Korean J Physiol Pharmacol
Vol 8: 111–115, April, 2004

Multiple Regulation of Roundabout (Robo) Phosphorylation in a Heterologous Cell System

Hwan Tae Park

Department of Physiology and Institute of Medical Science, College of Medicine, Dong-A University, Busan 602–714, Korea

Roundabout (Robo) is the transmembrane receptor for slit, the neuronal guidance molecule. In this study, the tyrosine phosphorylation of Robo was observed in Robo-transfected human embryonic kidney cells and developing rat brains, and found to be increased by the treatment with protein kinase A activator, forskolin. In contrast, protein kinase C activation by phorbol-12-myristate-13-acetate decreased the phosphorylation of Robo. Intracellular calcium was required for the tyrosine phosphorylation. Furthermore, the transfection of an Eph receptor tyrosine kinase dramatically enhanced the tyrosine phosphorylation. These findings indicate that the tyrosine phosphorylation of Robo is regulated by multiple mechanisms, and that Eph receptor kinases may play a role in the regulation of tyrosine phosphorylation of Robo in the rat brain.

Key Words: Robo, Slit, Axon guidance, Protein kinases, Tyrosine phosphorylation

INTRODUCTION

The development of neural connections in the central nervous system involves accurate guidance of axons to their final targets (Tessier-Lavigne & Goodman, 1996). Many studies in the past few years indicate that the combined works of attractive and repulsive guidance cues regulate proper axon pathfindings. Among them, diffusible guidance cues, such as netrins and slits, have been suggested to be capable of making attractive or repulsive gradients over distances, thereby directing the trajectory of migrating axonal growth cones (Serafini et al, 1996; Tessier Lavigne & Goodman, 1996; Kidd et al, 1999; Wong et al, 2002). *In vivo* genetic evidences obtained from knockout mice of the guidance cues have clearly indicated the importance of the diffusible guidance cues in the development of neuronal projections (Serafini et al, 1996; Bagri et al, 2002).

The secreted slit family proteins are repulsive guidance cues implicated in direction control of axon projection in worms, flies and mammals (Kidd et al, 1999; Li et al, 1999; Brose et al, 2000; Hao et al, 2001). Slits are involved in guiding olfactory axons of the olfactory bulb and midline crossing of several axons, including retinal axons in vertebrates (Li et al, 1999; Erskine et al, 2000; Bagri et al, 2002). In the spinal cord, slit plays a role in commissural axon crossing (Brose et al, 1999) and acts as a positive regulator of branching and elongation of dorsal root ganglion axons (Wang et al, 1999). The several actions of slits seem to require a well-conserved receptor, Roundabout (Robo)(Nguyen Ba Charvet & Chedotal, 2002).

Robo was discovered in a genetic screening for mutation affecting axon pathfinding in *Drosophila* (Kidd et al, 1998): Robo mutants exhibit an increased number of axons crossing and recrossing the ventral midline. The extracellular region of Robo contains five immunoglobulin domains and three fibronectin type III repeats (Kidd et al, 1998; Wong et al, 2002). The large intracellular region of Robo which determines the repulsive response to slit contains four identifiable conserved motifs, designated CC0, CC1, CC2 and CC3. A study with *Drosophila* demonstrated that the Abelson (Abl) tyrosine kinase and the Enabled protein (Ena) are involved in slit-Robo signaling (Bashaw et al, 2000). CC1 and CC2 motifs of Robo are important for its biochemical interaction with Ena, whereas the CC3 motif is important for its interaction with Abl. Cotransfection of Robo and constitutively active Abl results in significant tyrosine phosphorylation of Robo in Cos1 cells. Abl seems to be a negative regulator of Robo, because the tyrosine phosphorylation of Robo by Abl reduced the effect of slit on Robo (Bashaw et al, 2000). This finding indicates that the tyrosine phosphorylation of Robo might be an important mechanism regulating slit-Robo signalings.

