RESEARCH ARTICLE

Syk kinases are required for spinal commissural axon repulsion at the midline via the ephrin/Eph pathway

Nelly Noraz1,2,3,†, Iness Jaooin1,2,3, Camille Charoy1,2,3, Chantal Watrin1,2,3, Naura Chounlamountri1,2,3, Aurélien Benon1,2,3, Céline Mallevialle1,2,3, Hélène Boudin5, Jérôme Honnorat1,2,3,4, Valérie Castellanı̈1,2,3,* and Véronique Pellier-Monnin1,2,3,*

ABSTRACT

In the hematopoietic system, Syk family tyrosine kinases are essential components of immunoreceptor ITAM-based signaling. While there is increasing data indicating the involvement of immunoreceptors in neural functions, the contribution of Syk kinases remains obscure. Previously, we identified phosphorylated forms of Syk kinases in specialized populations of migrating neurons or projecting axons. Moreover, we identified ephrin/Eph as guidance molecules utilizing the ITAM-bearing CD3zeta (Cd247) and associated Syk kinases for the growth cone collapse response induced in vitro. Here, we show that in the developing spinal cord, Syk is phosphorylated in navigating commissural axons. By analyzing axon trajectories in open-book preparations of mouse embryos, we show that Syk kinases are dispensable for attraction towards the midline but confer growth cone responsiveness to repulsive signals that expel commissural axons from the midline. Known to serve a repulsive function at the midline, ephrin B3/EphB2 are obvious candidates for driving the Syk-dependent repulsive response. Indeed, Syk kinases were found to be required for ephrin B3-induced growth cone collapse in cultured commissural neurons. In fragments of commissural neuron-enriched tissues, Syk is in a constitutively phosphorylated state and ephrin B3 decreased its level of phosphorylation. Direct pharmacological inhibition of Syk kinase activity was sufficient to induce growth cone collapse. In conclusion, Syk kinases act as a molecular switch of growth cone adhesive and repulsive responses.

KEY WORDS: Syk kinases, Ephrin/Eph, Neural tube, Commissural axons, Repulsion

INTRODUCTION

The function of the non-receptor spleen tyrosine kinase (Syk) family members Syk and Zap70 (also known as ZAP-70) has been well characterized in cells of the hematopoietic lineage. They relay signals emanating from classical immunoreceptors such as TCR, BCR, NKR and FcR, thereby controlling their homeostasis and functional outcome. Immunoreceptors utilize a common signal transduction mechanism, which relies on conserved immunoreceptor tyrosine-based activation motifs (ITAMs) that are mostly present in the signal transducing subunits or as part of the recognition subunits themselves. Immunoreceptors can act as either activator when present with ITAMs or as inhibitor when present with immunoreceptor tyrosine-based inhibition motifs (ITIMs). ITAM-based signaling depends on the coordinated interplay of the Src and Syk kinase families. Upon ligand recognition and receptor clustering, tyrosines within the ITAM are dually phosphorylated by Src family kinases, providing a docking site for the tandem SH2 domains of the Syk family kinases. These early events allow the recruitment and activation of both kinase families and serve as a crucial link to adapter molecules [e.g. Lat and SLP-76 (Lcp2)] capable of organizing multiple signaling pathways in a spatiotemporal manner. Downstream signaling ensues principally via the Ras, DAG and calcium-dependent signaling pathways, as well as via actin dynamics regulatory molecules (Smith-Garvin et al., 2009).

Importantly, ITAM-based signaling has been extended to non-immunoreceptors. Indeed, many cell surface molecules that are not restricted in expression to hematopoietic cells have been shown to signal through an immunoreceptor-like pathway. These principally comprise adhesion molecules implicated in cell-pathogen, cell-cell or cell-extracellular matrix (ECM) interactions or receptors linked to inflammatory adaptive responses. Some of them contain an ITAM on their own cytoplasmic tail but lack, among such a molecule, an ITAM-transducing subunit in a co-optive manner to recruit and activate the Src/Syk kinases (Abram and Lowell, 2007; Ivashkiv, 2009; Kazerounian et al., 2011; Letellier et al., 2010). A striking feature of the diverse receptors utilizing ITAM-based signaling is their interplay with integrins at two intersected levels of regulation, referred as inside-out and outside-in (Jakus et al., 2007; Luckashenak and Clements, 2007). Overall, it appears that ITAM-based signaling typically does not function in isolation. Instead, it is enmeshed in the molecular network controlling cellular adhesion and chemotaxis as well as the functional outcome.

An increasing amount of data supports the involvement of ITAM- or ITIM-bearing molecules in neuronal functions, including dendrite and synapse formation, synaptic plasticity and behavior (Angibaud et al., 2012; Baudouin et al., 2008; Boulanger, 2009; Chuang and Lagenaour, 1990; Hamada et al., 2004; Kaifu et al., 2003; Kitano et al., 2002; Ogawa et al., 2007; Xu et al., 2010; Yamada et al., 2001). Consistent with a role for ITAM-based signaling in developmental events contributing to the establishment of neuronal connectivity, we showed in a previous study that Syk is phosphorylated on tyrosine residues representative of an active form of the kinase in regions of synaptogenesis and in specialized populations of migrating cells or projecting axons (Hatterer et al., 2011). Notably, since neurons are devoid of classical immunoreceptors, the neuronal function of ITAM-based signaling

1INSERM U1217, Institut NeuroMyoGêne, Lyon F-69000, France. 2CNRS UMR5310, Institut NeuroMyoGêne, Lyon F-69000, France. 3University Claude Bernard Lyon, Lyon F-69000, France. 4Hospices Civils de Lyon, Lyon F-69000, France. 5INSERM U1064, Institut de Transplantation Urologie-Néphrologie, Nantes F-44035, France.

*These authors contributed equally to this work

†Author for correspondence (nelly.noraz@inserm.fr)

N.N., 0000-0003-4076-9773

Received 28 July 2015; Accepted 15 April 2016
is likely to be linked to unrelated receptors that use it in a co-optive manner. Interestingly, we identified ephrin/Eph as adhesion molecules utilizing CD3ζ (also known as Cd247) as an ITAM-transducing subunit for the growth cone collapse response in vitro. Stimulation of hippocampal neurons with ephrin A1 induces the translocation of CD3ζ and associated Syk tyrosine kinases to EphA4 receptor clusters. In addition, genetic disruption of CD3ζ confers a non-permissive background to ephrin A1-induced repulsive signals in these cells (Angibaud et al., 2011).

