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Study of the spatio-temporal dynamics of guidance receptors during commissural axon navigation in the spinal cord

Aurora Pignata

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Aurora Pignata. Study of the spatio-temporal dynamics of guidance receptors during commissural axon navigation in the spinal cord. Neurobiology. Université de Lyon, 2018. English. NNT : 2018LYSE1286 . tel-02309060

HAL Id: tel-02309060

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N°d'ordre NNT : 2018LYSE1286

THÈSE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée au sein de
l'Université Claude Bernard Lyon 1

Ecole Doctorale N°340

Biologie Moléculaire, Intégrative et Cellulaire

Spécialité de doctorat :

Discipline : Neurosciences du développement

Soutenue publiquement le 10/12/2018, par :

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**Study of the spatio-temporal dynamics of guidance receptors during
commissural axon navigation in the spinal cord.**

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Merci 😊

Je remercie chaleureusement Dr. Sonia Garel et Dr. Alain Chédotal d'avoir accepté d'être rapporteurs de mes travaux de thèse et de prendre part à mon jury.

Je remercie également Dr. Olivier Thoumine et Dr. Olivier Raineteau qui ont suivi l'avancement de mon projet au cours des différents comités de suivi de thèse et qui ont accepté pour une dernière fois de discuter de mes travaux au cours de la soutenance.

Je tenais également à remercier Dr. Bénédicte Durand d'avoir accepté d'être présidente de mon jury de thèse.

Et voilà, la fin de cette merveilleuse aventure approche et me voici à penser à vous, les Castellani... une famille agrandie plus qu'une équipe...

Tout d'abord, je tiens à te remercier Valérie, du fond de mon cœur, pour tout ce que tu as fait pour moi au cours de ces nombreux années. Je me rappelle encore de tes cours, au premier semestre d'M1, quand je venais tout juste d'arriver en France et que tu me parlais de guidage axonal et de commissures... puis je suis arrivé dans le labo pour mon deuxième semestre M2 et on a préparé ensemble les oraux de BMIC et tu me disais que les couleurs de ma présentation rappelaient la déco d'une salle de bain des années '70 XD. Je tiens vraiment à te remercier pour les nombreuses occasions que tu m'as offertes, dès le début de ma thèse, de présenter mes travaux au cours des congrès et séminaires (5 fois ce n'est pas donné à tout le monde !). Merci pour ton massage des mains relaxant juste avant de présenter mes travaux au Cold Spring Harbor et pour m'avoir toujours soutenue, encouragé, motivé, passionné et surtout donné confiance...

Merci à Céline et Arnaud, qui m'ont encadré avec passion au cours de mon M2 en me donnant des bons avis et conseils, et qui m'ont assez vite fait attacher à ce projet ! Merci aussi pour tes retours et ton support sur le papier, Céline !

Je ne pourrais jamais arrêter de te remercier Julien ! Pour tous tes conseils, les discussions scientifiques et pour l'aide que tu m'as apporté avec le spinning au cours de cette thèse... Sans toi ça n'aurait été pas du tout la même chose !

Merci à toi Fred qui a toujours suivi le projet guidage avec grand intérêt ! Merci pour m'avoir donné la possibilité de réaliser mes enseignements en Génétique et en Embryologie et pour tes conseils pour la préparation des cours et des copies d'examen !

Merci à Servane, ma coloc de bureau 😊, de m'avoir toujours soutenue, écouté, donné des bons conseils et qui m'a bien remonté le moral cette été ! Merci pour ton investissement et pour l'enthousiasme que t'a toujours montré face aux découvertes de mon projet !

Merci à toi Ed... plus qu'un prof, un ami, un frère, une personne unique et adorable sur laquelle on peut toujours compter. Tu à fais beaucoup pour moi et je te garderai toujours dans mon cœur. Merci pour tes conseils, pour tes explications, pour les beaux moments rigolos passé dans le bureau et pour m'avoir accueilli plusieurs fois dans ta famille avec chaleur et amitié.

Merci à vous Karine K et Muriel, vous ne savez pas comment vous allez me manquer !!! Plus que des collègues, des amies ! On formait un bon trio de comaires ! Karine K merci pour ton optimisme et

l'énergie que tu dégage ! Une vraie centrale nucléaire ! Merci pour tes bons plans et pour tout l'aide que tu m'as apporté au cours de cette thèse ! Muriel merci pour ton support et pour tes conseils, pour les beaux moments passés ensemble à chanter « les petits boudins », pour ton humour et ton sarcasme qui me font toujours trop rigoler !

Merci à Karine T, Clélia et Loraine ! ça fut un plaisir de travailler avec vous et de partager des beaux moments de convivialité, avec nos discussions bio/écologiques et les conversations autour d'une tasse de thé XD

Merci au club de « los pollitos hermanos de la vega ! asi ! », Hugo, Thibo et Sarah, on en a des aventures à raconter ! XD XD Merci pour vos conseils, pour les belles soirées ensemble, pour nos craquages collectifs et les discussions de gros intellos ! Pour votre simplicité, votre bon cœur, votre bonne foi et votre bon foie (à bon entendeur XD). Merci à toi Dounia, pour ta bonne humeur, ton optimisme et ton soutien ! Tu es là depuis moins d'un an mais tout le monde t'adore déjà ! Merci Sophie pour ton intérêt vers mon projet ! ça m'a fait plaisir de te rencontrer et je te souhaite pleins de belles découvertes avec Fred !

Merci à la pollita Lorette ! Merci pour les beaux 3 ans passé ensemble, pour les moments rigolos, pour le soutien moral que tu m'as toujours donné dans les moments difficiles et les craquages après des longues journées de manip ! Je te souhaite vraiment le meilleur pour le future, copine !

Merci aux vieilles poulettes qui sont parties : Leila, Florie et Camille pour m'avoir accueilli dans l'équipe chaleureusement ! Et merci à toi Katrin pour ton amitié ! J'espère venir te voir bientôt à Berlin !

Je souhaite aussi remercier ma famille. Papiciulo et Madreuchka qui ont toujours cru en moi, qui m'ont supporté, conseillé, accompagné dans mes choix et que j'aime plus que tout au monde. Je suis tellement chanceuse d'avoir eu des parents comme vous ! Merci aussi à la famille de Turin et à la famille d'Alex pour votre support et votre bonne ambiance !

Merci aux copains du Flamenco et à la troupe FlamenKeando avec qui j'ai partagé des super moments pendant ces 5 ans, avec les cours et les spectacles ! Olé mis compañeros !

Merci aux loulous, Ivana, Jérôme, Ben, Serena, Anthony, William, Amélie, Perrine. Les meilleurs amis qu'on peut désirer ! Merci à Azza, Raph, Camille, Joel, Gweltaz et Céline, mes amis fifous.

Grazie agli amici di Torino ed ai Tarantini ! Non vedo l'ora di festeggiare la fine di tutta sta bella roba con voi, come sappiam ben fare !

Et pour finir... Merci Alex. Tout ça n'aurait peut-être jamais eu lieu sans toi. Jamais je ne finirai de te remercier pour tout le support, l'aide et la compréhension que tu m'as donné au cours de cette thèse. Merci de me montrer ton amour au quotidien, depuis presque 15 ans, et de toujours privilégier les gestes plutôt que les paroles.

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Résumé de la thèse en français

Les commissures forment un ensemble de connexions nerveuses assurant la communication entre les neurones de chaque hémipartie du système nerveux central des organismes à symétrie bilatérale. Au cours du développement embryonnaire, les axones des neurones commissuraux sont guidés au travers de la ligne médiane délimitant ces deux parties. Plusieurs sources de signaux de guidage aux effets attractifs et répulsifs agissent de concert pour organiser les trajectoires de ces axones tout au long de leur navigation.

Au niveau de la moelle épinière, les axones commissuraux traversent la ligne médiane dans un territoire ventral, la plaque du plancher (PP). Ils acquièrent une sensibilité à des signaux répulsifs exprimés par ce territoire dès lors que cette étape est franchie. Ces nouvelles propriétés sont nécessaires pour empêcher les axones de rebrousser chemin et retraverser, et également pour les pousser hors de ce territoire. Plusieurs couples de ligands/récepteurs médient ces forces répulsives au cours de la traversée de la PP. Les mécanismes qui sous-tendent l'acquisition de la sensibilité aux signaux répulsifs restent encore peu connus. Par exemple on ignore encore si les axons se sensibilisent à tous les signaux répulsifs en même temps, quand précisément ce switch de réponse se fait, et les contributions précises qu'apporte chacun de ces signaux. Une spécificité fonctionnelle est suggérée par l'analyse des phénotypes d'inactivation des gènes codant pour ces récepteurs chez la souris ou encore par des manipulations d'expression dans le modèle de l'embryon de poulet.

L'objectif de mes travaux de thèse a été de tester l'hypothèse selon laquelle la génération de spécificités fonctionnelles pourrait résulter de contrôles précis et distincts de la dynamique spatiale et temporelle des récepteurs de guidage à la surface du cône de croissance.

J'ai tout d'abord développé un dispositif de vidéomicroscopie adapté à l'enregistrement de cônes de croissance d'axones commissuraux accomplissant la traversée de la PP et la réorientation dans l'axe rostro-caudal qui lui succède, sur des moelles épinières en configuration de « livre ouvert ». Afin de visualiser l'adressage à la surface du cône de croissance, j'ai exploité une forme de GFP sensible au pH, la pHLuorin, dont les propriétés de fluorescence à pH neutre permettent un suivi spécifique du pool de surface des protéines

(Nawabi et al., 2010 ; Delloye-Bourgeois et al, 2014).

J'ai utilisé ce paradigme expérimental pour comparer la dynamique temporelle de 4 récepteurs clefs de la traversée de la ligne médiane: Nrp2, Robo1, Robo2 et PlxnA1, médiant les réponses aux divers signaux répulsifs de la PP. Les vecteurs d'expression de ces formes pHluo de récepteurs ont été introduits dans les neurones commissuraux de la moelle épinière par électroporation *in ovo*. L'analyse de la fluorescence, rapportrice de la présence du récepteur à la surface du cône de croissance, au cours de la navigation des axones commissuraux a révélé des différences notables dans la temporalité d'adressage membranaire de ces récepteurs. Par des approches de microscopie à super-résolution sur les livres-ouverts, j'ai pu également démontrer que les récepteurs PlxnA1 et Robo1, qui sont tous deux adressés à la membrane à différents temps de la navigation de la PP, occupent de plus des domaines distincts du cône de croissance.

J'ai ensuite adapté une technique d'électroporation à l'embryon de souris pour introduire les récepteurs-pHluo PlxnA1 et Robo1 dans les neurones commissuraux. Ces expériences ont montré que les séquences temporelles observées dans l'embryon de poulet sont conservées chez la souris. J'ai également réintroduit le récepteur Robo1 dans une lignée de souris présentant une invalidation des récepteurs Robo1 et 2 et montré que l'altération de la traversée de la PP caractéristique de cette lignée est abolie dans la population d'axones capables d'adresser le récepteur Robo1 à la membrane.

Au final, mes résultats démontrent que les axones commissuraux ne sont pas sensibilisés aux signaux répulsifs par la mise en œuvre d'un programme général. Au contraire, les récepteurs de guidage possèdent des profils de dynamiques temporelles spécifiques, et des domaines de distribution distincts dans le cône de croissance. Le contrôle de la dynamique d'adressage représente ainsi un mécanisme grâce auquel les cônes de croissance des axones commissuraux peuvent discriminer des signaux concomitants, en les fonctionnalisant à différents temps de la navigation de la moelle épinière.

English summary

During embryonic development, commissural axons are guided through the midline, crossing from one side of the CNS to the other one at specific time points and positions to project onto contralateral neurons. Several sources of attractive cues regulate their navigation. In addition, repulsive forces act at different steps to keep the axons along their path. In the developing spinal cord, commissural axons cross the midline in a ventral territory, the floor plate (FP). Commissural axons gain sensitivity to repellents present in the FP after their crossing. The setting of these novel properties is necessary for preventing the axons to cross back and also for pushing them towards FP exit.

Various ligand/receptor couples have been reported to mediate these repulsive forces. Whether commissural axons gain response to all the repulsive cues at the same time is not known. Whether these repulsive cascades have specific functions is suggested by different outcome of their invalidation in mouse models, but how are set these differences also remains unknown.

We hypothesized that the generation of functional specificities could be achieved through specific controls of the spatial and temporal dynamics of guidance receptors at the growth cone surface. During my PhD, I developed a set up for time-lapse imaging of “open book” spinal cords, to monitor the dynamics of guidance receptors in axons experiencing native guidance decisions across the midline. To visualize their cell surface sorting, receptors were fused to the pH-sensitive GFP, pHLuorin, whose fluorescence at neutral pH reports membrane protein pools (Nawabi et al, 2010; Delloye-Bourgeois et al, 2014), and were expressed in spinal commissural neurons through *in ovo* electroporation.

This paradigm revealed striking differences in the temporal dynamics of Nrp2, Robo1, Robo2 and PlexinA1, the receptors known to mediate the responsiveness to the major midline repellents referenced in vertebrates: Slit-Ns, Slit-Cs and Semaphorin3B. Moreover, using super-resolution microscopy, I could evidence that PlexinA1 and Robo1 are sorted in distinct subdomains of commissural growth cones navigating the floor plate. I also introduced the pHLuo-tagged receptors in the mouse embryo. These experiments showed that the temporal sequences established in the chick are conserved in the mouse, and that FP crossing in

Robo1/2 mutant embryos was rescued in growth cones that could achieve cell surface sorting of Robo1. Thus, my results show that guidance receptors for midline repellents have highly specific spatial and temporal dynamics. The generation of a temporal sequences of cell surface sorting thus represents a mechanism whereby commissural growth cones discriminate concomitant signals by functionalizing them at different timing of their spinal cord navigation.

INTRODUCTION

CHAPTER1: Axon guidance in the developing spinal cord.

The commissural circuitry of the spinal cord.

Commissural circuits are neuronal projections interconnecting both sides of the central nervous system (CNS) in bilaterians (Castellani, 2013)(Vulliemoz et al., 2005). In the spinal cord, commissural neurons allow the coordination of left-right movements and alternate/synchronized activities, conveying sensorial information to higher brain centers.

The spinal cord interneurons are a heterogeneous population divided in several pools, depending on their location, timing of birth and specific expression of transcription factors (Butler and Bronner, 2015).

