactic protein-1 [1], as well as in inflammatory bowel disease (IBD) this stimulation is considered as a novel protective mechanism that could promote intestinal epithelial wound healing [54]

### 1.3.3 EphB/EphrinB system in neuropathologies

#### 1.3.3.1 Pain

EphBs receptors and ephrinBs ligands are expressed at lower levels in mature nervous system (Figure 7a), but after traumatic or ischemic nervous system injury [1,26,55] and in prolonged MOR activation [56]is produced an upregulation in neurons, surrounding astrocytes and oligodendrocytes [13], affecting synaptic remodeling and plasticity[13], axon sprouting and repair processes [32]. Therefore, have been directly involved in the mediation of spinal nociceptive information and central sensitization [57], in dorsal root ganglia and in spinal dorsal horn neurons [8,58], contributing in sensory abnormalities in persistent pain states [56] and in the formation of an unfavorable environment for regeneration and functional recovery after CNS injury [15].

The mechanisms by which EphB/ephrinB system have been involved in the induction and persistence of pain could be principally due to changes in sensory neuron excitability which is produced by an increase in the firing of small-diameter sensory neurons that communicate noxious information to the dorsal horn of spinal cord and an increase in spinal synaptic plasticity [8,59] after inflammation or injury. Once that EphB/ephrinB system is activated can induced peripheral sensitization, which is manifests for hyperalgesia, and allodynia that later could produce central sensitization, which is a major cellular mechanism that converte acute nociceptive injury in chronic pain states [59].

Different molecules contribute to the development of peripheral sensitization inthe EphB/ephrinB system activation and the related downstream[59] , among them, MAPKs ,such as, ERK1/2, p38, JNK [59] and p-ERK5, p-CREB a nuclear transcription factor [60], phosphatidy linositol 3-kinase (PI3K) [61], protein kinase A (PKA) [62], protein kinase Cγ (PKCγ) [60] and calpain-1 and caspase-3 [63].

On the other hand, the activation of other kind of receptors have been related with EphB-mediated pain states principally N-methyl-D-aspartate receptor (NMDR) and toll-like receptor 4 (TLR4) [9,37].

Several studies have demonstrated that the induction of hypersensitivity and pain can occur by enhancing EphB-dependent effects on NMDAR function [37]. Forward signaling of EphB1 and EphB2 receptor have been considered as principal regulator of pain processes via NMDA receptors [37] in particular NR2B subunit (Figure 7b)[43,64]. EphrinB can phosphorylate a single tyrosine (p*Y504) in a highly conserved region of the fibronectin type III (FN3) domain of EphB2 receptor, moduling the EphB-NMDAR interaction in cortical and spinal cord neurons, enhancing, not only, NMDAR localization [37] but also sensitization of nociception in spinal cord synaptic efficiency which is modulated in an NMDR-dependent manner contributing to chronic neuropathic and inflammatory pain states [37] mediated via a MAPK–dependent mechanism [1].

While Toll-like receptor 4 (TLR4) contribute with EphrinB/EphB system in the induction of cancer pain in spinal cord [9].

Although, all EphB receptor are involved in pain processes, recently EphB1/ephrinB1 system has been considered as a principal complex involved in acute inflammatory pain [56], neuropathic pain [58,64,65], cancer pain , diabetic pain [66], opiate dependence and tolerance [67], and chronic ocular hypertension [68].

In a different studies, have been showed that peripheral nerve injury produced thermal hyperalgesia in wild-type (EphB1+/+) but not in EphB1 receptor homozygous knockout (EphB1-/-) and heterozygous knockdown (EphB1+/-) mice, as well as, the hyperexcitability in dorsal root ganglion neurons was prevented in EphB1-/- and EphB1+/- mice [69]. In addition, in chronically morphine-treated mice, the behavioral signs due to morphine administration were diminished in EphB1-/- mice; concluding that EphB1 receptors are required in physical dependence and tolerance of opiates [69], in the thermal and mechanical hyperalgesia and spontaneous pain in a variety of pain models [43],as well, as in microglial activation and in the up-regulation of keys proteins involved in pain such as pNR1, pNR2B, pSrc (Tyr418), pERK1/2, p-p38, p-JNK, pCaMKII, pCREB, pNR2B, c-fos [59,70,71].

In CCI and formalin pain models have observed that EphrinB1-Fc activate forward signaling of EphBs receptors [59]. The activation of EphBs by EphrinB1-Fc is fundamental during hyperalgesia and allodynia, since induce a dose-dependent increase of spinal Fos protein expression [61] as well as an increase expression of NMDA receptor and up-levels of peripheral and spinal phospho-MAPKs (Figure 7c) [59,71]. Furthermore, the precense of sensitizers molecules as TNFα in injury and/or inflammatory tissue could induce peripheral sensitization, which is an important neuronal mechanism underlying primary hyperalgesia at the site of injury or inflammation [59].

Forward signaling between EphB1 postsynaptic receptor expressed in neurons of the spinal cord and ephrinBs presynaptic expressed in pain sensory neurons [32] has demonstrated to be critical in the development of bone cancer pain (Figure 7d) [70]. In rats, tumor cell implantation (TCI) or spinal administration of ephrinB2-Fc (an endogenous ligand) can produce bone cancer–related thermal hyperalgesia, mechanical allodynia, and bone destruction, as well as, an increment in the expression of TLR4 and EphB1 receptors and IL-1β and TNF-α cytokines in astrocytes and microglial cells [9]. In addition, ephrinB2-Fc increase the phosphorylation of NR1 and NR2B receptors wich in turn also depend on the activation of EphB1 [70]. However, spinal administration of EphB2-Fc can relieve bone cancer pain and prevents or reverses pain behaviors, disminishing the activation of astrocytes and microglial cells; TLR4, EphB1 [9], NR1 and NR2B receptors, and c-Fos protein, Src protein within the N-methyl-D-aspartate receptor

complex, and the subsequent $Ca^{2+}$-dependent signals [70], and also increasing the activity of matrix metalloproteinase (MMP)-2/9 [9].

Concluding that spinal blocking or targeted mutation of EphB1 receptor could contribute in the treating bone cancer pain and TLR4 also could be a potential target for preventing or reversing bone cancer pain mediated by ephrinB-EphB receptor signaling [9]

In Diabetic neuropathic pain (DNP), streptozotocin (STZ) or alloxan causes significant activation of EphB1 receptor in the spinal cord, as well as, activation of astrocytes, microglial cells and IL-1β and TNF-α which are important in the pathogenesis of DNP [66]. However, spinal blocking EphB1 receptor activation can relieve DNP in diabetes induced rats by intraperitoneal injection of streptozotocin (STZ) inhibiting mechanical allodynia and the activation of the astrocytes, IL-1β and TNF-α in the spinal cord [66], concluding that EphB1 receptor activation in the spinal cord is critical to the maintenance, but not in the induction of diabetic pain [66].

Reverse signaling of EphB1 /ephrinB2 in rats can also contribute to retinal ganglion cells (RGC) apoptosis with chronic ocular hypertension (COH) in glial and neuronal elements in the retina which was accompanied by increased protein levels of phosphorylated Src and GluA2. High expression of EphrinB1 and EphB1 in monkeys have also been observed in mild-to-moderate glaucoma, as well as, in cultured astrocytes obtained from human glaucamatous patients, concluding that the upregulation of ephrinB/EphB signaling in the retina may be common in experimental and clinical glaucoma considering that attenuation of EphB/ ephrin B reverse signaling could be an appropriate way for preven the loss of RCGs in glaucoma [68].

On the other hand, the Opiates drugs are used in the treatment of moderate-to-severe post-operative and chronic pain, however, when opiates are administered simultaneously can activate the nocuous mechanism of a sensitization process causing pain hypersensitivity [57]. The involvement of EphB1 receptors in these processes have been observed in animal models which the scalating morphine treatment [70] and continuing infusion of remifentanil [57] significantly up-regulates expression of EphB1 receptor. In a rat hindpaw incisional model ephrin B1 ligands in spinal cord produce thermal hyperalgesia and mechanical allodynia

[57,70], whereas intrathecal administration of EphB2-Fc, used as a blocking reagent, revert the behavioral symptoms and neurochemical signs associated with chronic opiates treatment [70], as well as, reduce the levels of pNR2B, pERK and pCREB in the SC [67,69].

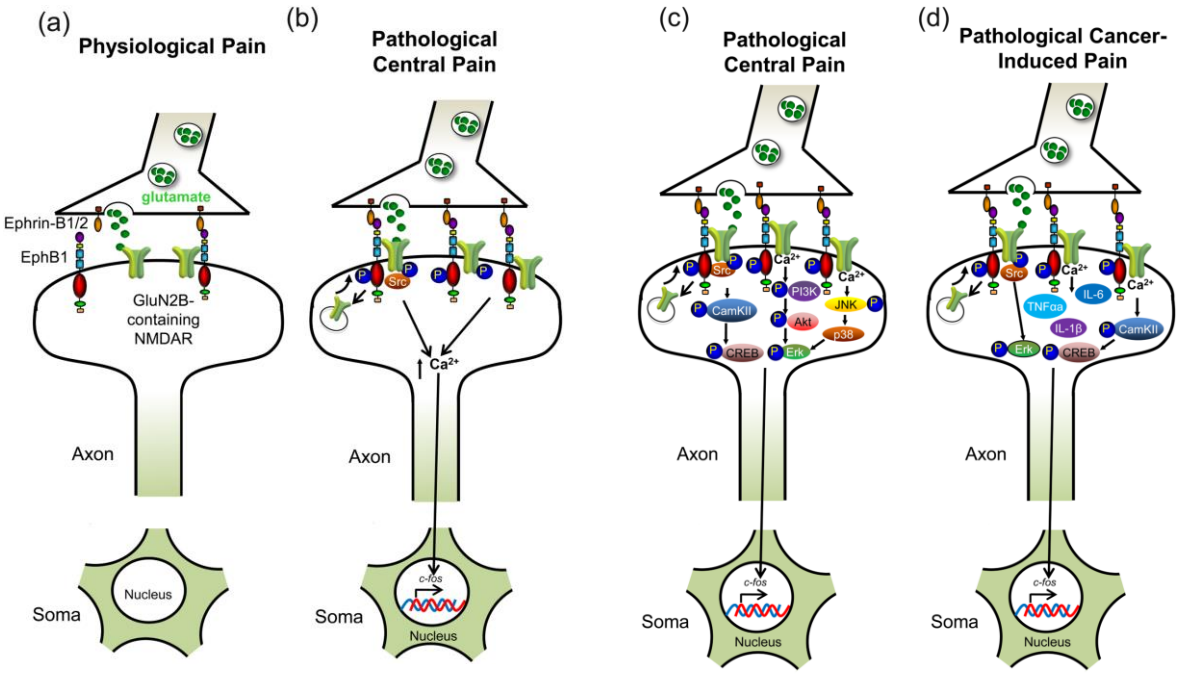


**Figure 7.** Involvement of ephrinB/EphB in neuropathic pain**. a**) low expression of ephrin-B and EphB1 in physiological pain conditions. **b**) In pathological central pain, both ephrin-Bs and EphB1 are upregulated. Activation of EphBs leads to recruitment and activation of Src and consequently NMDARs phosphorylation with an increasing of calcium influx leading to c-fos and CREB gene transcription. **c**) Pathological peripheral pain, shares with pathological central pain the activation of EphB and NMDR receptors, then the activation of other three signaling pathways caractherize this pain model. Src phosphorylates CamKII, which phosphorylates CREB causing nuclear translocation and Cyclic AMP Response Element (CRE) gene transcription. The activation of PI3K signaling pathway, which phosphorylates Akt and ERK proteins and consequently translocates to the nucleus to activate c-fos gene transcription; and JNK phosphorylated and activated can phosphorylate p-38 and converges to activate ERK. **d**) in Pathological cancer-induced pain shares the principal caractheristic of pathological central and peripheral pain. However, in cancer pain the phosphorylation of ERK directly by src kinase to activate gene transcription of c-fos and the up- regulation of inflammatory cytokines as TNFα, IL-6, and IL-1β can lead to hyperalgesia, and hyperexcitability of nerve afferents [8].

### 1.3.3.2 Neurodegenerative diseases

Aberrant synaptic activity is considered as a major pathological hallmark in neurodegenerative and psychiatric disorders [4]. Eph receptor and ephrin ligand are present in the development of the central nervous system and in the adult brain in regions of continued development as hippocampus and amygdala [31]

regulating synapse formation maintenance and plasticity [4] which are important in learning, memory formation and consequently in normal cognitive function.

Since, Ephs and ephrins play an important role in synaptic efficacy, regulating presynaptic transmitter release, postsynaptic glutamate receptor conductance and trafficking, synaptic glutamate reuptake, and dendritic spine morphogenesis [5,10,17], their deregulation have been directly involved in aberrant synaptic functions associated with cognitive impairment and in long-term memory formation, which can produce alterations of synaptic efficacy modifying neural transmission and morphology [36], producing brain disorders and diseases with memory impairment symptoms including trauma, stroke, epilepsy, psychiatric, anxiety and neurodegenerative diseases as Alzheimer disease, Parkinson disease and Amyotrophic lateral sclerosis (ALS) [18].

Furthermore, various Eph receptors and their ligands have been involved in the excitatory synapses in the hippocampus, including EphA4, EphB1, EphB2, EphB3, ephrinB2 and ephrinB3 [4]. EphB receptors can coordinate synapses due to a multiple upstream molecules, such as, Rho-GTPases GEFs (guanine-nucleotide exchanging factor) and GAPs (GTPase-activating proteins) [4]. In fact, they regulate actin cytoskeleton network through activation of GEF kalirin, intersectin or Tiam1-mediated activation of the Rho GTPases Rac1 and Cdc42 [4]. In addition, EphB2 can regulate the function of post- synaptic neurotransmitter receptors as NMDAR by the formation of co-clustering of NMDA receptor and specific postsynaptic proteins including calcium/calmodulin-dependent protein kinase II (CaMKII) and Grb1 [4].

Severe or sustained stress can result in changes of synaptic plasticity which can lead to behavioral changes associated with fear and anxiety disorders. The effects of EphB2 in NMDAR activation allow to think that EphB receptor plays an important role in bipolar disorder and cognitive functions [8].

Deficiency in serine protease neuropsin, an important protein expressed in amygdala and hippocampus, has been also involved in bipolar disorder and cognitive functions [72]. EphB2 and neuropsin are upregulated after stress in amygdala which results in cleavage of the EphB2 ectodomain [73]. Neuropsin-dependent cleavage of EphB2 increases the dynamics of the EphB-NMDAR interaction, likely explaining the changes

in NMDAR currents observed in neuropsin null mice [73] suggesting that targeting neuropsin-dependent cleavage of EphB2 is a potential strategy for treating stress-related and anxiety disorders [8].

Alzheimer's disease (AD) is considering a devastating disease in which is produced a permanent loss of memory and other cognitive functions causing progressive loss of synapses and neurons [32]. Senile plaques are a hallmark of AD, which are produced by proteolysis of amyloid precursor protein by the presenilin/γ-secretase intramembrane protease complex, generating the cytotoxic β amyloid (Aβ) peptides [32]. Soluble Aβ oligomers can decrease NMDA receptors affecting their equilibrium and activity at synaptic sites producing impairs synaptic plasticity and glutamatergic transmission (Figure 8) [74].

In mice models have demonstrated that beta-amyloid (Aβ) directly binds to the fibronectin repeats domains of EphB2 affecting its function and increasing its proteasomal degradation [74]. The reduction of hippocampal EphB2 levels produce memory impairment in early stages of AD due to a decreases of NMDA surface expression with a defective neuronal activation and reduction of LTP (Figure 8) [25]. However, since EphB2 expression levels can regulate the amount of NMDARs at synapses, the overexpression of EphB2 acts as a neuroprotector in hippocampal [74].

The involvement of Ephs an ephrins in neurogenesis suggests that could be used in the treatment of Parkinson's disease [75] which is other type of neurodegenerative disorder characterized by a deep loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc) accompanied by filamentous protein inclusions termed Lewy bodies (LBs) in the surviving neurons [76]. Jing and collaborators have demonstrated that the activation of EphA receptor in lateral ventricle by soluble form of the ephrin-A1 ligand (ephrin-A1 Fc) could promote regeneration of the brain dopaminergic neurons [75]

The involvement of EphB1/ ephrinB1 system in Amyotrophic lateral sclerosis (ALS), a fatal disease characterized by a progressive degeneration of motor neurons that produce the breakdown of neuron-glia communication and neuronal dysfunction and death [77], is due to the capacity of EphB1 receptor to produce the activation of protective phenotype of astrocytes in human and mouse ALS models[77]. EphB1 receptor is up-regulated in injured motor neurons, where, by the activation of ephrinB1 can induce STAT3 (activator

of transcription-3) activation which can regulate astrocyte activation [78], producing astrocyte transformation and consequently protective, anti-inflammatory or immunomodulatory pathways [77].

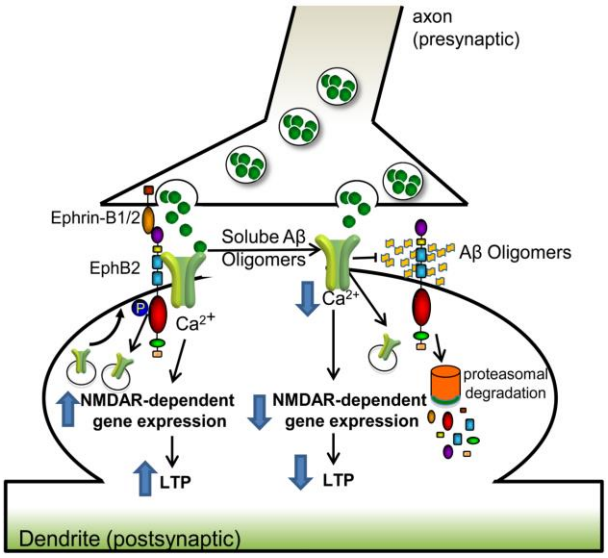


**Figure 8. EphBs in AD.** The EphB2-Aβ interaction inhibits receptor activation and causes internalization and degradation of both EphBs and NMDARs. Degradation of EphB receptors inhibits their ability to retain NMDARs on the membrane potentially though the EphB-NMDAR interaction. Fewer NMDARs on the cell surface leads to decreased calcium influx and none of the changes in gene transcription required for LTP [8]

## 1.4 Interactions between Eph/ephrin system with other receptors

The activation of Eph and ephrin proteins can produce a cross-regulation with other communication pathways producing molecular, functional, and genetic interactions with different cell surface signaling pathways controlling broaden range of function *in vivo* as cell survival, migration and differentiation [3,31,79]. Eph/ephrins can produce crosstalk with different cell surface receptor (tyrosine kinases receptors and g-coupled receptor), adhesion molecules, (integrins, claudins and cadherins) and ionotropic receptor (NMDA and AMPA receptors) (Figure 9)[79].