In this study, the molecular mechanism of the tyrosine phosphorylation of Robo in a heterologous cell system and in the developing brain was investigated. The results revealed the importance of several protein kinase systems and ephrin-Eph receptor kinase (EPH) in the tyrosine phosphorylation of Robo.

Corresponding to: Hwan Tae Park, Department of Physiology and Institute of Medical Science, College of Medicine, Dong-A University, 3-1, Dondaesin-dong, Seo-gu, Busan 602-714, South Korea. (Tel) 82-51-240-2636, (Fax) 82-51-246-6481, (E-mail) phwantae @daunet.donga.ac.kr

ABBREVIATIONS: EPH, eph receptor kinase; Robo, roundabout; PKA, protein kinase A; PKC, protein kinase A.

METHODS

Materials

Following antibodies were used: anti-HA, anti-myc (Santa Cruz), and anti-phosphotyrosine (4G10, Upstate Biotechnology). Anti-Robo antibody was provided by Dr. Y. Rao (Washington Univ.). Several pharmacological reagents were obtained from Calbiochem. The reagents for cell culture were purchased from Gibco, and other reagents were from Sigma.

Cell cultures

Human embryonic kidney (HEK) 293 cell lines were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Transient transfections were carried out by the calcium-phosphate methods. Ten g of plasmid DNA were transfected in a 100 mm dish. After overnight incubation, the transfected medium was replaced with fresh medium. The total protein extracts were prepared from the transfected cells after 2 days of transfection. The stably transfected HEK 293 cell lines were established with human HA-Robo-1 cDNA transfection. After transient transfection, neomycin (500 μg/ml) resistance clones were selected for 2 months. Several neomycin-resistant clones were analyzed with anti-HA Western blotting.

Western blot analysis and immunoprecipitation

Cells were lysed with boiled 2 × sodium dodecyl sulfate (SDS) sample buffer or RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 1protease inhibitor mixture (Roche Molecular Biochemicals)]. Protein extracts were separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. The membrane was immunostained with primary antibodies, and then washed with phosphate buffered saline (PBS). The blots were reacted with horseradish peroxidase conjugated secondary antibody (3000 : 1) and visualized with the enhanced chemiluminescence kit (Amersham). Some membranes were stripped with a buffer containing 100 mM mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 7.6), and then reprobed. All experiments were repeated at least three times with similar results.

For immunoprecipitation, RIPA lysates were precleared with protein A/G agarose for 1 h. The lysates were incubated with specific antibodies and protein A/G agarose beads for 3 h at 4°C. The samples were washed three times with RIPA buffer and then boiled in 2 × SDS sample buffer for 5 min. The samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies.

Rat brain extracts

The timed pregnant Sprague-Dawley rats were killed under ether anesthesia. The pups were decapitated and the brains were extracted. The brains were homogenized in glass tubes with RIPA buffer, and the homogenates were centrifuged at 13000 rpm for 30 min at 4°C, and the supernatant was used for the experiments.

RESULTS

Tyrosine phosphorylation of Robo in HEK 293 cells and developing rat brains

To examine whether Robo could have its tyrosine residue phosphorylated in heterologous systems, a HEK 293 cell line stably transfected with full length HA-Robo-1 cDNA was used, and protein extracts from the cells were immunoprecipitated with anti-HA antibody and immunoblotted with anti-phosphotyrosine. A tyrosine phosphorylated band with about 200 kDa molecular mass appeared in anti-HA immunoprecipitates (Fig. 1A). The immunoprecipitation with anti-myc antibody did not show any positive band. The straight Western analysis of cellular extracts with anti-HA antibody showed a clear expression of Robo, indicating that the tyrosine phosphorylation of Robo occurs in HEK cells.

