

To phosphorylate or not to phosphorylate

Selective alterations in tyrosine kinase-inhibited EphB mutant mice

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EphB tyrosine kinase receptors have been implicated in multiple developmental processes; however, the signaling mechanism underlying these events remains unclear. Through a triple knock-in mouse line for three neurally expressed EphBs, Sokis et al. demonstrated that EphB tyrosine kinase activity is required for axon guidance but does not influence synapse formation. This short communication highlights their study and appealing molecular approach that elucidated the functions of EphB tyrosine kinase during developmental events.

Eph/ephrin: A Signaling Network

During development, each cell has to find its exact target in the nervous system in order to establish functional connections. Among all the molecules implied in such a crucial task, the Eph family plays a major role by repelling extending axons and inducing growth cone collapse. Later in life, Eph also plays a role in maintaining and promoting synapse formation.^{1,2} The Eph family is the largest member of the Receptor Tyrosine Kinase (RTK) and was first characterized as orphan receptors from Erythropoietin-Producing Hepatocellular carcinoma (Eph).³ They can be divided into two families: EphA (A1 to A8) and EphB (B1 to B6) receptors. Their ligands, ephrins (EPh Receptor Interacting) can display two types of structures. Ephrin-A are glycosphosphatidylinositol (GPI) anchored and bind to EphA while ephrin-B have a transmembrane domain and bind to EphB.^{4,5}

Ephs regulate a diversity of cell–cell interactions in the nervous system⁴ and

can display bidirectional signaling via cytoplasmic tail containing multiple phosphorylation sites; a kinase and a SAM/PDZ binding domain (Fig. 1A). During forward signaling, the binding of ephrin to Ephs induces Eph oligomerization, tyrosine phosphorylation, and Eph kinase activation. Following receptor activation, several cytoplasmic proteins are recruited including proteins containing Src Homology 2 (SH2) domain that binds to phosphorylated tyrosine residues as a consequence of tyrosine kinase activity.⁶ Unlike the ephrin-B, little is known regarding ephrin-A reverse signaling. The phosphorylation of ephrin-B cytoplasmic tail recruits SH2 domain-containing proteins.⁷

Bidirectional signaling is involved in the development of hippocampus, corpus callosum, and optic chiasma. During visual system development in rodents, most retinal ganglion cell (RGC) axons from the retina cross the midline at the optic chiasm to form contralateral retinal projections.⁸ However, RGCs from the ventrotemporal region of the retina, which express EphB1 form ipsilateral projection because they are repelled by the ephrin-B2 expressed by the glia of the optic chiasma, and hence, do not cross the midline.⁹

Furthermore, EphB has been implicated in cortical and hippocampal synapse formation.¹⁰ Indeed, EphBs regulate maturation of postsynaptic sites by inducing spine morphogenesis and recruiting neurotransmitter receptors, like AMPAR and NMDAR. Also, EphB-knockout mice¹¹ display reduced excitatory synapse density and defect in dendritic spines and synapse development.

Keywords: Eph B, ephrin, tyrosine kinase, bidirectional signaling, PP1 analogue

Abbreviations: Eph, erythropoietin-producing hepatocellular carcinoma; Ephrins, Eph receptor interacting; RTK, receptor tyrosine kinase; Grb, growth factor receptor-bound protein; GPI, Glycosphosphatidylinositol; SH2, Src Homology 2; TK, tyrosine kinase; WT, wild-type