Among them, dl1 interneurons are located in the most dorsal position of the spinal cord, next to the roof plate (RP) and arise from a Math1-positive pool of progenitors, specified by the expression of Lhx2/Lhx9 transcription factors (Bermingham et al., 2001)(Lee et al., 1998). This population is composed of two types of interneurons: those establishing ipsilateral projections (dl1i) and those establishing commissural projections (dl1c)(Bermingham et al., 2001). DL1c neuron trajectories are highly stereotyped and have been well characterized in transgenic mice expressing LacZ or GFP under the control of Math1 promoter (Helms and Johnson, 1998). In mice, dl1 neurons arise from around E10 and, starting from this stage, they extend waves of axons towards the midline. Some of them have already crossed at E10.5, even if the majority complete this process at E12.5. At E13 most of commissural axons are navigating longitudinal post-crossing (Imondi and Kaprielian, 2001) (Imondi et al., 2007) (Jaworski et al., 2010)(Kadison and Kaprielian, 2004). DL1c axons first extend ventrally, away from the RP, navigating close to the pial surface which surround the spinal cord. Then, they brusquely change their orientation at mid-distance of the ventral border to navigate towards the floor plate, a central structure lying ventrally in mirror to the roof plate, somehow avoiding the motoneuron compartment. After FP crossing, commissural neurons turn rostrally without crossing again the midline and project longitudinally at different medio-lateral positions (Oppenheim et al., 1988) (Colamarino et al., 1995)(**Figure 1**).

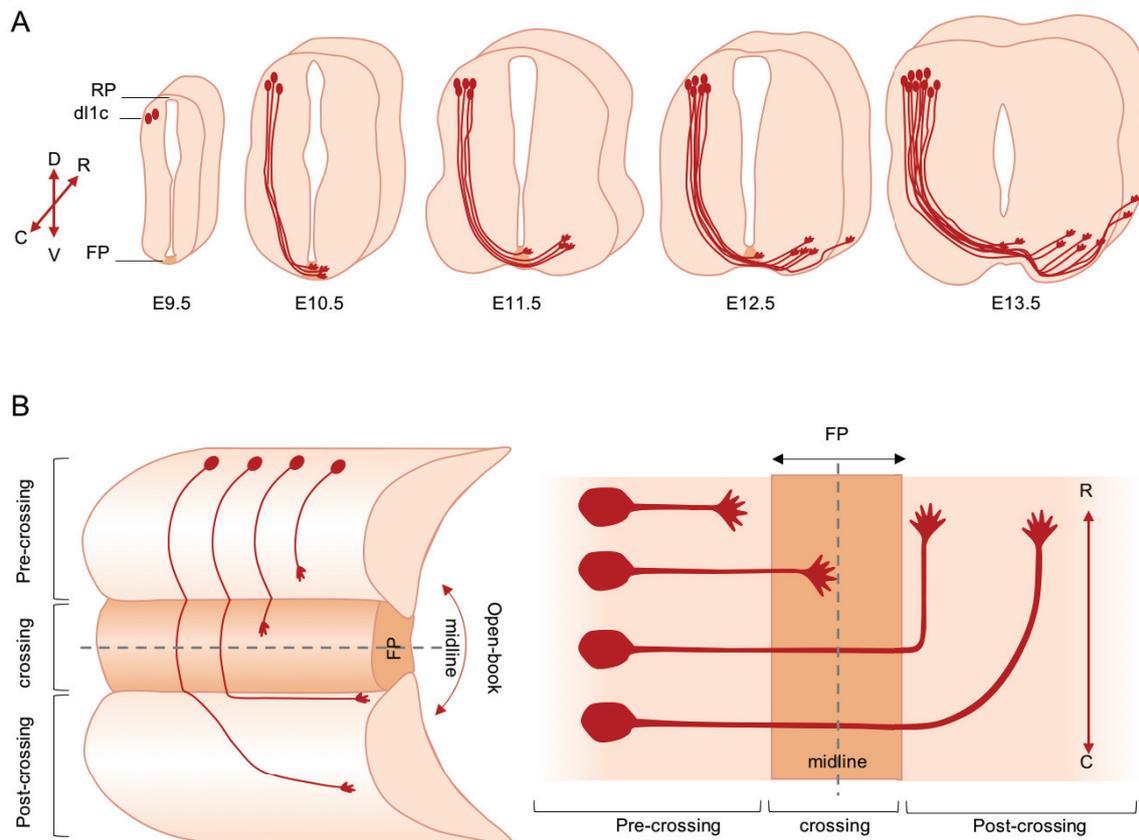


Figure 1: Schematic representation of commissural projections in the mouse (A) Temporal sequence of dl1c axon navigation in the spinal cord. R: Rostral, C: Caudal, D: Dorsal, V: Ventral, RP: Roof Plate, FP: Floor Plate. The first dl1c neurons are born around E9.5 in the dorsal horn of the spinal cord and start to extend their axons across the midline around E10.5. Between E10.5 and E12.5 several waves of follower dl1c neurons project their axons across the midline. By E13.5 all dl1c axons navigate post-crossing longitudinal routes. (B) Schematic view of an open-book preparation illustrating the 3 compartments crossed by commissural axons: the pre-crossing, where the dl1c cell bodies are located, the FP, in which dl1c axons cross the midline, and the post-crossing, in which dl1c axons turn rostrally and choose between ventral and lateral longitudinal trajectories. Adapted from Pignata *et al.*, 2016.

The crisis of positive chemotropism

During embryonic development, commissural axons extend along specific pathways to reach their final targets. At the axon edge, the growth cone, equipped of guidance receptors, sense the surrounding environment, being attracted towards or repelled away from the guidance cues expressed by “guidepost” tissues. This chemotropism theory, proposed by Ramon Y Cajal, has been the textbook model over the last past decades, and guidance cues have been

traditionally classified as having short-range or long-range activities depending on their secreted or membrane-bound structure and the distance at which they can elicit a guidance response over the growth cones (Dickson, 2002)(Tessier-Lavigne and Goodman 1996). During the past decades, several long-range attractive cues have been described in the spinal cord. One of the most important textbook example of long-range attractive cue is Netrin1, a member of the Netrin family related to the laminin superfamily, and secreted by FP cells (Kennedy et al., 1994). Others, such as the morphogen SHH (Sonic Hedgehog) acting *via* SMO (Smoothened) and BOC (Brother of CDO) receptors (Charron et al., 2003) (Okada et al., 2006), and VEGF, acting *via* Flk receptor (Ruiz de Almodovar et al., 2011) have been proposed to collaborate with Netrin1 to guide commissural axons toward the FP.

The long-range effect of chemoattractants has been predominantly established by *ex vivo* assays in which commissural tissues were co-cultured at a distance of FP piece of tissues, soluble molecules or transfected cells expressing guidance factors. As it was expected, deletion of chemoattractants or their respective receptors in mouse models was observed to alter the ability of commissural axons to reach the FP, with axons exhibiting defasciculation, growth arrest or invading ectopic territories (Serafini et al., 1996)(Charron et al., 2003)(Ruiz de Almodovar et al., 2011)(Fazeli et al., 1997).

Recent studies, based on the generation and analysis of conditional mouse lines, have revisited the view that Netrin1 released by the FP has a long-range attractive effect. Rather they suggest a different mode of action of Netrin1 during commissural axon extension. Intriguing observations have pioneered these works. In embryos lacking Netrin1 in the FP or in which Netrin protein is membrane tethered and does not diffuse anymore, the formation of ventral commissure occurs regularly whereas, in hypomorph and full null mutants, the commissural tract is severely diminished and disorganized (Serafini et al., 1996)(Dominici et al., 2017) (Varadarajan et al., 2017)(Bin et al., 2015)(Brankatschk and Dickson, 2006). This suggested to the authors the existence of an additional source of Netrin1 in the spinal cord. Indeed, the authors removed Netrin1 from the ventricular zone (VZ) and could observe a high disorganization of commissural axons, invading the VZ, and a severe reduction of ventral commissure, phenocopying Netrin1 hypomorph. Netrin1 mRNA was detected in the progenitors of the VZ (Kennedy, 2006)(Varadarajan et al., 2017) as described previously (Kennedy et al., 1994) (Serafini et al., 1996) but, for the first time, an expression of the protein

was detected along the pial surface, co-localizing with laminin and progenitor basal end feet (Dominici et al., 2017)(Varadarajan et al., 2017). The authors raised a model whereby Netrin1 would be exocytosed by progenitor terminals, rapidly associating with the extracellular matrix and acting as a short-range guidance cue for commissural axons rather than a long-range one. Studies of cell biology are now needed to test this model.

How are these new fascinating discoveries consistent with previously reported findings? Long range attractive properties of Netrin1 have been largely documented *in vitro* (Kennedy et al., 1994)(Serafini et al., 1996) (Kennedy, 2006).

In terms of commissure width, VZ-Netrin1^{-/-} have a strong phenotype that doesn't completely phenocopy the full Netrin mutant (Dominici et al., 2017). Possibly, FP-Netrin can rescue the crossing of a small amount of fibers.

Beyond the mode of action of Netrin1, a direct link between long-range chemoattraction and commissural axon navigation towards the FP is quite complicated to make in this system. For instance, dorsal spinal cord explants grow ventrally even without FP tissues around and, in chick, the removal of notochord sections, inducing loss of FP development, does not prevent commissural axons to reach the FP (Morales, 2018)(Kennedy et al., 1994)(Placzek et al. 1990)(Yamada et al., 1991). Moreover, in mice carrying the Danforth's short tail-mutation, causing loss of FP at caudal positions, commissural axons still reach the FP and cross it (Bovolenta and Dodd, 1991). All these evidences suggest that long-range chemo-attractants might not be the main forces directing axon guidance in the vertebrate spinal cord.

Guidepost territories instructing commissural axon navigation through repulsive cues

The idea that repulsive interactions could be important forces canalizing growth cones in their appropriate route is quite ancient (M. Abercrombie, 1970)(Kapfhammer and Raper, 1987). Already in the 80's, Kapfhammer and collaborators suggested that in the nervous system, diffusible signals, selective substrate modifications, differential adhesion and contact-mediated inhibition of locomotion could be important mechanisms involved in the interactions between the growth cone and specific tissues that, rather than acting as passive barriers, would dynamically shape growth cone morphology and pathways (Kapfhammer et

al., 1986). For instance, pioneer studies showed a collapse response of dorsal root ganglion (DRG) axons treated *in vitro* with suspensions of membranes from embryonic brain. It was also demonstrated that DRG growth cones interact with mesenchymal cells of the dermis, while completely avoiding epidermal cells (Raper and Kapfhammer, 1990)(Vema, 1985). Other studies suggested that dermomyotome/ectoderm and notochord tissues, flanking DRGs in the embryo, produce diffusible repellents that channel the sprouting of DRG axons (Keynes et al., 1997).

Considering the developing spinal cord, chemorepulsion by surrounding tissues has also been demonstrated for motoneurons (MN) extending their axons from the neural tube to the periphery (Bonanomi and Pfaff, 2010). Indeed, the first steps of MN axon outgrowth from the neural tube are dependent on the sequential exposure to repulsive signals delivered by the FP (Kim et al., 2015)(Kim et al., 2017), which propel axons away from the midline, ventral mesenchyme, which channel MN axons on their way toward the anterior-half somite (Gallarda et al., 2008) and limb tissues, which also organize through repulsive signaling the positioning of spinal nerves (Luria et al., 2008).

Thus, spinal cord tissues provide important repulsive forces driving axon navigation, propelling axons away from guidepost cells, confining axon navigation in specific territories and channeling axons along defined trajectories (Ducuing et al., 2018). Thus, although historically, the FP has been considered as the principal intermediate target for commissural axons, many other cell-types participate in shaping commissural circuitry. The different forces described in the next paragraphs are summarized in the **Figure 2**.

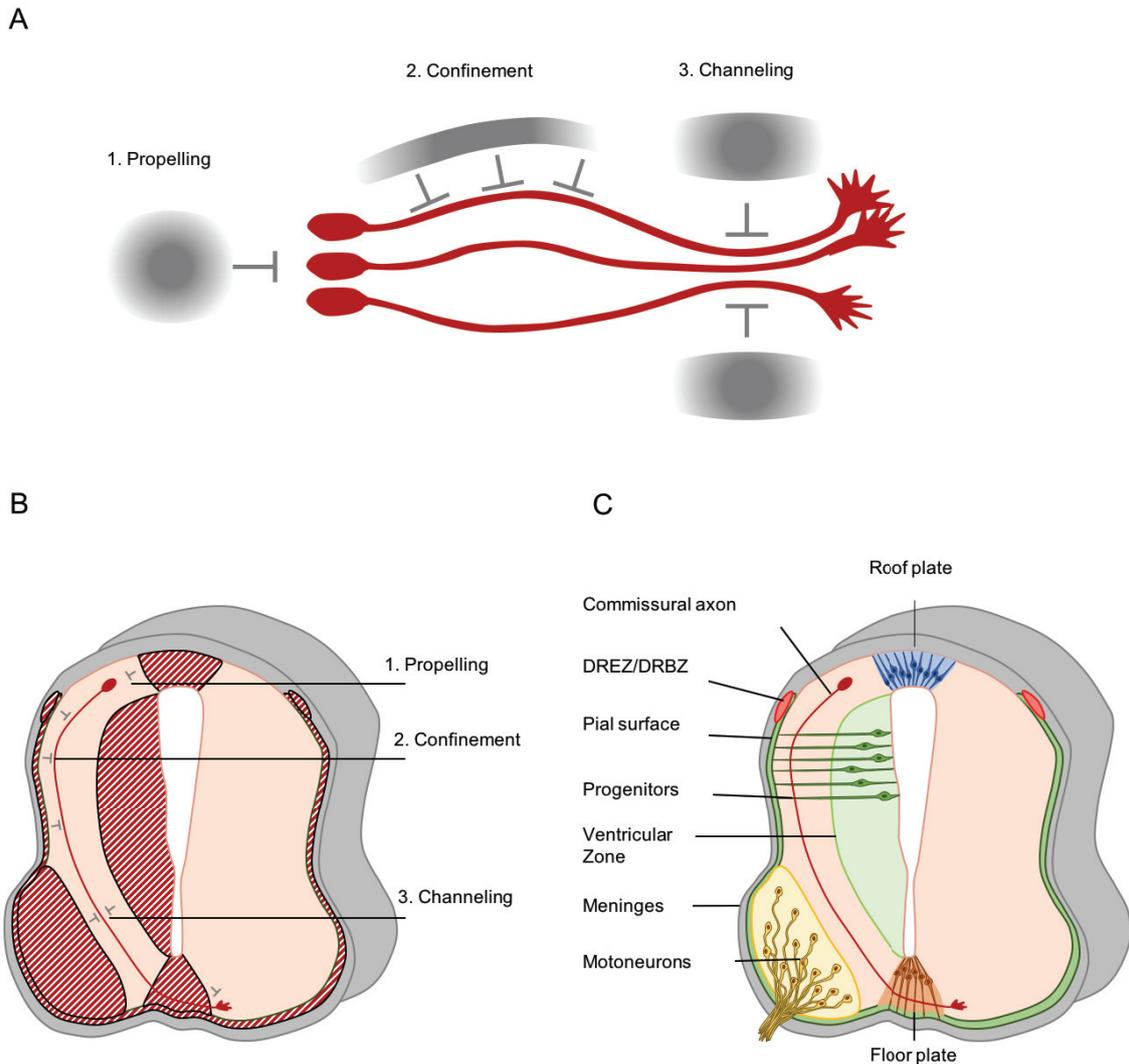


Figure 2: Repulsive forces patterning commissural axon navigation in the vertebrate spinal cord. (A) Modes of action of repulsive forces during axon navigation. (B, C) Schematic representation of the guidepost repulsive territories of the spinal cord. Commissural axons extending towards the midline first perceive propelling repulsive signals emanating from the Roof Plate and navigate close to the pial surface. At this location, they are confined in their path by signals derived from DREZ/DRBZ and pial surface. At mid-distance from the FP, axons turn brusquely, projecting towards the FP. At this level axons are kept together by channeling cues emanating from motoneurons and the ventricular zone. As axons reach the FP, they contact the basal lamina and navigate across the basal processes of FP glial cells, becoming progressively sensitive to repulsive cues which they did not perceive before. After FP crossing, axons turn in rostral direction at various distance from the FP, responding to longitudinal gradients of cues. During post-crossing navigation axons are likely to respond to the same repulsive territories encountered during the pre-crossing step which can again propel, confine and channel their trajectories. Adapted from Ducuing *et al.*, 2017.