In the nervous system, to form precise neuronal networks axons navigate through a complex environment guided by the concerted action of guidance molecules. Eph receptor tyrosine kinases and their membrane-bound ligands, the ephrins, have been shown to play a crucial role in the processes of axon targeting. This arises from their ability to regulate cell-cell interactions, generating adhesive and repulsive responses (Egea and Klein, 2007; Pasquale, 2008). In the immune system, ephrin/Eph-associated functions fit well with the extended ITAM-based paradigm. Ephrin/Eph interactions positively or negatively regulate leukocyte adhesive properties as well as chemotactic responses (Funk and Orr, 2013). In addition, ephrin/Eph signaling affects leukocyte functional outcomes relying on classical immunoreceptors, including integrins (Arvanitis and Davy, 2008; Pitulescu et al., 2010; Sharfe et al., 2008).

In light of these findings, we have studied the role of Syk kinases during spinal cord midline crossing, a well-characterized model of axon guidance in which ephrin/Eph have been implicated (Kadison et al., 2006).

RESULTS

Syk is phosphorylated in projecting commissural axons of E11-13 mouse embryos

In the developing mouse spinal cord, commissural axons project from the dorsal region to the floor plate and cross it before projecting to their contralateral synaptic targets in the caudorostral axis. This crossing decision is under exquisite spatial and temporal regulation. Growing commissural axons initially respond to attractive signals, but once across the floor plate repulsion dominates, allowing axons to leave and preventing them from re-entering the floor plate (Dickson and Zou, 2010).

To gain insight into the phosphorylation status of Syk kinases during this process, transverse sections of E11.5 and E13.5 embryos were stained with anti-βIII tubulin, a marker of differentiating neurons, and two antibodies recognizing phosphorylated forms of Syk YS525/S526 and Syk YS525/Zap70 Y319. Notably, phosphorylation on these tyrosines has been shown to support an activator role (Brdicka et al., 2005; Zhang et al., 2000). The two phosphorylation-specific antibodies presented a similar pattern of labeling on E11.5 and E13.5 embryos (Fig. 1A,B; N.N., unpublished). Phospho-Syk/Zap70 labeling was readily detected in different axonal populations comprising commissural axons, sensory nerve fibers in the dorsal root entry zone and motor axons originating from the developing motor columns. A similar pattern of expression was also obtained using Syk/Zap70-specific antibodies but with much lower intensity (N.N., unpublished). No obvious staining was observed in control conditions (Fig. 1C). In commissural neurons, Syk kinases appeared phosphorylated in precrossing axons extending from the cell bodies residing in the

![Fig. 1. Syk kinases are phosphorylated in outgrowing commissural axons.](image)
dorsal regions of the spinal cord, in crossing axons at the floor plate forming the ventral commissure, and in postcrossing axons coursing in the ventral as well as the lateral funiculi (Fig. 1A,B). Syk expression and phosphorylation were confirmed by western blot on tissue extracts prepared from the most dorsal part of E12.5 open-book preparations, enriched in commissural neurons (Fig. 1D).

Syk kinases are required in vivo to expel commissural axons from the midline
To investigate the role of Syk kinases in commissural axon guidance, we generated Syk−/−; Zap70−/− double-knockout (dKO) mouse embryos. Whereas the Zap70 knockout is viable and leads to a block in T cell differentiation at the CD4+ CD8+ double-positive stage (Kadlecek et al., 1998), Syk knockout mice die around birth with marked petechiae due to blood-lymphatic shunts, and fewer than 10% of Syk−/− mice survive postnatal day 2 (Turner et al., 1995). Therefore, Syk−/−; Zap70−/− embryos were generated by intercrossing Syk−/−; Zap70−/− animals and commissural axon trajectory was examined on E13.5 spinal cord open-book preparations. Dil crystals were applied to the most dorsal part of mutant or wild-type (WT) open-book preparations and after 2 days the trajectory of commissural axons was analyzed (Fig. 2A,B).

In dKO embryos, precrossing axons were apparently normally fasciculated and reached the midline. Moreover, they entered the

---

**Fig. 2.** Syk kinases are required to expel commissural axons from the midline. (A) Schematics of spinal cord open-book preparations with anterograde labeling of commissural axons after insertion of Dil crystals in the dorsal part. As observed on E13.5 WT and dKO open-book preparations, the different trajectories are represented as: (1) ‘normal’ when commissural axons crossed the floor plate and turned rostrally; (2) ‘stalling’ when commissural axons were arrested in the floor plate; (3) ‘defective turning’, which includes ‘premature turning’ when commissural axons turned prematurely before crossing the floor plate, ‘R/C defects’ when commissural axons turned in an aberrant rostrocaudal direction, and ‘no turning’ when commissural axons projected straight without turning after crossing the floor plate; and (4) ‘back crossing’ when commissural axons recrossed the floor plate. (B) Images illustrating examples of fiber tract trajectories observed from one inserted Dil crystal. Images are focused on axons crossing the floor plate, since most of abnormal trajectories (arrowheads) were observed at this particular step. As an example, in B phenotype (3), although several fiber tracts with a defective turning or a stalling phenotype are observed we counted one defective turning phenotype and one stalling phenotype. (C) For each open-book (embryo), the percentage of each phenotype was determined and results are represented as mean percentage±s.e.m. WT, n=21 embryos (84 crystals, 156 counted phenotypes); dKO, n=13 embryos (62 crystals, 143 counted phenotypes). Two-tailed Student’s t-test, except for the back crossing phenotype for which a Mann–Whitney test was applied. **P<0.01. D, dorsal; V, ventral; C, caudal; R, rostral; FP, floor plate. Scale bar: 30 µm.
midline showing no significant premature turning or premature stalling/knotting. These observations indicated that the molecular machinery supporting growth cone dynamics is intact in Syk family kinase-deficient cells and that Syk kinases are dispensable for growth cone responsiveness to floor plate-derived attractive signals. By contrast, dKO embryos presented obvious late pathfinding errors (Fig. 2C). Compared with WT animals, the percentage of fiber tracts with a ‘normal’ pathfinding phenotype was significantly reduced in dKO embryos (57.5±2.9% versus 41.0±4.6%, P<0.01). The proportion of fiber tracts showing a ‘defective turning’ phenotype did not differ between the two groups (P>0.05). However, dKO embryos presented a higher percentage of fibers in ‘stalling’ (39.0±4.1% versus 23.0±3.5%, P<0.01) and, strikingly, 3.0±1.4% of them were observed to be ‘back crossing’, a phenotype never seen in WT animals. These results indicated that Syk kinases are required to expel commissural axons from the floor plate and to prevent them from recrossing the midline. Among the guidance molecules described in the literature as participating in such repulsive functions, namely Slits (Long et al., 2004), ephrins (Kadison et al., 2006) and semaphorins (Nawabi et al., 2010; Zou et al., 2000), we favored the ephrin/Eph pathway because we had data showing that, in hippocampal neurons, Syk kinases are involved in ephrin A1-induced growth cone collapse (Angibaud et al., 2011). On that basis, the possible involvement of Syk kinases downstream of the ephrin/Eph pathway in commissural neurons was explored.