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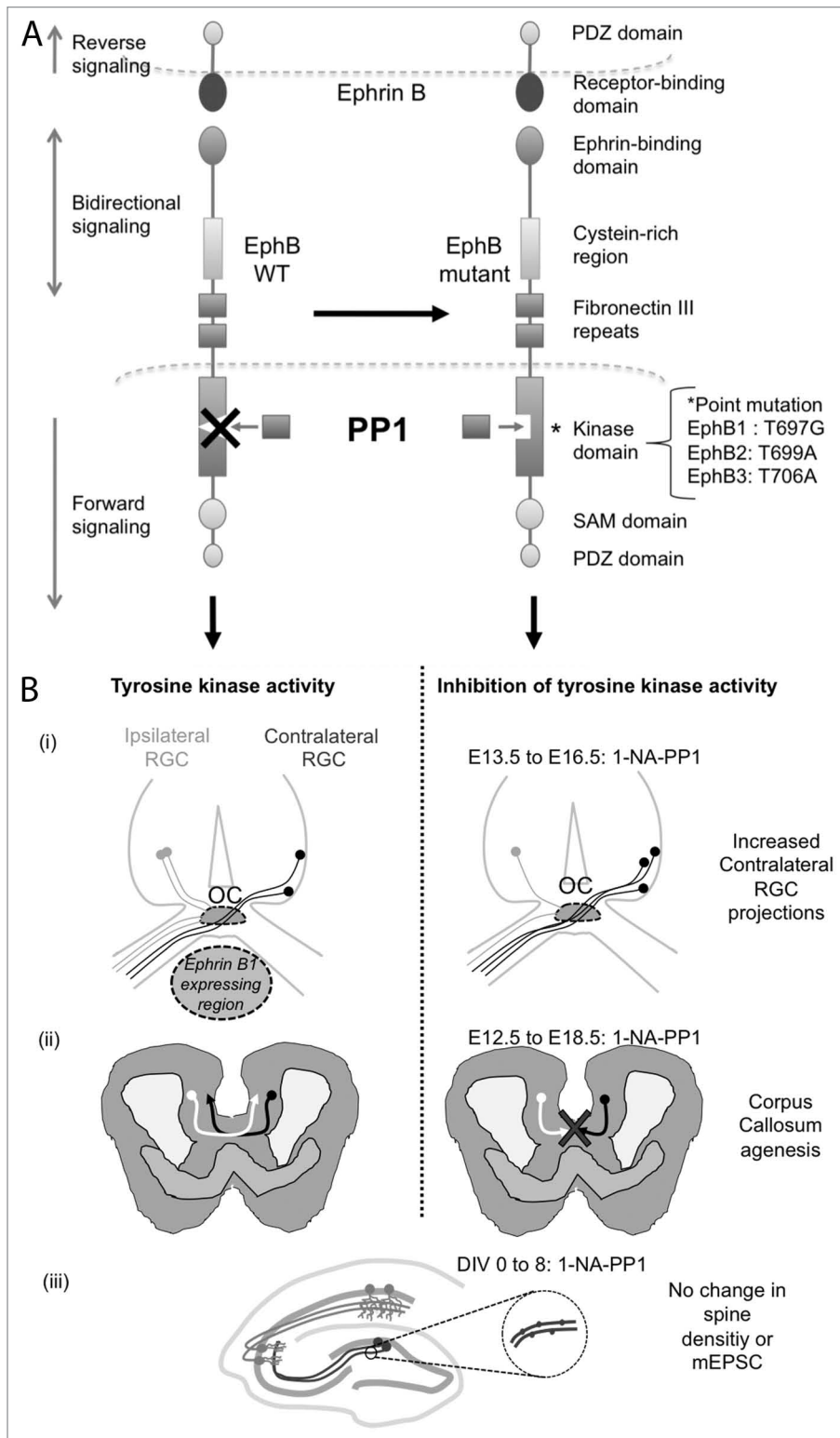


Figure 1. Schematic representation of AS-EphB triple knock-in mutants compared with WT at both structural and functional levels. **(A)** Bidirectional signaling by ephrin along with wild-type and analog-sensitive EphB receptors. The ligand and receptor are both phosphorylated. Following EphB receptor binding, tyrosine kinase activity is activated along with other kinase-independent mechanisms, e.g., PDZ domain. However, in mutant Eph B, tyrosine kinase activity is inhibited by PP1 analogues. **(B)** Roles of EphB tyrosine kinase (TK) activity during developmental events in analog-sensitive EphB triple knock-in mouse line treated with PP1 analogs for specified times. (i) Retinal axon guidance requires EphB receptor tyrosine kinase signaling. In the optic chiasm (OC), there was a reduction in ipsilateral radial glial cells (RGC) projections. (ii) EphB receptor TK signaling regulates corpus callosum formation. Defective corpus callosum formation leads to a gap in dorsal midline region following PP1 analogs treatment. (iii) In the dentate gyrus (DG) of hippocampal slices, Ephrin B receptor TK is not required for synaptogenesis. No differences observed in dendritic spine formation.

receptor during specific developmental events. The authors addressed whether kinase activity of EphBs expressed under physiological conditions are required for axon guidance and synapse development. The investigation was performed *in vivo* and *in vitro* by utilizing a novel chemical and genetic approach.

Making a Point: Sensitization of Tyrosine Kinase Activity

To assess the role of the TK activity in EphB/ephrin-B signaling, a point mutation was inserted in EphB receptors at the level of an access site to the ATP-binding pocket (Fig. 1A). This mutation sensitizes EphB to PP1 (4-Amino-5-[4-methylphenyl]-7-[t-butyl]pyrazolo[3,4-d]-pyrimidine) and their analogs, which can specifically and reversibly block the TK activity, thus providing a temporal control. Three different knock-in mice were generated, each carrying the analog-sensitive mutation (AS) in EphB1, EphB2, and EphB3, respectively. Triple knock-in animals (TKI) were obtained by crossing together AS-EphB1, 2, and 3 and showed no abnormality in the absence of PP1. To assess the role of TK activity, PP1 was added at different time points (see Fig. 1B

Nevertheless, these roles of EphB kinase activity have not been studied *in vivo* when EphB and ephrins-B are expressed at endogenous levels and remain to be clarified. Another aspect is the questions regarding the roles of TK (Tyrosine kinase) and SAM/PDZ domains in signal

transduction, which could not be clarified by classical studies. Indeed, up to now, no strategy was available to discriminate between those two signaling pathways and attributing a specific role for each.