Repulsive propelling forces.

Spinal cord roof plate (RP) is considered the first important territory involved in orienting commissural axons ventrally and is the first dorsal structure to differentiate and therefore influence the differentiation of all other dorsal populations in the embryo. The RP constitutes a barrier that no axon can cross until E16.5 when a dorsal commissure forms (Comer et al., 2015). Differentiated RP cells express specific markers, like BMPs and WNTs, which specify several classes of dorsal interneurons, regulating their proliferation, migration and guidance (Chizhikov and Millen, 2004)(Le Dréau and Martí, 2012). BMPs, in particular GDF7:BMP7, acting *via* BMPRII-BMPRIB receptors expressed on dl1c, provide the propelling force pushing commissural axons ventrally (Butler and Dodd, 2003)(Augsburger et al., 1999). Moreover, RP cells secrete Draxin which was shown to act as a chemorepellent *in vivo* through DCC receptor (Ahmed et al., 2011) (**Figure 3**).

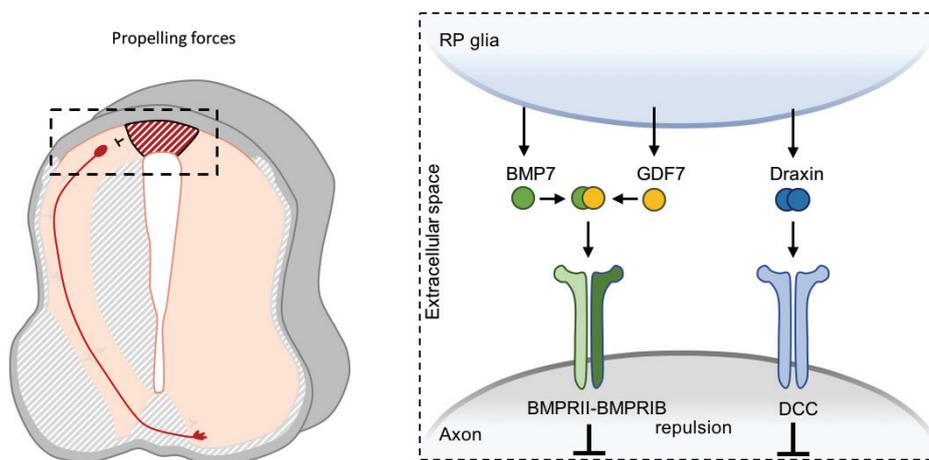


Figure 3: Propelling forces of the Roof Plate. RP glial cells secrete BMP7 and GDF7, two BMP family members, which bind to BMPRII-BMPRIB receptors and repel axons toward the ventral spinal cord. RP glia also secrete Draxin which repels axons *via* DCC receptor. Adapted from Ducuing *et al.*, 2017.

Repulsive confining forces.

Commissural axons navigating the spinal cord must remain confined to this territory. Conversely, MN axons project their axons out from the CNS through the Motor Exit Point (MET), while sensory axons of the DRG enter the spinal cord *via* the Dorsal Root Entry Zone (DREZ). Confinement of commissural axons is operated by DREZ/dorsal root bifurcation zone (DRBZ), meninges and the pial surface. Meninges envelop the brain and the spinal cord and act as a barrier during lifetime, controlling exchanges between the CNS and outside. They are composed of fibroelastic cells and blood vessels. Their role in the confinement of pre-crossing spinal cord axons *in vivo* has not been demonstrated yet, but *in vitro* studies suggest that this multi-layer structure can secrete cues able to repel different neuronal populations (Suter et al., 2017). DREZ/DRBZ represents another important confinement territory of spinal cord. The DREZ is constituted of a fenestrated basal lamina, that guarantees continuity between the CNS and the surrounding tissues, and is also constituted by boundary cap cells which prevent neuronal soma to exit the CNS (Vermeren et al., 2003). DRBZ originates later on, when sensory axons send their axons towards the spinal cord and form this zone in direct apposition to the DREZ (Altman and Bayer, 1984). Some studies suggested that Netrin-1, expressed between E11.5 and E12.5 in DRBZ (Gao et al., 2015) and acting *via* DCC and UNC5C receptors, might play a role in preventing commissural axon exit from the spinal cord. Indeed, in mouse embryos mutants for these molecules, a strong invasion by ectopic axons is observed in DREZ and DRBZ supporting a role in axon confinement (Gao et al., 2015). Draxin is another potential candidate cue providing confinement at DREZ/DRBZ, since its expression is also detected along the pial surface and in the DREZ (54). Moreover, glycoproteins, such as dystroglycan, which is detected along the pial surface might play an essential role in confining axons. Dystroglycan is an important scaffold for ECM proteins, such as Laminin, and in the visual system this interaction was shown to modulate the response of axons to Netrin-1 by switching the response from attractive to repulsive (Höpker et al., 1999). Therefore, a similar interaction might occur at the pial surface and set this territory as a repulsive barrier (**Figure 4**).

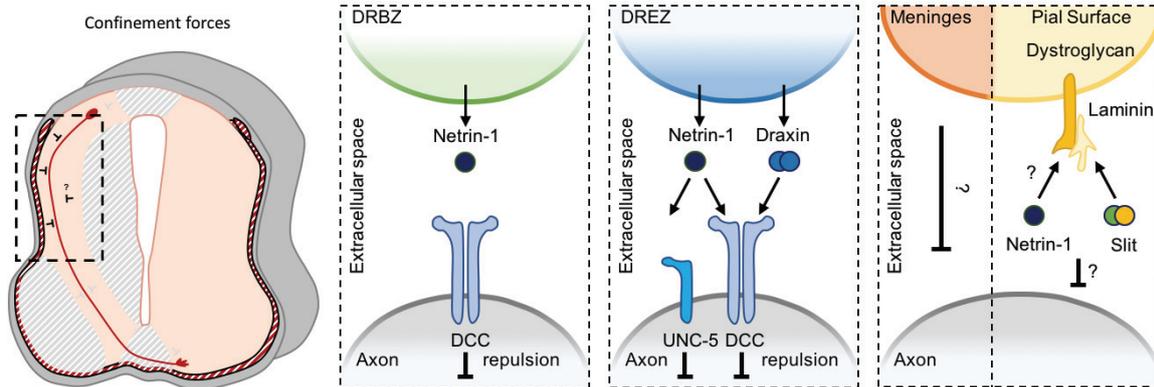


Figure 4: Confinement of commissural axons. DREZ and DRBZ cells secrete Netrin-1 that induces repulsion *via* DCC or *via* DCC/UNC5 heterodimer. DREZ cells also secrete Draxin that repels axons *via* DCC. Signals originating from meninges and the pial surface could also contribute in confining axons within their path but still remain unidentified. Dystroglycan glycoprotein might play a role after binding to laminin, switching the response to Netrin-1 from attraction to repulsion and therefore rendering the Pia a repulsive territory for commissural axons. The interaction between Slit and Dystroglycan along the pial surface might also be involved in confinement. Adapted from Ducuing *et al.*, 2017.

Repulsive channeling forces.

Once reaching the ventral half of the spinal cord, pre-crossing commissural axons break their circumferential navigation, along the spinal cord border, to re-orient medially and navigate towards the FP. At this point, two main channeling territories come into play to constrain commissural axons to navigate in between as a compact bundle: the VZ and the MN domain. The VZ is constituted by bipolar progenitor cells extending one process connecting the pial surface and the other one connecting the central canal (Taverna *et al.*, 2014). During embryonic neurogenesis, these cells undertake interkinetic nuclear migration and give rise to post-mitotic neurons which detach from the apical border and migrate in the mantle. As described in the previous chapter, some recent studies proposed that progenitor cells produce Netrin-1 and transport this cue *via* their basal processes, releasing it at the basal lamina (Dominici *et al.*, 2017)(Varadarajan *et al.*, 2017). This mechanism would keep dl1 along the pial surface border, preventing the axonal invasion of the VZ. The mode of action by which Netrin-1 prevents VZ ingrowth is still unclear. Moreover, in mice lacking Netrin-1 in the VZ, even if a strong invasion of the VZ by dl1 occurs, a not negligible number of axons still follow

the right pathway, suggesting that Netrin-1 may not be the sole cue conferring non-permissive properties to the VZ.

Indeed, various proliferative regions of the developing brain were reported to secrete repulsive factors, for example in the cerebral cortex, where they prevent invasion by cortical and thalamic axons (Leyva-Díaz and López-Bendito, 2013). Furthermore, transcripts encoding repulsive molecules such as Semaphorins, Eph/Ephrins and Slits have been found to be expressed in spinal cord VZ in chick and mouse (Moret et al., 2007)(Sanyas et al., 2012) (Arbeille et al., 2015)(Laussu et al., 2017) (Püschel et al., 1996) (Philipp et al., 2012).

The MN domain is constituted of cells originating from a ventral pool of progenitors at around E9.5 in mouse. This domain comprises distinct and adjacent MN pools expressing a panoply of repulsive cues that could be involved in channeling pre-crossing commissural axons, such as several members of Semaphorin, Ephrin and Slit families (Philipp et al., 2012)(Brose et al., 1999; Moret et al., 2007)(anyas et al., 2012)(Imondi et al., 2000). Recently, another molecule, NELL2, has been found expressed by MNs (Jaworski et al., 2015). *In vitro* studies suggested that NELL2 exerts a repulsive effect on commissural neurons via Roundabout3 (Robo3) and, *in vivo* removal of NELL2 and ROBO3 caused a strong defasciculation of commissural tract, with axons invading MN columns (Jaworski et al., 2015). The repulsive forces involved in channeling are represented in **Figure 5**.

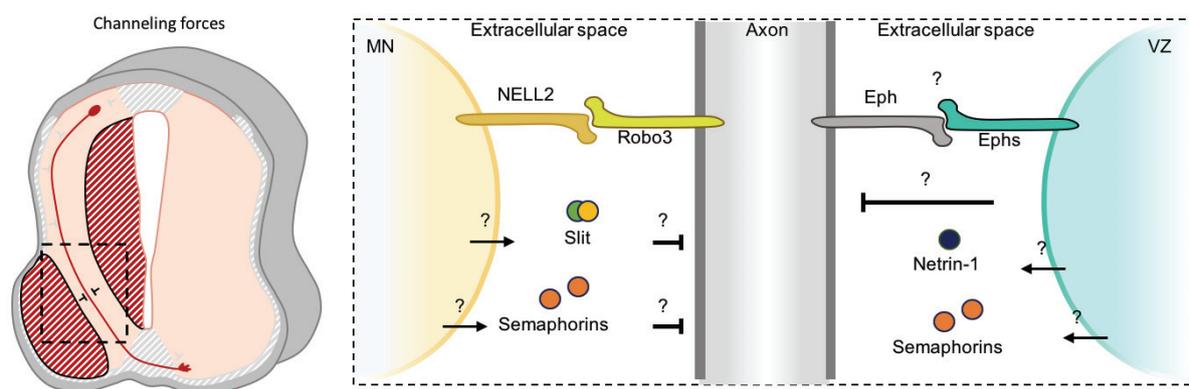


Figure 5: Channeling of commissural axons. MN and VZ are both non-permissive zones for commissural axons. MN express NELL2, which acts *via* Robo3 for repelling axons from the MN domain, and secrete Slits and Semaphorins which might be involved in axon channeling too.

Unidentified molecules provided by the VZ might also be involved: Semaphorins, Eph/Ephrins and Netrin-1 produced by progenitor cells are good candidates for axon channeling. Adapted from Ducuing *et al.*, 2017.

Therefore, despite known expression of a panoply of potent repellents, only few of them have been implicated in the navigation of pre-crossing commissural axons. Conditional mouse models to target the deletion of repulsive candidates in specific cell-types would greatly help in understanding how repulsive forces shape commissural trajectories.

FP crossing: how navigating a repulsive field.

The spinal cord floor plate (FP) has probably been the most studied guidepost territory in the field of axon navigation. FP is constituted by a set of non-neural cells, the glia, which exert in the spinal cord of all vertebrates a fundamental influence on neuronal differentiation and on the organization of ipsilateral and contralateral axon tracts. FP glia cells have a morphology quite similar to that of RP cells and other progenitors: they own two processes, one attached to the apical side towards the central canal and the other one which is attached to the basal lamina (Campbell and Peterson, 1993). During FP crossing, commissural axons form close contacts with glial basal processes, and are completely wrapped in these structures (Yaginuma *et al.*, 1991) (**Figure 6**). Commissural axons and glial cells establish complex *cis* and *trans* interactions *via* adhesion molecules. For instance, it has been demonstrated that specialized junctional contacts form between FP processes and commissural growth cones and are mediated by hetero-*trans*-interactions between growth cone nectin-1 and FP nectin-3 (Okabe *et al.*, 2004). Such contacts would guarantee the transmission, from the glial cells to the cones/axons, of specific signals instructing signaling cascades into the growth cones that could *in fine* impact on guidance receptor distributions (Campbell and Peterson, 1993)(Gainer, 1977). FP cells express a large panoply of adhesion molecules which are absent from the surrounding neural tissues, for instance p84 (Chuang and Lagenaur, 1990), polysialylated (PSA) N-CAM (Griffith and Wiley, 1991), GP90 (Ranscht and Dours, 1989) and F-spondin (Tzarfati-Majar *et al.*, 2001)(Klar *et al.*, 1992). In particular, the adhesion molecule NrCAM expressed by FP cells was found to interact with Axonin-1/TAG-1 expressed by commissural growth

cones. This interaction is thought to be important for commissural axons to proceed for FP ingrowth since, its inhibition prevents FP crossing, inducing a premature turning of axons in the ipsilateral compartment of the spinal cord (Stoeckli and Landmesser, 1995). The expression of adhesion molecules by the commissural growth cone and its axon shaft is finely controlled during the navigation. For instance, in the mouse, it has been showed that commissural axons express high levels of TAG-1 and low levels of L1 as they project ventrally and cross the FP and that, after FP crossing, the expression of these adhesion molecules is inverted, TAG-1 being down-regulated and L1 up-regulated. As a result, this switch would allow the commissural growth cones to respond differently to the surrounding adhesion partners on each side of spinal cord (Seeger, 1993). The nature of the binding partners before and after midline crossing as well as the functional outcome that these changings produce remain undetermined. The principal molecules mediating FP-commissural growth cone interactions are presented in **Figure 6**.