Despite, this chapter will be focalized principally in the interaction of Eph/ephrin system with cell surface receptor as ionotropic, tyrosine and g-coupled receptor and their different downstream molecules that are activated, I have believed interesting also review the interactions formed between Eph/ephrin system with other proteins.

Eph receptor can attenuate Ras-MAP- Kinase signaling downstream of other receptors, such as, integrins [80,81] affecting integrin-mediated cell communication with the extracellular environment [11]. Endogenous ephrin-B2 expressed in melanoma cells is associated with β1-integrins and their interaction can promote cell adhesion and migration, suggesting a role in tumor progression [11].

PI3K–AKT signaling has been involved in Eph kinase-independent activities and in cell migration *in vitro* and *in vivo*. However, in phosphatase and tensin homologue (PTEN)-deficient tumour cells, in which AKT and mTOR are active, the expression of EphA2 can suppres AKT phosphorylation and cell migration [31]

Crosstalk between EphB/ephrin-B and Wnt signaling has also been reported. Both EphB receptors and B-type ephrins produce their signal through components of the noncanonical Wnt pathway [11]. This interaction produce endocytic removal of EphB receptors from the cell surface, whereas canonical Wnt signaling increase EphB transcripts and decrease ephrin-B transcripts [11], for instance, in intestinal crypts an increasing WNT expression can induce EphB2 and EphB3 expression while represses ephrin B1 and ephrin B2 expression resulting cell–cell repulsion [31].

Eph receptor expression and ephrin-dependent activation, in cell-surface localization, can also be regulated by E-cadherin. E-cadherin can be drive to the cell surface by EphB signaling thus promoting the formation of epithelial adherents junctions and enabling EphB/ephrin-B-dependent cell sorting [11] which is important during vascular and neural remodeling [12]. In addition adherent junction can be disturb when EphB-ephrin-B binding is inhibited[11].

Croostalk between EphA2 or ephrin-B1 with claudins has been implicated in the regulation of cell adhesion and intercellular permeability. Furthermore, claudins can activate ephrin-B1 tyrosine phosphorylation independently of EphB receptors [11].

Besides, EphBs may also interact with acetylcholine receptors (nAChRs) regulating neurotransmitter receptor function [8], in addition, since EphB2 can interact with TNF-α could be considered as neuroprotector [82].

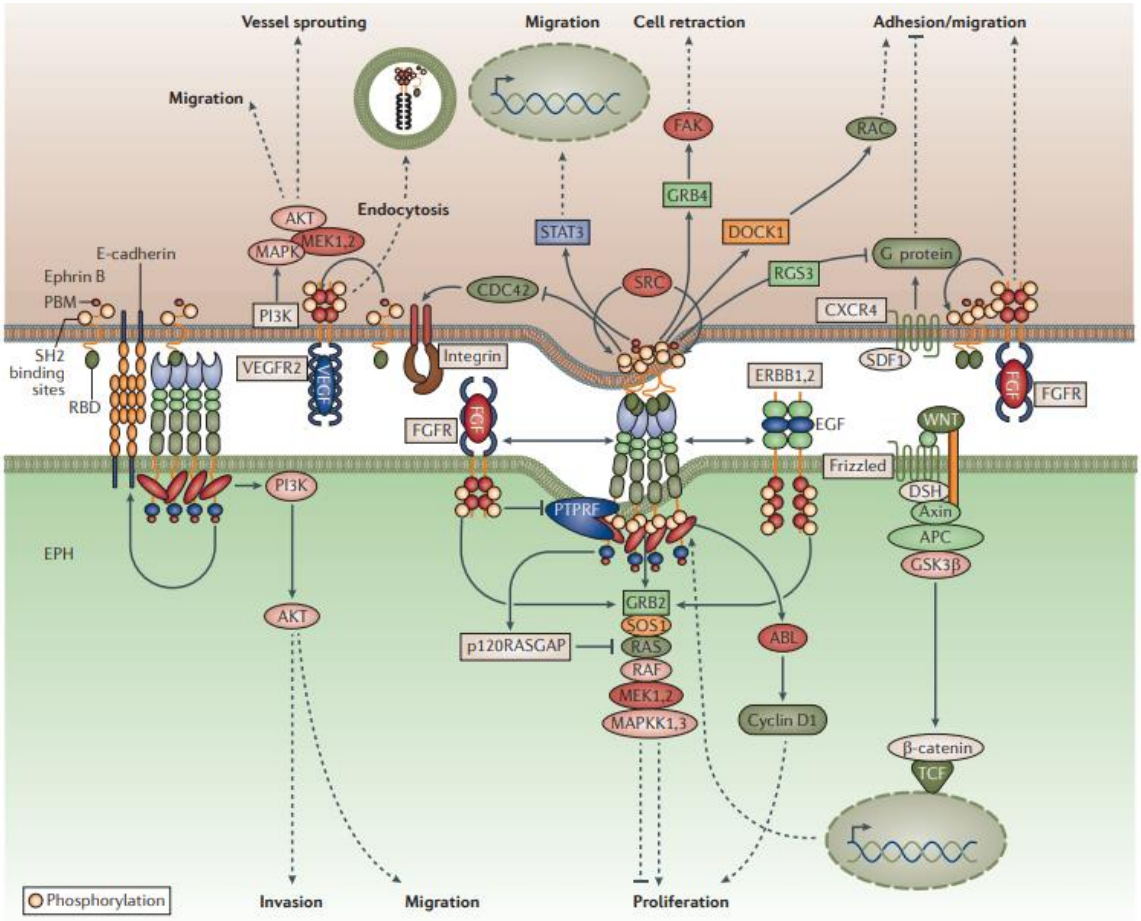


**Figure 9**. Eph and ephrin B crosstalk with cell signalling pathways [31]

### 1.4.1 Ion channels receptors

#### 1.4.1.1 NMDR

Previously have seen that the interaction between NMDRs and EphBs receptors contribute in pathological states such as long-lasting mechanical hypersensitivity,[37] in different pain states, [1] opiate dependence and tolerance [57],as well as, anxiety disorders and Alzheimer's disease[8].

Therefore, during this chapter, a summary of how the cross-communitation between both receptor is produced will be reported.

N-methyl-D-aspartate receptors (NMDARs) are essential in synapses [11]. Sinaptic localization, function and signaling of NMDARs can be regulated by intracellular scaffolding proteins (PSD-95), extracellular interacting proteins (neuroligin-1) and EphB receptors. In this way, crosstalk formed between EphB and NMDA receptors can control localization, function, and signaling of NMDARs [37]. This interaction have an important role in neural plasticity, in adult nervous system, regulating the development of glutamatergic synapses [83–85].

Ephrin-B induce activation of EphB2 or EphB1 receptors [36,86] which by its tyrosine Y504 in the intracellular domain interact with the NMDAR [37]activating and enhancing its functions [8]. Src family kinases interacting with EphB2 [11] activates both subunits NR1 and NR2 of NMDARs [83] resulting in $Ca^{2+}$ influx activation and subsequent stimulation of various $Ca^{2+}$-dependent proteins, such as $Ca^{2+}$/calmodulin-dependent kinase (CaMKII), extracellular signal-regulated kinase (ERK) (Figure 10) [38,69,87] and Grb10 [6]. Furthermore the increase of intracellular calcium in turn promotes proteolytic degradation of EphB2, demonstrating that Eph levels can be regulated by intracellular calcium independently of ephrin binding [11].

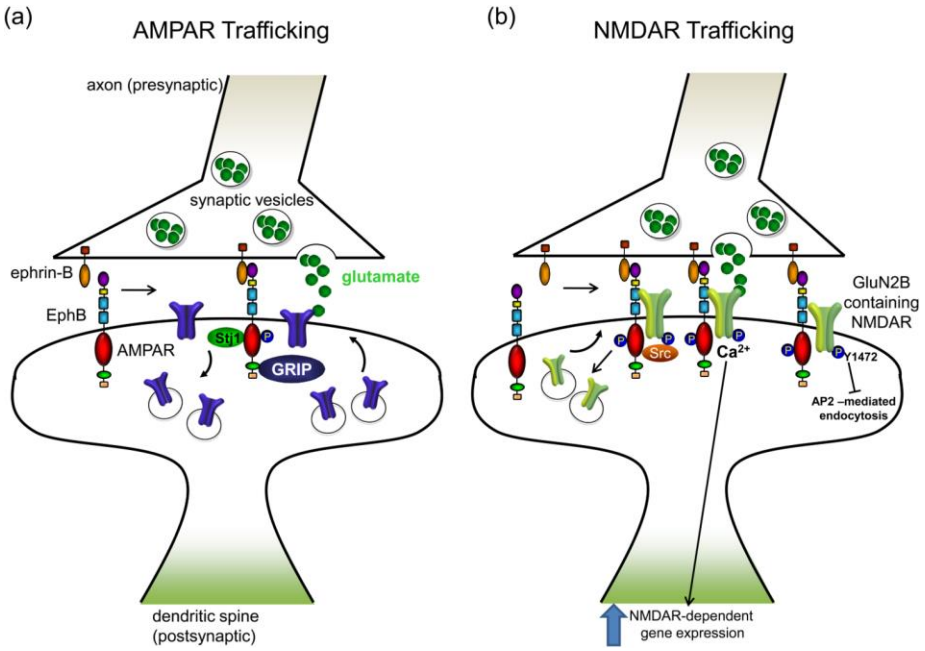


**Figure 10.** Cross-intercation between EphBs and glutamate receptor. **a**) After binding ephrin-B ligand, EphBs directly interact with NMDARs to regulate their synaptic surface localization and function. After activation, EphBs recruit Src kinase to phosphorylate GluN2B subunit at Y1472 tirosine blocking binding of the AP2 complex and clathrin mediated endocytosis. **b)** EphBs regulate AMPAR trafficking through a PDZ-dependent interaction with GRIP1 and indirect interactions with synaptojanin-1 (Stj1), a phosphatidylinositol 5′-phosphatase. If an EphB receptor is interacting with GRIP1, kinase activation by ephrin-Bs promotes AMPAR insertion into the membrane from the recycling pool. Alternatively, EphB kinase activation by ephrin-Bs can also promote AMPAR internalization by phosphorylation of Stj1, which activates clathrin-mediated endocytotic mechanisms [8].

#### 1.4.1.2 AMPAR

Is well known that excitatory synaptic transmission is also mediated by AMPA-type glutamate receptors which are cell surface receptor and regulate neuronal activity and intermolecular interactions in synapses [6]. During learning, release of glutamate from presynaptic neurons activates AMPA receptors (AMPARs) depolarizing postsynaptic neuron [36]. Scaffolding proteins, such as, postsynaptic density protein 95 (PSD-95) and glutamate receptor-interacting protein 1 (GRIP1), synaptic adhesion molecules, such as, EphBs and neuroligins, and epidermal growth factor receptor family (ErbBs) have been involved in these processes [8]. EphrinB2 and EphB2 have involved in the stabilization and regulations of AMPARs at the cellular membrane [36]. In cultured neurons, EphB2 and AMPARs can associate their PDZ-adaptor proteins with C-Kinase 1 (PICK1) and glutamate receptor-interacting protein 1 (GRIP1) (FIGURE 10) [4].

GRIP1 promotes AMPAR surface retention, in fact, when this interaction does not occur is abolished the colocalization of EphB2 and AMPA receptors [4] while PICK1 acts to remove AMPARs from the cell surface but this interaction is poor understand [8]. Furthermore, EphBs can control AMPAR trafficking by ephrin-B and kinase activation of EphB2 receptors [8,23]. AMPARs can be internalizate by EphB2 receptors throught activation of synaptojanin 1 EphB2 – mediated , this activation is involved in clathrin- mediated endocytosis (Figure 10) [8]. Besides, reverse signaling EphB/ephrinB2 by the increase of cellular level of phosphatidylinositol 4,5-bisphosphate and transferrin uptake can be involved in AMPA glutamate receptor endocytosis in hippocampal neurons [74].

### 1.4.2 Cell surface receptors

#### 1.4.2.1 Crosstalk with others RTKs

Ephs also have the capacity of interact with other RTKs (tyrosine kinases receptors), in particular with erbB/EGFR family members [12], Ryk receptors [86], fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDFGR) [7].

EGFR can interact ligand- independent with EphA2, EphA3 and EphB4 [12]. EphA2 is considered as an effector to promote cell motility and proliferation even independently of ephrin stimulation [11]. Moreover, at cell-cell contacts activation can alterate the cellular response to EGFR modulating the vesicular trafficking of its receptor [88] and consequently cell migration [12].

The activation of Eph receptor can trapped EGFR in Rab5-positive early endosomes inhibiting Akt-dependent vesicular recycling, alterating the distribution of EGFR activity, wich can suppress EGF-promoted Akt signaling, thereby inhibiting cell migration [88].

Fibroblast grown factor (FGFR) and Eph/ephrin signaling pathways present an agonistic relation since FGFR and EphA4 can transphosphorylate each other and activate common downstream signaling pathways in mammalian cells (Figure 11a) [11,79].

The activation of EphA4 can promot cell proliferation and migration on FGF 2-mediated cells which are accompanied by mitogen-activated protein kinase (MAPK) [79] and Akt phosphorylation [89]. Furthermore, Rac1 and Cdc42 proteins can increase the expression of EphA4 in malignant gliomas which accelerate

glioma cell proliferation and migration through promoting the FGFR1 signaling pathway, increasing in turn MAPK pathway [89]. Therefore, the potentiation of EphA4-FGFR1 complex acts enhancing malignant phenotype in gliobastomas [3].

On the other hand, also ephrin-B1 can interact with FGFR in cis mode, inducing tyrosine phosphorylation of FGFR which in turn inhibited the ability of ephrin-B1 to induce blastomere dissociation [79]

Other cross-interaction between RTKs is produced by vascular endothelial growth factor (VEGF) and EphA2 receptors. In bovine retinal endothelial cells, forward signaling ephrin A1/EphA2 receptor can inhibit VEGF-induced phosphorylation of VEGF receptor 2 (VEGFR2), producing a reduction in the VEGF-induced angiogenic cell activity [90] suggesting that the VEGFR activity is dependent of EphA2 receptor. VEGF can also upregulates the expression of EphA2 in microvascular endothelial cells (HBMEC) through the independent activation of PI3K/Akt and ERK1/2 signaling pathways[91].

Furthermore, in mouse and zebrafish models have demonstrated that ephrin-B2 can also interact with VEGF signalling pathway, promoting sprouting behaviour and motility in the angiogenic endothelium. Ephrin-B2 is involved in angiogenic and lymphangiogenic control though the regulation of the internalization and signalling activity of VEGFR2 during physiological and pathological angiogenesis [92]

Finally, RYK an atypical RTK receptor has been involved with EphB receptor since both receptors are engaged in the regulation of axon guidance and craniofacial development (Figure 11a). In mouse models, homozygous deletion of Ryk resulted in craniofacial defects, similar to the observed in Eph-B2/Eph-B3-deficient embryos. Therefore is thought that Eph receptors could phosphorylate RYK regulating the migration of neuroprogenitors [79]

#### 1.4.2.2 Crosstalk with GPCRs

G- coupled receptors are transmembrane receptors which could form crosstalk with Eph receptors. In fact, Eph/ephrin signaling and CXCR4/ SDF-1 signaling can regulate processes involving cell chemotaxis [79].

Eph receptor and ephrin ligands, as well as, G-protein–coupled receptor CXCR4 and its unique SDF-1 chemokine ligand can regulate cell movement. The stimulation of EphB2 and EphB4 receptors can enhances SDF-1-induced chemotaxis in endothelial cells, required for extracellular matrix–dependent

endothelial cell clustering and activating synergic pathways, as AKT proteins, which regulate endothelial movement and morphogenesis of blood vessels (Figure 11a) [93].

Furthermore, ephrinB cytoplasmic domain can bind to the PDZ-dependent signaling pathway, for instance, to PDZ-containing RGS3 protein which is a regulator of G-protein-coupled receptor signaling and a scaffold protein PAR-3 [16]. Ephrin-B reverse signaling can inhibited SDF-1-induced chemotaxis of cerebellar granule cells which is PDZ-RGS3-mediated (Figure 11b) [79].

Furthermore, the synergic activation of G protein–coupled receptor Kiss1 (Kiss1R) and Eph receptors can suppress EGFR vesicular recycling, inhibiting EGF-promoted migration in *Pten*−/− mouse embryonic fibroblasts and in MDA-MB-231 triple-negative breast cancer cells [88].

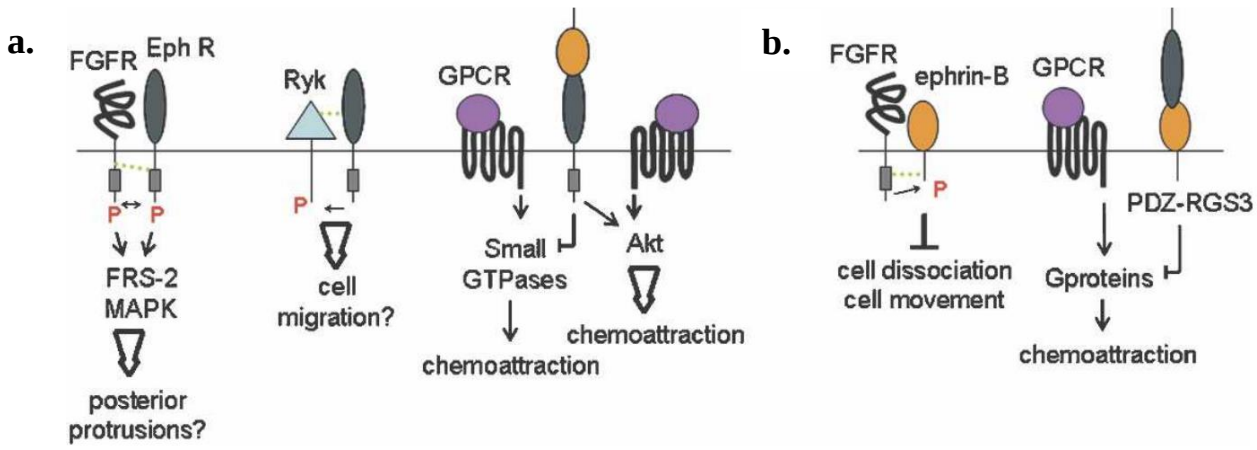


**Figure 11.** Interactions with cell surface receptors. **a)** Eph receptors interact with FGFR in agonistic interaction producing cell migration; with Ryk receptors the agonistic interaction allows the axon guidance and craniofacial development, whereas the interaction with chemokine receptors can regulate chemotaxis. **b)** Ephrins interact with FGFR in agonistic manner regulating cell movement and with chemokine receptors producing chematttraction in cerebellar granule cells [79].

