RESEARCH ARTICLE

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

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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 Syk−/−; Zap70−/− 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 most lacking such a motive, 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 emmeshed 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...
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 Y525/526 and Syk Y352/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. (A,B) Transverse sections of E11.5 (A) and E13.5 (B) mouse spinal cords were immunostained for phospho-Syk (P-Y525/526) or phospho-Syk/Zap70 (P-Y352/319) and βIII-tubulin. (C) Immunostaining in control conditions with omission of primary antibodies. Asterisk, dorsal root entry zone; arrow, precrossing axons; arrowhead, postcrossing axons. FP, floor plate. Scale bars: 50 µm. (D) Western blot performed on total tissue extracts obtained from the dorsal part of E12.5 open-book preparations (CNeu, 30 µg protein) and splenocytes of adult mice (Spleno, 5 µg protein). Membranes were blotted with anti-Syk (N19) and anti-phospho-Syk/Zap70 (P-Y352/319) antibodies. Erk1 and Erk2 were used as loading controls. CNeu, commissural neurons; spleno, splenocytes.
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 (Ctr), commissural neurons presented 26.9±5.4% collapsed growth cones, and the addition of the control, 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*−/−; Zap70*−/− 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 Dec− 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 fraction (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\( \pm \)0.51 in dKO versus 1.17\( \pm \)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 addition. Cultures were treated as before with control Fc or eB3 recombinant proteins, quickly washed, fixed and permeabilized. eB3 clusters were visualized using Alexa 488-conjugated streptavidin in green and
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

Fig. 4. Syk kinases are part of events leading to eB3 surface clearance.
(A) EphB2 immunolabeling of spinal cord transverse sections of E12.5 WT and dKO mouse embryos. Arrow, precrossing commissural axons; arrowhead, postcrossing commissural axons. FP, floor plate. (B) Representative western blot illustrating EphB2 expression in commissural neuron-enriched tissue extracts of E12.5 WT and dKO embryos. Gapdh was used as loading control. (C) Commisural neurons isolated from E12.5 WT spinal cords were cultured for 48 h and treated for 5 min with Fc or eB3. Growth cone DIC images, with EphB2 receptor surface labeling (green) and eB3 bound fraction (streptavidin, red). (D) Quantitative analysis of the number of eB3 clusters present on the surface of collapsed (C) and non-collapsed (NC) growth cones (GC) in WT and dKO cultures treated as in C. Data resulting from three independent experiments are compiled in a box plot. Mann–Whitney test. (E) Western blot analysis of EphB2 Y594 phosphorylation in tissue extracts treated for 5 min with Fc or eB3. Quantification of signal intensities obtained from three independent experiments is depicted as mean index±s.d. Two-tailed Student’s t-test. *P<0.05. Scale bars: 50 µm in A; 20 µm in C.
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.

Altogether, our results suggest a model whereby Syk kinases are activated in commissural axons at the precrossing stage but, upon floor plate crossing and exposure to midline cues, including ephrin B3, Syk kinases are dephosphorylated, allowing the repulsive
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 Shp3 (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 preparation and then fixed in 4% paraformaldehyde (PFA) for 2 h and kept in PBS at 4°C. Four to eight small Dil crystals (Molecular Probes) were inserted in the most dorsal part of one hemiscut to achieve anterograde labeling of commissural axons. The different trajectories summarized in Fig. 2A were observed after 48 h by conventional fluorescence microscopy and were categorized as normal (28%), ten stalling (40%), eight defective turning (32%) and zero (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 control Fc or ephrin B3-Fc recombinant proteins (R&D Systems, Q15768, P01857) that had been previously preclustered 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). Preclustered ephrin B3-Fc and control Fc or monoclonal ephrin B3 (0.5 µg/ml) were applied 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-ylaminol]glyridine-3-carboxamide hydrochloride) and Picatan (6-[4-[1-(2,3,5-trihydroxyphenyl)ethenyl]benzene-1,2-diol] (Sigma-Aldrich) at the indicated concentrations for 5 min. As control for inhibitor, cells were treated with culture medium supplemented with vehicle DMSO. Neurons were then washed in PBS and fixed in 4% PFA, 2% sucrose for 10 min. Growth cones were visualized by polymerized actin staining using Alexa Fluor 488-phalloidin (Molecular Probes). Growth cones with two or fewer filopodia were considered as collapsed. The state of growth cones was scored under blinded condition.

**Immunofluorescent labeling**

**On tissue sections**

Tissue sections were obtained from E11-13 embryos as previously described (Hatterer et al., 2011). For immunostaining, sections were incubated in blocking buffer PBS-T (0.3% Triton X-100) containing 3% BSA for 1 h at RT and then with primary antibodies overnight at 4°C. After three washes in PBS-T, sections were incubated with appropriate Alexa Fluor-conjugated antibodies for 1 h at RT. After that, washed in PBS-T, sections were treated with 0.1 µg/ml DAPI (4’,6-diamidino-2-phenylindole dihydrochloride) for 5 min to label the nuclei and then mounted in Fluor Preserve reagent (Calbiochem). For control experiments, samples were treated following the same procedure but primary antibodies were omitted.

**On cultured cells**

Co-immunostaining of EphB2 receptor and ephrin B3 cell-bound fraction was performed on live cells. Commissural neurons were treated as described for the collapse assay with preclustered ephrin B3-Fc and control Fc (2.5 µg/ml) for 5 min at 37°C, washed in cold PBS and kept on ice. Cells were labeled for 15 min using goat anti-EphB2 antibody, washed and further incubated for 30 min with Alexa Fluor 488-conjugated anti-goat secondary antibody and Alexa Fluor 546-conjugated streptavidin to visualize the ephrin B3 cell-bound fraction. After washing, cells were fixed in 4% PFA for 10 min. Coverslips were mounted in Fluor Preserve reagent (Calbiochem).

To quantify the number of ephrin B3 clusters at the surface of growth cones, cells were treated in the same manner (5 min at 37°C), washed in cold PBS, fixed in 4% PFA, 2% sucrose for 10 min and incubated with Alexa Fluor 488-conjugated streptavidin for 30 min at RT. A region of interest (ROI) corresponding to the growth cone area was defined based on differential interference contrast (DIC) images. 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.

Co-immunostaining of ephrin B3 clusters present at the cell surface and phospho-Syk was performed sequentially. Cells treated as previously described (5 min at 37°C) were washed in cold PBS and fixed in 4% PFA, 2% sucrose. They were then permeabilized in blocking buffer for 1 h and exposed to rabbit anti-phospho-Syk Y348 antibodies overnight at 4°C. After washing in PSB-T,
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 Axios 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, or 30 min at 37°C). After three washes in TBS-T, membranes were incubated for 1 h at RT with horseshadish 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.

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Supplementary information

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

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