During FP navigation, the axons gain responsiveness to repulsive cues to which they were previously insensitive. This sensitization is thought to prevent the axons from turning back, from re-crossing the midline and to propel them out of the FP, in the contralateral side. Such repulsive forces involve several couples of ligand-receptors summarized in **Figure 7**, which I will present in details in the next paragraphs, as they are at the core of my thesis work.

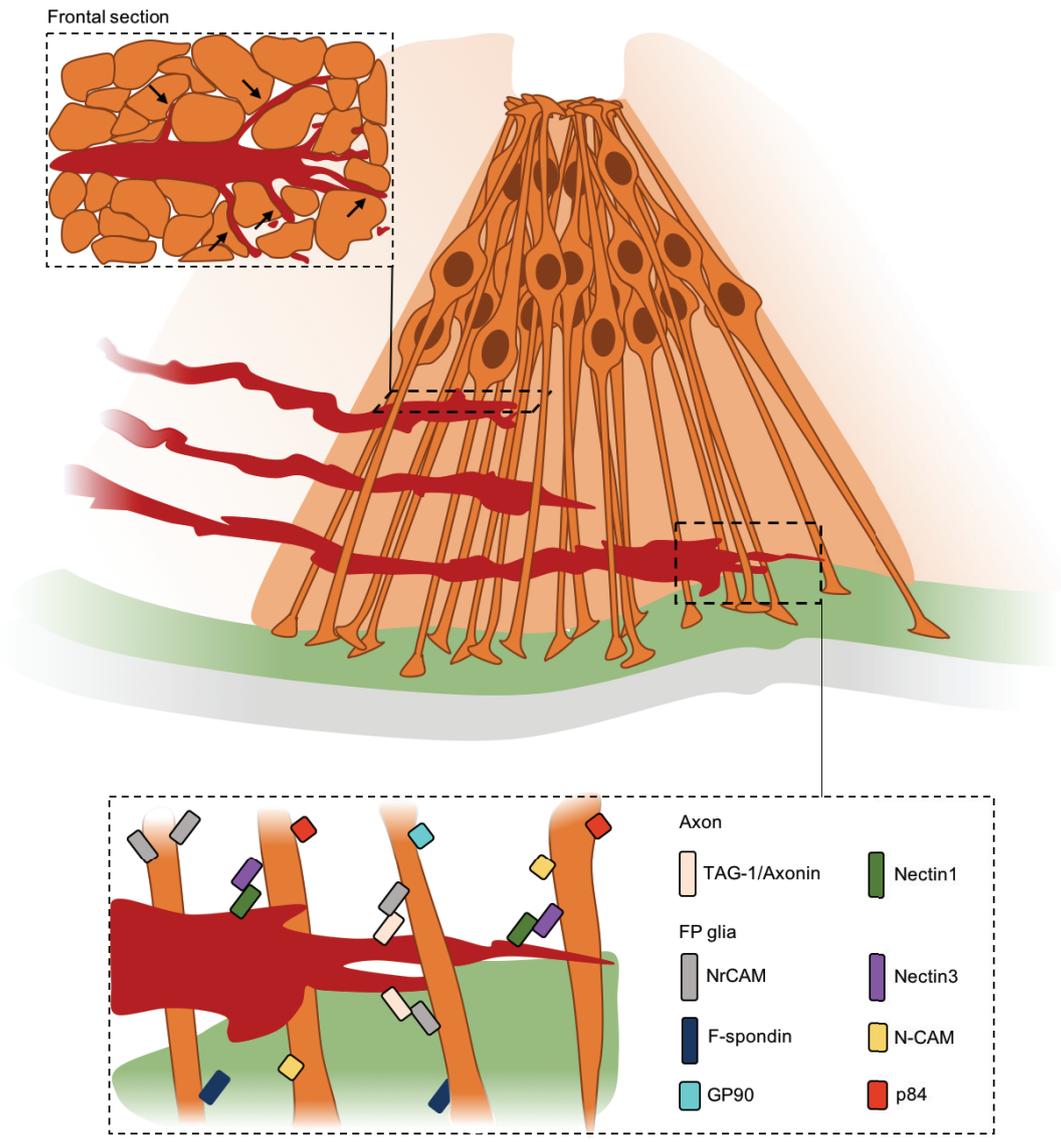


Figure 6: Adhesive interactions between the FP glia and commissural axons. FP is constituted by a set of non-neural cells, the glia, that extends two processes, one attached to the apical side towards the central canal, and the other one attached to the basal lamina. The panel at the top left represents a frontal section showing close interactions of the growth cone with glia basal process, and how it is enwrapped by these structures during FP navigation. Bottom panel: molecular partners involved in the adhesive interactions between the growth cone and the glia. To simplify the drawing, only some glial processes were represented.

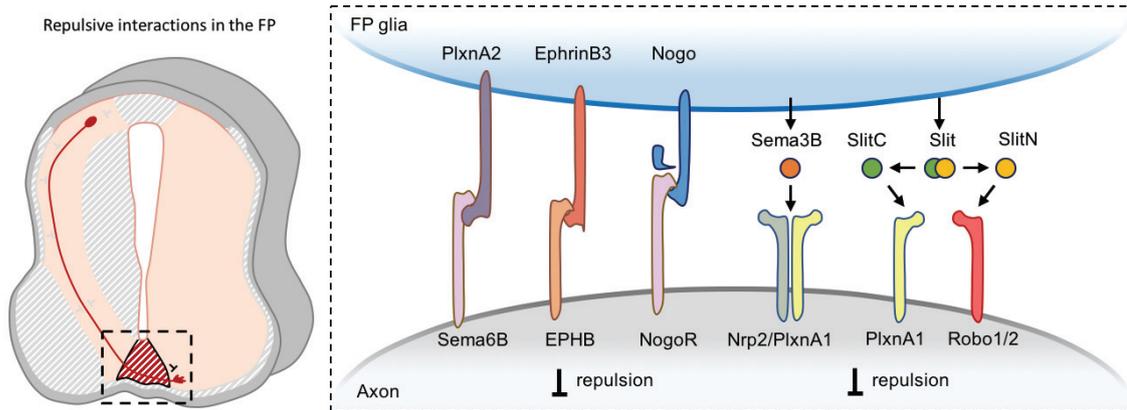


Figure 7: Repulsive interactions in the FP. During FP crossing, commissural axons become progressively sensitive to repulsive signals. PlxnA2, EphrinB3 and Nogo at the surface of FP glia interact with Sema6B, EPHB3 and NogoR, respectively, and induce a repulsive response of the growth cone. In parallel, FP glia secrete repellent guidance cues such as Sema3B, that interact with Nrp2/PlxnA1 receptors, and Slits, which are cleaved into N-ter and C-ter fragments, both eliciting a repulsive response of the growth cone. Slit-N fragment acts *via* Robo1/2 receptors whereas SlitC fragment binds to PlxnA1. Adapted from Ducuing *et al.*, 2017.

The Slit/Robo signaling.

The Slit-Robo signaling was discovered in fly to control midline crossing of commissural axons in the ventral cord. Slits are secreted proteins of about 200 kDa with a conserved structure between vertebrates and invertebrates. *Drosophila* express just a single Slit gene, whereas vertebrates express three Slits (Slit1-3). At its N-terminus, the Slit protein has four leucine-rich-repeat (LRR) domains followed by six EGF-like domains, one laminin G-like domain and three EGF domains. At the C-terminus Slit contains a cysteine knot domain (CTCK). Slit proteins form dimers *via* hydrophobic their LRR4 domain. By the time of spinal commissural navigation in the FP, Slit1 mRNA is detected in the FP both in chick, rat and mouse, whereas Slit2 is expressed in the FP but also in the lateral compartments of the spinal cord (Brose *et al.*, 1999)(Mambetisaeva *et al.*, 2005). In rat, Slit3 has a more dynamic expression since it is expressed both by the FP cells and in lateral compartments at E11 but, at E13, its expression is restricted to the FP (Brose *et al.*, 1999).

Slit proteins mediate their functions via Roundabout (Robo) receptors. The Robo family comprises three proteins in *Drosophila* and four in mammals, Robo1, Robo2, Robo3 (also known as Rig-1) and Robo4 (also called Magic Roundabout), however this latter is not expressed within the spinal cord (Kidd et al., 1998a). Robos are single-pass transmembrane proteins most of which constituted by five immunoglobulin-like (Ig) and three fibronectin type III (FN3) repeats, a transmembrane domain and a cytoplasmic domain lacking of catalytic activity. The cytoplasmic domain of Robos is generally poorly conserved, except for CC0-3 (cytoplasmic conserved) motifs which interact with different signaling proteins.

Robos have very comparable ectodomains and mostly diverge in their cytoplasmic domain. Robo1 contains each of the four cytoplasmic conserved regions (CC0-CC3), whereas Robo2 and Robo3 lack CC2 and CC3. Therefore, it has been suggested that the specific repulsive functions of Robos rely on their cytoplasmic regions. In *Drosophila*, a series of chimeric receptors of Robo1 (which can prevent crossing of commissural axons in the ventral cord) and Robo3 (which cannot) were generated with reciprocal exchange of their cytoplasmic domains. Such experiments revealed that rescue of midline crossing can be obtained with a chimeric construct having the cytoplasmic region of Robo1 but not that of Robo3. Further experiments allowed the identification of the cytoplasmic motif involved in preventing midline crossing, which is located within the cytoplasmic region surrounding the CC2 motif (Spitzweck et al., 2010). Comparable *in vivo* experiments were performed with Robo (mediating repulsion) and Fra (mediating attraction upon Netrin binding), with the aim to define which domain encodes the nature of the response. The chimeras were composed of Robo ecto/Fra endo or Fra ecto/Robo endo domains and revealed involvement of Robo cytoplasmic sequence in eliciting a repulsive response and not an attractive one (Bashaw and Goodman, 1999). Therefore, the cytoplasmic domain of a guidance receptor can dictate the nature of the guidance response.

FRET experiments have suggested that Robos form dimers at the cell surface and that this property depends on the extracellular domain of the protein (Zakrys et al., 2014). Robos bind to the LRR2 domain of Slits *via* their first Ig domain (Morlot et al., 2007). Moreover, heparan sulfate can bind both Robo and Slit to increase the stability of their interaction (Hussain et al., 2006). Both Slit-FL and SlitN can bind to Robo1/2 but not to Robo3, and elicit a repulsive

response (Ba-Charvet et al., 2001). Robo3, also called Rig-1, has been considered a divergent member of Robo family, since in mouse models its loss completely prevents commissure formation in the spinal cord, contrarily to Robo1/2 loss which rather causes stalling phenotypes in the FP (Sabatier et al., 2004). Diverse Robo3 isoforms, having distinct functions and expression patterns have been described, for instance Robo3A and Robo3B which differ in their N-termini or Robo3.1 and Robo3.2. Robo3.1 is expressed at the pre-crossing stage and was proposed to silence Slit repulsion and to enhance FP attraction. The Robo3.2 isoform was reported by one group to be specifically expressed in post-crossing axons and to stimulate repulsion by Slits (Chen et al., 2008).

The Sema3/Nrp/PlexinA signaling.

The Semaphorin (Semas) family includes highly phylogenetically conserved secreted and transmembrane proteins. In vertebrates, almost twenty different Semas have been described, all containing a Sema domain that mediates the association of these proteins with their receptors, belonging to the Plexin (Plxns) family. All Semas are in addition characterized by the presence, at the N-terminal region, of Plexin-Semaphorin-Integrin (PSI) domains. Many studies in the past suggested that Semas, in isolation, dimerize to function as repulsive guidance cues, and that this interaction occurs *via* the sema-domains (Klostermann et al., 1998)(Koppel and Raper, 1998). Class-3 Semas (Sema3A-Sema3G) are all secreted molecules containing the so-called “basic tail” provided by a disulfide bridge which reinforces the binding between two Semas. In the mouse embryo, Sema3B mRNA is highly detected in the FP and in ventral progenitor cells lying the central canal (Zou et al., 2000). Sema3B protein in a Sema3B-gfp mouse line could be detected by anti-GFP labeling in FP glial cells. It was shown to act as a key repulsive cue for commissural axons at the midline (Zou et al., 2000) but also to control, once released by the FP in the cerebrospinal fluid, the orientation of progenitor divisions (Arbeille et al., 2015).

The major receptors of Semas are Plxn family members which include, in vertebrates, nine proteins (PlxnA1-A4, PlxnB1-B3, PlxnC1 and PlxnD1)(Tamagnone and Comoglio, 2000). These proteins are single-pass transmembrane proteins containing an extracellular region composed by ten domains. Specifically, at the N-terminus, they contain a sema domain followed by

Plexin-Semaphorin-Integrin (PSI) and Immunoglobulin-Plexin-transcription (IPT) domains. Moreover, PlxnAs contain a conserved transmembrane helical domain and a cytoplasmic region containing a GTPase-activating (GAP) domain with a Rho GTPase-binding (RGB) domain. The extracellular domain has a characteristic ring architecture and interacts in *cis* with the ectodomain of another PlxnA, resulting in inhibition of receptor activation (Kong et al., 2016). In this configuration, the cytoplasmic domains of PlxnAs are far from each other and cannot elicit a signaling response. After Sema binding, the cytoplasmic regions of Plxns get closer and dimerize, which enable fast activation of downstream signaling (Kong et al., 2016). Some studies suggested that upon Sema binding to Plxn ectodomain, a coiled-coil structure forms in the juxtamembrane cytoplasmic region that would initiate the activity of the Plxn GAP domain (Bell et al., 2011). However, we have still scarce knowledge on the structural changes that the extracellular binding of Sema induces on the transmembrane domain and therefore how the extracellular and the intracellular activities of the receptors are linked remains unclear. The activation of PlxnA receptors induces a series of intracellular events that regulate microtubule/actin dynamics and endocytosis, leading to the disassembly of cytoskeleton elements, substrate detachment and retraction of the growth cone (Zhou et al., 2008)(Mambetisaeva et al., 2005).

Many Semas can bind directly to Plxns *via* their Sema domain whereas several secreted Semas, such as the Sema3s, bind to Plxn with low affinity and require co-receptors, Neuropilin1 (Nrp1) or Neuropilin2 (Nrp2) which also are single pass transmembrane proteins, to stabilize the interaction. The association between Plxns and Nrps form an active holoreceptor complex, whose composition can vary according to the different Semas and the cell-types (Tran et al., 2007)(Nawabi et al., 2010). In the context of commissural axon navigation, genetic deletion in the mouse of Sema3B, Nrp2 and PlxnA1 all resulted in defective midline crossing. Various defects were reported, such as stalling and caudal instead of rostral turning (Zou et al., 2000) (Nawabi et al., 2010).

The SlitC/PlexinA signaling.