To examine whether the Robo tyrosine phosphorylation of Robo could be observed in rat brain and regulated during development, total protein lysates were extracted from

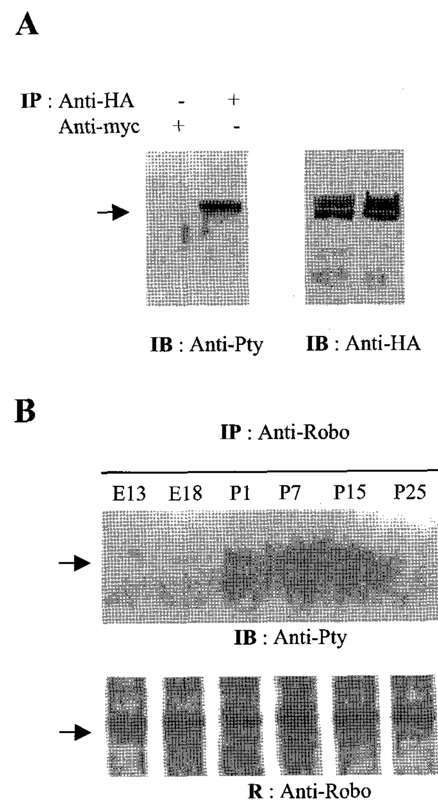

Fig. 1. The tyrosine phosphorylation of Robo in transfected HEK cells and rat brains. (A) The protein extracts from HA-Robo-transfected HEK cells were immunoprecipitated (IP) and immunoblotted (IB) with anti-phosphotyrosine antibody (anti-Pty, left panel). The protein extracts were immunoblotted with anti-HA antibody to show the expression of Robo in transfected-HEK cells (right panel). Arrow indicates Robo. (B) The immunoprecipitation of protein extracts from the brains of various ages of rats (E; embryonic, P; postnatal) shows the developmental regulation of the tyrosine phosphorylation of Robo (upper panel). Among two tyrosine phosphorylated bands, the upper one is rat Robo. The same membrane was reprobed with anti-robo antibody. R; reprobing.

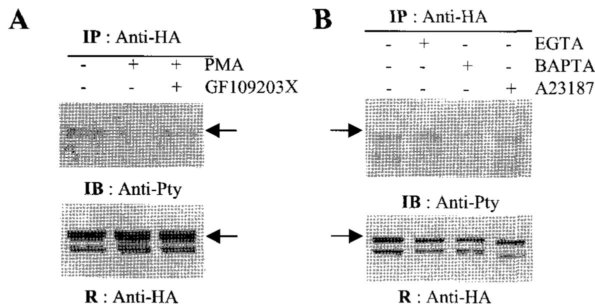

Fig. 2. The role of PKC and calcium on the tyrosine phosphorylation of Robo. The protein extracts from HA-Robo-transfected HEK cells were immunoprecipitated (IP) and immunoblotted (IB) with anti-phosphotyrosine antibody (anti-Pty). The same membrane was reprobed with anti-HA antibody (R). (A) PKC activation by PMA treatment (0.2 μM) reduced the tyrosine phosphorylation of Robo, and the PMA-induced decrease of the tyrosine phosphorylation was blocked by the pretreatment with GF109203X (2 μM). (B) Intracellular calcium chelation by BAPTA/AM (20 μM) reduced the tyrosine phosphorylation of Robo, whereas neither EGTA (3 mM) nor calcium ionophore A23187 (10 μM) had any effect.