The recent paper by Soskis et al., aims specifically at elucidating the role of EphB

for details) during development in afferent structures in wild-type (WT) and triple knock-in showing PP1 sensitivity.

Embryonic cortical neurons from AS-EphB triple knock-in and WT mice were stimulated with clustered Ephrin B1, which resulted in similar tyrosine phosphorylation levels. However, these phosphorylations were blocked in cells from AS EphB triple knock-in mice pre-incubated with PP1 analogs (e.g. 1-NA-PP1). In addition, the phosphorylation of downstream TK substrate, Grb2, was blocked in the presence of PP1 analog but not in WT mice.

In retinal explants from AS EphB triple knock-in the number of growth cone collapse by clustered ephrin-B2 was significantly reduced following PP1 analogs pre-treatment. In addition, pregnant AS EphB triple knock-in mice treated with 1-NA-PP1 produced embryos with reduced ipsilateral retinal projections along with corpus callosal agenesis (Fig. 1B, i, ii). In hippocampal slices, there were no differences between spine densities (Fig. 1B, iii) and excitatory synapses functionality between populations from AS EphB triple knock-in and WT after chronic 1-NAPPI treatments.¹²

Complexity in EphB Signaling

This study demonstrated the successful development of a transgenic mouse model in which it was possible to specifically inhibit kinase activity of EphB *in vitro* and *in vivo*. Using this model, it was possible to show that EphB TK activity is directly involved in the formation of ipsilateral retinal projections and corpus callosum. On the contrary, the EphB kinase signaling was not required for the formation of excitatory synapses, but may possibly be involved in other processes such as synaptic plasticity. These observations led the authors to favor a model where EphB TK activity is required for repulsive interactions, e.g., axon guidance, but is not required for adhesive interactions, e.g., axon fasciculation or synaptogenesis.¹² In addition, AS EphB triple knock-in displays a specific phenotype of great interest in human pathology.¹³ In lines with this data, mutations in the ephrin-B1 gene result in craniofrontonasal syndrome

(CFNS) in humans.¹⁴ Besides a number of craniofacial anomalies including cleft palate, craniofrontonasal dysplasia, and craniosynostosis, this syndrome includes neurological defects such as agenesis of the corpus callosum (ACC) and mental retardation.

While the role of EphB kinase activity has been further clarified, several questions remain unanswered. Another issue pertains as to how EphB and ephrin-B can have distinct and opposing effects; such as those observed where ephrin-B has to regulate attraction and repulsion of neural crest cells during their migration.¹⁵ Moreover, the precise role of the balance between EphB and ephrin-B signaling underpinning these diverse functions requires further elucidation. Even if it seems clear that EphB kinase activity is necessary for axonal guidance at the level of the corpus callosum and optic chiasma, the role of reverse signaling in synaptogenesis has not been addressed in this particular study. Accordingly, it is possible that synaptogenesis is mediated through reverse signaling rather than kinase-independent mechanisms. Indeed, both reverse and forward signaling can be found at the level of spine and synapse formation,^{1,2,16} and impairing TK activity has no effect on reverse signaling.

Interestingly, in addition to the normal signals through ephrin-B, this EphB receptor has been observed to show some crosstalk with EphA and ephrin-A. Therefore, a compensatory mechanism could be imagined through EphA/ephrin-B signaling in the event of synapse formation, as seen in thalamocortical axonal guidance.¹⁷ To rule out this eventually, further investigation on spine formation involving EphA knockouts would be required.

Furthermore, targeting specifically the TK signaling could allow highly selective therapeutic approach, for instance, in axonal regeneration, as Eph play a role in axonal guidance. EphB signaling is also known to play a role in cancer, notably in colorectal cancer, where its activation suppresses tumors. Considering this and the specific targeting of the AS-EphB, the identification of downstream effectors of EphB TK would be possible and give insights in new therapeutic strategies.

Finally, one could also enquire if spine formation indeed involves PDZ domain signaling. Assessing such specific functions would require other strategies. For instance, ephrinB1 Δ V mutant construct harbors a critical deletion of the conserved C-terminal valine in the PDZ-binding domains of ephrin-B1 and ephrin-B2.¹⁸ A similar approach could be used to generate PDZ-deficient AS-EphB triple knock-in mutants. Nevertheless, this will not tell us much about the PDZ downstream cascade. However, knocking-down the multi-PDZ domain scaffold protein GRIP1 by RNA interference in hippocampal neurons caused a loss of dendrites associated with mislocalization of EphB2 and KIF5. This phenotype can be rescued by overexpression of the EphB2 extracellular domain. Altogether, these results strongly suggest an important role for the PDZ domain of EphB and its binding partner GRIP1 in dendrite morphogenesis.¹⁹

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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