Slits were shown processed by proteases. The cleavage of Slit2, and probably of Slit1, generates an N-terminal fragment (Slit2N, 140 kDA) and a C-terminal fragment (Slit2C 55-60

kDA)(Brose et al., 1999). The functions of Slits were thought to be mediated by the full-length and the N-terminal fragment since it contains the binding sequence to Robos (Delloye-Bourgeois et al., 2015). Consequently, the Slit-C fragment has been considered inactive until our laboratory found it exerts a repulsive action, that turned out to be mediated by PlexinA receptors, and especially PlexinA1 in the context of the spinal commissure.

Therefore, this put Plxn1A as a receptor shared by Sema3B and Slit-C. However, the mechanisms leading to PlxnA1 activation and to the consequent repulsive effect are different between the two ligands. Our laboratory found that Slit-C but not Sema3B induces the phosphorylation of the tyrosine residue Y1815 in PlxnA intracellular domain. Mutating this residue interferes with Slit-C but not Sema3B activity. Moreover, Slit-C-PlxnA1 binding is independent of the Robos, but when interacting with Nrp2, PlxnA1 cannot bind to Slit-C anymore (Delloye-Bourgeois et al., 2015).

Analysis of mouse models gave useful information for discriminating the functions ensured by these different repulsive signaling. Indeed, spinal commissural axons recrossing the midline were observed in Slit 1-3 triple and PlxnA1 mutants, but neither in Sema3B nor in Robo mutants, suggesting that the PlxnA1-SlitC might be primarily responsible for preventing midline re-crossing (Long et al., 2004)(Nawabi et al., 2010).

Nevertheless, and beyond the midline barrier setting, it remains very unclear whether these different repulsive signaling have redundant or complementary functions. For example, many questions remain on the spatial distribution of the ligands within the FP, which highlight the nature of the growth cone behavior they control. Slits exhibit heterophilic binding with many molecules of the extracellular matrix, such as Neurexins, type IV Collagens, glypican and syndecan (Blockus and Chédotal, 2016). For instance, in mammals, Slit-FL and SlitN bind mostly to the cell surface whereas SlitC was reported associated to the extracellular matrix (Brose et al., 1999). In particular, Slit-FL and Slit-C can potentially bind to dystroglycan in the FP and the basal lamina, whereas Slit-N doesn't have this property because it lacks the dystroglycan-binding laminin G domain (Wright et al., 2012). In *Drosophila*, Slit protein distribution is altered in syndecan mutants, supporting that heparan sulfate glycosaminoglycans can localize Slit in specific territories (Johnson et al., 2004). Our laboratory

also has several evidences from ongoing work supporting that Slit fragments distribute differently in the spinal cord.

Additional interactions occurring in the FP implicate B-class Ephrins which can act as ligands or receptors mediating forward or reverse signaling, respectively (Cowan and Henkemeyer, 2002). In the FP, glial cells express ephrin-B3 and commissural axons Eph-B3 receptors (Kadison, 2006). The commissure is impaired in mice lacking ephrin-B3 or Eph receptors (Kadison, 2006). It remains unclear whether the predominant function of the Ephrin/Eph signaling is, as in other commissural systems, to prevent the crossing of ipsilateral axons, or if it contributes in addition to repel commissural axons after the crossing.

Nogo, mainly known as a ligand inhibiting axon regeneration, has also been reported expressed by radial glia and Nogo receptor (NogoR) by spinal commissural axons (Wang et al., 2010). Blocking NogoR was found to induce axon stalling in the FP, therefore resulting in a reduced number of commissural fibers reaching the spinal cord contralateral side (Wang et al., 2010).

Repulsive propelling forces after crossing.

Following their crossing, commissural axons turn rostrally and navigate longitudinally to the FP. Rostral turning of the growth cone is made possible by a rostral to caudal gradient of SHH and Wnt morphogens, SHH being concentrated more caudally and Wnt more rostrally. The attractive activity of SHH in the first half of the spinal cord navigation exerted *via* SMO and BOC receptors is turned to repulsion after the crossing via a switch of commissural receptor towards HHIP (Hedgehog interacting protein)(Charron et al., 2003)(Okada et al., 2006)(Bourikas et al., 2005). This switch appears to also activate simultaneously the sensitivity to Wnt5, whose gradient attracts commissural growth cones towards the rostral direction. This cross-talk is mediated by SHH/SMO-dependent downregulation at transcriptional level of Shisa2, a protein which inhibits glycosylation of the Wnt receptor Frizzled3 (Fzd3), thereby resulting in increase of Frizzled3 sorting at the cell surface (Onishi and Zou, 2017).

In parallel to these repulsive forces expelling commissural axons from the FP, many studies have reported the fundamental role played by cell adhesion molecules. For instance, in the chick embryo, MDGA2, a cell adhesion molecule of the IgCAM Superfamily, was found to promote commissural axon extension along the longitudinal axis. This protein is expressed in both commissural and ipsilateral projecting interneurons at HH24-HH26, a developmental stage at which ipsilateral and commissural axons are navigating the same compartment, the VF. Ipsilateral knock-down of MDGA2 prevented the longitudinal growth of commissural axons, a phenotype phenocopied by commissural ablation of MGDA2. Moreover, the demonstration that this protein is able to form homophilic trans-interactions suggested a mode of action whereby MGDA2 mediates the fasciculation of post-crossing axons with the ipsilateral axons navigating the VF. According to this model, ipsilateral axons would serve as a substrate for commissural axon elongation (Joset et al., 2011). Furthermore, in the chick embryo, a role of a subfamily of IgCAMs, the Necls/SynCAMs, has been described at the post-crossing step. In particular, it has been suggested that Necl3 produced by the FP induces the clustering in *cis*, of Necl2 and Necl3 receptors at the commissural growth cone surface. This complex can interact with FP Necl3, and triggers, in combination with Shh and Wnt gradients, the rostral turning at the FP exit (Niederkofler et al., 2010). The repulsive propelling forces at the FP exit are represented in **Figure 8**.

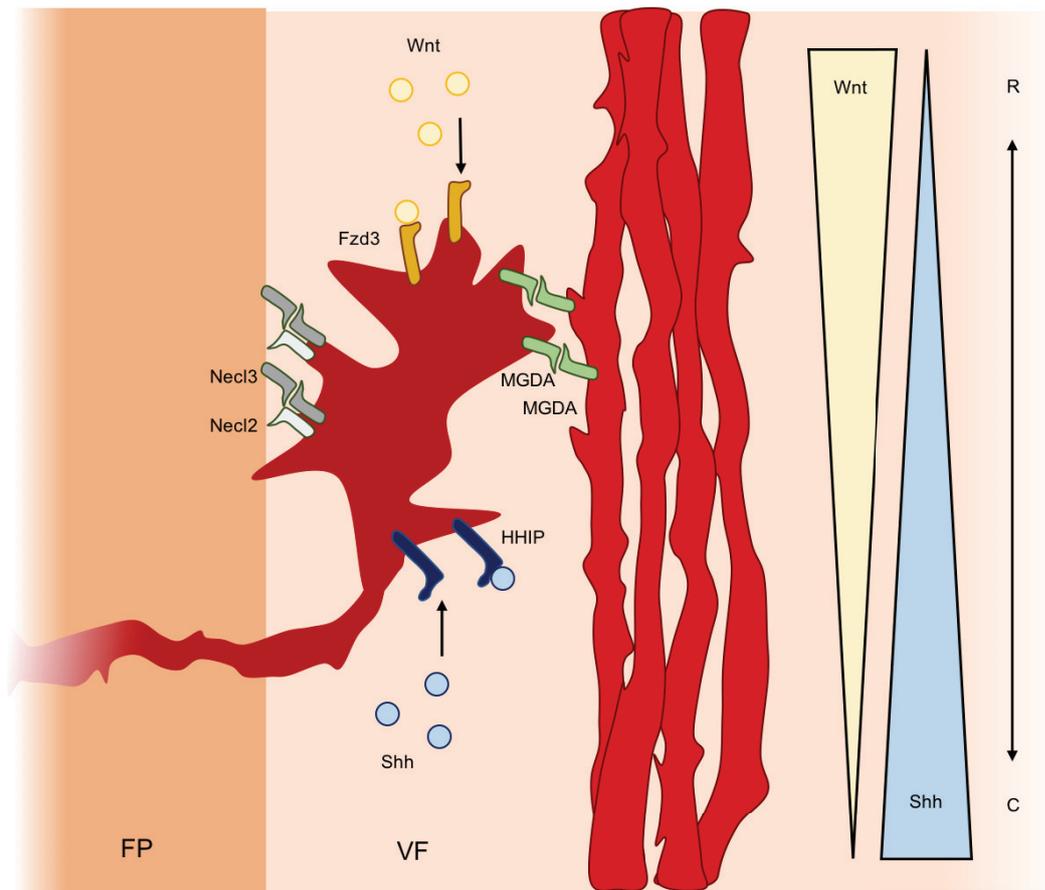


Figure 8: Propelling forces after FP crossing. Both soluble cues and adhesive interactions participate to growth cone propulsion away from the FP. Rostral turning of the growth cone is triggered by rostro-caudal gradients of Shh and Wnt morphogens, Shh being more concentrated at the caudal side and inducing *via* HHIP receptor a repulsive response that prevent growth cones from turning caudally, and Wnt, being more concentrated at the rostral side and inducing an attractive response of the growth cone *via* Fzd3 receptor. In parallel, several couples of adhesive molecules are involved in the growth cone propulsion, for instance Necl3- Necl2/Necl3 *trans* interactions between the FP glia and the growth cone and MGDA-MGDA *trans* interactions between the growth cone and the post-crossing fibers navigating the VF.

Repulsive channeling forces after crossing.

Once the rostro-caudal direction is selected, commissural axons segregate in several tracts at different medio-lateral positions. This process of bundle selection depends both on the perception of repulsive guidance cues and on the interaction between adhesion molecules expressed by the growth cone and the surrounding environment (**Figure 9**).

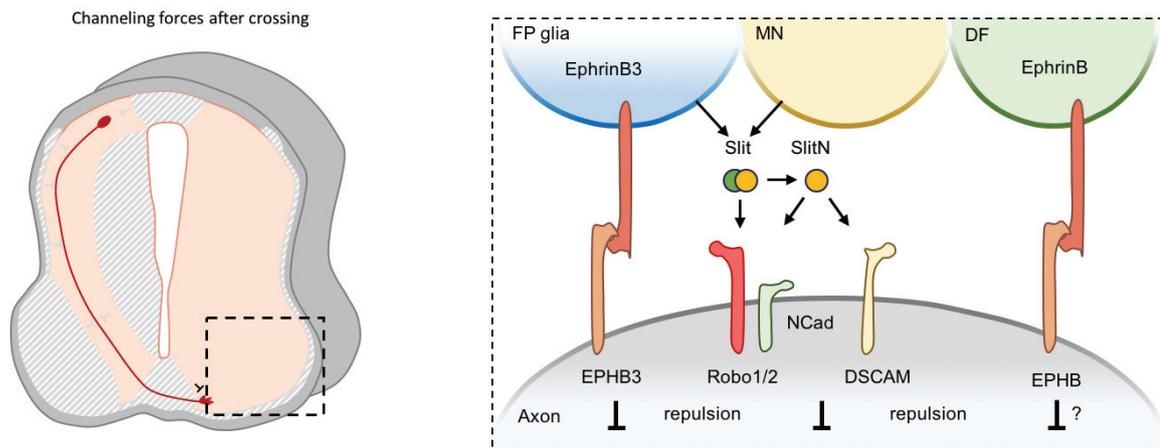


Figure 9: Channeling forces after FP crossing. At the post-crossing stage, commissural axons form two main tracts, the VF and the LF. The trans-interaction occurring between FP glia EphrinB3 and EPHB3 receptor participates in assigning the medio-lateral position of post-crossing axons. Slits provided by the FP glia contribute to axon sorting within the two bundles through its interaction with Robo1/2 receptors. The involvement of Slits secreted by MN in post-crossing bundle selection has not been proved yet. The involvement of EphrinB-EPHB signaling in post-crossing axon channeling has been suggested in the past but it needs to be demonstrated *in vivo*.

In the *Drosophila* nerve cord, Slits acting as long-range cues are thought to organize the longitudinal tract positioning, by forming a medio-lateral gradient from the midline source (Rajagopalan et al., 2000). However, and mainly due to the lack of appropriate tools to characterize their expression profile, there are still no direct evidence, *in vivo*, for such a medio-lateral gradient of Slit proteins during the formation of post-crossing longitudinal pathways. Slit processing might be required for the longitudinal path since substitution of Slit by uncleavable Slit form, while not affecting midline crossing, was reported to have profound impact on the longitudinal axon tracts. This study further suggested that this post-crossing function is mediated by binding of Slit-N to Dscam and Robo receptors (Alavi et al., 2016). Manipulations of Robo receptors in commissural axons also support a post-crossing role of Slits (Rajagopalan et al., 2000)(Simpson et al. 2000). According to these models, Robo2 and Robo3 are involved in the lateral navigation since their loss causes a shift of the tracts closer to the midline whereas their forced expression in axon tracts that express only Robo, repositions them further away from the midline (Rajagopalan et al., 2000)(Simpson et al.

2000). This “robo code” would specify the broad lateral zone in which axon tracts project and then a “fasciculation code” would refine axon positioning *via* local cues produced by the lateral tracts, through the involvement of adhesion molecules like Fasciclin II.

An analogous Robo code for lateral positioning has been suggested in vertebrates. During the time-window of FP and post-crossing navigation, mRNAs for Robo1 and Robo2 have been detected in overlapping populations of commissural neurons. Commissural neurons expressing Robo2 lie their cellular body at more lateral positions than those expressing Robo1 (Brose et al., 1999). Many cellular bodies produced mRNAs for both receptors, suggesting that commissural axons can concomitantly express Robo1 and Robo2. After crossing, commissural axons organize in two main tracts navigating in the ventral and the lateral funiculi (VF and VL). Antibodies directed against the extracellular domains of Robo1 and Robo2 revealed their expression primarily in the post-crossing segments of commissural axons. Moreover, Robo1 and Robo2 antibodies labeled overlapping but distinct post-crossing territories of the spinal cord: the VF with anti-Robo1 and the LF with anti-Robo2 (Long et al., 2004). The analysis of mouse models, mutant for these receptors, supports that Robo1 and 2 control the mediolateral sorting of post-crossing commissural axons, and that the two receptors have specific contributions. At E11.5 when the commissures are forming, the LF appeared significantly thicker in Robo1 mutants and thinner in Robo2 mutants, compared to WT. The correspondence between Robo expression and mediolateral position of the axons is quite complex to infer, because axons can express Robo1 and Robo2 only, or both. Moreover, Robo expression levels also vary, which also likely influences the strength of the response. As a result, the interpretation of single and double Robo1 and Robo2 mutant phenotypes is rather uncertain, and not consistent between studies. For example, thickening of the VF consequent to Robo1 loss was reported in a first study, but not seen in a second one (Long et al., 2004)(Jaworski et al., 2010). In mice lacking Robo3, in which all commissural axons stay ipsilateral, the loss of Robo1 and Robo2 generates similar defects to those observed in Robo1^{-/-}, Robo2^{-/-} and Robo1/2^{-/-} mutants, indicating that the organization of the lateral navigation by Slit-Robo signaling is not conditioned by midline crossing.