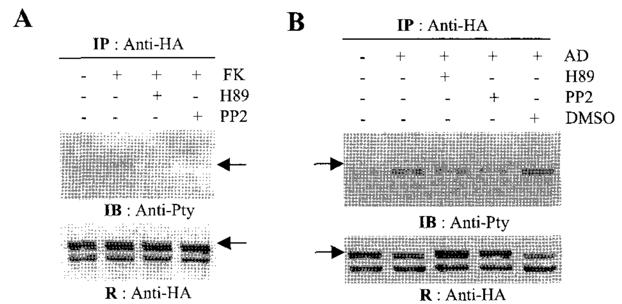

Fig. 3. The role of PKA and adenosine on the tyrosine phosphorylation of Robo. The protein extracts from HA-Robo-transfected HEK cells were immunoprecipitated (IP) and immunoblotted (IB) with anti-phosphotyrosine antibody (anti-Pty, left panel). The same membrane was reprobed with anti-HA antibody (R). (A) PKA activation by forskolin (FK, 10 μM) increased the tyrosine phosphorylation of Robo. The forskolin-induced tyrosine phosphorylation of Robo was blocked by the pretreatment with PKA inhibitor, H89 (10 μM) or tyrosine kinase inhibitor, PP2 (2 μM). (B) Adenosine treatment (AD, 200 μM) increased the tyrosine phosphorylation of Robo. The effect of adenosine was inhibited by H89 and PP2, but not by DMSO.

various developmental stages of rat brains, and proteins were immunoprecipitated with a polyclonal anti-Robo antibody, and the immune complexes were immunoblotted with an anti-phosphotyrosine antibody. As shown in Fig 1B, the tyrosine phosphorylation of Robo was observed in developing brains, especially from postnatal day 1 and 7. The phosphorylation level was very low in the prenatal and adult brains. Reprobing the membrane with anti-Robo showed similar expression level of Robo during the developmental period. This finding indicates that the tyrosine phosphorylation of Robo is developmentally regulated in the rat brain.

The role of protein kinase C (PKC) and calcium in the tyrosine phosphorylation of Robo

PKC and calcium play important roles in the regulation of axon guidance (Hong et al, 2000; Xiang et al, 2002; Nishiyama et al, 2003). Therefore, a question of whether these signaling molecules are involved in the tyrosine phosphorylation of Robo was tested using several pharmacological agents. The treatment with phorbol-12-myristate-13-acetate (PMA, 200 nM), a strong PKC activator, inhibited the Robo tyrosine phosphorylation, and the inhibitory effect of PMA was blocked by the cotreatment with PKC inhibitor, GF109203X (Fig 2A). This finding suggests that PKC pathway decreases the Robo tyrosine phosphorylation by either inhibiting tyrosine kinase systems or increasing the tyrosine phosphatase activity.

The increase of intracellular calcium by the treatment with calcium ionophore, A23187 (10 μM), did not affect the tyrosine phosphorylation, whereas the chelation of intracellular calcium by BAPTA (20 μM) almost completely inhibited the tyrosine phosphorylation (Fig. 2B). The chelation of extracellular calcium level by the addition of 3 mM EGTA in the medium did not significantly alter the level of tyrosine phosphorylation of Robo. Therefore, the proper maintenance of intracellular calcium level seems to be required for the tyrosine phosphorylation.

The role of protein kinase A (PKA) in the tyrosine phosphorylation of Robo

Next, the possible role of PKA in the tyrosine phosphorylation of Robo was investigated. As seen in Fig 3A, the tyrosine phosphorylation of Robo was increased by the treatment with the activator of adenylyl cyclase, forskolin (20 μM). The increase was dose-dependent, and it occurred within 5 min, persisting until 30 min. The forskolin-induced tyrosine phosphorylation was blocked by the specific PKA inhibitor, H89 (10 μM), showing that the increase of the Robo tyrosine phosphorylation by forskolin was caused by the activation of PKA. It was previously reported that Src family kinases are involved in the tyrosine phosphorylation of Robo (Jeon et al, 2003). The forskolin-induced increase of the tyrosine phosphorylation of Robo was blocked by the pretreatment with a Src family kinase inhibitor, PP2, implicating the requirement of Src family kinases in the PKA-induced Robo tyrosine phosphorylation.

Adenosine is a neurotransmitter that increases the activity of PKA through A2B adenosine receptor which is endogenously expressed in HEK 293 cells (Gao et al, 1999). Therefore, a possible involvement of the adenosine receptor activation in the tyrosine phosphorylation of Robo was tested. As expected, adenosine treatment (200 μM) increased the tyrosine phosphorylation in HEK 293 cells in 5 min, and this effect was blocked by the pretreatment with either H89 or PP2 (Fig. 3B). This finding indicates that both PKA and Src family kinases are involved in the adenosine-induced tyrosine phosphorylation of Robo.