Syk kinases are required for ephrin B3-induced growth cone collapse

In the developing spinal cord, ephrins are expressed in glial cells present at the floor plate and EphB receptors are expressed in commissural neurons (Imondi et al., 2000; Jevince et al., 2006). In addition, whereas similar crossing errors were reported in mice lacking EphB receptors (EphB1, EphB2 and EphB3) or ephrin B3, mice expressing a form of ephrin B3 truncated in its cytoplasmic domain, and thereby unable to transduce signals, presented no abnormalities (Kadison et al., 2006), thus confirming that EphB forward signaling rather than ephrin B3 reverse signaling controls commissural axon midline crossing. Besides, ephrin B3 acts on commissural neurons as a growth cone collapsing factor, as shown previously using E13 spinal cord explants (Imondi et al., 2000). Therefore, in a first set of experiments, an ephrin B3-induced growth cone collapse assay was set up. Recombinant ephrin B3-Fc was added to 48 h cell cultures of commissural neurons isolated from E12.5 or E13.5 WT spinal cords and, after 30 min, growth cone collapse was quantified (Fig. 3A). Since ephrin oligomerization is required for collapsing activity (Davis et al., 1994), ephrin B3-Fc recombinant proteins were clustered with an anti-Fc antibody before assaying. At basal level (Ct), commissural neurons presented 26.9±5.4% collapsed growth cones, and the addition of the control protein, which comprises an Fc fragment only, did not change growth cone behavior (30.9±3.6%, P>0.05). By contrast, and as expected, the clustered ephrin B3 (eB3) but not the monomeric forms (eB3m) induced a marked collapse (eB3, 67.8±4.6% versus 30.9±3.6%, P<0.001; eB3m, 34.7±3.5% versus 30.9±3.6%, P>0.05). Notably, eB3 caused rapid growth cone collapse, reaching a maximum within 5 min, as demonstrated in the timecourse assay (Fig. 3B).

In a second set of experiments, the collapsing effect of ephrin B3 was assessed on commissural neurons isolated from dKO embryos. As shown in Fig. 3C, whereas 67.8±4.6% of growth cones were collapsed in WT cells after eB3 treatment, doubly deficient cells remained unresponsive with a collapse of 34.1±3.6%, similar to the control Fc condition of 36.7±12.8% (P>0.05). Such a defect was linked to Syk deficiency rather than to any particular feature of the genetic background, since in Syk<sup>−/−</sup>, Zap70<sup>−/−</sup> mice from the same litter the response to eB3 was restored with 63.7±3.5% collapse (P<0.001). Neurons cultured from dKO embryos did not present any morphological peculiarities. Furthermore, the degree of spontaneous collapse observed in the control Fc condition (Fig. 3C), the culture content of Dcc<sup>+</sup> cells (a marker of commissural neurons), as well as the cell viability were similar between dKO and WT animals (Fig. 3D). Taken together, these results showed that signaling via Syk kinases is required for ephrin B3-mediated commissural neuron growth cone collapse.

Syk kinases are involved in proximal events leading to ephrin B3 cleavage and/or ephrin B3/EphB2 endocytosis

Regarding the EphB receptor(s) mediating the repulsive function of ephrin B3 in commissural neurons, we identified EphB2 as one candidate. In spinal cord sections of E12.5 embryos, EphB2 labeling was found in commissural axons crossing the floor plate and its expression was confirmed by western blot on commissural neuron-enriched tissue extracts (Fig. 4A,B). EphB2 was also observed in commissural neurons cultured in vitro. The signal was detected in clusters of variable size distributed all over the surface of the cells (Fig. 4C, top) and was observed in the majority of cells. To address whether ephrin B3-induced growth cone collapse relies on EphB2, commissural neurons were treated with ephrin B3-Fc recombinant proteins preclustered with an anti-Fc biotinylated antibody. A double surface labeling was performed with Alexa 546-conjugated streptavidin to visualize the cell-bound eB3 fragment (red) and with anti-EphB2 primary plus anti-goat Alexa 488-conjugated secondary antibodies to visualize EphB2 receptor (green). After 5 min at 37°C, the cell-bound fraction of eB3 almost always colocalized with EphB2. Notably, both eB3 and EphB2 staining appeared as large dots representative of ephrin B3/EphB2 complex formation (Fig. 4C, middle panel). Such ephrin B3-induced EphB2 receptor clustering identified EphB2 as a clear candidate in mediating commissural neuron growth cone collapse. Nonetheless, EphB1 and EphB3, which have a similar pattern of expression to EphB2 on spinal cord sections (Imondi et al., 2000; Jevince et al., 2006), remain possible candidates.