In vertebrates, sources of Slit other than the FP might account for the post-crossing navigation, in particular, motoneurons express all three Slits in mice, rat and chick. This source of Slit could

constrain post-crossing axons expressing Robo1 at medial trajectories, and those expressing Robo2 at more lateral positions, between the lateral basal lamina and the motor column. Experiments to evidence this channeling function still lack. Up to now, this motoneuron source of Slit has been shown to organize the phrenic motor axon tract also expressing Robos, through an autocrine mode of action (Jaworski and Tessier-Lavigne, 2012). Further investigation of the possible role of motoneuron-derived Slit could be achieved by analysis of commissural trajectories in mouse embryos mutant for the Homeobox Gene Hb9, which plays an essential role in MN differentiation (Arber et al., 1999) or in chick embryos by mechanic removal of the limb, which results in MN apoptosis. Targeted deletion of Slits in motoneurons could also be undertaken to specifically assess their role (Wilson and Stoeckli, 2012).

Slit/Robo signaling appears to act in concert with the Eph/Ephrin signaling in channeling post-crossing axons. At E13, in the mouse embryo, the expression patterns of B-class ephrins and B-class Eph receptors are complementary: B-class ephrins are expressed on the lateral border of the FP and in the dorsal funiculus whereas B-class Eph receptors are highly expressed on axonal segments that have crossed the midline but absent on commissural axons navigating toward the FP (Imondi et al., 2000). *In vitro*, the interaction between EphB-ephrinB was observed to induce a collapse of commissural growth cones whereas its blockade resulted in an aberrant invasion by commissural axons of spinal cord dorsal regions, in which axon growth is normally avoided (Imondi and Kaprielian, 2001). These evidences suggested that the expression of ephrins in the dorsal funiculus and at the FP borders constrains the trajectory of post-crossing commissural axons expressing Eph receptors in a corridor (Imondi and Kaprielian., 2001)(Imondi et al., 2000). However, a following study, based on *in vivo* analysis did not support a prominent role of this signaling since mice lacking EphB or ephrin-B members had unaffected contralateral projections (Kadison, 2006). Conversely, this signaling turned out to be essential for proper midline crossing, since its loss resulted in aberrant trajectories at the vicinity of the midline (Kadison, 2006). It remains to be tested whether other Eph/Ephrin couples contribute to pattern the post-crossing navigation.

The longitudinal sorting of post-crossing commissural axons might also be dependent on interactions between guidance receptors and cell adhesion molecules. For instance, N-cadherin (Ncad) is involved in the longitudinal tract positioning in *Drosophila* (Iwai et al., 1997)

and Robo receptors are capable of inactivating Ncad-mediated adhesion in a variety of systems (Rhee et al., 2007)(Rhee et al., 2002)(Wong et al., 2012). In the chick model, over-expression of Ncad prevents the formation of the LF, which is also the result of Robo signaling loss. However, perturbing simultaneously Robos and Ncad, rescues this phenotype. The same outcome is produced when inactivating Robo function in Ncad conditional knock-out mice. These findings thus suggest that Robo-mediated inhibition of Ncad is required for the correct formation of longitudinal tracts.

To which extent post-crossing axons of commissural neurons join axonal tracts formed by their homologous ipsilateral neurons is not well determined. In the chicken embryo, homophilic interactions do form between axons of the same subtype, for instance dl1 commissural axons, which project contralaterally across the midline, fasciculate in the LF with dl1 ipsilateral axons (Hivert, 2002). Consequently, it is conceivable that ipsilateral axons, which establish their tracts prior to the arrival of the correspondent contralateral population, pioneer the path. Many ipsilateral and contralateral populations share the same guidance receptors, differing by the commissural-specific silencing mechanisms that operate to enable midline crossing.

In the mouse, if such homotypic fasciculation exists, it would impact on the interpretation of the post-crossing defects of mutant models. They could be due to fasciculation with ipsilateral axons aberrantly positioned than to loss of responsiveness to guidance cues. In this direction, some studies in the past have even proposed a model according to which Robo receptors would sort axons in specific compartments through homophilic interactions, thus not being fundamentally different from any other adhesion molecules such as Fasciclin II (Hivert, 2002). Population-specific deletions could help discriminating the mode of action of the Robos. In the chicken embryo, *Atoh1* and *Neurog1* were used to drive the expression of truncated variants of Robo having dominant-negative properties in dl1 and dl2 commissural neurons, respectively (Reeber et al., 2008). These experiments resulted in a failure of post-crossing dl1 and dl2 axons in projecting away from the FP after unilateral electroporation of Robo1 truncated variants. Surprisingly, the authors also found that after the unilateral electroporation, post-crossing axons of either side of the spinal cord failed to project away from the FP. More specifically affecting the guidance of ipsilateral axons would allow assessing whether they contribute to the post-crossing navigation of commissural axons.

As a summary of this chapter, **Figure 10** presents the different phenotypes of commissural axon guidance observed in the mouse models.

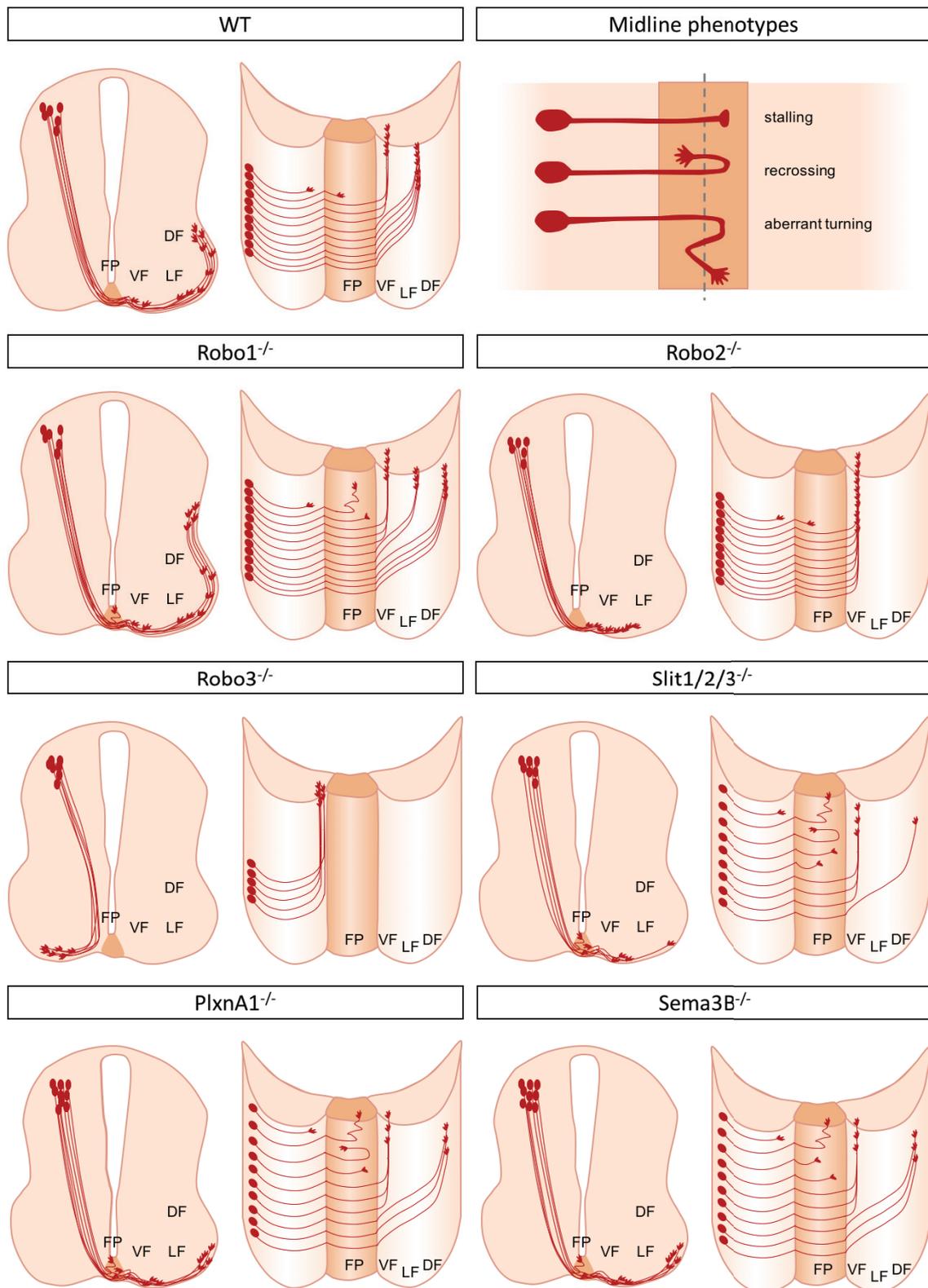


Figure 10: Summary of the principal commissural axon phenotypes observed in mutant mouse models. WT: commissural axons extend towards the midline and then, after crossing turn rostrally, selecting medio-lateral pathways. In mice mutants for guidance receptors and repulsive cues, the principal phenotypes consist in growth cone stalling at the FP entry, within the FP and at the exit, midline re-crossing and aberrant turning within the FP. *Robo1*^{-/-}: these mutants display aberrant axon turning and stalling during FP navigation and a thickening of the LF-DF. These mutants do not display re-crossing. *Robo2*^{-/-}: these mice do not exhibit navigation defects during FP crossing but present a strong thinning of the LF. *Robo3*^{-/-}: These mutants completely lack commissures. *Slit1/2/3*^{-/-}: Slit triple mutant display phenotypes which are more severe than those displayed by the simple mutants. They have all the midline phenotypes and also a thin LF. *PlxnA1*^{-/-}: as Slit triple mutants, these mice display all the midline phenotypes however, the condition is less severe than that of Slit triple mutants. *Sema3B*^{-/-}: these mutants display stalling and aberrant turning at the FP but do not exhibit midline re-crossing.

CHAPTER2: Molecular mechanisms regulating commissural axon sensitivity to the FP guidance cues.

During spinal cord navigation, growth cones adapt step by step their sensitivity and their responses to surrounding external cues. If one can easily conceive how growth cones can be repelled away from a territory releasing repulsive cues, it is more challenging to understand how commissural growth cones can navigate a territory such as the floor plate which produce repulsive cues all over their navigation. To make this navigation possible, it has been shown that commissural growth cones only perceive the repellents after they have crossed midline. I will present in this chapter the principal mechanisms regulating the sensitivity of commissural growth cones to the guidance cues orchestrating their navigation in the vertebrate spinal cord and *drosophila* ventral cord.

In both vertebrates and invertebrates, growth cone sensitivity to midline repellents must be tightly regulated in space and time, since a premature response of growth cones prevents contacts with the intermediate target, whereas a delayed response results in stalling. During the whole period of commissural axon extension towards the midline and exit from this structure (from the first axon waves of pioneers at E9.5 to the latest one at E12.5) (Pignata et al., 2016), the glial FP cells present repulsive cues to growth cones. This suggests that commissural axons actively silence their response to repellents until they have reached the FP

and that during crossing they turn-on their sensitivity to these signals. This growth cone sensitization is achieved *via* precisely controlled modifications of the guidance receptor repertoire at the plasma membrane. This goes through both loss of pre-existing receptors and gain of novel receptors, although the latter phenomenon appears from the literature to be more prevalent. Various mechanisms contribute to these changing of receptor composition, ranging from modifications at transcriptional levels to direct targeting of the protein. They include regulation of receptor synthesis, trafficking, extracellular sorting, changing of their interactome profile or stability modification.

Silencing and sensitization to Slits.

In both invertebrates and vertebrates, the pre-crossing silencing to midline Slits is crucial for appropriate midline crossing and has been described to occur *via* a control of the surface expression of Slit receptor Robo. To do so, invertebrates and invertebrates adopt different post-translational strategies, requiring specific molecular interactions. In *Drosophila*, Robo1 surface sorting is regulated by Commissureless (Comm) (Kidd et al., 1998b) which is transiently expressed in commissural but not in ipsilateral projecting neurons. At the pre-crossing stage, Comm addresses Robo1 to the endosomal-lysosomal compartment whereas, during crossing, Comm is down-regulated via a yet unknown pathway, which results in Robo1 surface expression (Keleman et al., 2002)(Keleman et al., 2005). Moreover, a second mechanism, complementing the Comm-mediated silencing in pre-crossing has been described to prevent Robo1 that would start reaching the cell-surface before the crossing to confer premature sensitivity to midline Slits. This mechanism relies on a *trans*-interaction between Robo1 at the surface of commissural growth cones and Robo2 expressed at the surface of midline glial cells (Evans et al., 2015).

An ortholog of Comm has not been found in the vertebrate genome, which raised the evidence that other mechanisms ensure the pre-crossing silencing to Slits. Immunohistochemical detection of Robo1/2 has shown that the receptors are weakly detected in pre-crossing axons, and become enriched at the post-crossing stage. In contrast, Robo3 is found mainly in pre-crossing axon segments. Robo3 was initially thought to bind Slits, thus possibly competing with Robo1/2 for binding, and silencing the responsiveness of the growth cone by failing to

activate downstream cascade (Mambetisaeva et al., 2005). However, this hypothesis has been invalidated with the recent findings that Robo3 in mammals has lost the Slit binding (Zelina et al., 2014). Another model has been proposed in which Robo3 would trigger Robo1/2 degradation. However, this mechanism has only been addressed *in vitro* (Li et al., 2014). Distinct spliced isoforms of Robo3 have been reported, depending on whether the splicing affects the N-terminal or C-terminal domain of the protein. Robo3.1 and Robo3.2 are two C-terminal isoforms that have been proposed involved in Robo1/2 regulation. Robo3.1, which is the isoform having restricted pre-crossing expression, was proposed to engage in *cis*-interaction with Robo1/2 at the plasma membrane, the complex formation resulting in silencing of the receptor signaling activity (Chen et al., 2008). These models rely on the presence of Robo receptors in pre-crossing axons, which is uncertain. Beyond, a recent study in the chick embryo rather proposes that at the pre-crossing stage, Robo1 transcript is silenced by a microRNA, miR-92 (Yang et al., 2018). Whether this mechanism is coupled to that implicating Robo3 is not known. Overall, how is silenced Slit sensitivity in vertebrates, and whether all vertebrates engage the same type of regulations remain largely puzzling.