EPH38 receptor tyrosine kinase increased tyrosine phosphorylation of Robo

EPH is the receptor tyrosine kinase for ephrins, and ephrin-EPH interaction is important for proper development of several axonal projections such as retinal axons (Kullander & Klein, 2002). Thus, the possible function of EPH in the Robo tyrosine phosphorylation was explored.

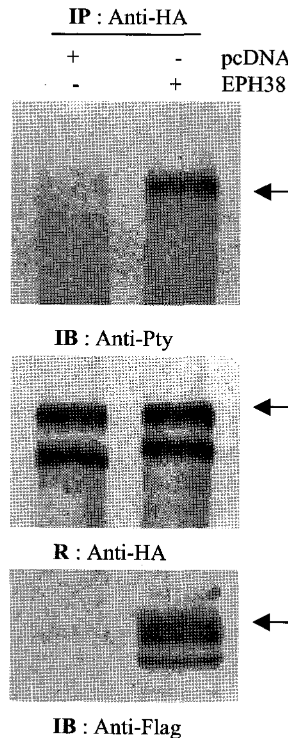

Fig. 4. The effect of transfection of EPH38 on the tyrosine phosphorylation of Robo. The transfection of Flag-EPH38 cDNA, not the transfection of empty vector (pc-DNA), into HEK cells expressing Robo induced a dramatic increase of the Robo tyrosine phosphorylation (upper panel). Western blot with anti-Flag antibody (lower panel) showed the expression of transfected Flag-EPH38 in HEK cells.

As shown in Fig 4, the transfection of EPH38 (Xenopus EPH-B2 homologue) cDNA dramatically enhanced the Robo tyrosine phosphorylation, whereas the transfection of pcDNA vector did not affect at all. In addition, the increased Robo tyrosine phosphorylation was also observed in EPH-A5 transfected cells (data not shown). This finding indicates that EPH directly or indirectly phosphorylates Robo in HEK cells.

DISCUSSION

The data presented in this study provide a novel functional relationship of several signaling molecules in the tyrosine phosphorylation of Robo. I have established a heterologous cell system to dissect the molecular mechanism of the Robo tyrosine phosphorylation in eukaryotic cells. Since there are several tyrosine residues in the intracellular region of Robo which can be phosphorylated (Kidd et al, 1998), identification of the tyrosine residues phosphorylated by several signaling pathways and the functional importance of each tyrosine residue remain to be elucidated.

The direction of axonal growth is regulated not only by extracellular guidance cues, but also by the intracellular concentration of cAMP, cGMP and calcium (Ming et al, 1997; Song et al, 1998; Song et al, 1998; Nishiyama et al, 2003). An attractive guidance cue also acts as a repellent in certain situations *in vitro* and *in vivo*. For example, netrin, an attractant for the axons of *Xenopus* spinal neurons, repels spinal axons in the presence of high concentration of cAMP (Ming et al, 1997). The repulsive response of *Xenopus* spinal axons to semaphorin changes into attraction to the same cue, depending on the level of intracellular cGMP (Song et al, 1998). In addition, slit, an repellent, attracts muscle progenitor cells during development in *Drosophila* (Kramer et al, 2001). Therefore, it seems that an axonal response to a guidance cue is largely modulated by the intracellular condition of signaling molecules. The present study showed that the tyrosine phosphorylation of Robo is changed depending on the activity of PKA and PKC, indicating the possible role of a tyrosine kinase/phosphatase system in the regulation of axonal response to a guidance cue. It is likely that PKA or PKC modulates the reaction of neurons to slit by altering the tyrosine kinase or phosphatase systems which regulate the tyrosine phosphorylation of Robo.