## 1.5 Eph receptor as a therapeutic target

Ephs and ephrins play an important role in different pathological conditions, whereby currently are considered promising therapeutic target for the development of therapies that can help in the treatment of human pathologies [94] without affecting other normal physiological processes in which are required Eph receptors [95]

So far, have been developed drugs that agonize or antagonize Ephs/ephrins signaling [36] which are being used as anticancer therapeutics modulators of synaptic plasticity and controller of regenerative and pathological neoangiogenesis, bone homeostasis and remodeling, immune modulation, glucose homeostasis and stem cell biology [31]

Different strategies have been developed to modulate Eph/ephrin signal transduction. For instance, recombinant monomeric proteins and chimeric proteins containing the extracellular domain of an Eph receptor or ephrin fused to the Fc portion of an antibody (Figure 12) [96] which due to the promiscuity of Eph receptor and ephrin ligands are not considered as selective inhibitors of Eph and ephrins [31]. Therefore, more selective targeting agents have been investigated [96], among them include, peptides that bind ephrin-binding pocket of Eph receptors, and small molecules that bind the Eph receptor extracellular or kinase domain [96], as well as, biological molecules, such a,s antibodies that can recognize specific extracellular epitopes of Ephs and ephrins binding to the ephrin-binding pocket or the ATP-binding pocket and siRNAs, miRNAs that also can bind Eph receptor and ephrin ligands (FIGURE 12) [94].

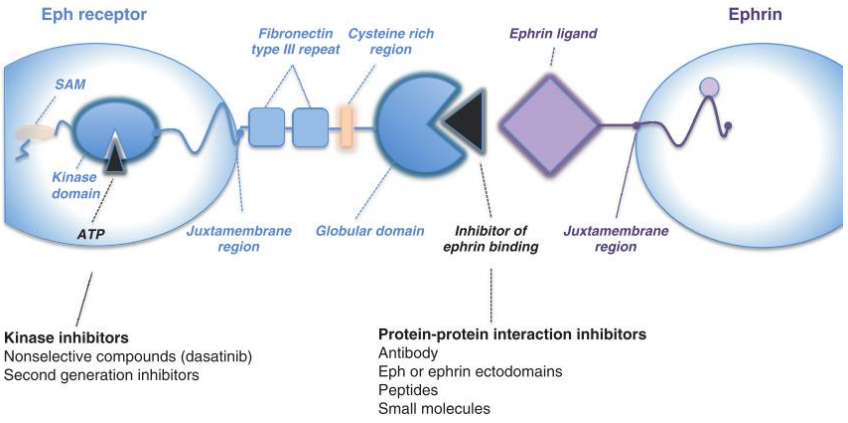


**Figure 12.** Eph receptor as a therapeutic target. Forward signaling can be inhibited by kinase inhibitors, whereas Eph forward and reverse signaling can be blocked by protein–protein interaction inhibitors (PPIs) including antibodies, peptides or small molecules [22].

## 1.5.1 Molecules that interfered with EphrinB/EphB signaling

### 1.5.1.1 Soluble form of EphB receptors as part of fusion protein

Fc fusion protein or chimeric protein can block the interactions of Eph and ephrin proteins which are upregulated in several pathological processes [31].Recombinant Eph/ephrin ECDs proteins are widely used as soluble molecules that can activate or inhibit forward signaling, reverse signaling, or both [32]. These ECDs molecules can couple with high affinity to the fragment crystallizable (Fc) region of human IgG [69] or albumin [32]. The administration of the extracellular domain of Eph or ephrin ECDs fused to Fc can disrupt Eph/Ephrin signaling interaction [1]. For instance, after nerve injury or formalin injection [69], the spinal administration of EphB1/B2-Fc can induce the downregulation of EphB receptors through the up regulation of matrix metalloproteinase and the downregulation of molecular pain inducers [70], alleviating spontaneous pain, thermal hyperalgesia, mechanical allodynia and opiate-resistant in rodent models [32,97]. While, Ephrin ECDs fused to Fc can activate Eph forward signaling, but in some cases also promote Eph degradation loosing Eph signaling activities [32].

In addition, Eph/ephrin Fc proteins can also compete with their endogenous counterpartsreducing their signaling ability [32].

On the ther hand, EphB4 act as a tumor suppressor in colon, breast and prostate cancers whereby have been developed EphB4–human serum albumin (HSA) fusion protein which has been tested in colon, lung, breast,

glioma, melanoma and prostate tumours with promising results entering in Phase I clinical trials in patients with refractory or metastatic solid tumors [31].

Recently, have been developed multivalent ephrin ECD bioconjugates with precise geometrical configurations which were generated by incorporation into nanostructured biomaterials. These nanoparticles can accurately modulate Eph/ephrin oligomerization in target cells and thus signaling output [32].

#### 1.5.1.2 Small molecules

Agonist and antagonist small molecules that targeting Eph receptors bind the ephrin-binding domain and the ATP-binding pocket in the Eph kinase domain [96] with a modest binding affinities in the micromolar range [96].

The small molecules development for disrupt Eph/ephrin system are, lithocholic acid (LCA), salicylic acid derivatives, polyphenols from green tea and polyphenol metabolites, doxazosin (a clinically used α1-adrenoceptor antagonist), peptide analogs mimicking ephrin G-H loop [22].

Lithocholic acid inhibits ephrin binding to both EphA and EphB receptors in biochemical assays, for example, with EphA2 behaves as a reversible competitive inhibitor, with a Ki value of ~50 μM [96] preventing ephrin-induced EphA2 and EphB4 phosphorylation at a similar concentration [22]. Furthermore, LCA also can prevent apoptosis in adult cardiomyocites by the inhibition of the kinase activity of EphA2 [98].

Recently, UniPR126 (PCM126) a derivative molecule of LCA have been developed which act as antagonists of the EphA2 receptor, since can disrupt EphA2–ephrin-A1 interaction with an IC50 of 2.0 mM, as well as preventing EphA2 activation and cell retraction in human prostate adenocarcinoma cells [99].

Futhermore, UniPR139 and UniPR502 molecules derivates of LCA have also been developed which act as a competitive and reversible antagonist of all Eph receptors reducing both ephrin-A1 and -B1 binding to EphAs and EphBs receptors in low micromolar range. Principally, UniPR139 can inhibit ephrin-A1-dependent EphA2 activation producing an antiangiogenic effect, inhibiting HUVEC tube formation and VEGF-induced vessel formation *in vitro* [100].

On the other hand, among the salicylic acid derivatives that have been identified 4-(2,5- dimethyl-1H-pyrrol-1-yl)-2-hydroxybenzoic acid can inhibit ephrin binding to the EphA2 and EphA4 receptors with IC50 of 10 mM in binding assays [96]. Other compound obtained was a disalicylic acid furanyl derivative which is a noncompetitive inhibitor of the EphA4–ephrin-A5 interaction [22].

Doxazosin has also been recently identified as a novel small molecule agonist of the EphA2 and EphA4 receptor which can inhibit the activation of Akt and ERK activities EphA2-dependent in prostate cancer, breast cancer and glioma cells suppressing aberrant migration [22].

#### 1.5.1.3 Peptides

Agonist and antagonist peptides can also be used in the modulation of Eph/ephrin signaling response with high selectivity and binding affinity towards the ligand-binding domain (LBD) of individual Eph receptors[32,95,101].and as in Eph receptor LBD is extracellular, peptides that bind this domain do not need to cross the plasma membrane solving a major problem encountered in the use of peptides that bind to intracellular targets [94].

These peptides contain similarity sequence with 15 amino acid motifs found in the G-H loop of the ephrin-B ligands which is the region that mediate high- affinity interaction with the EphB receptors [95].

Although peptides and small molecules can be used with the same purpose, even more, peptides present several advantages over small molecules, among them , peptides cover the chemical space between small molecules and biologic drugs [102], can bind with high affinity to protein interfaces even in the absence of the highly concave pockets preferred by small molecules (being particularly effective in the inhibition of protein-protein interactions due to their larger size), and also present low toxicity [94].

However, peptides can also present some disadvantages, including their potentially poor pharmacokinetic parameters and oral bioavailability. Since, for *in vivo* assays, peptides needs high resistance to plasma proteases and persistence in the blood circulation peptides have been bind with unnatural amino acids in process as cyclization, PEGylation or inclusion into nanoparticles, obtaining novel peptides with reductive digestionbeing metabolically stable and prolonging peptide lifetime in the circulation [31].

Phage display has been the approach used to identify peptides that bind to Eph receptors, which have identified a series of dodecameric peptides that target the ligand binding domains of several Eph receptors with low micromolar range and with high affinity by a single Eph receptor [2,14,31].

Furthermore, 3 linear peptides identified contain 2 cysteines (separated by 4 or 7 intervening residues) enabling cyclization through formation of a disulfide bond. Cyclic peptides formed are promising class of Eph receptor-targeting agents, due to their conformations present higher binding affinity and specificity as well as better metabolic stability [94].

To date, the crystal structures of only 4 peptides in complex with the EphA2, EphA4, EphB2 or EphB4 LBDs have been solved, revealing that peptides can bind to the ephrin-binding pocket in a variety of orientations [94].

YSA, SWL and derivative peptides are agonists that can promote **EphA2** tyrosine phosphorylation as well as EphA2 degradation [31,101].

KYL, VTM, APY peptides are antagonists that can inhibit ephrin-induced **EphA4** activation in cultured cells and mouse hippocampal slices. Blockage of the EphA4 LBD complex 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 [31].

Notably 8 of the 13 peptides identified by panning on EphB2 also bound to EphB1, suggesting a particularly close similarity between the ephrin binding pocket of both receptors [94].

However, SNEW antagonist peptide can inhibits selectively ephrin-B2/**EphB2** interaction in ELISA assay with an IC50 value of 15 μM [95] whereas EWLS antagonist peptide binds selectively to **EphB1** inhibiting ephrin-B2/EphB1 interaction with an IC50 value of 10 μM [95,103].

TNYL peptide is the best inhibitor of ephrin-B2/**EphB4** signaling, even though its potency was only 50-150 μM for the biotinylated and non-biotinylated versions. In addition, if RAW sequence in the C-terminal extension of TNYL formed TNYL-RAW, peptide increased potency compared to TNYL [31]. Furthermore, TNYL-RAW peptide possesses very short half-life in cell culture medium and plasma, suggesting high susceptibility to proteolytic degradation and clearance from blood circulation. Therefore the conjugation to

40 kDa branched polyethylene glycol (PEG) polymer or nanoparticles, the fusion to the Fc portion of an antibody, and the complexation of the biotinylated peptide with streptavidin have been used to inhibit peptide degradation and rapid blood clearance [95,96].

Finally, since EphB2 and EphB4 can promote tumorigenesis by interacting with ephrin-B ligands, the use of respective antagonist peptides can block human umbilical vein endothelial (HUVEC) cell retraction caused by ephrin-induced activation indicating the ability of the peptides to counteract the cell shape changes and anti-migratory effects mediated by the EphB2 and EphB4 receptors, opening the possibility of using antagonist peptides for cancer therapy [31].

#### 1.5.1.4 Kinase inhibitors

Tyrosine kinases inhibitors (TKI) can bind to the ATP- binding pocket of Eph receptors with nanomolar affinity and some of them are being evaluated for their potential effects on oncogenic Eph function [31].

Inhibitors of EphB4 have been identified, principally NVP-BHG712 molecule, discovered by Novartis , and identified by computer design using a model of the EphB4 kinase domain, can inhibit EphB4 autophosphorylation and vessel formation *in vivo* after oral administration [32,96]. XL647 molecule,other tyrosine kinases inhibitor developed by Exelixis, California USA, is a novel second generation ATP-competitive small-molecule inhibitor of epidermal growth factor receptor (EGFR), ERBB2, VEGFR2 [31] which also can block EphB4 phosphorylation that was reached the clinical Phase II for the treatment of non-small-cell lung cancer (NSCLC) with non-favorable results.[32]

Dasatinib is other multi-targeted kinase which inhibit EphA2, EphB1, EphB2 and EphB4 kinases and reduces EphA2 expression. The second-generation and third-generation of Src and Abl inhibitors nilotinib and bosutinib, respectively are other kind of TKIs with *in vitro* Eph- inhibitory properties which inhibits EphB1, EphB2 and EphB4 [31]

#### 1.5.1.5 Monoclonal antibodies

Activating and inhibitory monoclonal antibodies that recognize Eph/ephrin ECDs with high binding affinity and specificity modulate the Eph/ephrin system [11,32]

During the last years have been developed monoclonal antibodies with particular interest on EphA2, EphB2, EphB4, and ephrin- B2 which have shown to be efficacy in mouse tumor xenografts as antiangiogenic/anticancer agents and in some cases in combited with anti-VEGF therapy [32]

EphA2 was the first Eph to be considered for therapeutic antibody development [31]. In a panel of agonistic monoclonal antibodies (agonistic mAbs), mAb EA5 was able to reduce microvascular density and tumor growth and increase survival in ovarian cancer xenografts [104], as well as, inhibit breast cancer xenograft growth and metastasis [105]

Genentech company also developed anti-EphB2 mAb 2H9 which antagonizes EphB2–ephrin B1 interactions and causes internalization of non-phosphorylated EphB2 in color cancer [106].

Because of the essential roles of EphB4 and ephrin B2 in embryonic vasculogenesis and tumor angiogenesis, Vasgene (California, USA) and CNIO Biotechnology (Madrid, Spain) developed mAbs for this proteins [31]. Vasgene's mAbs caused EphB4 degradation, inhibiting tumor angiogenesis and growth, and, in combination with bevacizumab, caused tumor regression in xenografted mice [107] whereas single-chain (scFv) anti-ephrin B2 antibodies reduced tumor growth by inhibiting formation of tumor blood and lymphatic vessels [108]

#### 1.5.1.6 siRNAs or antisense oligonucleotides

Other type of direct therapeutic approach developed for modulate Eph/ephrin system is the regulation of the gene expression using small interfering RNA or antisense oligodeoxynucleotides [46]

siRNAs or antisense oligonucleotides have been used for inhibit EphA2 or EphB4 responses. These molecules have the capacity of dowregulate the expression of both receptors, inhibiting the malignant cell behavior *in vitro* and tumor growth in vivo [46].

Delivery of EphA2 siRNA to tumors, using neutral liposomes, inhibits tumor growth and metastasis in mouse models of ovarian cancer. Furthermore, EphA2 siRNA can be combined with focal adhesion kinase (FAK) or with paclitaxel chemotherapy [109].

On the other hand, siRNA and antisense oligodeoxynucleotides for targeted knockdown of EphB4 expression can lead to poor survival and increased apoptosis of breast cancer cells, as well as, can suppress

tumor growth in a murine tumour xenograft model [110]. Furthermore, ephrinB2 siRNA has been administered in mice model blocking EphB1 receptor and therefore inihibiting neuropathic hyperalgesia and morphine dependence [69], as well as, EphB1 siRNA reduced drastically EphB1 receptor in DAOY cell model reducing cell migration and enhancing cellular sensitization to radiation therapy [45]

### 1.5.2 Eph receptor – binding molecules conjugated in the delivery of drugs, toxins or imaging agents

Because of the high expression of the Eph receptors in many tumors, molecules that bind Eph receptors represent attractive targets for the delivery of several molecules to cancer tissue [46]

Several chemotherapeutic drugs, toxins, deliver imaging agents, radioisotopes and nanoparticles have been conjugated to Eph or ephrin antibodies and Eph peptides with the aim of cause receptor-mediated internalization [32,46]. Furthermore, Eph receptors and ephrins could be considered as entry receptors for viruses [32].

Linear and cyclic peptides used with these conjugated molecules can enhance the selective delivery of agents to tumors overexpressing specific Eph receptors, as well as, enable tumor visualization for early detection and diagnostic purposes, monitor the effectiveness of therapy and image-guided surgery [94].

EphA2 and EphB4 are principally considered as attractive target for their widespread expression in both cancer cells and tumor vasculature [46].

EphA2-specific YSA agonistic peptide and its derivates were conjugated to paclitaxel through a triazole ester linker, enhancing the efficacy of paclitaxel in a PC3 prostate cancer mouse xenograft model without signs of toxicity but with poorly bioavility in mouse xenografts [32,94]. Furthermore, YSA peptide was fused to the homodimer p19 siRNA-binding protein to deliver functional siRNAs inside of ovarian cancer cells with high levels of EphA2, leading to siRNA-mediated gene knockdown [94].

Due to TNYL-RAW peptide possess high binding affinity by EphB4 and slow dissociation rate has been well suited for cancer imaging [94].EphB4 antibodies and the high-affinity EphB4-targeting peptide TNYL-RAW have been used to deliver imaging agents and therapeutic nanoparticles to mouse tumor xenografts

[32].EphB4-binding peptide TNYL-RAW was conjugated with polyethylene glycol (PEG) and with radioisotopes (Cu)[64] to identify EphB4-overexpressing cells in small animal biodistribution [31]. Besides, [64]Cu-DOTA-TNYL-RAW, a promising radiotracer for PET-computed tomography (PET-CT) imaging, was successfully used to image EphB4-positive PC3 prostate cancer and CT26 colon cancer cells in mouse tumor xenografts [94].