In a number of assay systems, ephrin/Eph interactions have been shown to positively or negatively regulate growth cone adhesive properties. Importantly, both responses rely on Eph receptor clustering and are determined individually through receptor intracellular signaling. Adhesion is associated with low Eph kinase activity, prolonged ephrin binding and Eph clustering. By contrast, repulsion is associated with high Eph kinase activity, ephrin proteolytic cleavage and/or endocytosis, as well as cytoskeletal collapse of cell protrusions, both processes resulting in surface proteolysis of the ligand (Janes et al., 2012; Pasquale, 2005). In dKO neurons treated with eB3, collapse was impaired. A possible reason is a defect in the initial ephrin/Eph binding and clustering events. To address this possibility, WT and dKO neurons were treated as before and the state of collapse, as well as the number of eB3 clusters present on the growth cone surface, were compared. As expected, in WT cells, growth cone collapse was accompanied by rapid surface clearance of eB3 ligand (Fig. 4C, bottom) and was observed in the majority of cells, whereas prolonged eB3 binding and EphB2 clustering were a hallmark of the non-collapsed growth cones (Fig. 4C, middle). In cultures established from dKO embryos, most of the growth cones were non-collapsed but presented eB3 clusters at the cell surface. Importantly, the number of clusters that formed in neurons lacking Syk kinases was not significantly different from WT cells (14.15±1.50 in dKO versus 13.71±1.11 in WT for non-collapsed growth
cones, \( P > 0.05 \); and 2.85±0.51 in dKO versus 1.17±0.23 in WT for collapsed growth cones, \( P > 0.05 \); Fig. 4D), indicating that ephrin B3 binding and clustering occurred normally in dKO cells. In agreement with these observations, EphB2 was expressed normally in dKO embryos (Fig. 4A,B). To conclude, Syk kinases are dispensable for prolonged ephrin B3 binding and EphB2 clustering, which are likely to confer growth cone adhesive properties, but are instead involved in proximal signaling events leading to ephrin B3 cleavage and/or ephrin B3/EphB2 endocytosis.

We next asked whether EphB2 activation might be defective in dKO mice. Upon ephrin binding, Eph receptors become phosphorylated on tyrosine residues present in the juxtamembrane segment of the protein. Phosphorylation promotes kinase activity by disrupting intramolecular inhibitory interactions and generates docking sites for signaling partners, ultimately leading to repulsion (Pasquale, 2005). Therefore, the phosphorylation status of EphB2 upon ephrin B3 binding was examined in WT and dKO animals. Fragments of commissural neuron-enriched tissues freshly obtained from E12.5 open-book preparations were incubated for 5 min at 37°C with preclustered control Fc or ephrin B3-Fc (eB3) and protein lysates were blotted with anti-phospho-EphB2 juxtamembrane Y594 (Fig. 4E). As expected, eB3 led to a rapid increase in EphB2 phosphorylation in WT treated tissues. By contrast, in the absence of Syk kinases, the level of EphB2 phosphorylation remained unchanged. These findings indicate that, in dKO animals, axon guidance defects are associated with impaired EphB2 proximal signaling.

Ephrin B3-induced collapse is associated with a decrease in Syk phosphorylation

We next looked for changes in the level of Syk phosphorylation and/or its recruitment to EphB2 clusters upon ephrin B3-Fc (eB3) and protein lysates were blotted with anti-phospho-EphB2 juxtamembrane Y594 (Fig. 4E). As expected, eB3 led to a rapid increase in EphB2 phosphorylation in WT treated tissues. By contrast, in the absence of Syk kinases, the level of EphB2 phosphorylation remained unchanged. These findings indicate that, in dKO animals, axon guidance defects are associated with impaired EphB2 proximal signaling.
phospho-Syk using anti-phospho-Syk Y348 primary with anti-mouse Alexa 555-conjugated secondary antibodies in red (Fig. 5A). In control Fc-treated cells, phospho-Syk was readily detected; staining appeared as puncta of various sizes and intensities distributed throughout the cell, as well as along the length and tip of filopodia (Fig. 5A, left). After eB3 treatment, the overall phospho-Syk signal intensity decreased, although in a non-significant manner (Fig. 5A, bar chart). eB3 clusters were found in close proximity to phospho-Syk labeling (Fig. 5A, right). However, since collapse was accompanied by a rapid surface clearance of ephrin B3, the proximity of phospho-Syk to EphB2-engaged receptor was essentially only observable on non-collapsed cells. Consequently, a link between the state of Syk phosphorylation and growth cone collapse could not be established using this approach.

To determine whether Syk activity was required for ephrin B3-mediated growth cone collapse, the experiment was repeated in the presence of 1 µM BAY 61-3606 (Bay-61), a pharmacological inhibitor that competes for the Syk ATP-binding site. Strikingly (Fig. 5B, left), WT neurons exposed to Syk inhibitor for 5 min showed a marked collapse response [62.2±1.9% versus 29.7±3.5% in DMSO (Bay-61 vehicle), P<0.01]. As a control for inhibitor specificity and potential toxicity, the same treatment was applied to cells lacking the kinases. In this case, Syk inhibitor did not induce any changes in the percentage of collapsed cells compared with DMSO (28.3±1.7% versus 28.0±0.3%, respectively, P>0.05). To confirm these results and address the question of whether the collapse response depends on the level of Syk kinase activity, a dose-response experiment was performed using two Syk inhibitors, namely Bay-61 and Piceatannol. Percentages of collapsed growth cones were dose dependent (Fig. 5B, middle and right). Therefore, it appears that in cultured commissural neurons Syk is constitutively activated and that directly decreasing Syk kinase activity to a given level is sufficient to rapidly induce growth cone collapse. These results also suggest that Syk inhibition is a general requirement for growth cone collapse.

In a last set of experiments, EphB2/Syk interaction as well as the phosphorylation state of Syk upon ephrin B3 binding was examined on freshly isolated tissue fragments enriched in commissural neurons. As reported previously (Charoy et al., 2012; Nawabi et al., 2010), this approach has the advantage of allowing neurons to be assessed in their physiological context. EphB2 was immunoprecipitated and associated proteins were blotted with anti-Syk antibody (Fig. 5C). A band corresponding to highly phosphorylated Syk kinase (see upper
band in total lysates blotted with anti-Syk antibodies) was observed in EphB2 immunoprecipitate (IP, Eph) but not when IgG was omitted (no) or in isotype control IgG (iso) conditions, providing evidence of EphB2/Syk interaction. To examine the phosphorylation status of Syk upon ephrin B3 binding, fragments of commissural neuron-enriched tissues were incubated at 37°C with preclustered control Fc or eB3 and protein lysates were blotted with anti-Phospho-Syk antibodies. eB3 treatment did not alter the level of Syk (Fig. 5D, middle). According to our previous observations on embryonic spinal cord sections (Fig. 1A,B) or freshly isolated tissues (Fig. 1D), Syk is present in phosphorylated forms in the control Fc condition. eB3 treatment decreased the Syk phosphorylation level, with a pronounced effect after 5 min incubation, whereas phospho-Syk was still diminished but to a lesser extent after 30 min (Fig. 5D). Thus, eB3 induced a rapid and probably reversible inhibition of Syk phosphorylation. Taken together, these results indicated that in commissural neurons Syk kinases are constitutively activated and that ephrin B3 induces growth cone collapse through Syk kinase inhibition.