The present study also showed that the *Xenopus* homologue of EPH-B2 (and EPH-A5) enhanced the tyrosine phosphorylation of Robo, when both proteins were co-expressed in HEK cells. However, it remains still unknown whether EPH directly or indirectly phosphorylates Robo. Recently, hetero-dimerization of the receptors for different guidance cues has been reported. For example, Robo binds to Deleted in Colorectal Cancer, the netrin receptor (Stein et al, 2001), and L1 cell adhesion molecule forms a receptor complex with neuropilin, the semaphorin receptor (Castellani et al, 2000). It is possible that direct interaction between EPH and Robo is responsible for the tyrosine phosphorylation of Robo by EPH. Alternatively, Src family kinases may mediate the EPH-induced Robo tyrosine phosphorylation, because Src family kinase inhibitors reduced the Robo tyrosine phosphorylation caused by several stimuli (this study), and Src family kinases are important mediator of intracellular EPH signaling (Davy et al, 1999; Yu et al, 2001; Steinle et al, 2002). We are now in the process to investigate this possibility.

Even though the role of the tyrosine phosphorylation of Robo has been analyzed in *Drosophila* axon guidance (Bashaw et al, 2000), the exact function of the tyrosine phosphorylation of Robo in the mammalian brain has not yet been defined at all. As shown in the present study, the tyrosine phosphorylation of Robo is developmentally regulated and, interestingly, the phosphorylation reaches a peak at around postnatal day 1~7. Therefore, it is tempting to speculate that the tyrosine phosphorylation of Robo may not be important for axon guidance, since the axonal outgrowth usually occurs during embryonic period. It has recently been shown that slit is a regulator of dendritic branching (Godenschwege et al, 2002; Whitford et al, 2002), and this phenomenon occurs mainly after the axon outgrowth was accomplished during development. It is possible that the tyrosine phosphorylation of Robo might be a regulatory mechanism of dendritic branching by slit. In addition, novel roles of slit, such as angiogenesis and cell migration have recently been demonstrated (Wu et al, 2001; Park et el, 2002; Park et al, 2003; Wang et al, 2003). Thus, it is necessary to elucidate the role of tyrosine phosphorylation of Robo in slit-induced dendritic branching, angiogenesis and chemotaxis inhibition.

ACKNOWLEDGEMENTS

This work was supported by the Dong-A University Research Fund of 2003. We thank Dr. Yi Rao and Jane Wu (Washington University Medical School) for several materials for this study.

REFERENCES

Bagri A, Marin O, Plump AS, Mak J, Pleasure SJ, Rubenstein JL, Tessier-Lavigne M. Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. *Neuron* 33: 233−248, 2002

Bashaw GJ, Kidd T, Murray D, Pawson T, Goodman CS. Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell* 101: 703−715, 2000

Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessier-Lavigne M, Kidd T. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96: 795−806, 1999

Castellani V, Chedotal A, Schachner M, Faivre-Sarrailh C, Rougon G. Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* 27: 237−249, 2000

Davy A, Gale NW, Murray EW, Klinghoffer RA, Soriano P, Feuerstein C, Robbins SM. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. *Genes Dev* 13: 3125−3135, 1999

Erskine L, Williams SE, Brose K, Kidd T, Rachel RA, Goodman CS, Tessier-Lavigne M, Mason CA. Retinal ganglion cell axon guidance in the mouse optic chiasm: expression and function of robos and slits. *J Neurosci* 20: 4975−4982, 2000

Gao Z, Chen T, Weber MJ, Linden J. A2B adenosine and P2Y2 receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. cross-talk between cyclic AMP and protein kinase c pathways. *J Biol Chem* 274: 5972−5980, 1999

Godenschwege TA, Simpson JH, Shan X, Bashaw GJ, Goodman CS, Murphey RK. Ectopic expression in the giant fiber system of Drosophila reveals distinct roles for roundabout (Robo), Robo2, and Robo3 in dendritic guidance and synaptic connectivity. *J Neurosci* 22: 3117−3129, 2002