Since, nanoparticles can protect peptides of a rapid degradation and clearance from the blood circulation [94], the TNYL-RAW peptide has been conjugated with various nanoparticles for controlled its delivery to EphB4-positive cells. TNYL-RAW peptide was conjugated through a PEG linker to hollow gold nanospheres which in turn were loaded with the chemotherapeutic drug doxorubicin. After the treatment with these molecules can be seen complete regression of most tumors without systemic toxicity. Targeting EphB4 with the cTNYL-RAW peptide can enhance laser-controlled chemo photothermal therapy of tumors through a single gold nanoparticle delivery system [94]. Furthermore, TNYL-RAW peptide conjugated with polymeric micellar nanoparticles labelled with the near-infrared dye indocyanine 7 (Cy7)[111] allowed multimodal whole-body imaging of tumor-bearing mice by single- photon emission computed tomography and near- infrared microscopy, being this technique promising for the development of non-invasive strategies for visualizing tumor lesions [31].

Nanoparticles, were also used with YSA peptide which was attached through a PEG linker to polyaspartic acid and coated on anisotropic gold nanoparticles (nanorods). This molecule was explored for both aims imaging with near infrared light and photothermal cancer therapy [94]

Finally, YSA peptide can be encoded by the adenovirus genome which is considered as a vector for gene therapy and vaccination. Due to YSA contains only natural amino acids can bind adenovirus and internalized through EphA2 activation, which is promising for adenoviral transduction of EphA2-positive cancer cell [94].

### 1.5.3 Vaccine based immunotherapy

The high expression of Eph receptors in tumors have considered the possibility to use Eph-derived peptides as anticancer vaccines [32]. EphA2 and EphB6- derived peptides have been tested raising an immune response, promoting the activation of cytotoxic T-lymphocytes and destroying glioblastomas [31,32].

EphB6 peptides can induce cytotoxic T cells activation from human leukocyte antigen A2-positive (HLA-$A2^{+}$) in glioma patients whereas EphA2 peptide-pulsed dendritic cell vaccines that induce natural killer, CD4+ and CD8+ T cells which inhibit growth and metastasis in mouse EphA2+ tumor models [111,112].

EphA2 peptide has been studied in phase I/II trial in recurrent glioma, evaluating the safety of and clinical response to pulsed dendritic cell vaccination with glioma-associated antigens, demostrating measurable CD8+ T cell and clinical responses in 58% of patients. Furthermore, it has been assessed the effect of dasatinib on the immune-suppressive tumor microenvironment, in which trigger tumor cell EphA2 degradation, thereby improving CD8+ T cell recognition of cell surface EphA2 and enhancing the killing of remaining EphA2+ tumor cells [111,112].

# 2. Aim of the research

Opioid receptors are members of the G protein-coupled receptor (GPCR) family. The most important class of opioid receptors in clinic are Mu opioid receptors (MOR), which can be activated by both endogenous and exogenous opioids in central and peripheral nervous system, where they produce a significant analgesia through different signaling events such as protein-protein interactions between PKC/PKA and JNK proteins [113] as well as inhibition of adenylyl cyclase activity [113] and activation of p42/p44 MAPK phosphorylation [114].

Morphine and related Mu opioid receptors (MOR) agonists are still among the most widely used pain killers, in fact morphine is one of the most prescribed and effective drug used in the treatment of acute and chronic pain conditions [115]. However, the presence of side effects as the promotion of analgesic tolerance, dependence and hyperalgesia [116] by repetitive or prolonged MOR activation,[67] as well as the low effectiveness in chronic pain states which are accompanied by sustained glial activation and altered neuron-glia cross-talk,[117] limit their use in clinical therapies.

The cellular and molecular mechanisms involved in these phenomena are complex and may implicate several factors, as receptor desensitization [118] or activation of other receptor systems and intracellular signaling proteins, as well as neuronal alterations produced by prolonged MOR activation or after nerve injury which could activate certain molecules that are important during development but are inactivates in matured nervous system.

On the other hand, the tyrosine kinases Eph receptors and their membrane-anchored ligands ephrins are expressed in the nervous system where their interactions normally initiated by cell-cell contact eliciting a bidirectional signaling. Forward signaling produced in receptor expressing cells

and reverse signaling produced in ligand expressed cells can regulate through neuron-glia communication [18] several physiological and pathological functions.

Physiological functions as modulation of axon guidance, neurogenesis, synaptic transmission, synaptogenesis and neuronal migration in developing nervous system [8,13] and synaptic plasticity in learning and memory formation in mature nervous system [36] involve Eph/ephrin system. Moreover, alterations in Eph/ephrin system, principally type B receptors and ligands, has been implicated in different pathological processes as inhibition of neural repair after traumatic injury as well as in tolerance and dependence to opiates, and in pain states of different etiology [31] in which opioids administration and principally morphine is the major drug therapy used.

Different conditions as tissue injury, tibia bone cavity tumor cell implantation (TCI) [70] as well as different pain models including chronic constriction injury (CCI), dorsal rhizotomy [65], carrageenan, formalin [61], L5 distal crush injury [64] and microglial activation following PNL (partial sciatic nerve ligation or Seltzer model) [43] have showed the involvement of EphB/ephrin B system in the modulation of inflammatory pain, neuropathic pain, diabetic pain and bone cancer pain [66,70]. Furthermore, in these pain models it has been observed that the expression levels of ephrin-B1, ephrin-B2 and EphB1 are upregulated in dorsal root ganglia (DRG) neurons and spinal cord,[58,64,65] where the activation of EphBs receptors (forward signaling) by ephrinBs lead to peripheral sensitization characterized by the induction of thermal hyperalgesia and mechanical allodynia [71] which increase the synaptic plasticity that can induce central sensitization, converting acute nociceptive injury in chronic pain states [59].

During the last years different research groups demonstrated the key role of EphB1 receptor in different pain processes; in fact, it has been observed that after nerve injury only wild-type (EphB1+/+) mice showed thermal hyperalgesia and mechanical allodynia and in addition the

activation of EphB1 receptors by ephrinB1-Fc ligand produced a dose- and time-dependent thermal and mechanical hyperalgesia accompanied by the up-regulation of PI3K protein and c-Fos activation expression [61]. Activation of mitogen-activated protein kinases (MAPKs), particularly p42/p44 MAPK, which in turn can produce inflammatory cytokines such as IL-1β, IL-6 and TNF-α [119] was also reported.

Likewise, chronic morphine or remifentanil exposure significantly up-regulates expression of EphB1 receptor and N-methyl-d-aspartate receptor (NMDR) in mouse spinal cord;[57,69] therefore, EphB signaling has also been involved in the development of opiates dependence, analgesic tolerance, hyperalgesia and withdrawal symptoms [116].

At the moment, EphB receptor blockers as EphB1-Fc or EphB2- fc molecules [56,58] or ephrinB2 siRNA [64] or targeted mutation of EphB1 receptor (EphB1$^{-/-}$) [69] have been used with the aiming at preventing the effects of EphBs receptors in different pain states as in inflammatory and neuropathic pain, [64,69] in order to inhibit spinal activation of MAPKs, in bone cancer pain [119] induced by tumor cell implantation (TCI) alleviating mechanical allodynia and reducing the expression of spinal inflammatory cytokines and in chronic morphine or remifentanil treatment [57,67]abolishing their behavioral symptoms and neurochemical signs; all these findings suggest that the modulation of EphB1 signaling could be used as an option for pain treatment and pathological processes associated with opiates administration.

Furthermore, the activation of Eph receptor and ephrin ligands can be dependent and independent of each other through the activation of bidirectional signaling or crosstalk with other signaling systems respectively. Thus either way leading to the activation of several signaling pathways which may be implicated in a vast array of functions [30,46]. Different studies have revealed the capacity of receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) to produce

cross-communication between them (transactivation), which is an important pathway where RTKs utilize GPCR signaling molecules to transduce signals which is initiated by both and is mediated by specific kinase dependent cascades [120].

Since Lombardo Sara confirmed that EphB1 and MOR activation presented antagonist effects in the activation of p42/44 MAPK phosphorylation and considering all above-mentioned findings, the aim of the preset research is:

- To confirm that the activation of EphB1 receptor by ephrinB1-Fc ligand may counteract the morphine-mediated activation of p42/p44 MAPK phosphorylation.
- To elucidate the molecular mechanisms and signaling pathways involved in the functional crosstalk between EphB1 and MOR through their effects on p42/p44 MAPK activation.
- To determine if blocking the pathway involved in the occlusive crosstalk could lead to rescue p42/p44 MAPK phosphorylation produced by morphine.

Finally, to validate the role of EphB1 signaling in the occlusive crosstalk between EphB1 and MOR, as a potential pharmacological target considering that it could contribute to the ineffectiveness of opioid analgesics and to their side effects. Therefore, the development of molecules that can inhibit selectively EphB1 receptor could represent future innovative pain therapies.

Koolper and collaborators,[95] in fact, through phage display approaches obtained linear peptides sequences for EphB1 receptor antagonists, from which we developed four novel EphB1 peptidomimetic in order to:

- Evaluate the capacity of four peptides to block or at least reduce the intracellular signaling induced by ephrinB-induced EphB1 activation.

# 3. Materials and Methods

## 3.1 Plasmid and reagents.

EphB1 (pCMV6-AC-GFP) plasmid was purchased from OriGene Technologies (Rockville, USA). Ephrin B1- Fc was obtained from R&D System (Minneapolis, USA) and morphine hydrochloride was from Boehringer Ingelheim limited (Bracknell – UK).

EphB1 (5F10) Mouse mAb monoclonal antibody, anti rabbit-phosho p42/44 MAPK, anti rabbit p42/44 antibody and phospho-PKC (pan) (βII Ser660) antibodies were purchased from Cell Signaling Technology, whereas β-actin polyclonal antibody and peroxidase-conjugated secondary antibodies anti-rabbit or anti-mouse or anti-goat were purchased from Santa Cruz Biotechnology.

Cultures media were obtained from Thermo fisher scientific. PP2 (selective inhibitor of Src-family tyrosine kinases), Gö6983(PKCα/δ/β inhibitors), LY294002 (PI3Kα/δ/β inhibitors), Polyethylenimine branched (PEI Transfection Reagent), BSA (bovine serum albumin), TNF – α (tumor necrosis factor), Phorbol 12-myristate 13-acetate (PMA), and Forskolin were purchased from Sigma (Steinheim, Germany).

Cayman chemical cyclic AMP Elisa kit were obtained from Chaymanchem Ellsword Rd, Ann Arbor Mi, USA.

[D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO) was purchased from Bachem (Weil-am-Rhein, Germany) and [$^{3}$H]-DAMGO from GE Healthcare (Milan, Italy).

All other reagents of analytical grade, or of the highest purity available, were purchased from Sigma or Roche.

## 3.2 Cell culture.

Human embryonic kidney HEK-293 cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 1% nonessential amino acids, 1% L-glutamine and 100 U/ml antibiotic-antimycotic. Human neuroblastoma SHSY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM high-glucose) supplemented with 10% FBS, 1% L-glutamine and 100 U/ml penicillin

streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$. All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA).

## 3.3 Western Blots analysis in SHSY5Y cell model

For these experiments, we used SHSY5Y undifferentiated and differentiated cells which endogenously expressing both receptors EphB1 and Mor [121]; for the differentiation of SHSY5Y cells, we used Phorbol 12- myristate 13- acetate (PMA) at a concentration of 16 nM for 5 days.

Furthermore, undifferentiated and differentiated SHSY5Y cells were exposed to TNF-α at a concentration of 10ng/ml for 48 hours.

Hence, during this research we used three different experimental settings: SHSY5Y native cells, PMA-differentiated SHSY5Y cells and PMA-differentiated SHSY5Y cells exposed to TNF-α.

For Western blots analisys, SHSY5Y cells in the three states were incubated in serum-free medium for 16–18 h and subsequently treated with ephrinB1-Fc (EphB1 receptor soluble agonist) at a concentration of 1μg/ml and/or morphine (Mor agonist) at a concentration of 1μM. Besides, pharmacological tools as PKC, Src, PI3K inhibitors were used to selectively inhibit specific signal transducers.

Total protein lysate was obtained by homogenization cell in MAPK buffer lysis (50mM Tris-Cl, 300mM NaCl, 1mM EDTA, 1mM $Na_3VO_4$, 1mM NaF, and 10% glycerol) with 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); 15μg of total protein lysates were used to analyzed p42/44 MAPK expression, the lysates were denaturated at 95°C for 5 min and then loaded and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 20 mA for 4 hr. MagicMark™ XP Western Standard (Invitrogen) served as a molecular weight standard. Blots were transferred to nitrocellulose membranes (Whatman, Middlesex, UK) for 1.5 hour at 400 mA. Membranes were blocked with 5% BSA-TBST 0,1% for 1 hour at 23°C and incubated overnight at 4°C with the following primary antibodies: primary polyclonal rabbit anti p42/44 MAPK (1:1000) or anti phospho-p42/44 MAPK (1:1000) in 5% BSA-TBST 0,1% overnight at

4°C. Later membranes were washed 3 x 5 min in TBST 0,1% and incubated with peroxidase conjugated secondary antibodies anti-rabbit at a dilution of 1:8000, for 1 hour at 23°C and then were washed for 3×5 min in TBS-T 0,1%. Membranes were developed and analyzed as a described for Bedini and collaborators [122].

The same protocols were performed to determinate changes in protein levels of EphB1 receptor and PKC protein, where the total protein lysates were extracted by T-per buffer lysis (Pierce Thermo Fisher Scientific) with protease and phosphatase inhibitors cocktail and the homogenates were shaking for 10 minutes at 4°C and then centrifugates at 10000 g for 10 minutes at 4°C. The supernatants were collected and protein concentration was measured as above described. In the case of EphB1 protein were loaded 50 µg of total protein lysate and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis whereas for PKC protein were used 15µg of total protein lysates and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were incubated with primary monoclonal mouse anti EphB1 (1:1000) or phospho PKC (1:1000) or goat polyclonal anti actin (1:1000) overnight and then used anti– mouse or anti-rabbit or anti-goat secondary antibodies respectively.

## 3.4 Cells transfection and Western Blot analysis in HEK- 293 cell model.

HEK-293 cells (no expressing endogenously EphB1 and MOR receptors) [121] were plated in 6-well dishes and at 50–60% confluence were transiently transfected with both plasmid EphB1 (1,5 µg/well) using PEI Transfection Reagent. HEK-EphB1 cells were cultured in serum-free medium for 16–18 h and then treated with EphrinB1-Fc (1µg/ml) and/or different concentration of possible EphB1 antagonist peptides for 15 minutes. Total protein lysates were obtained by T-per buffer lysis (Pierce Thermo Fisher Scientific) with protease and phosphatase inhibitors cocktail and the homogenates were shaking for 10 minutes at 4°C and then centrifugates at 10000 g for 10 minutes at 4°C. The supernatants were collected and protein concentration was measured by BCA assay (Pierce); 15 µg of total protein lysates were used to analyzed p42/44 MAPK expression as previously explained.

## 3.5 Saturation binding assays

SH-SY5Y cells (both undifferentiated and differentiated) were used for saturation binding assays, in order to quantify MOR receptor protein levels. Cells membranes were prepared by homogenizing cells in 1 M Tris–HCl buffer, pH 7.4, containing 0,5 M EDTA, 1 mM dithiothreitol, and 1 mM benzamidine, with a Polytron homogenizer. After centrifugation (2000 rpm for 10 min at 4°C), supernatants were centrifuged (18 000 g for 30 min at 4°C) and the pellet was resuspended in 1 M Tris–HCl buffer, pH 7.4, containing 5 mM $MgCl_2$. Protein concentration was determined by BCA assay (Pierce). For saturation binding experiments, cells membranes (20µg/tube) were incubated in 100 mM Tris–HCl, pH 7.4, containing 0.3% bovine serum albumin with increasing concentrations of [$^3$H]-DAMGO (5-500 nM). Non-specific binding was determined in the presence of DAMGO (500µM). After 90 min incubation at 25°C, bound ligand was isolated by rapid filtration on Whatman GF/B filters. Filters were previous activated with 0.3% polyethylenimine and washed with ice-cold 1M Tris–HCl buffer, pH 7.4, and finally scintillation fluid for 24 h before counting. Triplicate determinations were made for each experiment. Data were analyzed as explained by Bedini and collaborators [123]

## 3.6 Total RNA preparation and real time-polymerase chain reaction (RT-PCR) analysis

Changes in mRNA levels of EphB1 and Mor receptors in SHSY5Y undifferentiated, PMA-differentiated or PMA- differentiated cells exposed to TNF- α were evaluated by RT-PCR. Cells were treated and then collected and centrifuged (500 g for 5 min) and rinsed with phosphate-buffered saline (PBS). Total cellular RNA was extracted using Trizol® reagent (Invitrogen) and digested with RNAse free DNAse (Invitrogen) for 15 min at 25 °C according to the manufacturer's instructions; 2 µg sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RT-PCR was employed for relative quantification of mRNA transcripts: the StepOne™ RT-PCR System (Applied Biosystems) was used in accordance with the

manufacturer's instructions. To amplify EphB1 cDNA, a sense primer (5′-GACTGACGATGATTACAAGTCAGAGC-3′) and an antisense primer (5′-AGATGGCCACCAAGGACACA -3′) amplifying a 101bp fragment, producing a fragment (1953-2053bp) from GenBank Accession no. NM_004441.4 at 50 µM final concentration. To amplify hMOPr cDNA, a sense primer (5'-CTGGGTCAACTTGTCCCACT-3') and an antisense primer (5'-TGGAGTAGAGGGCCATGATC-3') were used at 2.5 nM final concentration for amplifying a 146-bp fragment (327–472 bp) from GenBank Accession no. NM_000914.MOR [123]. As a control, a 169 bp fragment of the L19 ribosomal protein was amplified using a sense primer (5′-CTAGTGTCCTCCGCTGTGG-3′) and an antisense primer (5′-AAGGTGTTTTTCCGGCATC-3′) at 5 nM final concentration, producing a fragment (62–230 bp) from GenBank Accession no. BC062709. Three amplifications follow the same protocol explained by Baiula and collaborators [123].

Relative expression levels of RT-PCR products were determined using the ΔΔCt method [124]. Each sample was tested in triplicate and the mean Ct was used in the ΔΔCt equation as described by Baiula and collaborators [125].