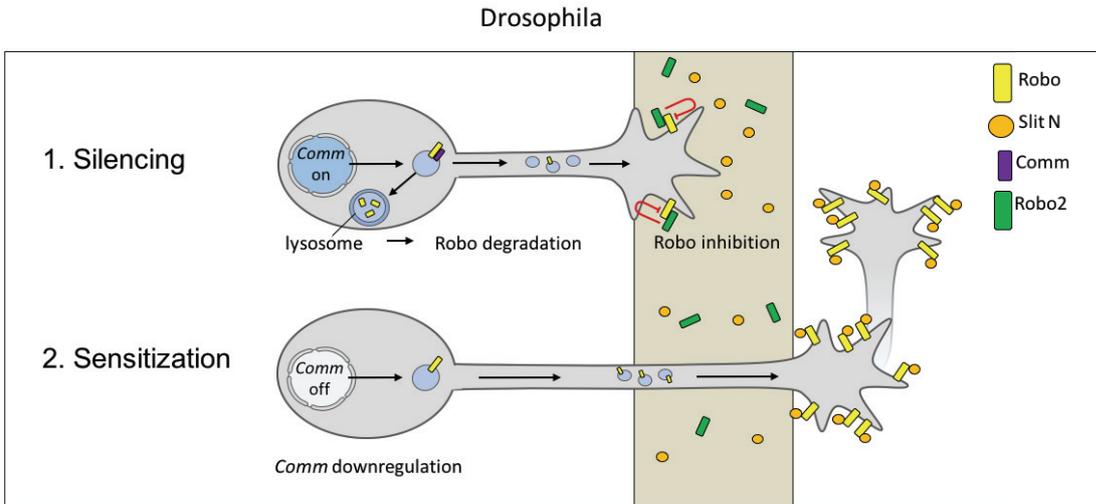
The sensitization to Slits also remains unclear, with debated models. During crossing, Robo3.1 would be replaced by Robo3.2, through a mechanism implicating local synthesis triggered by a FP signal (Colak et al., 2013), this latter isoform acting as an agonist to switch on responsiveness to Slits (Chen et al., 2008). Surprisingly, However, no model of this Robo3.1 to Robo3.2 switch has been proposed as well as a mechanism by which Robo3 would sense Slit gradient, triggering Robo1/2 repulsion. Surprisingly, Robo3.2 variation is poorly conserved and might only exist in rat and mouse (and not in other mammals or other species) (Friocourt and Chédotal, 2017), which makes it difficult to generalize these findings. Recent results indeed bring a radically different mode of action of Robo3, which relies on its binding to DCC. According to this study, Robo3 would be involved in Netrin mediated-growth cone attraction toward the midline (Zelina et al., 2014).

The gain of sensitivity to Slits is suggested to occur “simply” because of increase of Robo levels. In the chicken embryo, trafficking of vesicles carrying Robo receptors was reported regulated upon the crossing. More precisely, RabGDI (a highly conserved regulator of vesicle trafficking) and Calsynthenin1 (protein involved in anterograde vesicular transport, interacting with

Kinesin motor) were found to collaborate in triggering at right time Robo surface expression (Philipp et al., 2012)(Alther et al., 2016). What are the mechanisms triggering the up-regulation of RabGDI and does the FP provide a checkpoint control are still open questions.

The findings that Robo proteins undergo proteolytic cleavage open additional possibilities to this already very complex picture. Controlling this cleavage could indeed represent an efficient way for regulating growth cone sensitivity to Slits. Cleavage by metalloproteases has first been reported in *Drosophila*, and shown to be essential for the recruitment of downstream signaling molecules. Moreover, an uncleavable form of Robo could not rescue midline stalling phenotypes in Robo mutant mice (Coleman et al., 2010). It has also been reported that Robos undergo a second cleavage by gamma-secretases which leads to the translocation of intracellular RoboC fragment to the nucleus (Seki et al., 2010), suggesting that Robo not only acts as a receptor but also as a nuclear signaling molecule. After binding Slit, Robos are addressed to endosomes and recycled. Interestingly, Robo sorting to endosome represents a fundamental step for the recruitment of adaptor molecules and the initiation of a signaling cascade (Chance and Bashaw 2015). It would be interesting to extend these findings in the context of vertebrate axon guidance given that cleavage by metalloproteases was reported in the *Drosophila* nerve cord and processing by gamma-secretases in cancer cells. For example, this could be investigated by developing genetic tools based on “dark-to-bright” reporters that would reveal cleavage events through a fluorescent signal. How regulation of Robo cleavage to control growth cone sensitivity to Slits could be achieved? This obviously could go through the regulation of metalloprotease activity but also of Robo conformation. Some studies have suggested that the control of Robo cleavage depends on the folding of its juxtamembrane (JM) domain. After having revealed the crystal structure of this portion, the authors have suggested that in absence of Slit, the JM linker has a compact folding which prevents metalloprotease cleavage. Slit binding to Robo would generate a tension at the site of JM containing the linker. This would expose the cleavage site to metalloprotease (Barak et al., 2014). Thus, such micro-tension at the growth cone surface could be essential for exposing or not Robo to the proteases. The molecular mechanism that are involved in silencing and sensitization to Slits are illustrated in **Figure 11**.

A



B

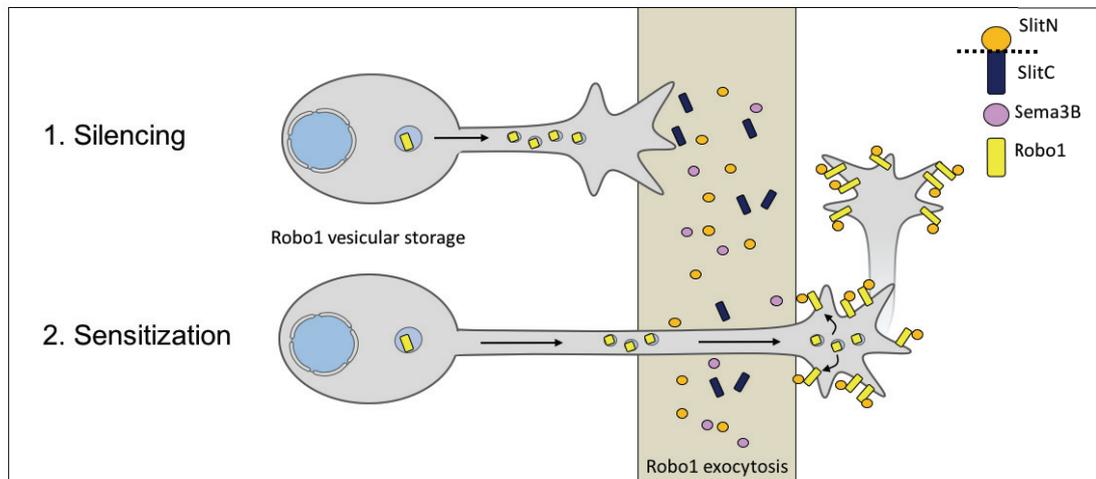
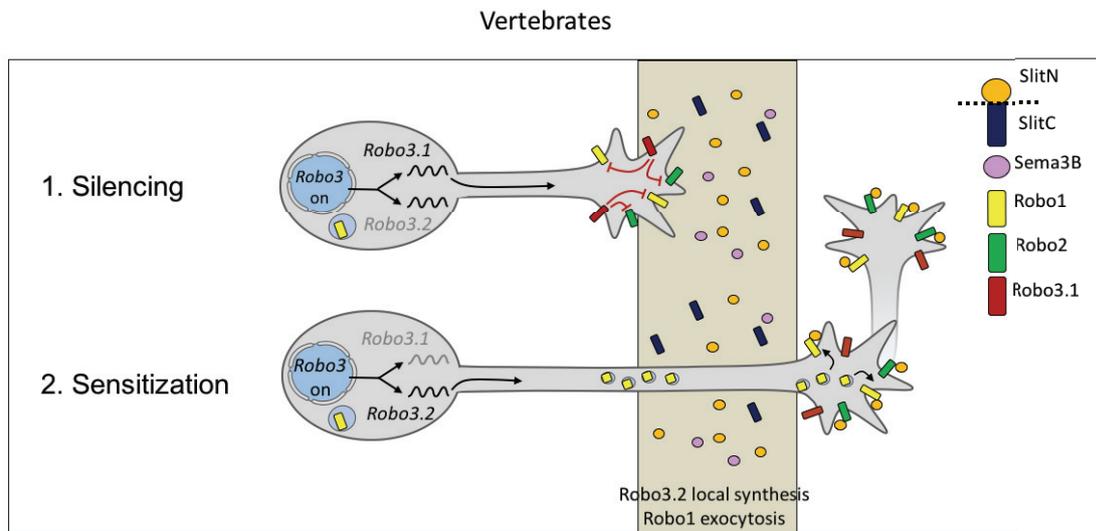


Figure 11: Mechanisms mediating Robo1 silencing and sensitization to Slits in *drosophila* and vertebrates. (A) In *drosophila*, Robo in pre-crossing is targeted to lysosomes by Comm protein. During crossing, Comm is downregulated, allowing Robo to reach the cell surface and transduce Slit signaling. A second mechanism, occurring in parallel to Comm-mediated silencing, involves Robo2 expressed by glial cell, which interacts *in trans* with Robo1 at the growth cone surface, blocking its interaction with FP Slit. (B) Upper panel: in mouse embryo, upon crossing Robo3.1 is replaced by Robo3.2 which acts as an agonist of Robo1-Slit signaling. This model lacks further confirmation. Lower panel: in chick embryo, the cell surface sorting of Robo1 is triggered by the transcriptional up-regulation of RabGDI.

Silencing and sensitization to the Semaphorins.

The Sema3B/Nrp2/PlxnA1 signaling is also regulated to enable midline crossing.

Our laboratory reported that the silencing is achieved through regulated processing of PlxnA1 by calpains. These proteases bear the particularity to process their targets rather than degrading them, thus modifying for example their interactome profile (Carragher and Frame, 2002)(Araujo et al, 2018). Calpain was shown to generate two PlxnA1 fragments from the integral protein. PlxnA1 and Nrp2 transcripts were detected in the dorsal spinal cord over the FP navigation period but at protein levels, only Nrp2 protein expression was consistent in pre-crossing axon segments. PlxnA1 signal was in contrast strongly enriched in crossing and post-crossing axons. A series of experiments raised a model in which PlxnA1 processing by calpains prevents the protein to reach the surface. Upon exposure to FP signals identified as being NrCAM and GDNF, calpain activity would be inhibited and PlxnA1 conformed for surface sorting (Nawabi et al., 2010)(Charoy et al., 2012). Because PlxnA1 also mediates the response to FP Slit-C (Delloye-Bourgeois et al., 2015), such regulation might also act as a trigger of Slit-C sensitivity.

This model was challenged by a study reporting PlxnA1 expression and sensitivity of commissural axons at the pre-crossing stage (Hernandez-Enriquez et al., 2015). The labeling was achieved with a home-made antibody recognizing the C-terminal domain of PlxnA1, which thus prevented the distinction of intracellular and surface pools. The antibody thus likely detected both integral and processed PlxnA1. Lack of commissural axon sensitivity to Sema3B has been reported by three independent labs (Delloye-Bourgeois et al., 2015)(Nawabi et al., 2010)(Zou et al., 2000)(Parra and Zou, 2010)(Tran et al., 2013), and despite our effort we could

not obtain a response to *Sema3B* using the protocol described in this study (unpublished data presented in the result section). The authors observed that specific removal of *Nrp2* in the FP induces reduction of commissure thickness, a phenotype that was rescued by removal of *PlxnA1*. In the subsequent model proposed in this study, FP-*Nrp2* would trap *Sema3B* in the FP to prevent its diffusion and to make it inaccessible to commissural neurons over the crossing period. Sensitization would then be achieved via transcriptional downregulation of *Nrp2*. This model implies that midline crossing by commissural axons occurs in synchrony, which is not supported by time course analyses. Rather many studies provided evidence for successive generation of commissural neurons and waves of axon growth across the midline from E10 to E13.5 in the mouse (Nawabi et al., 2010)(Pignata et al., 2016)(Brown et al., 2001). Finally, we quantitatively compared the *Sema3B* distribution profile in the ventral spinal cord of *Sema3B*-GFP knock-in embryo wild-type or mutants for *Nrp1/2* using GFP antibody staining. We found no difference of distribution patterns between the two conditions (unpublished observations, see result section). Despite these discrepancies, it remains that *Nrp2* in FP cells, as well as *PlxnA1*, could play indispensable roles during midline crossing, through modalities that remained to be clarified.

The regulation of growth cone sensitivity to *Sema3B* and to closely related *Sema3*, *Sema3F*, was also reported to be regulated by *Shh* signaling, via downregulation of PKA and second messenger activity (Parra and Zou, 2010). Experiments in our lab could not reproduce the SHH-mediated sensitization of commissural axons to *Sema3B* while they did confirm their sensitization to *Sema3F*. Beyond these differences that might rely on different experimental paradigms, *Sema3F* likely has a role distinct of that of *Sema3B*, given that it is not expressed by the FP but by adjacent motoneurons, at least in the mouse (Zou et al., 2000)(Püschel et al., 1996)(Parra and Zou, 2010). Nevertheless, the roles of second messengers in these regulations are likely important, given their broad implication in axon guidance, but further investigations are needed to clearly delineate them.

Additional mechanisms uncovered in the chicken embryo model and involving other *Sema* and *Plxn* proteins have been reported to come into action to switch-on the sensitivity to *Sema* repellents, especially *Sema6B*. *Sema6B* expressed by commissural axons was proposed to interact in *cis* with *PlxnA2* thus preventing the interaction between this *PlxnA2* and *Sema6B*

in *trans* from the FP. Upon reaching the FP, this *cis* complex would be destabilized by the presence of the PlxnA2 expressed by FP glial cells, and the resulting *trans* interaction between FP-PlxnA2 and growth cone Sema6B would then trigger a repulsive behavior (Andermatt et al., 2014). Thus, these studies illustrate the diversity of mechanisms underlying the regulation of commissural axon sensitivity to FP Semas. The molecular mechanisms that are involved in silencing and sensitization to Sema3B and SlitC are illustrated in **Figure 12**.