Hao JC, Yu TW, Fujisawa K, Culotti JG, Gengyo-Ando K, Mitani S, Moulder G, Barstead R, Tessier-Lavigne M, Bargmann CI. C. elegans slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. *Neuron* 32: 25−38, 2001

Hong K, Nishiyama M, Henley J, Tessier-Lavigne M, Poo M. Calcium signalling in the guidance of nerve growth by netrin-1. *Nature* 403: 93−98, 2000

Jeon ES, Bae KW, Park HT. Fyn tyrosine kinase-mediated tyrosine phosphorylation of Roundabout (Robo), the slit receptor. *Mol Brain Res* (submitted)

Kidd T, Bland KS, Goodman CS. Slit is the midline repellent for the robo receptor in Drosophila. *Cell* 96: 785−794, 1999

Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* 92: 205−215, 1998

Kramer SG, Kidd T, Simpson JH, Goodman CS. Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. *Science* 292: 737−740, 2001

Kullander K, Klein R. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 3: 475−486, 2002

Li HS, Chen JH, Wu W, Fagaly T, Zhou L, Yuan W, Dupuis S, Jiang ZH, Nash W, Gick C, Ornitz DM, Wu JY, Rao Y. Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. *Cell* 96: 807−818, 1999

Ming GL, Song HJ, Berninger B, Holt CE, Tessier-Lavigne M, Poo MM. cAMP-dependent growth cone guidance by netrin-1. *Neuron* 19: 1225−1235, 1997

Nguyen-Ba-Charvet KT, Chedotal A. Role of Slit proteins in the vertebrate brain. *J Physiol Paris* 96: 91−98, 2002

Nishiyama M, Hoshino A, Tsai L, Henley JR, Goshima Y, Tessier-Lavigne M, Poo MM, Hong K. Cyclic AMP/GMP-dependent modulation of Ca2+ channels sets the polarity of nerve growth-cone turning. *Nature* 424: 990−995, 2003

Park HT, Wu J, Rao Y. Molecular control of neuronal migration. *Bioessays* 24: 821−827, 2002

Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Chien CB, Wu JY, Urness LD, Li DY, Robo4 is a vascular-specific receptor that inhibits endothelial migration. *Dev Biol* 261: 251−267, 2003

Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87: 1001−1014, 1996

Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, Poo M. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* 281: 1515−1518, 1998

Stein E, Tessier-Lavigne M. Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science* 291: 1928−1938, 2001

Steinle JJ, Meininger CJ, Forough R, Wu G, Wu MH, Granger HJ. Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. *J Biol Chem* 277: 43830−43835, 2002

Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. *Science* 274: 1123−1133, 1996

Wang B, Xiao Y, Ding BB, Zhang N, Yuan X, Gui L, Qian KX, Duan S, Chen Z, Rao Y, Geng JG. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell* 4: 19−29, 2003

Wang KH, Brose K, Arnott D, Kidd T, Goodman CS, Henzel W, Tessier-Lavigne M. Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell* 96: 771−784, 1999

Whitford KL, Marillat V, Stein E, Goodman CS, Tessier-Lavigne M, Chedotal A, Ghosh A. Regulation of cortical dendrite development by Slit-Robo interactions. *Neuron* 33: 47−61, 2002

Wong K, Park HT, Wu JY, Rao Y. Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev* 12: 583−591, 2002

Wu JY, Feng L, Park HT, Havlioglu N, Wen L, Tang H, Bacon KB, Jiang Zh, Zhang Xc, Rao Y. The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors. *Nature* 410: 948−952, 2001

Xiang Y, Li Y, Zhang Z, Cui K, Wang S, Yuan XB, Wu CP, Poo MM, Duan S. Nerve growth cone guidance mediated by G protein-coupled receptors. *Nat Neurosci* 5: 843−848, 2002

Yu HH, Zisch AH, Dodelet VC, Pasquale EB. Multiple signaling interactions of Abl and Arg kinases with the EphB2 receptor. *Oncogene* 20: 3995−4006, 2001