## 3.7 cAMP measurement

The secong messenger Adenosine 3'5' cyclic monophosphate cAMP responses were examinated by using Cayman chemical cyclic AMP Elisa kit, according to the manufacturer's recommendations (Chaymanchem Ellsword Rd, Ann Arbor Mi, USA).

Cyclic AMP Elysa Kit used is based on the competition between free cAMP and cAMP-acetylcholinesterase (AChE) conjugate (cAMP tracer) for a limited number of cAMP-specific rabbit antibody binding sites which bind to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well.hen using Hellman's reagent that contain substrate of (AChE), the plate is measured spectrophotometrically at 412 nM where the intensity of colour is proportional to the amount of cAMP tracer bound to the well which is inversely proportional to the amount to free cAMP.

SHSY5Y native cells were treatment with forskolin an activator of adenylyl cyclase at 10 μM for 15 min and morphine 1 μM fo 15 min or ephrinB1-Fc 1 μg/ml for 15 min or both ligands for 15 min, then were scraped off in cold phosphate-buffered saline, centrifuged and the pellet homogenised with 0.1M HCl, and centrifuged at 10,000g for 15 minutes at 4°C to obtain the supernatant. Then 100 μL of the diluted cAMP standard solution was loaded to 96-well plates whereas 50μl of the supernatant obtained above was mixed with 50 μL of cAMP tracer and loaded to 96-well plates followed the recommendations of manufacturer's and incubated for 18 hours. All standards and samples were run in duplicate. After the incubation the plates were empty and rinse with wash buffer and then treated with Ellman's reagent for 90 -120 min then Ellman's reagent were removed and measured spectrophotometrically at 420nm. The cAMP values obtained from the absorvance measurements were converted by the total protein amount expressed as percentage of cAMP. Data analysis was performe as explained in https://www.caymanchem.com/pdfs/581001.pdf.

## 3.8 Statistical analysis

In RT-PCR sample was amplified in triplicate; in the other experiments were repeated at least three times. All data are presented as mean ± SEM for the number of experiments indicated. Statistical significance was determined by Newman–Keuls test after Anova using GraphPad Prism, version 3.0 (GraphPad Software Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

# 4. Results

In this investigation, I used two different cell models, the first human neuroblastoma cell line SHSY5Y due to is the most cell model used in neuroscience and neuroblastoma research [126]. These cells express several human-specific proteins and protein isoforms that would not be inherently present in rodent primary cultures [127], among them opioid receptors [128] and some tyrosine kinases receptors.

Previously, Lombardi S in Prof. Spampinato laboratory has evaluated and confirmed the expression of EphB1 and MOR receptors in human neuroblastoma cell line SHSY5Y and the presence of an occlusive croostalk between both receptor EphB1 and MOR which dependent of the physiological state of neuronal cells [121].

Hence, my reserach was focused in elucidated the downstream molecules and mechanism involved in the occlusive interaction, as well as, possible antagonist peptides that could inhibit the croostalk between both receptors.

## 4.1 Downstream molecules involved in occlusive crosstalk observed following the co-administration of morphine and ephrinB1-Fc ligands.

Previously, it has been explained that EphB1 agonist (ephrinB1-Fc) and MOR agonist (morphine) receptors induce a time-dependent increase of p42/44 phosphorylation only when these ligands are administered as single agents whereas their co-administration occluded p42/p44 MAPK activation [121].

Furthermore, Mu opioid receptors (MOR) eliciting a significant analgesia through different signaling events, such as, adenylyl cyclase inhibition and activation of p42/p44 MAPK phosphorylation [113,129] whereas the activation of ephrinB1/EphB1 system can produce diverse pain states through the activation of MAPK mechanism principally by the phosphorylation of p42/44 MAPK [42,59,71]. The phosphorylation of p42/44 MAPK plays an important role either in analgesia and in pain states being a convergence point for evaluating the interaction between EphB1 and MOR.

Hence, elucidate the signaling pathways involved in p42/44 MAPK modulation ephrinB1-Fc- and morphine-mediated in SHSY5Y cells is an important step for explain the appearance of occlusive crosstalk after of the co-admistration of MOR and EphB1 ligands.

### 4.1.1 Signaling pathways involved in p42/p44 MAPK activation elicited by ephrinB1 Fc- and Morphine in SHSY5Y native cells.

Inhibitors of several signaling pathways involved in p42/44 MAPK activation were used. Gö6976 (5 μM) a PKC inhibitor which only inhibits classical isoforms, PP2 (5 μM) a selective inhibitor of Src-family tyrosine kinases and LY294002 (10μM) a reversible competitive PI3K inhibitor were administered before ephrinB1-Fc or morphine stimulation.

SHSY5Y cells starved for 16-18 hours, were pre-treated for 30 min with PKC or Src inhibitors and 60 min with PI3K inhibitor before of the stimulation of MOR and EphB1 receptors by morphine (1μM) and Ephrin B1-Fc (1μg/mL) ligands respectively for 15 min. By western blots, I have evaluated p42/44 MAPK phosphorylation using anti p-p42/44 and anti p42/44 antibodies.

Thus, I observed that p42/44 MAPK activation EphB1-mediated was not abrogated by the PKC inhibitor (Gö6976) and Src inhibitor (PP2) while PI3K inhibitor LY294002 was able to inhibit p42/44 MAPK activation. This suggest that p42/p44 MAPK activation by ephrinB1-Fc is PKC- and Src-independent but PI3K signaling pathway plays an important role in p42/44 activation. (Figure 13a)

Conversely, p42/44 MAPK activation MOR-mediated was abrogated by the PKC inhibitor (Gö6976), but not by Src inhibitor (PP2) and PI3K inhibitor (LY294002) suggesting that PKC signaling pathway contributes in p42/p44 MAPK activation by morphine, whereas Src and PI3K are not involved in this pathway (Figure 13 b).

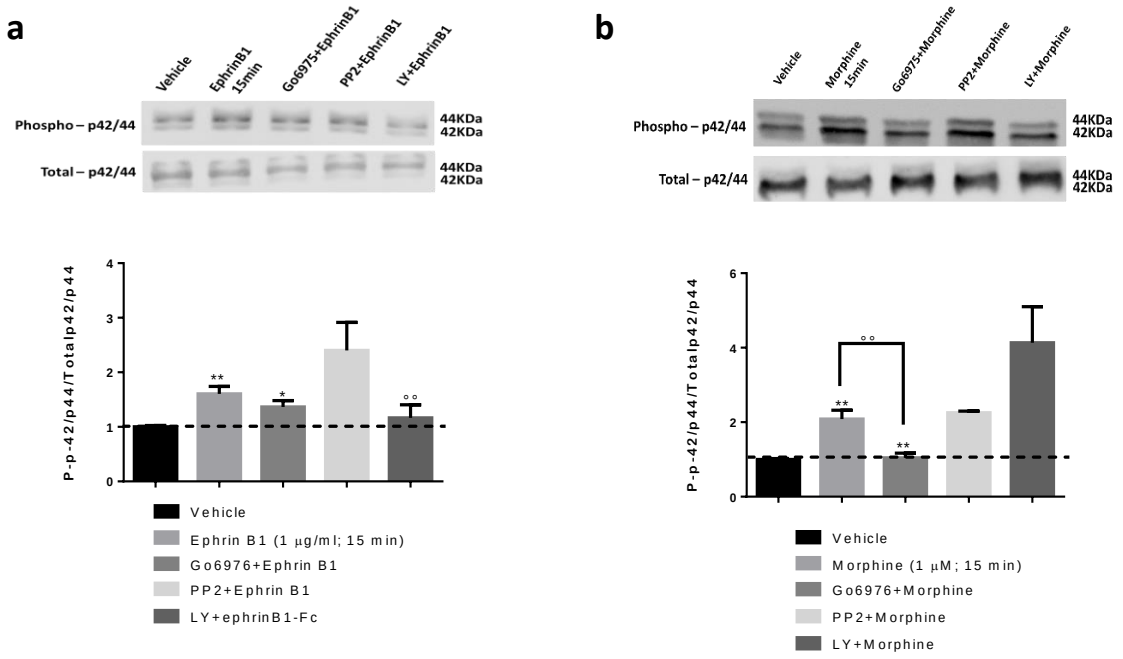


**Figure 13: Signaling pathway involved in p42/p44 MAPK activation by ephrinB1-Fc and Morphine. (a)** Western blot analysis of p42/44 MAPK phosphorylation levels in SHSY5Y native cells treated with Gö6976 (5 mM) or PP2 (5 mM) 30 min and LY294002 60 min prior ephrinB1-Fc administration (1µg/ml; 15 min). **(b)** Western blot analysis of p42/44 MAPK phosphorylation levels in SHSY5Y native cells treated with Gö6976 (5 mM) or PP2 (5 mM) 30 min and LY294002 60 min prior morphine administration (1µM; 15 min). SHSY5Y native cells were considered as control group. Densitometric analysis of the bands is shown (mean ± SEM of five independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. **=p<0.01 vs vehicle, *=p<0.05 vs vehicle; °°=p<0.01 vs ephrinB1-fc or morphine.

### 4.1.2 PI3K signaling pathway is involved in the occlusive croostalk produced by the co-administration of morphine and ephrinB1-Fc ligands.

The occlusive croostalk observed by the concomitant administration of both agonist (morphine and ephrinB1-Fc) lead to reduce p42/44 MAPK phosphorylation, likely due to the activation of ephrinB1/EphB1 system [121]. Therefore, blocking the interaction between EphB1 receptor with Ephrin B1 ligand could restore the principal signaling pathways involved in the analgesic effects produced by MOR agonist as morphine.

Since previously have found that morphine can activate p42/44 MAPK via PKC pathway and that the activation of p42/44 MAPK ephrinB1-Fc mediated is PI3K-dependent, SHSY5Y native cells were treated

with PKC and PI3K inhibitors to evaluate the effects of both inhibitors in the occlusive croostalk previously evidence.

By western blot analysis I assessed p42/44MAPK proteins expression. SHSY5Y cells starved for 16-18 hours were pre-treated with PKC inhibitor for 30 min and PI3K inhibitor for 60 min before concomitant administration of morphine and ephrinB1-Fc ligands for 15 min. I observed that the co-administration of ephrin-B1-Fc and morphine does not show a synergistic effect caused by EphB1- and MOR-activated signaling pathways on p42/44 MAPK phosphorylation (Figure 14); thus, confirming previous results.

Futhermore, the inhibition of PKC signaling pathway by Go6976 inhibitor is not able to abolish the effect produced by the co-admistration of both ligands whereas when PI3K signaling pathway was inhibited by LY294002 interestengly the occlusive croostalk produced is completely abolished and the effects of morphine in the activation of p42/44 MAPK seem to be restored (Figure 14). Thus, suggesting that the activation of EphB1 could block the normal activation of MOR receptor producing the occlusive crosstalk.

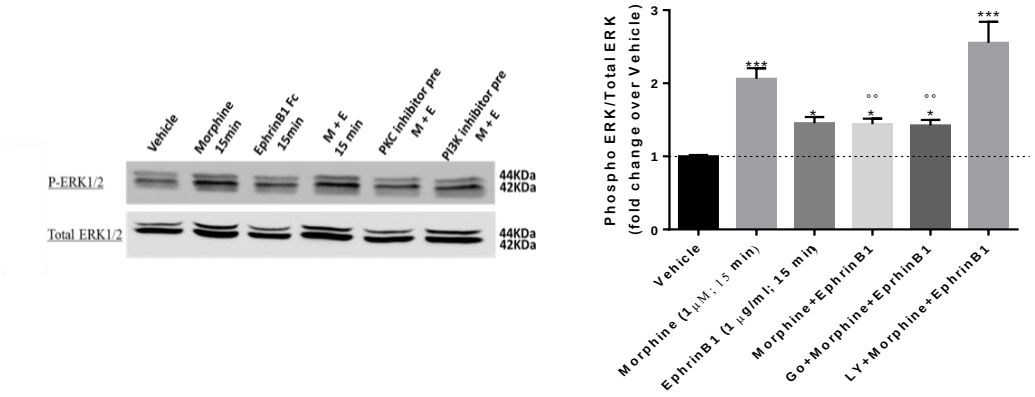


**Figure 14: Blocking PI3K signaling pathways restores the effects of morphine in the activation of p42/44 MAPK**. Representative western blots of the modulation p42/44 MAPK phosphorylation in SHSY5Y native cells treated with Gö6976 (5 mM) 30 min and LY294002 60 min prior to the co-administration of EphrinB1-Fc (1µg/ml; 15min) and morphine (1µM; 15 min). Densitometric analysis of the bands are shown (mean ± SEM of sixteen independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. SHSY5Y native cells were considered as control group. n=16, *** = $p<0.001$ vs vehicle, * = $p<0.05$ vs vehicle and °°= $p<0.01$ vs morphine.

### 4.1.3 Blocking PI3K signaling pathway also can recovers completely the activation of PKC protein.

Morphine can activate p42/44 MAPK phosphorylation via the protein kinase C (PKC)-dependent pathway [130] regulating Mu opioid receptor signaling [131].

We explored the involvement of ephrinB1/EphB1 system in the modulation of PKC protein. Thereby, by western blot analysis I assessed the changes in PKC phosphorylation when PI3K signaling pathway was inhibited.

SHSY5Y cells starved for 16-18 hours were pre-exposed to LY294002 inhibitor of PI3K pathway 60 min before of the treatment with ephrinB1-Fc (1μg/ml) or morphine (1 μM) or with both ligands for 15 min. I observed that, as well as p42/44 MAPK phosphorylation is restored when PI3K signaling pathway is inhibited, also PKC phosphorylation was totally recovered in comparison to cells co-treated with morphine and ephrinB1 ligands (Figure 15), suggesting that the co-activation of EphB1 MOR receptors inhibit the effects of morphine on mor receptors.

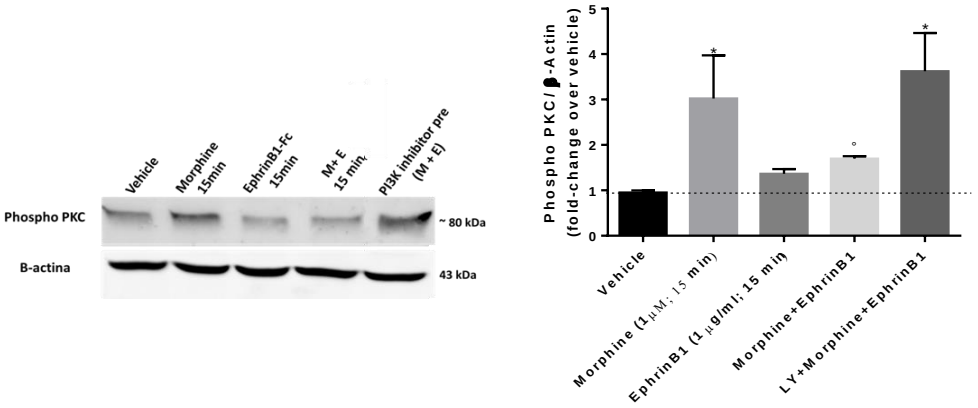


**Figure 15: Blocking ephrinB1/EphB1 pathway restored PKC phosphorylation.** Representative western blot analysis of PKC phosphorylation in SHSY5Y native cells treated with LY294002 60 min prior co-administration of EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min). Densitometric analysis of the bands is shown (mean ± SEM of three independent experiments); the amount of PKC protein was normalized to β-actin protein. SHSY5Y native cells were considered as control group. n=3, * = $p<0.05$ vs vehicle and °= $p<0.01$ vs morphine.

### 4.1.4 cAMP expression is involved in antagonist interaction between MOR and EphB1 receptors.

I have determinated thereafter, if the activation of ephrinB1/EphB1 system is involved in the block of morphine effects on MOR receptors. However, the mechanism by which this block could be produced is unknown.

To assay, one of the possible mechanism contributing to the lack of morphine effects on MOR receptors and considering that morphine reduced cAMP levels through the stimulation of Gi protein α-subunit [132] and that intracellular cAMP changes are mediated through the modulation of adenylate cyclase activity, I have decided to evaluate changes in cAMP content in cells after treatments.

I used Forskolin an activator of adenylate cyclase enzyme that can generates cAMP from ATP, raising intracellular cAMP levels. Thus, as showed in Figure 16, in SHSY5Y native cells forskolin (10μM, 15 min) increased the expression of cAMP whereas morphine reduced the forskolin effects. Treatment with ephrinB1-Fc does not produce significant changes in cAMP, but interestingly when the cells were co-treated with both ligands (morphine and ephrinB1) was produced an increase in the levels of cAMP. This effect probably is produced due to morphine does not capable to inhibit adenylate cyclase likely by the activation of ephrinB1/EphB1 system.

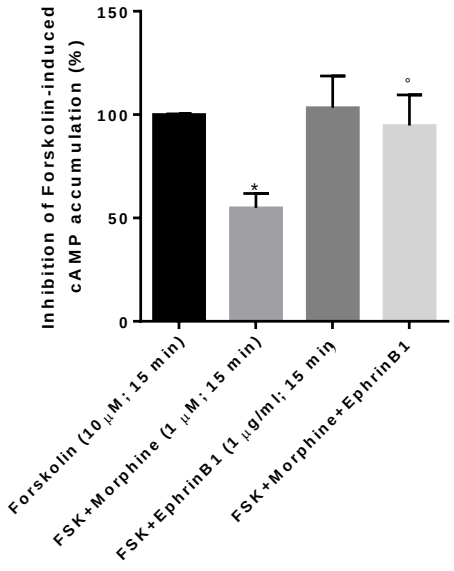


**Figure 16: Inhibition of cAMP accumulation induced by forskolin is involved in occlusive crosstalk produced by the co-administration of morphine and ephrinB1-Fc ligands**. SHSY5Y native cells were treated with forskolin (10μM, 15min) and morphine (1μM, 15min) or ephrinB1-Fc (1μg/ml, 15min) or both ligands. cAMP levels were measured using Cayman chemical cyclic AMP Elisa kit according to the manufacturer's recommendations. The results are presented as percentage of inhibition of cAMP accumulation. n=3, * = $p<0.05$ vs vehicle and °= $p<0.05$ vs morphine.