Fig. 5. Ephrin B3-induced collapse is associated with a decrease in Syk phosphorylation. (A) Commissural neurons cultured for 48 h were treated for 5 min with control Fc or eB3 at 2.5 µg/ml. Green labeling represents eB3 bound fraction (streptavidin, living cells) and red labeling represent phospho-Syk Y348 (permeabilized cells). Scale bars: 5 µm. Quantification of phospho-Syk signal intensities obtained from three independent experiments with more than 16 cells analyzed per condition are presented as mean percentages±s.e.m. (B) Cultures of commissural neurons derived from WT or dKO embryos were incubated for 5 min in the presence of Syk pharmacological inhibitors (Bay-61 and Piceatannol) at the indicated concentrations and the percentage of growth cone collapse was determined. As control for inhibitor vehicle, cells were treated with culture medium supplemented with DMSO (D-5.0 and D-20 correspond to control for Bay-61 5 µM and Piceatannol 20 µM, respectively). Experiments were performed in triplicate with at least 100 growth cones analyzed per coverslip and were repeated two or three times. Bar charts show the mean percentage±s.d. (C) EphB2 (Eph) was immunoprecipitated from tissue extracts and associated proteins were blotted with anti-EphB2, anti-Syk (clone 01) or anti-Gapdh antibodies. Omitted IgG (no) and isotype IgG (iso) conditions were used as controls. For total lysates, 30 µg protein, corresponding to 6% of IP extracts, was loaded on the gel. (D) Western blot analysis of phospho-Syk/Zap70 Y352/319 and Syk (clone 01) in tissue extracts treated with Fc or eB3. Erk1, Erk2 and Gapdh proteins were used as loading controls. Quantification of signal intensities obtained from three independent experiments is depicted as mean index±s.d. *P<0.05, **P<0.01, ***P<0.001. Two-tailed Student’s t-test.
function of the floor plate to operate. Notably, because Syk dephosphorylation is likely to occur locally at the growth cone, it would not be detected on spinal cord sections by immunolabeling.

**DISCUSSION**

**Syk activity and growth cone responsiveness to guidance cues**

We have shown in this study that Syk kinases are required for ephrin B3-mediated commissural neuron growth cone collapse, and we collected data implicating EphB2 in this process. These findings extend our previous work performed on hippocampal neurons reporting that the ITAM-bearing CD3zeta molecule and associated Syk kinases are required for ephrin A1/EphA4-induced collapse (Angibaud et al., 2011). Although the use of two couples of ephrin/ Eph lead to a similar outcome, major differences were observed between the two populations of neurons regarding the level of Syk phosphorylation and the kinetics of collapse. In hippocampal neurons, Syk phosphorylation was low at basal level and increased upon ephrin ligand binding; collapse was slow (45 min; Fig. S1) (Angibaud et al., 2011; Hatterer et al., 2011). By contrast, in commissural neurons, Syk phosphorylation was constitutively elevated and decreased upon ephrin binding, although the difference did not reach statistical significance; collapse was rapid (5 min). Furthermore, unlike hippocampal neurons, Eph clusters were preformed at basal level in commissural neurons. Altogether, these observations suggest that the Syk level of activation relies on Eph receptor clustering, which does not necessarily depend on ephrin ligand binding, and that collapse requires Syk to reach a threshold of activation. Such requirements could account for the slow and rapid collapse kinetics observed in these two populations. In support of this idea of an activation threshold, impacting Syk kinase activity through application of Syk pharmacological inhibitors resulted in distinct outcomes. Applied to hippocampal neurons in which, at basal level, Syk activation is under the threshold, Syk inhibitor had no collapsing effect per se. Moreover, by preventing the ephrin-induced increase of Syk activation, Syk inhibitor antagonized growth cone collapse. By contrast, applied on commissural neurons in which, at basal level, the activation threshold is essentially reached, Syk inhibitor readily induced collapse and the collapsing response relied on the relative level of Syk kinase activity. It is therefore tempting to speculate that Syk acts as a molecular switch of growth cone attractive and repulsive responses. Interestingly, in T cells, a graded and switchable Syk-dependent response to ephrins has been reported (Kawano et al., 2012). The authors described an ephrin-concentration-dependent switch from promotion to inhibition of TCR-induced proliferation, with high concentrations of ephrin negatively impacting T cell proliferation. Notably, the switch was associated with strong Eph receptor activation and inhibition of the Src (Lck) and Syk (Zap70) kinase activities through the recruitment of the phosphatase Shp1 (Ptpn6) (Kawano et al., 2012).

Consistent with Syk kinases being downstream of Src kinases in ITAM-based signaling, simply decreasing Src activity using a pharmacological inhibitor is equally sufficient to induce growth cone collapse in commissural neurons (N.N., unpublished). Actually, whereas there is little evidence in the literature of Syk kinases determining growth cone behavior, a causal link between the Src state of activation and growth cone behavior has been elegantly demonstrated (Yam et al., 2009; Robles et al., 2005). These findings illustrate the graded and switchable nature of Src/Syk-dependent growth cone responses and fit well with the idea that coupling Syk kinase activation with inhibition constitutes an integrated molecular readout utilized by guidance molecules to change growth cone behavior.