## 4.2 Occlusive crosstalk in PMA-differentiated SHSY5Y cells.

SHSY5Y cells can be differentiated to mature neuron-like phenotype [127] and for this reason are considered a suitable cell model for studying neuronal differentiation [133]. In fact depending on media conditions can express different phenotypes such as cholinergic or dopaminergic/adrenergic [133]. Phorbol 12-myristate 13-acetate (PMA), a differentiation agent, can stimulated SHSY5Y neuroblastoma cells towards neuronal differentiation by blocking cell growth and inducing neurite outgrowth [134,135].

Lombardi S, previously evaluated the effects of the co-administration of morphine and ephrinB1-Fc in PMA-SHSY5Y differentiated cells. She observed that the conconmitant administration of ephrin-B1-Fc and morphine ligands no longer occludes p42/p44 MAPK activation [121]. Furthermore, cell differentiation produced changes in mRNA and protein levels of MOR and EphB1 receptors, the levels of Mor receptors were increased in comparison to SHSY5Y native cells whereas the levels of EphB1 receptors either in mRNA and protein were drastically decreased [121].

Hence, in agreement whith this previous research and considering that in SHSY5Y native cells the inhibition of PI3K pathway lead to restore p42/44 MAPK and PKC phosphorylation morphine-mediated I decided to investigate the effect of inhibit PI3K pathway after differentiation with PMA for five days.

### 4.2.1 Blocking PI3K signaling pathway in PMA-differentiated SHSY5Y cells does not affect p42/44 and PKC phosphorylation.

After differentiation with PMA (16nM) for 5 days, SHSY5Y- differentiated cells were starved for 16-18 hours in serum free medium and then exposed to ephrinB1-Fc (1μg/ml) or morphine (1 μM), or both ligands for 15 min and pre-exposed to LY294002 inhibitor of PI3K pathway 60 min before of the treatment with ephrinB1-Fc (1μg/ml) or morphine (1 μM) for 15 min.

By western blot analysis of p42/44 MAPK and PKC proteins, I observed that p42/44 MAPK phosphorylation was increased by co-treatment with both ligands which could be due to high transcription and protein expression of MOR receptors and low expression of EphB1 receptors (Figure 17a), confirming the results observed by Lombardi, S, as the administration of ephrinB1-Fc to PMA-differentiated SHSY5Y cells no longer occludes morphine-mediated activation of p42/p44 MAPK phosphorylation.

Besides, blocking PKC and PI3K pathways under co-administration of both ligands it was showed that the inhibition of PKC pathway does not allow full activation of MOR receptor; obviously, MOR activation is PKC-dependent whereas inhibition of PI3K pathway does not alter the normal activation of p42/44 MAPK phosphorylation by morphine (Figure 17a) since as I have described before the lower expression of EphB1 receptor is not able to block MOR activation.

A similar trend was observed by western blots of PKC phosphorylation (Figure 17b), where the downregulation of EphB1 receptor following the inhibition PI3K pathway does not modificate MOR activation after the co-administration of morphine and ephrinB1-Fc ligands, thus, allowing me to think that the activation of EphB1/Ephrin B1 system is really involved in the low effectiveness of the MOR agonist.

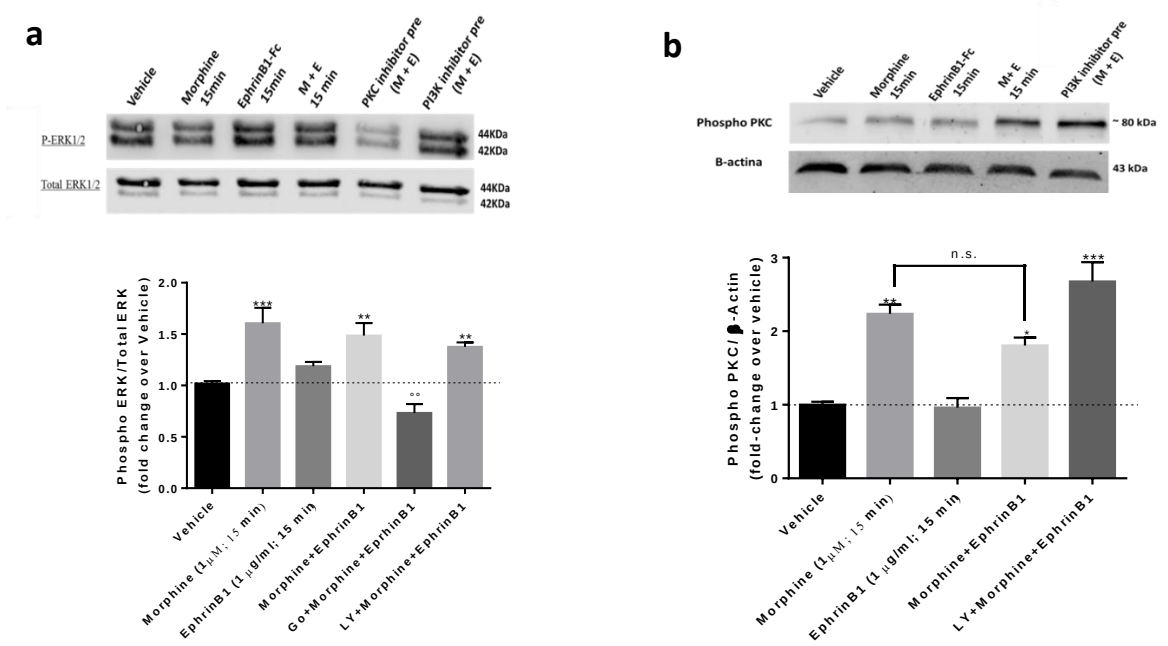


**Figure 17: p42/44 MAPK and PKC phosphorylation are not altered by the co-administration of morphine and ephrinB1-Fc ligands. a)** Representative western blot analysis of p42/44 MAPK phosphorylation in PMA-differentiated SHSY5Y cells treated with ephrinB1-Fc (1μg/ml) or morphine (1 μM), or both ligands for 15 min and pre-exposed to Gö6976 (5 mM) 30 min and LY294002 60 min to the co-administration of EphrinB1-Fc (1μg/ml and morphine (1μM) for 15 min **b)** Western blot analysis of PKC phosphorylation in PMA-differentiated SHSY5Y cells treated with ephrinB1-Fc (1μg/ml) or morphine (1 μM), or both ligands for 15 min and pre-exposed to LY294002 60 min prior to the co-administration of EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min). Densitometric analysis of the bands is shown (mean ± SEM of six independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK whereas the mounts of PKC protein were normalized to β-actin protein. PMA-differentiated SHSY5Y cells were considered as control group n=6, ***= $p<0.001$ vs vehicle, ** = $p<0.01$ vs vehicle, * = $p<0.05$ vs vehicle and °°= $p<0.01$ vs morphine.

## 4.3 Occlusive crosstalk in PMA-differentiated SHSY5Y cells exposed to a pro-inflammatory stimulus

So far, I have observed that the concomitant activation of EphB1 and MOR receptor block p42/44 MAPK and PKC activation in SHSY5Y native cells. However, the inhibition of a main signaling pathway involved in the EphB1 receptor activation can abolish the block of the activation of MAPK and PKC phosphorylation. Since SHSY5Y native cells can be exposed to a differentiating stimulus in a celular model resembling mature neurons *in vivo* and that under this condition the activation of EphB1 receptor is not capable to block morphine effects, I decided to further assess the occlusive croostalk in PMA-differentiated

SHSY5Y cells by the administering tumor necrosis factor (TNF-α), a proinflammatory stimulus, which play a key role in development of central sensitization and persistence pain [117,136].

### 4.3.1 MOR and EphB1 expression is altered in PMA-Differentiated SHSY5Y cells exposed to TNFα.

In order to assess any significant change in mRNA and protein levels of EphB1 and MOR receptors in PMA-differentiated SHSY5Y, cells were exposed to pro-inflammatory stimuli for mimicking the neuroinflammatory response that contributes to different persistence pain states among them neuropathic pain.

For this reason, SHSH5Y cells during the last 48 h of differentiation were treated with TNF-α at concentration of 10 ng/ml. Then the expression of EphB1 and MOR transcripts were measured by Real time PCR (as explained in materials and methods) whereas protein levels of EphB1 and MOR were analyzed by western blot and saturation binding assay, respectively.

I observed that, the exposure of TNF-α (10 ng/mL) to PMA-differentiated SHSY5Y cells produces the alteration of EphB1 and MOR receptors in comparison to SHSY5Y native cells and differentiated SHSY5Y cells.

As regards, EphB1 receptors it was observed that the exposure of TNF-α up-regulate dramatically the levels of mRNA and protein in comparison to control cells (Figure 18a, 18b).

Whereas, transcript Mor levels were more up-regulated in comparison to SHSY5Y native and differentiated cells and their protein levels present the same trend of increase that in PMA-differentiated cells (Figure 18c, 18d).

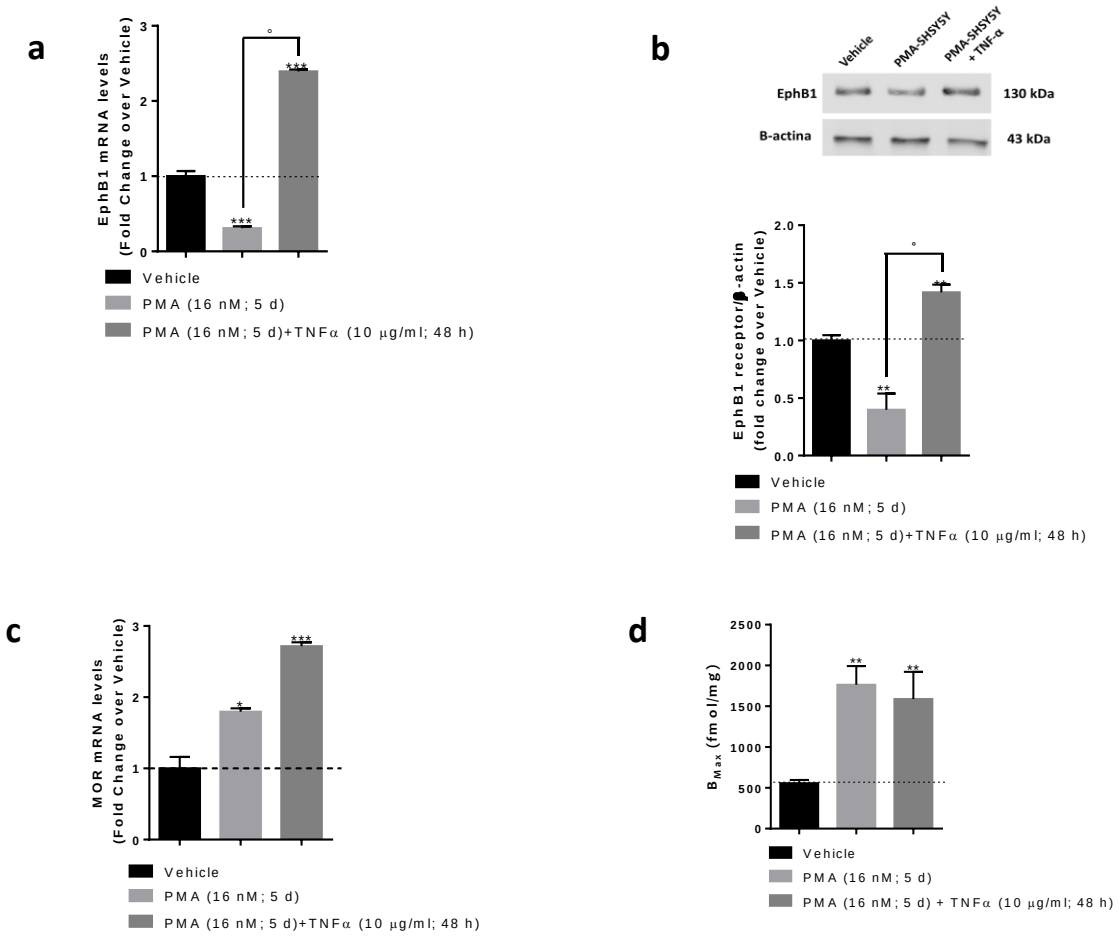


**Figure 18: Exposition to TNF-α produced changes in EphB1 receptor and MOR expression in PMA-differentiated SHSY5Y cells a)** Real-time PCR of EphB1 in PMA- differentiated SHSY5Y cells exposed to TNF-α, mRNA levels of EphB1 receptor were significantly increased in comparison with SHSY5Y native cells and PMA – differentiated SHSY5Y cells **b)** EphB1 protein levels present the same pattern **c)** mRNA levels of MOR receptors are significantly increased **d)** while MOR protein levels present the same pattern that PMA-differentiated cells. mRNA levels of EphB1 and MOR were calculated using the ΔΔCt method as described in materials and methods. For protein levels of EphB1 was performed a densitometric analysis of the bands and the amount of EphB1 receptors were normalized to β-actin. For MOR protein levels was performed a Saturation binding assays which were conducted using [$^3$H] DAMGO on cell membranes, a single-site receptor binding model provided the best fit for data analysis, Bmax values were estimated from non-linear regression analysis. Values are mean ± SEM of at least six independent experiments. SHSY5Y native cells were considered as control group *** = p<0.001, ** = p<0.01, * = p< 0.05 vs undifferentiated cells; ° = p< 0.01 vs PMA- differentiated cells; n=6

### 4.3.2 Occlusive crosstalk occurs in PMA-differentiated SHSY5Y cells exposed to TNF-α.

Previously, I have seen that exposition of SHSY5Y- differentiated cells to pro-inflammatory stimuli as TNF-α, has altered the expression of EphB1 and MOR receptors, mainlly EphB1 expression was dramatically up-regulated in comparison to SHSY5Y undifferentiate and differentiate cells.

To evaluate, if the occlusive croosstalk could be restored since EphB1 receptors expression are more up-regulated in comparison that SHSY5Y native cells, SHSY5Y- differentiated cells exposed to TNF-α were starved for 16-18 hours and then treated with ephrinB1-Fc (1μg/ml) or morphine (1 μM), or both ligands for 15 min.

By western blots analysis, I observed that the co-administration of both ligands led to restore the occlusive crosstalk in p42/44 MAPK activation (Figure 19), due to the high EphB1 receptors expression, thus, confirming once again that ephrinB1/EphB1 system activation is involved in p42/44 MAPK inhibition when is activated at the same time that MOR receptors.

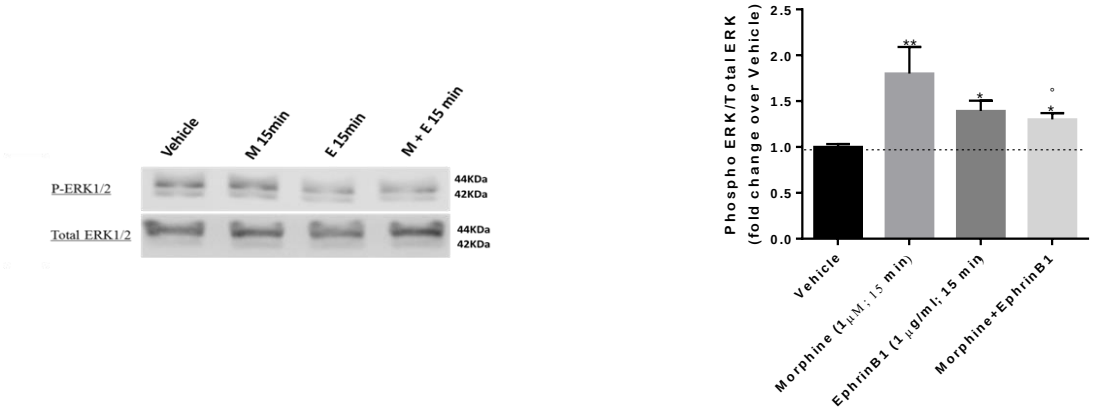


**Figure 19**. **Ephrin B1 Fc– mediated occlusion on p42/p44 MAPK phosphorylation is restored, in PMA-differentiated SHSY5Y cells exposed to TNF – α.** Western blot analysis of p42/44 MAPK phosphorylation levels in PMA-differentiated SHSY5Y cells exposed to TNF-α, un-treated or treated with morphine (1μM; 15 min) EphrinB1-Fc (1μg/ml; 15min), or co-treated with both EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min). PMA-SHSY5Y cells exposed to TNF-α were considered as control cells. Densitometric analysis of the bands is shown (mean ± SEM of four independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. * = $p<0.05$ vs vehicle; n=4

### 4.3.3 TNF-α added to PMA-differentiated SHSY5Y cells blocks the signaling pathway involved in ephrinB1/EphB1 system activation and recues p42/44 MAPK and PKC activation produced by morphine.

Previously, I have seen that the exposition of PMA-differentiated cells to TNF-α restored the antagonist interaction between MOR and EphB1 receptors as those in native SHSY5Y cells.

I decided to employ PMA-differentiated SHSY5Y exposed to TNF-α cells for evaluate if the inhibition of PI3K pathway may also to restore p42/44 MAPK and PKC phosphorylation.

To this aim, SHSY5Y differentiated cells exposed to TNF-α were starved for 16-18 hours and pre-treated whith PI3K inhibitor for 60 min before of the concomitant administration of morphine and ephrinB1-Fc ligands for 15 min. Then I assessed the modulation of p42/44MAPK and PKC proteins by western blot analysis.

I observed that likewise in SHSY5Y native cells, blocking the signaling pathway responsible of EphB1 activation allowed the complete activation of p42/44 MAPK (Figure 20a) and PKC proteins (Figure 20b) which are important downstream proteins involved in analgesics effects of morphine.

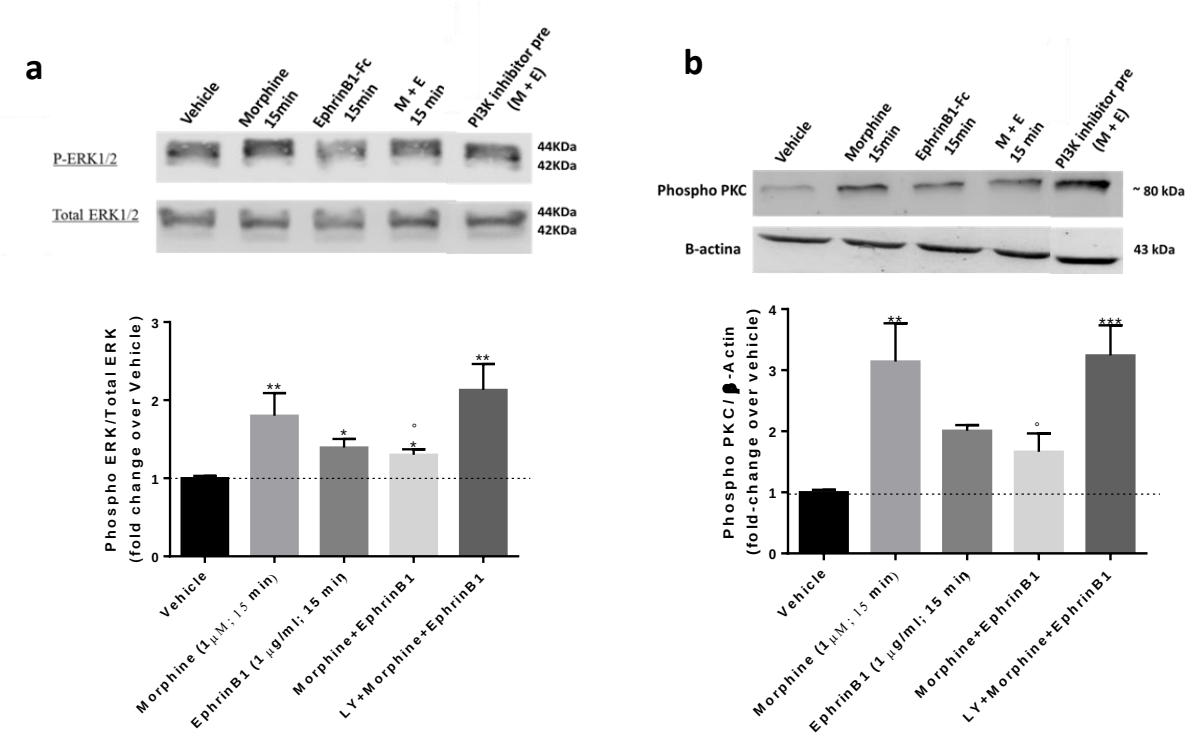


**Figure 20**. **Blocking PI3K pathway in PMA-differentiated SHSY5Y cells exposed to TNF – α the normal activation of p42/44 MAPK and PKC proteins were restored**. **a)** Western blot analysis of p42/44 MAPK phosphorylation levels in PMA-differentiated SH-SY5Y human neuroblastoma cells exposed to TNF-α, treated with morphine (1μM; 15 min) or EphrinB1-Fc (1μg/ml; 15min), or co-treated with both EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min) and pre-exposed to LY294002 60 min to the co-administration of EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min) **b)** Western blot analysis of PKC phosphorylation in PMA-differentiated SHSY5Y cells exposed to TNF-α treated with ephrinB1-Fc (1μg/ml) or morphine (1 μM) , or both ligands for 15 min and pre-exposed to LY294002 60 min prior to the co-administration of EphrinB1-Fc (1μg/ml; 15min) and morphine (1μM; 15 min). Densitometric analysis of the bands is shown (mean ± SEM of four independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK whereas the mounts of PKC protein were normalized to β-actin protein. PMA- differentiated SHSY5Y cells exposed to TNF- α were considered as control group. n=4, ***= $p<0.001$ vs vehicle, ** = $p<0.01$ vs vehicle, * = $p<0.05$ vs vehicle and °= $p<0.05$ vs morphine.

## 4.4 Blockade of EphB1 receptor activation by novel peptide antagonist

So far, we have observed that the inhibition of signaling pathways involved in EphB1 receptor activation ephrinB1-Fc-mediated restored morphine effects on MOR receptors.

Several studies have demonstrated that EphrinBs/EphBs signaling pathway is a key regulator of physiologic and pathologic processes underlying pain and other CNS diseases; thus, suggesting that may act as a new molecular target for pain prevention and relief [70]. Particularly, it has been demonstrated that reducing EphB1 expression can alleviate spontaneous pain, thermal hyperalgesia, mechanical allodynia, and opiate-resistant

pain in rodent models suggesting that antagonist targeting EphB1 receptor could represent a novel class of analgesics for the treatment of difficult-control chronic pain [32].

In this context, the development of agents that bind to the extracellular ephrin-binding pocket of these receptors show promise for medical applications. Koolper and collaborators through phage display found different sequence of peptides which have the capacity to bind EphB receptors selectively. I selected EWLSPNLAPSVR linear sequence, because present 100% specificity to EpB1 receptor [95], and from this linear sequence, four different sequence of linear peptide were synthetized by Profesor Alessandra Tolomelli at the Department of Chemistry of the University of Bologna. These peptides were characterized by evaluating their ability to modulate EphB1 receptor either as a single agent or with the previous administration of EphB1 agonist (ephrinB1-Fc).

Considering that the activation of p42/44 MAPK in response to EphB1 stimulation is involved in several pathological processes, I retained important firstly, to assay the ability of these peptides to modulate p42/44 MAPK phosphorylation by itself and to counteract ephrin B1-Fc-mediated activation.

For tested these peptides I used human embryonic kidney cell line (HEK-293) due to has been greatly used as an expression tool for recombinant protein and also for its incredible use in stably transfected forms to study a variety of cell-biological questions in neurobiology [137].

### 4.4.1 Administration of EphB1 binding peptides in HEK-EphB1 cells do not activate p42/44 MAPK activation.

I tested four different linear sequence peptides EWL or Tripeptide, SPNLA or Pentapeptide, EWLSPN or Hexapeptide and EWSPNLA or Octapeptide.

HEK-EphB1 stably transfected cells were plated in 6-well dishes until 50–60% of confluence and then starved of serum for 16-18 hours and treated for 15 min with different concentrations of each peptide.

I observed that none of these compounds have the capacity to activate or inhibit p42/44 MAPK phosphorylation on its own (Figure 21 a, b, c, d).

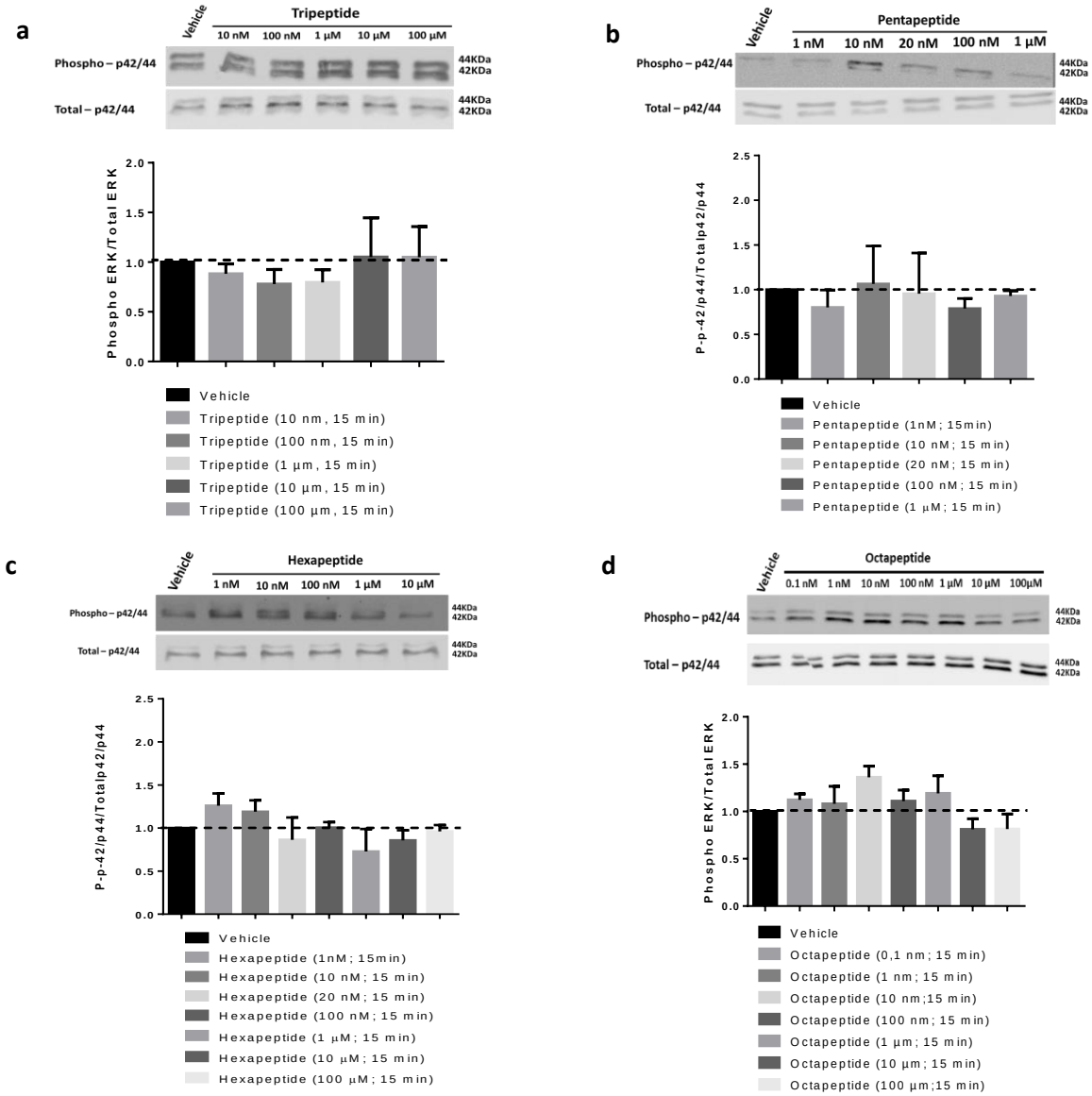


**Figure 21. Effects of the different linear peptides on p42/44 MAPK phosphorylation when are administered as a single agent in HEK-EphB1 cells**. Representative Western blot analysis of the effects of four different linear peptides in p42/44 MAPK phosphorylation levels. HEK-EphB1 cells treated for 15 min with different concentration of **a)** EWL tripeptide, **b)** SPNLA pentapeptide, **c)** EWLSPN hexapeptide, **d)** EWLPNLA octapeptide. HEK-EphB1 cells were considered as control cells. Densitometric analysis of the bands is shown (mean ± SEM of four independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. n=4.

### 4.4.2 Effects of Tripeptide and pentapeptide on p42/44 MAPK phosphorylation mediated by ephrinB1-Fc in HEK-EphB1 cells.

Previuos studies have demonstrated that EphrinB1-Fc induced hyperalgesia accompanied by activation of MAPKs (mitogen-activated protein kinases -dependent), mainly p42/44 MAPK proteins,[58,71] and that previously Lombardi Sara has observed that the exposure for 15 min of HEK-EphB1 cells to Ephrin B1 Fc (1μg/ml) produce a great activation of p42/44 MAPK [121]. I evaluated if the first two peptides synthesized possess the ability to counteract the effect of ephrinB1-Fc in the p42/44 MAPK phosphorylation.

HEK-EphB1 stably transfected cells were plated in 6-well dishes until 50–60% of confluence and then starved of serum for 16-18 hours and pre-treated for 15 min with different concentration of tripeptide and pentapeptide and then treated with ephrinB1-Fc (1μg/ml) for 15 min.

By western blot, the effects of both peptides in the modulation of p42/44MAPK phosphorylation were evaluated.

I have observed in both cases an increase in the phosphorylation of p42/44 MAPK due to the effect of ephrinB1-Fc, in other words tripeptide (Figure 21a) and pentapeptide (Figure 21b) did not counteract ephrinB1-Fc-mediated activation of p42/p44 MAPK.

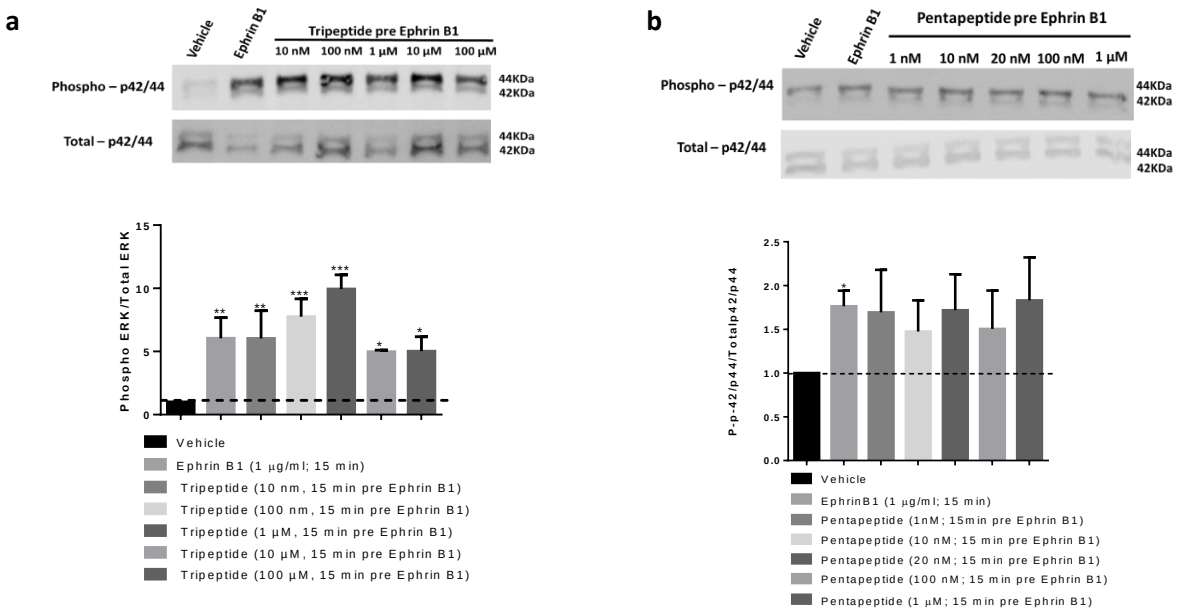


**Figure 21. Effects of tripeptide and pentapeptide on p42/44 MAPK phosphorylation mediated by EphrinB1-Fc in HEK-EphB1 cells.** Representative Western blot analysis of p42/44 MAPK phosphorylation levels HEK-EphB1 cells treated or untreated with ephrinB1-fc (1µg/ml) for 15 min and differenten concentration of **a)** Tripeptide, **b)** Pentapeptide for 15 min. HEK-EphB1 cells were considered as control cells. Densitometric analysis of the bands is shown (mean ± SEM of three independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. n=3. ***= $p<0.001$ vs vehicle, ** = $p<0.01$ vs vehicle, * = $p<0.05$ vs vehicle.

### 4.4.3 Effects of hexapeptide on p42/44 MAPK phosphorylation mediated by ephrinB1-Fc in HEK-EphB1 cells

The peptide sequence of the hexapeptide is derivative from EWLSPNLAPSVR which selectively can inhibit EphB1 receptor with $IC_{50}$ of 10µM [95]. By using HEK-EphB1 cells, I evaluated if this peptide may modulate ephrinB1-Fc-mediated p42/44 MAPK phosphorylation.

Cells were pre-incubated for 15 min with various concentrations of hexapeptide and then treated with ephrinB1-Fc at 1µg/mL for further 15 min to activate EphB1 receptor (Figure 22a). By western blot analysis, I observed that hexapeptide inhibits p42/44 MAPK phosphorylation ephrinB1-Fc-mediated at higher concentration. Indeed, hexapeptide can counteract ephrinB1-Fc-mediated activation of p42/44 phosphorylation with $IC_{50}$ 1,21 µM (Figure 22b).

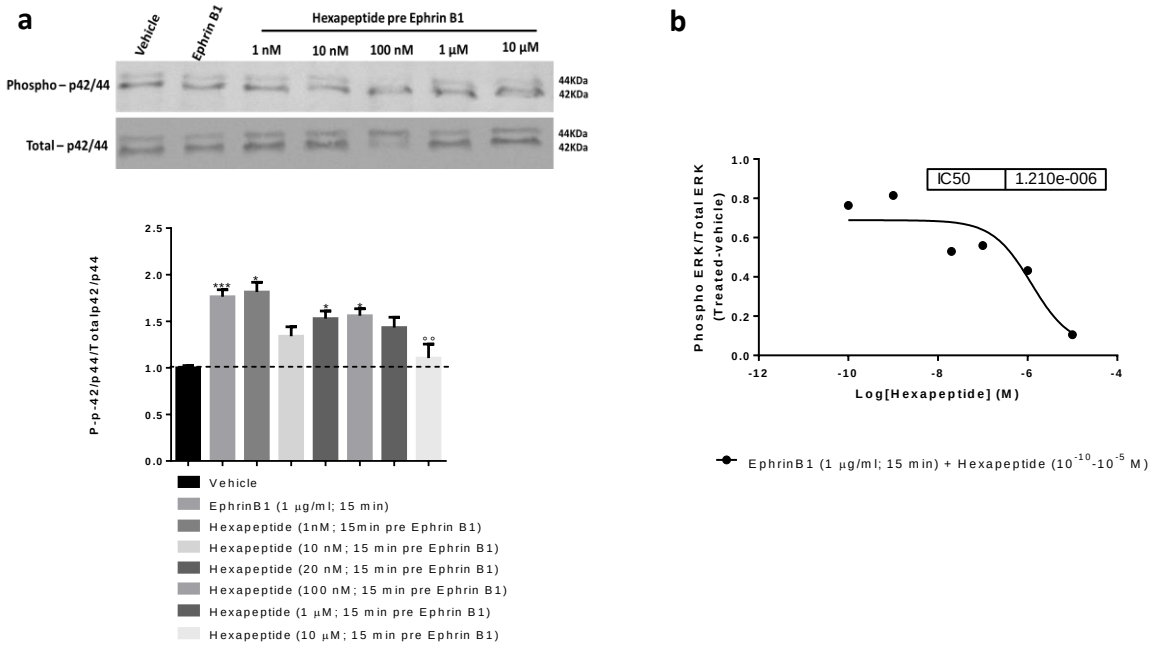


**Figure 22. Effects of hexapeptide on p42/44 MAPK phosphorylation mediated by EphrinB1-Fc in HEK-EphB1 cells. a)** Representative Western blot analysis of p42/44 MAPK phosphorylation levels, cells were pre-treated with ephrinB1-Fc (1µg/ml; 15 min) with or without different concentration of hexapeptide for 15 min. HEK-EphB1 cells were considered as control, **b)** $IC_{50}$ value for hexapeptide**.** Densitometric analysis of the bands is shown (mean ± SEM of seven independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. n=7. ***= $p<0.001$ vs vehicle, * = $p<0.05$ vs vehicle and °° = $p<0.01$ vs ephrinB1.