**Proper floor plate navigation relies on Syk kinases**

We showed in this study that in navigating commissural axons Syk is phosphorylated when precrossing axons are attracted to the floor plate. However, by analyzing commissural axon trajectory in spinal cord open-book preparations of dKO embryos, we found that Syk kinases are dispensable for axon attraction to the midline but are required for proper exit. Importantly, consistent with the coordinated activation of Src/Syk kinases in ITAM-based signaling, similar results have been reported for Src kinases. Phospho-Src staining of spinal cord sections of mouse embryos revealed a Syk-like pattern of activation (Yam et al., 2009), and recent studies performed in *Drosophila* showed that Src family kinases are dispensable for midline attraction of commissural axons but antagonize midline crossing (O’Donnell and Bashaw, 2013). Whether this also applies to rodents remains to be determined, but these findings are nonetheless in agreement with *in vitro* data showing that Src/Syk kinases confer growth cone responsiveness to repulsive signals. However, they conflict somewhat with prevailing models positioning Src kinases downstream of guidance cues known to mediate attraction to the midline (i.e. netrin, Shh and VEGF) (Meriane et al., 2004; Ruiz de Almodovar et al., 2011; Yam et al., 2009).

Growth cone advance depends on the repeated formation and dissociation of transient contacts between adhesion molecules and the ECM or adjacent cells. Referred to as adhesion turnover, this process depends on tension forces created by physical links between adhesion molecules and the internal dynamics of the cytoskeletal network (Thoumine, 2008). Adhesion turnover can be schematically deconstructed into sequential phases. (1) The assembly phase consists of initial ligand-receptor binding and physical attachment to the cytoskeletal network, thus putting it under tension, and is associated with weak adhesion (slip bond). (2) The stabilization phase allows reinforcement of cytoskeleton-adhesion molecule links and is associated with strong adhesion (catch bond). (3) The disassembly phase, in which linkages between adhesion receptors and the cytoskeleton are dismantled or broken (Choquet et al., 1997; Thoumine, 2008). Several lines of data have provided evidence that, although recruited to the adhesion point, Src and Syk kinases are not necessary for adhesion assembly but rather play a role in adhesion stabilization and/or disassembly (Felsenfeld et al., 1999; Fincham and Frame, 1998; Suter and Forscher, 2001; Webb et al., 2004; Abram and Lowell, 2009; de Virgilio et al., 2004; Minanti et al., 1998). Therefore, in order to discuss the role of Src/Syk kinases in growth cone guidance, their involvement in adhesion turnover needs to be considered.

On the basis of what we have learned from the extended ITAM-based paradigm (i.e. its interplay with adhesion molecules) one can speculate that guidance cues that mediate attraction towards the midline increase the binding capability of growth cones, which in turn leads to downstream Src/Syk activation. By impacting on adhesion stabilization/disassembly, Src/Syk kinases could positively regulate the direction and speed of growth cone progression towards the midline. However, upon crossing, inhibitory signals intersect with activating signals, leading to Src/Syk inhibition and growth cone repulsion. Src kinases phosphorylate both immunoreceptor activation (ITAM) and inhibition (ITIM) motifs. ITIMs allow the recruitment of phosphatases such as Shp1, Shp2 (Ptpn11) or Shpi (Inpp5d) that decrease the phosphorylation of various substrates, including those crucial in adhesion/migration (Bryceson et al., 2009; Oh et al., 1999; Pereira and Lowell, 2003; Underhill and Goodridge, 2007; Wang et al., 2013). The coordinated induction of activating and inhibitory molecules could act in an integrated manner to regulate the strength of adhesion. That said, in Syk−/−; Zap70−/− animals, simple adhesion might be sufficient to relay the attractive properties of guidance cues;
the growth cone would still progress towards the midline, although at low speed. However, the fact that Syk activation might be a prerequisite to gain sensitivity to repulsive signals could account for midline repulsive defects observed in dKO animals.

**Integration of guidance signals by ITAM-based signaling**

We showed in this study that ephrins are obvious candidates in driving Syk-dependent switching at the midline. However, comparing the collapse response to Slit2/3 and to semaphorin 3B (Sema3B) in WT and dKO commissural neurons in culture, we found that both require signaling via Syk kinases (N.N., unpublished). These observations support the idea that the interpretation of signals emanating from diverse guidance cues as being repulsive relies on Syk kinase activity. Although all present at the midline, ephrin B3 and Slit2/3 or Sema3B plausibly act in different spatial and temporal windows. Expressed at the floor plate by glial cells, ephrin B3 acts with short-range activity, whereas Slit2/3 and Sema3B are secreted factors with potentially longer range activity. Combinations and sequences of ligand accessibility and guidance receptor availability are likely to play crucial roles in providing spatial and temporal coordination of guidance signals at the floor plate (Dickson and Zou, 2010; Kaplan et al., 2014; Nawabi and Castellani, 2011). In addition, the common use of Syk signaling would provide an efficient way to ensure the integration of the various guidance forces.

In conclusion, ITAM-based signaling might constitute an integrated molecular readout of environmental information allowing the proper coordination between attraction and repulsion in the context of a complex array of guidance cues.

**MATERIALS AND METHODS**

**Animals**

The generation and genotyping of mutant mice and animal procedures are described in supplementary Materials and Methods. Animal care and procedures were conducted according to European Community Council Directive 2010/63/UE and the French Ethical Committee.

**Antibodies**

Antibodies and reagents are described in Table S1.

**DIL staining on spinal cord open-book preparations**

Wild-type and mutant mouse embryos (E13.5) were collected in cold PBS and dissected in cold Hank’s Balanced Salt Solution (HBSS), 6.5% glucose ( Gibco). Neural tubes were isolated and prepared in an open-book preparations by dissecting the most dorsal part containing commissural fibers, we counted one for the ensemble of fiber tracts with a particular phenotype. For each open-book, the percentage of the different phenotypes corresponding to three times the background value was chosen manually and the quantified using ImageJ software (NIH). For individual growth cones, at a threshold corresponding to the growth cone area was defined based on differential interference contrast (DIC) images, and the number of ephrin B3 clusters was quantified using ImageJ software (NIH). For individual growth cones, a threshold corresponding to three times the background value was chosen manually and the cluster number was determined using the ‘analyze particles’ function.

**Primary culture of spinal cord commissural neurons**

Commissural neurons were obtained from E12.5 spinal cord open-book preparations by dissecting the most dorsal part containing commissural neuron cell bodies in Neurobasal medium (Gibco). Tissues were then incubated in a mixture of trypsin (5 mg/ml) and DNase (0.1 mg/ml) at 37°C for 30 min and washed in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) containing 10% fetal calf serum (FCS) (First Link). Cells were dissociated by trituration through Pasteur pipettes. The dissociated cells were plated onto coated glass coverslips (25 µg/ml poly-L-lysine and 20 µg/ml laminin; Sigma) at 26,000 cells/well in a 24-well plate. Commisural neurons were cultured in Neurobasal medium (Gibco) supplemented with 1% B27 (Gibco), 0.1 µg/ml netrin 1 (R&D Systems), 0.5 mM L-glutamine (Gibco), 50 U/ml penicillin (Gibco) and 50 µg/ml streptomycin (Gibco).

**In vitro collapse assay**

After 2 days in culture, commissural neurons were treated with controlFc or ephrin B3-Fc recombinant proteins (R&D Systems, Q15768, P01857) that had been previously preincubated with an anti-human Fc biotinylated antibody (Jackson ImmunoResearch) at a 1:2 ratio in plain Neurobasal medium for 2 h at room temperature (RT). Preincubated ephrin B3-Fc and control Fc or monomer ephrin B3 (0.5 µg/ml) were added and incubated at 37°C for the indicated period of time. In some experiments, commissural neurons were treated with the Syk inhibitors Bay-61-2-[7-(3,4-dimethoxyphenyl)]imidazo[1,2-c]pyrimidin-5-ylaminopyridine-3-carboxamide hydrochloride (Jackson ImmunoResearch) at 0.1 µg/ml DAPI (4′,6-diamidino-2-phenylindole function).
cells were incubated with Alexa Fluor 488-conjugated streptavidin and Alexa Fluor 555-conjugated anti-mouse antibodies in blocking solution for 1 h at RT. To quantify phospho-Syk signal intensities, an ROI delimiting the cell surface was defined based on DIC images. Using ImageJ, a threshold corresponding to three times the average signal intensity of cells treated with control Fc was applied on each cell and the resulting values, corresponding to the total phospho-Syk-labeled area, were divided by the cell area.

To determine the percentage of Dcc− cells and cell viability, commissural neurons cultured for 48 h were fixed in 4% PFA, 2% sucrose and labeled with anti-Dcc antibodies and DAPI (0.1 μg/ml). Cell viability was quantified based on DAPI nuclear staining. Cells with small, bright and pyknotic nuclei were counted as dead, whereas those with large and dim nuclei presenting visible nucleoli were counted as alive.

Images were acquired with a Zeiss Axio Imager.Z1 fluorescence microscope and analyzed using Axiovision software 4.7. Digitized images were processed for adjustment of brightness and contrast with ImageJ and Adobe Photoshop CS image editing software.

Western blots

Spinal cords from E12.5 embryos were prepared in an open-book configuration and the dorsal part was further dissected to isolate commissural neurons. Tissue fragments were lysed in buffer comprising 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 60 mM octyl glucoside, protease and phosphatase inhibitors. For immunoprecipitations, precleared lysates containing 500 μg protein were incubated overnight with 2 μg anti-EphB2 or control IgG followed by 2 h with protein G-Sepharose beads (Millipore). After five washes in lysis buffer, pellets were resuspended in Laemmli buffer. In some experiments, tissue fragments were incubated with preclustered ephrin B3-Fc or control Fc at 2.5 μg/ml for 5 or 30 min at 37°C. After one wash in PBS, pellets were resuspended in Laemmli buffer. Proteins were resolved on 9% SDS-PAGE and transferred to nitrocellulose membranes (PerkinElmer Life Sciences). Membranes were blocked for 30 min in TBS-T (150 mM NaCl, 20 mM Tris, 0.1% Tween 20, pH 7.5) containing 5% milk and blotted overnight at 4°C with primary antibodies. After three washes in TBS-T, membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies (Amersham). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (ThermoFisher Scientific). Phospho-Syk or phospho-EphB2 signal intensities were quantified using ImageJ and corrected for protein loading using Gapdh. Results are presented as index values relative to control conditions.

Statistical analyses

A Student’s t-test (Microsoft Excel) was performed, except for results presented in Fig. 2C for the back crossing phenotype and Fig. 4C for which a Mann–Whitney test (AnaStats, www.anastats.fr) was applied.

Acknowledgements

We thank E. Schweighoffer and V. L. Tybulewicz (Division of Immune Cell Biology, MRC National Institute for Medical Research, London) for providing the Syk−/−; Zap70−/− knockout mice; J. Falk (Inserm U1217 CNRS U5310, INMG, Lyon) for critically reading the manuscript; J. M. Vicat, J. Gondoluci, A. Chamousset and M. Lavigne (Aixes, Faculté Rockfellker, Lyon) for animal care; and C. Bardel Danjean (CNRS UMR5588, Laboratoire de Biométrie et Biologie Evolutive, Villeurbanne) for statistical analyses.

Competing interests

The authors declare no competing or financial interests.

Author contributions

V.C. brought expertise on the model of axon guidance during spinal cord midline crossing. H.B., V.C., N.N. and V.P.-M. contributed to the formulation and design of the experiments. A.B., C.C., N.C., I.J., N.N., C.M., V.P.-M. and C.W. performed experiments or data analysis. N.N. and V.P.-M. wrote the manuscript with contributions from H.B., J.H. and mainly V.C.

Funding

This work was supported by Institut National de la Santé et de la Recherche Médicale (Inserm), Centre National de la Recherche Scientifique (CNRS), Université Claude Bernard Lyon 1 and Université de Nantes. The work of the team of V.C. was conducted within the framework of the Labex CORTEX and Labex DevWeCAN of Université de Lyon, within the program ‘Investissements d’Avenir’ [ANR-11-IDEX-0007] operated by the French National Research Agency [Agence Nationale de la Recherche (ANR)] and Fondation pour la Recherche Médicale (FRM).

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.128629/-/DC1

References


Fincham, V. J. and Frame, M. C. (1998). The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. 17, 81-92.