### 4.4.4 Effects of octapeptide on p42/44 MAPK phosphorylation mediated by ephrinB1-Fc in HEK-EphB1 cells.

Previously, I observed that tripeptide and pentapeptide does not inhibit the effects of ephrinB1-Fc in p42/44 MAPK phosphorylation whereas that hexapeptide at a 1,21 µM of concentration inhibits this activation. Then, I decided to assay larger peptide than hexapeptide named octapeptide.

HEK-EphB1 cells were subjected to dose-response experiments in which cells were incubated for 15 min to various concentration of octapeptide and treated for 15 min with ephrinB1- Fc (1 µg/mL) to monitor the possible antagonistic activity of the peptide.

By western blot analysis, I observed that p42/44 MAPK phosphorylation decreases whenever the peptide concentraction increases (Figure 23a) demonstrating that effectively longer peptide is more potent antagonist than hexapeptide with $IC_{50}$=0,76µM (Figure 23b).

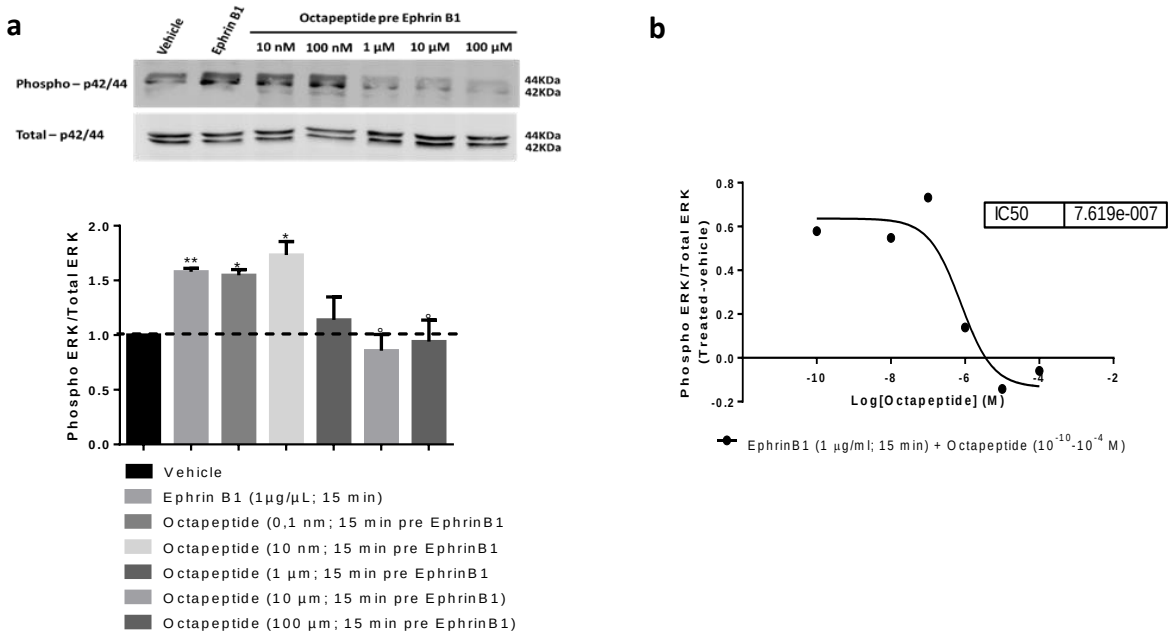


**Figure 23. Effects of octapeptide on p42/44 MAPK phosphorylation mediated by EphrinB1-Fc in HEK-EphB1 cells. a)** Representative Western blot analysis of p42/44 MAPK phosphorylation levels, cells were pre-treated with ephrinB1-Fc (1µg/ml; 15 min) with or without different concentration of octapeptide for 15 min. HEK-EphB1 cells were considered as control cells **b)** $IC_{50}$ value for hexapeptide**.** Densitometric analysis of the bands is shown (mean ± SEM of four independent experiments); the amount of p42/44 MAPK were normalized to total p42/44 MAPK. n=4. **= $p<0.01$ vs vehicle, * = $p<0.05$ vs vehicle and ° = $p<0.05$ vs ephrinB1.

# 5. Discussion

This investigation report for the first-time evidence that support the involvement of EphB1 receptor signaling in an occlusive crosstalk with mu opioid receptors (MOR), which could be implicated in the ineffectiveness of analgesic effects morphine-mediated as well as in the onset and maintenance of different chronic pain states and in the development of dependence and tolerance to opioids. Furthermore, I have found two possible EphB1 receptor peptide antagonists which may provide a novel strategy to reduce the negative impact of ephrin system activation on pain perception and opioid efficacy.

EphB/ephrinB system possess the capacity to produce bidirectional signals either pre- or postsynaptically, activating a wide variety of signal transduction pathways[138] which through neuron-glia communication [60,59,62] can communicate with a variety of other cell surface proteins.
In the mature nervous system, the upregulation of ephrinB and EphB1 receptor in nociceptive dorsal root ganglion (DRG) neurons and in spinal dorsal horn (DH) neurons after nerve injury and bone cancer, as well as after the long exposure to opiates, have been involved in various types of pain; even to those accompanied by long-lasting alterations of the excitability of spinal neurons, [69,65,67] and in physical dependence and tolerance to opiates respectively [70].
Activation of EphB1 receptors by ephrinB1-Fc ligand produce a dose- and time-dependent thermal and mechanical hyperalgesia, accompanied by the increased activation of PI3K [61] and mitogen-activated protein kinases (MAPKs), particularly p42/p44 MAPK [59]. Spontaneous pain, thermal hyperalgesia, mechanical allodynia, and opiate tolerance were inhibited using EphB1 blockers, suggesting that antagonist targeting EphB1 receptor could represent a novel class of analgesics for the treatment of painful neuropathies [32].
Opioid receptors are members of the G protein-coupled receptor (GPCR) family, being the mu opioid receptor (MOR) the most important relevant. Opioid receptors can be activated by both endogenous and exogenous opioids in central and peripheral nervous system [113]. Morphine and related MOR agonists are among the most widely used pain killers which produce analgesia trough the inhibition of adenylyl cyclase activity [113] and activation of p42/p44 MAPK phosphorylation [114]. However, chronic exposure to opioids may result in different relevant side effects (tolerance, dependence and hyperalgesia) and ineffectiveness analgesic affects.

There some studies that demonstrate evidence tahta G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) can cross-communicate each other and activate overlapping signaling pathways. For instance, the functional interactions between the EGF receptor (tyrosine kinase receptor) and dopamine D2-like receptors (G protein-coupled receptor ) where the signaling of D3R is selectively inhibited by EGFR stimulation [139]

Therefore, considering that EphB receptor activation produce hyperalgesia through the activation of p42/p44 MAPK whereas morphine by the activation of p42/p44 MAPK produce their analgesic effects, the presence of a potential inverse relation between the activation of the EphB-ephrinB system and the analgesic effects produced by MOR agonists was studied.

This investigation was previously initiated at University of Bologna by Lombardi Sara, who found a potential cross-talk between EphB1 and MOR. She observed that the activation of EphB1 and MOR receptors by their agonists ephrinB1-Fc and morphine respectively in undifferentiated SHSY5Y cells induced a time-dependent increase p42/44 MAPK phosphorylation whereas their co-administration occluded p42/p44 MAPK activation, suggesting that EphB receptor activation by ephrinB1 could prevent the contemporary activation of MOR-mediated intracellular signaling pathways. Such crosstalk, as well as EphB1 and MOR expression, were modified in neuronal cells underwent to in vitro differentiation and under these conditions the occlusive interaction observed disappear and morphine can produce p42/44 MAPK phosphorylation [121].

These previous data allow me to continue this investigation and confirm that EphB1 receptor activation is involved in the reduced effects elicited by morphine as well as I elucidated any signaling pathways and mechanism responsible for the functional crosstalk between EphB1 and MOR receptors.

Therefore, the principal findings of this research are the following:

- MOR activation by morphine is blunted when EphB1 receptor is simultaneously engaged by ephrinB1.
- The p42/44 MAPK phosphorylation ephrinB1-Fc-mediated activation is PI3K- dependent.
- Inhibition of PI3K signaling pathway EphB1-mediated may rescue morphine-dependent intracellular signaling.
- The occlusive crosstalk triggered by EphB1 receptor activation could be also determined at the cAMP levels.

- In differentiated neuronal cells, blocking PI3K signaling pathway does not affect p42/44 and PKC phosphorylation.
- Differentiated neuronal cells exposed to TNF-α moderately up-regulate MOR and dramatically up-regulate EphB1 receptor expression. Under these conditions, p42/44 MAPK phosphorylation is due to only EphB1 activation and the occlusive crosstalk is restored.
- Blocking signaling pathway involved in ephrinB1/EphB1 system activation in differentiated neuronal cells exposed to TNF-α, recover p42/44 MAPK activation morphine-mediated.
- Hexapeptide and octapeptide tested in HEK-EphB1 cells can counteract ephrinB1-Fc-mediated activation of p42/44 MAPK phosphorylation. These peptides seem to be an EphB1 receptor antagonist with an $IC_{50}$ of 1.21 µM and 0.8 µM.

Agonist of EphBs and MOR receptors induces the activation of several downstream intracellular signaling system, including p42/44 MAPK phosphorylation which play important roles in neuronal function [140]. Phosphatidylinositol 3- kinase (PI3K), protein kinase A (PKA) act as the downstream factors of ephrinB-EphB signaling which are required for p42/44 MAPK activation [61,141] (Figure 24a), whereas morphine can regulate Mu opioid receptor signaling through the activation p42/44 MAPK phosphorylation using as downstream molecule protein kinase C (PKC) [131,130] (Figure 24b) and in addition via the modulation of intracellular level of adenylate cyclase activity cAMP-dependent, in normal conditions morphine can reduce cAMP levels through the stimulation of Gi protein α-subunit [132] (Figure 24b).

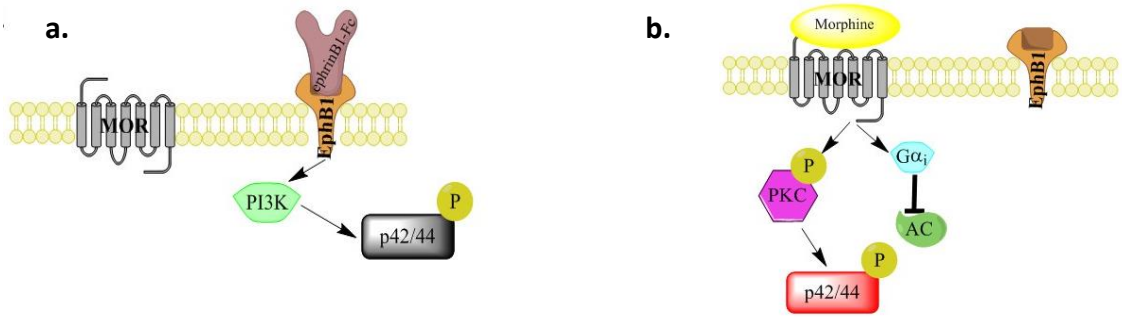


**Figure 24**. a) activation of EphB1 receptor by ephrinB1-Fc trigger the phosphorylation of p42/44 MAPK using as a downstream signaling pathway PI3K proteins. b) morphine produces their analgesic effects via phosphorylation of PKC and p42/44 MAPK proteins and reducing cAMP levels.

The occlusive crosstalk observed by the concomitant administration of both agonists (morphine and ephrinB1-Fc) lead to reduced p42/44 MAPK phosphorylation morphine-mediated, likely due to the activation of ephrinB1/EphB1 system[121]. Considering that the activation of EphB1 receptor by EphrinB1-Fc is PI3K- dependent, the block of the signaling pathway involved in EphB1 receptor signaling restore the analgesic effects produced by morphine (Figure 25). Furthermore, the inhibition of AC (Adenylyl cyclase) mediated by morphine is prevented if EphB1 is simultaneously activated by ephrin, suggesting that EphB1 receptor signaling through the activation of PI3K pathway which in turn block morphine effect on cAMP levels hamper the induction of opioid mediated intracellular signaling (Figure 25).

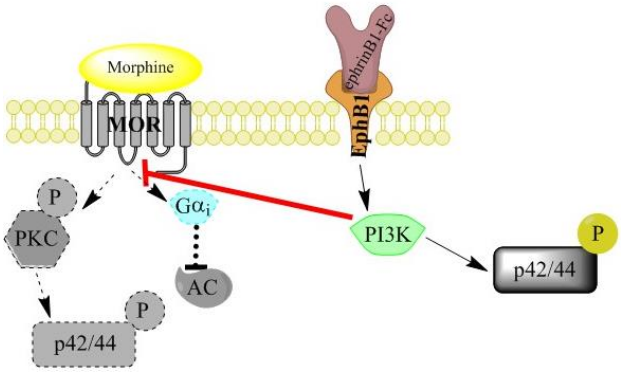


**Figure 25**. Co-administration of ephrinB1-Fc and morphine ligands occludes p42/p44 MAPK activation which is produced by the activation of PI3K downstream signaling pathway responsible of the activation of EphB1 receptor signaling. Once that PI3K signaling pathway is activated EphB1 receptor can interact with MOR receptors via the modulation of cAMP levels. Dashed lines demonstrate that under EphB1 activation morphine does not produce the activation of p42/44MAPK as well as loses the capacity to reduce cAMP levels.

Phorbol 12-myristate 13-acetate (PMA) can stimulated SHSY5Y neuroblastoma cells to neuronal differentiation converting cells to mature neuron-like phenotype [134,127]. Under this condition EphB1 and MOR receptors protein and mRNA levels undergo changes in their expression, particularly, EphB1 receptor is dramatically down-regulated and in these conditions EphrinB1-Fc administration no longer occludes morphine-mediated activation of PKC and p42/p44 MAPK phosphorylation (Figure 26).

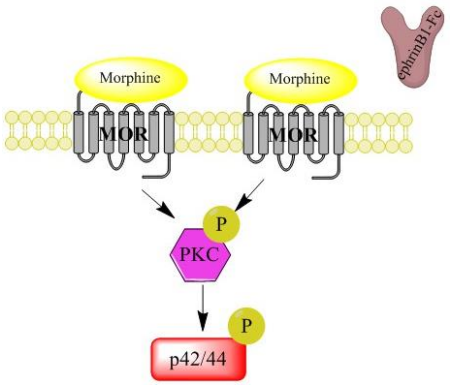


**Figure 26**. Co-administration of morphine and ephrinB1-Fc ligands in differentiate neuronal cells does no produce p42/44 MAPK and PKC phosphorylation alterations.

Administration of pro-inflammatory stimuli as TNF-α for mimicking the neuroinflammatory response that contributes to different persistent pain states in differentiated neuronal cells determined a significant increase in protein and mRNA levels of EphB1 receptor; under these conditions EphrinB1 Fc– mediated occlusion on p42/p44 MAPK phosphorylation is promptly restored. Such crosstalk was abolished blocking the signaling pathway responsible of EphB1 activation, allowing the complete activation of p42/44 MAPK and PKC proteins (Figure 27) which are important downstream proteins involved in analgesics effects of morphine, suggesting that EphB1 signaling activation is involved completely in the block of morphine effects which depend on the physiological state of neuronal cells.

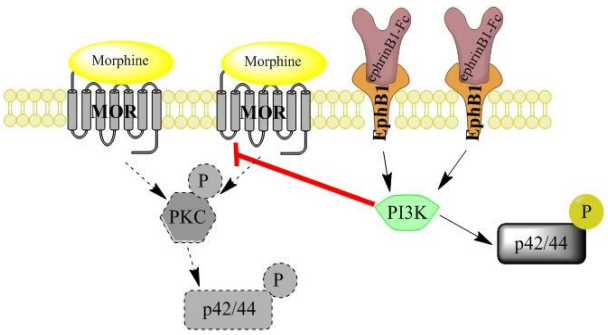


**Figure 27**. Exposition of differentiated neuronal cells to TNF-α increase the expression of EphB1 receptor and under these conditions restore the antagonist interaction between Mor and EphB1 receptors. Dashed lines show that activation of signaling pathway responsible of EphB1 activation block the activation of p42/44 MAPK and PKC activation morphine-mediated.

At these regards, the two novel EphB1 receptor peptide antagonists tested in this research can counteract ephrinB1-Fc-mediated activation of p42/44 MAPK, suggesting that hexapeptide and octapeptide are novel EphB1 receptor antagonists and could provide a novel strategy to reduce the negative impact of ephrin system activation on pain perception and opioid efficacy.

Considering that EphB receptors [62,56] and opiate system [87] can modulate NMDA receptor function, further studies are needed to determinate if EphB1 receptor activation is directly involved ineffectiveness of analgesic effects morphine-mediated or maybe is necessary NMDA receptor activation to produce these effects, in addition tested the capacity of the two novel EphB1 antagonist peptides to counteract ephrinB1-induced attenuation of MOR-dependent signaling in neuronal cells co-expressing MOR and EphB1 receptors is necessary to confirm that effectively these antagonist peptides can rescue morphine-mediated responses

# 6. Conclusions

- Activation of EphB1 receptor does not allow that morphine to trigger PKC-dependent signaling events downstream of MOR receptor.
- Morphine administered as single agent inhibit adenylyl cyclase and induce PKC-dependent p42/44 MAPK phosphorylation in neuronal cells co-expressing MOR and EphB1 receptor, regardless the differentiation or inflammatory state.
- EphB1 activation by ephrinB1 ligands is PI3K-dependent which is capable to blunt the MOR-dependent signaling induced by morphine in undifferentiated neuronal cells, as well as in differentiated cells subsequently exposed to pro-inflammatory stimuli, due to the expression of MOR and EphB1 receptor are at similar levels.
- Morphine triggers MOR-dependent signaling in differentiated neuronal cells regardless the co-administration of ephrinB1, due to the significant EphB1 receptor down-regulation and MOR up-regulation.
- The two novel Hexapeptide and Octapeptide assayed in this study, may represent two potential novel EphB1 receptor peptide antagonists as they effectively counteracted ephrinB1-dependent EphB1 receptor activation.

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