Supplementary Materials and Methods

Mice and genotyping

Wild-type C57BL/6 mice (Janvier Montpellier, France) were used in this study. Syk heterozygous and Zap70 homozygous mutant mice (Syk+/-; Zap70+/-), on a C57BL/6 background (at least 10 backcrossing), were a kind gift from V. L. Tybulewicz (Division of Immune Cell Biology, MRC National Institute for Medical Research, London). Homozygous double-mutant embryos lacking both Syk and Zap70 (Syk-/-; Zap70-/-) were generated by crossing Syk+/-; Zap70-/- mice. Embryos were genotyped by PCR. For the wild-type Syk allele we used primers a (5'-AGAGAAGCCCTGCCCATGGAC-3') and b (5'-GTCCAGGTAGACCTTTGGGC-3') and for the mutant Syk allele we used primers b and c (5'-GAGACTAGTGAGACGTGCT-3'). For Zap70, primers a (5'-GCACATATGCACTGTCCCTGGTCTA-3') and c (5'-GGGTCGCTGTAGGGACTCTCGTACA-3') were used for the wild-type allele and primers a and b (5'-TGGCTACCCGTGATATTGCTGAAGA-3') were used for the mutated allele. Pregnant mice were sacrificed under deep anesthesia induced by halothane inhalation and the embryos were taken out by Caesarean section at the indicated day of gestation, the day of conception corresponding to E0.5. Mice were bred and maintained in Transgenic Animal Care Facility (ALECS) in Lyon, France. Animal care and procedures have been conducted according to the European Community Council Directive 2010/63/UE and the French Ethical Committee.
**Fig. S1**

Syk and phospho-Syk levels in commissural neurons (A) and hippocampal neurons (B). Syk level of expression was analyzed by Western-bLOTS performed on freshly isolated cells obtained from the dorsal part of E12.5 open-book preparations (Com), from the hippocampus of E18 embryos (Hip) or from the spleen of adult mice (Spleno). Gels were loaded with the indicated quantities of proteins and membranes were blotted with anti-Syk Ab (N19). Syk levels of expression in commissural neurons and hippocampal neurons were estimated by semi-quantitative immunoblot analysis at respectively 19.4±1.2 and 24.6±2.1 fold lower than that of splenocytes, which express a fair amount of the protein. Phospho-Syk level in those two populations of neuronal cells was assessed by immunolabeling on cultured cells, after 3 days for hippocampal neurons and 2 days for commissural neurons, using an anti-P-Syk Y525/526 Ab. As illustrated, commissural neurons presented at basal level consistently higher phospho-Syk that hippocampal neurons. Scale bar: 20µm.
Table S1: Primary and secondary antibodies and reagents. IF: Immunofluorescence; WB: Western Blot; IP: Immunoprecipitation; C: used for Ctr-Fc and ephrinB3-Fc recombinant proteins preclusterization at a concentration ratio of 1 (recombinant proteins) for 2 (biotinylated anti-human).

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Species</th>
<th>Reference</th>
<th>Use</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syk (N-19)</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology SC-1077</td>
<td>IF</td>
<td>1/100</td>
</tr>
<tr>
<td>Syk (clone 01)</td>
<td>Mouse</td>
<td>BioLegend #626201</td>
<td>WB</td>
<td>1/1000</td>
</tr>
<tr>
<td>Phospho-Syk Y525/526</td>
<td>Rabbit</td>
<td>Cell Signaling #2711</td>
<td>IF</td>
<td>1/300</td>
</tr>
<tr>
<td>Phospho-Syk Y348</td>
<td>Mouse</td>
<td>BD Biosciences #558167</td>
<td>IF</td>
<td>1/100</td>
</tr>
<tr>
<td>Phospho-ZAP Y319-Syk Y352</td>
<td>Rabbit</td>
<td>Cell Signaling #2701</td>
<td>IF</td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WB</td>
<td>1/1000</td>
</tr>
<tr>
<td>EphB2</td>
<td>Goat</td>
<td>R&amp;D Systems AF467</td>
<td>IF</td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WB</td>
<td>1/1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IP</td>
<td>2μg</td>
</tr>
<tr>
<td>βIII tubulin</td>
<td>Mouse</td>
<td>Sigma Aldrich T8660</td>
<td>IF</td>
<td>1/3000</td>
</tr>
<tr>
<td>Phospho-EphB2 Y594</td>
<td>Rabbit</td>
<td>Abcam ab61791</td>
<td>WB</td>
<td>1/1000</td>
</tr>
<tr>
<td>Control IgG</td>
<td>Goat</td>
<td>Life Technologies #02-6202</td>
<td>IP</td>
<td>2μg</td>
</tr>
<tr>
<td>DCC</td>
<td>Mouse</td>
<td>BD Biosciences #554223</td>
<td>IF</td>
<td>1/500</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>Rabbit</td>
<td>Cell Signaling #9102</td>
<td>WB</td>
<td>1/1000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>Millipore MAB374</td>
<td>WB</td>
<td>1/10000</td>
</tr>
<tr>
<td>Alexa Fluor 488/555 anti-mouse</td>
<td>Goat</td>
<td>Molecular Probes A10684/A21427</td>
<td>IF</td>
<td>1/2000</td>
</tr>
<tr>
<td>Alexa Fluor 488/555 anti-rabbit</td>
<td>Goat</td>
<td>Molecular Probes A11034/A21430</td>
<td>IF</td>
<td>1/2000</td>
</tr>
<tr>
<td>Alexa Fluor 488 anti-goat</td>
<td>Donkey</td>
<td>Molecular Probes A11055</td>
<td>IF</td>
<td>1/1000</td>
</tr>
<tr>
<td>HRP conjugated anti-mouse/rabbit</td>
<td>Goat</td>
<td>Jackson I. #115-036-003, # 111-036-003</td>
<td>WB</td>
<td>1/10000</td>
</tr>
<tr>
<td>Biotinylated anti-human</td>
<td>Donkey</td>
<td>Jackson I. #115-065-008</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Reference</th>
<th>Use</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 488-phalloidin</td>
<td>Molecular Probes A12379</td>
<td>IF</td>
<td>1/40</td>
</tr>
<tr>
<td>Streptavidin Alexa Fluor 488</td>
<td>Molecular Probes S11223</td>
<td>IF</td>
<td>1/700</td>
</tr>
<tr>
<td>Streptavidin Alexa Fluor 546</td>
<td>Molecular Probes S11225</td>
<td>IF</td>
<td>1/700</td>
</tr>
</tbody>
</table>