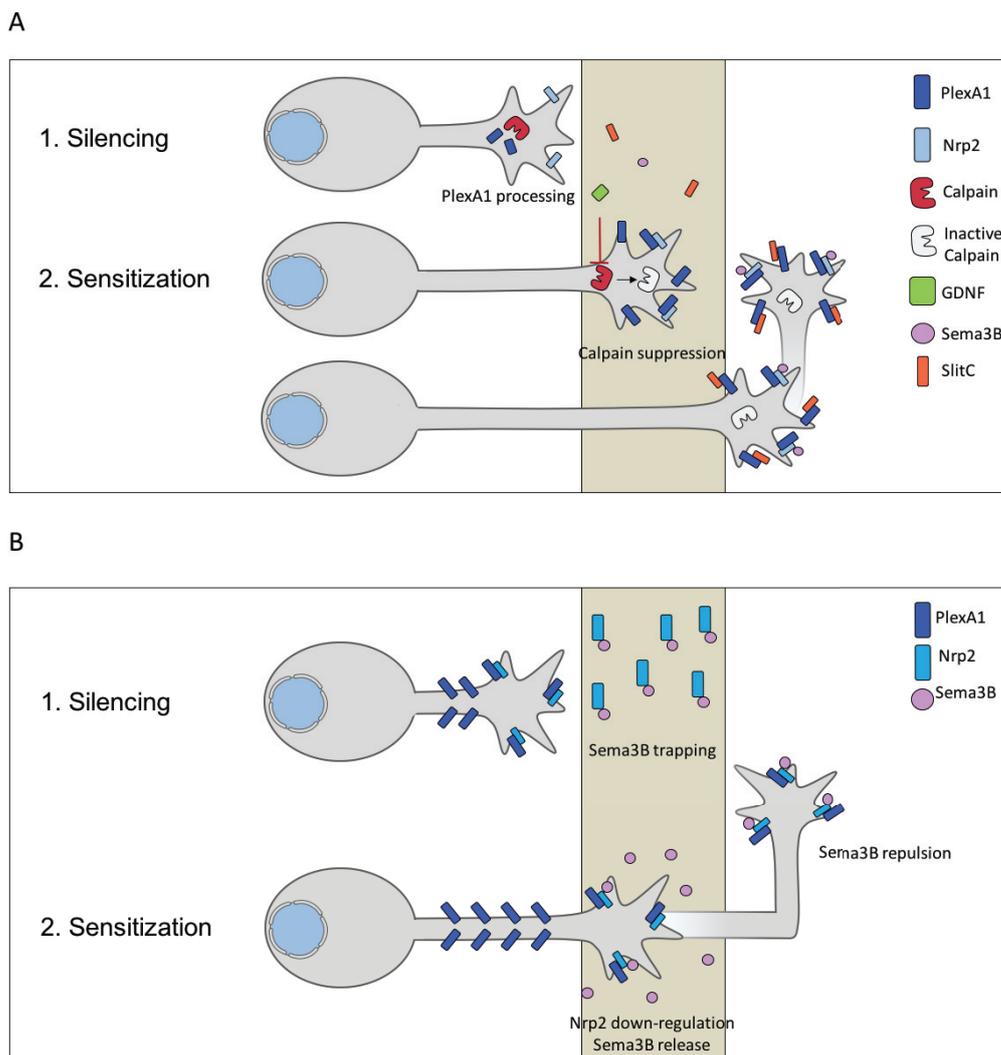


Figure 12: Silencing and sensitization of PlxnA1 to Slits and Sema3B. (A) Model for regulation of Sema3B signaling in the mouse spinal cord suggested by previous work of our laboratory. Calpain cleaves PlxnA1 during the pre-crossing navigation, preventing receptor cell surface sorting. During crossing, GDNF blocks Calpain activity, allowing the receptor to reach the surface and to respond to SlitC, and to Sema3B when associated with Nrp2. (B) Alternative

model for the regulation of Semaphorin 3B signaling suggested by T. Tran lab. In this model PlexinA1 and Neuropilin 2 are both expressed at the cell surface in the pre-crossing compartment, but the sensitivity to Semaphorin 3B is prevented because the ligand is trapped by Neuropilin 2 expressed by glial cells. After the crossing, at E13, Neuropilin 2 is downregulated, leading to Semaphorin 3B release and allowing repulsion.

Silencing of attractive cues.

The expression of Netrin or Shh persists in the FP during midline crossing and the post-crossing navigation, which suggested that the responsiveness to these attractants might be regulated over time. Early works consisted in grafting ectopic FP in spinal open books, either encountered by axons navigating towards the endogenous FP or after their crossing. The authors observed that pre-crossing commissural axons were deflected towards the ectopic FP whereas post-crossing axons ignored it, supporting that crossing of endogenous FP induced them to lose their sensitivity to FP attractants (Zou et al., 2000). *In vitro* experiments performed with *Xenopus* spinal neurons have suggested a mechanism by which Slit treatment would induce a silencing of Netrin response (Stein and Tessier-Lavigne, 2001). The growth cones of these neurons were not able to turn anymore towards focal Netrin source when concomitantly exposed to Slits. It was further proposed that this desensitization occur via Slit-induced interaction between the intracellular domains of Robo1 and DCC (Stein and Tessier-Lavigne, 2001). Cross-talks between Netrin and Slit have also been described in the developing brain to regulate the pathfinding of thalamocortical projections (Bielle et al., 2011).

Modulations of the sensitivity to the other reported FP attractants were also reported to occur *via* regulations at receptor levels. Likewise, SHH effect is switched from attraction to repulsion by changing of activated receptors, with SMO and BOC receptors mediating attraction (Charron et al., 2003)(Okada et al., 2006) and HHIP mediating repulsion (Hedgehog interacting protein) (Bourikas et al., 2005). It has been showed that the induction of HHIP expression occurs after the interaction of SHH with Glypican1. Indeed, Glypican1 appears required for the transcriptional regulation of HHIP (Wilson and Stoeckli, 2013) interestingly suggesting that Shh acting as an attractant in pre-crossing, induces the expression of its own repulsive post-crossing receptor (Bourikas et al., 2005)(Wilson and Stoeckli, 2013). Alternatively, other studies proposed that the switch from attraction to repulsion by SHH cue would depend on an intrinsic timer that would change the responsiveness of axons through accumulation of 14-

3-3 intracellular scaffolding proteins. Using *in vitro* assays, the authors found that during the first two days of culture, the growth cones were attracted by SHH and from the third day they got repelled by SHH. This behavior was correlated with a modulation of 14-3-3 proteins. These latter are major constituents of the growth cone and can control the spatial and temporal activity of the proteins they bind to. Increase of these protein levels was shown to result in reduction of PKA activity, resulting in repulsive (Yam et al., 2012).

These studies enlighten the diversity and complexity of the modulations that take place in the growth cones to drive their guidance decisions over the FP crossing and post-crossing navigation. They also illustrate that beyond the regulations of ligand and receptor expressions at transcriptional levels, many important regulatory pathways are operating at post-translational levels. The diversity of growth cone responses to guidance cues emerges from the control of the dynamics of guidance receptors and signaling molecules and the generation of asymmetries that will drive the growth cone steering. I will present in the next chapter the principal characteristics of the growth cone machinery that underlie these modulations.

CHAPTER 3: Generating polarized molecular distribution in the axon.

The growth cone: a machinery for generating molecular polarity and localized signals.

Structural and functional compartmentalization of proteins are major feature of the neurons (Katsuki et al., 2011). For example, their ability to organize highly complex molecular patterns in their axon is remarkably demonstrated by the formation of nodes of Ranvier, axonal microdomains in which sodium channels form clusters, spaced by myelin gaps arranged at regular distances along the axon shaft to ensure fast propagation of the action potential (Kaplan et al., 1997). As well, the axon initial segment (AIS), the part proximal to the soma, has a specific molecular composition in cell adhesion molecules, scaffolds and ion channels, needed to initiate the action potential (Nelson and Jenkins, 2017). The synapse is another major illustration of molecular compartmentalization capability of the neuron, for instance reflected by specific pre- and post-synaptic arrangements of cell adhesion molecules, neurotransmitter receptors and scaffolding proteins (Sheng and Kim, 2011)(Choquet and Triller, 2013)(Ribeiro et al., 2018). Compartmentalization of molecules in the axons has been shown controlled both by intrinsic and extrinsic mechanisms. For example, while AIS assembly

is an intrinsic process, the formation of nodes of Ranvier also requires extracellular glial-derived signals (Nelson and Jenkins, 2017).

Structural and functional compartmentalization also underlies major properties of the navigating axon. The growth cone possesses a highly complex machinery to direct different components at different places and to regulate their dynamics depending on the context and the decision to make. It is a structurally heterogeneous organ whose composition in cytoskeletal and cytoskeleton-interacting proteins varying from the front to the rear. This polarity has been demonstrated by multiple work to be crucial for growth cone adhesion to the substrate, motility and directional growth (Omotade et al., 2017). The cytoskeleton components of the growth cones, fibrillary actin (F-actin) and microtubules (MT) define three main compartments along the front-rear axis: the peripheral domain, the central domain and the transition domain. Bundled MT occupy the axon shaft and the growth cone central domain whereas F-actin composes the peripheral domain of the growth cone. The two domains are delineated by the Transition zone, containing contractile structures regulating both actin and microtubules. The central domain also differs from the peripheral one in that it is also enriched in cellular organelles such as exocytotic vesicles and mitochondria. Conversely, actin nucleating factors and regulators are distributed in the peripheral domain. For instance, Ena/VASP are actin-associated proteins directly involved in growth cone remodeling after guidance receptor activation (Drees and Gertler, 2008). These proteins concentrate in filopodia tips and in lamellipodia where they increase filament length regulating some aspects of actin dynamics such as the rate of G-actin incorporation and the protection of F-actin from polymerization-terminating capping proteins. The dynamics of the growth cone cytoskeleton is also polarized. F-actin polymerizes at the growth cone edge, and de-polymerizes at the basis of the lamellipodia. Such retrograde flow of F-actin is at the basis of growth cone advancement (Omotade et al., 2017).

Compartmentalized signaling in the growth cone.

On top of this front-rear compartmentalization, the growth cone establishes dynamic polarity to achieve guidance decisions. The turning behavior of a growth cone indeed results from the generation of asymmetric activities that reflect the gradient of external guidance cues. These

asymmetries are created through polarized trafficking and activation of signaling molecules and also through polarized dynamics of cytoskeleton elements (Itofusa and Kamiguchi, 2011). For example, asymmetric elevation of calcium across the growth cone intracellular space reflecting the presence of a guidance molecule on one side results in an attractive response. In contrast, calcium release from the endogenous endoplasmic reticulum located in the central domain, elicits a repulsive response. Growth cone turning away from an exogenous guidance cue is manifested by loss of F-actin whereas an attractive turning depends on F-actin polymerization at the growth cone site closest to the attractive signal (Omotade et al., 2017).

The growth cone accumulates multiple signaling molecules to transduce the information provided by the guidance cues into a directional change. Several cascades downstream of Slits and Semaphorins targeting actin and microtubule dynamics have been characterized, which interestingly, are framed by the structural polarity of the growth cone. I briefly illustrate here some representative examples.

Signaling to actin.

Robo activation was shown to result in recruitment of Ena/VASP, Ena interacting with MRL proteins to promote actin polymerization, membrane protrusion and adhesion and with DAAM1 proteins to promote filopodia formation (Matusek et al., 2008)(Quinn et al., 2006). The actin-binding proteins ERMs (Ezrin, radixin, Moesin) physically connect actin cytoskeleton with cell adhesion and guidance receptors at the plasma membrane. Their loss reduces the area of the growth cone, disorganizes F-actin and induces lamellipodia retraction. L1CAM forms a complex with Nrp1, and *in vitro*, it has been suggested that Sema treatment induces a temporary inactivation of ERMs resulting in collapse of the growth cone (Mintz et al., 2008). The tyrosine kinase Abelson (Abl) is another actin-binding protein which was shown to directly bind and phosphorylate Robo (Bashaw et al., 2000). Moreover, Abl also binds and phosphorylates other substrates, including actin regulators such as Ena/VASP, and GEFs/GAPs proteins regulating the activation and the inactivation of Rho family proteins. Abl and Ena play opposite roles in Robo signaling, Abl antagonizing Robo-mediated repulsion signaling via phosphorylation, whereas Ena mediating part of Robo repulsive response (Bashaw et al., 2000).

The sub-membranous cortical spectrin network, which inter-connects the plasma membrane proteins with F-actin allowing a rapid cytoskeletal remodeling is also implicated in the growth cone response to repulsive cues (Garbe et al., 2007). In addition, rapid actin-F remodeling wouldn't be possible without rapid regulations of Myosin II actin-associated protein *via* the direct binding to Rho GTPases. For instance, *in vitro* Sema3A treatment was shown to induce a rapid redistribution of Myosin II coincident with F-actin depolymerization. Mical redox enzymes are an additional class of actin-binding proteins that can exert a direct depolymerizing action over F-actin. MICALs were discovered as effectors downstream of Plexins (Terman et al., 2002). Finally, some actin-binding proteins also act over G-actin, sequestering the monomers and possibly limiting actin polymerization. This is for instance the case of CAP proteins, found to act downstream of Robos and forming complexes with other actin regulatory proteins such as Abl and Profilin (Wills et al., 1999). Overall these examples illustrate how guidance receptors generate signals within the actin domain.

Signaling to microtubules.

Microtubules (MTs) occupy the central domain of the growth cone, and its dynamics is the target of several repulsive signaling activated within the growth cone. As for actin, many MT binding proteins have been described to act downstream of guidance receptors, regulating various aspects of MTs dynamics. Some of them bind the MT fraction extending in the distal actin region of the growth cone, at the growing ends (+TIPs), as for example CLASP proteins which are involved in MT stabilization and rescue. Some studies have suggested that *Orbit/MAST* (the *Drosophila* homologue of CLASP) participate to midline expulsion, operating in Robo/Slit signaling through Abl protein to stabilize MTs at side of the growth cone exposed to the highest concentrations of Slit (Lee et al., 2004a). These results appear counterintuitive if we consider that Slit might cause collapse of the growth cone processes exposed to high doses. However, some MT-stabilizing proteins such as MAP1B protein also have been specifically detected on the same side of the growth cone facing the repulsive source (Mack et al., 2000). Insights could come from better characterization of the activity of MT-destabilizing proteins and how it polarizes in growth cones exposed to focal sources of repulsive cues.

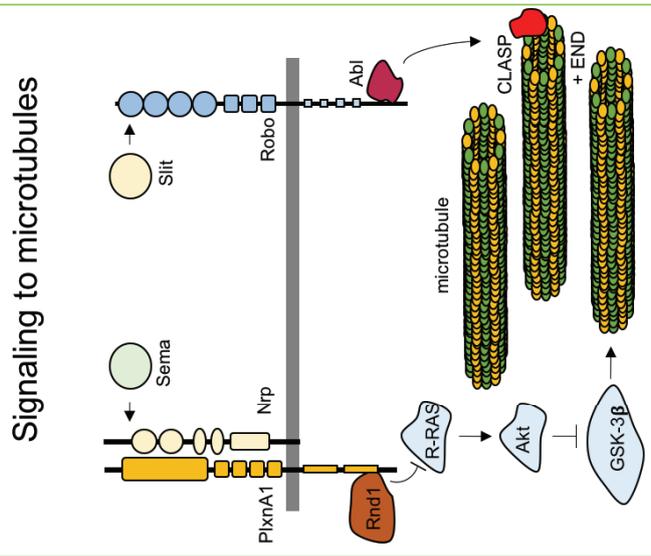
Rho family of small GTPases.

Finally, main targets of activated guidance receptors are Rho-family of small GTPases (Hall and Lalli, 2010). Signaling downstream of PlxnA1 involves Rho GTPase that, *via* Rho-associated kinase (ROCK)-mediated activity, leads to an increase in actin contractility (Gallo, 2006). Simultaneously, the binding of Rac GTPase to PlxnA1 may induce conformational changes increasing the endocytosis of the receptor that is required in Sema3A-induced growth cone collapse (Jurney et al., 2002). PlxnA1 downstream signaling has also a direct effect on adhesion. After Sema3A stimulation, PlxnA1 recruits the Rho GTPase Rnd1, this interaction resulting in the inactivation of proteins regulating integrin-mediated cell adhesion, such as R-Ras (Oinuma, 2004). Precise regulation of Rac GTPases is also necessary for proper Robo signaling and for the appropriate cytoskeleton rearrangements occurring during growth cone repulsive responses (Hu et al., 2005). The Slit/Robo signaling also regulates growth cone adhesion properties. For instance, activation of Robo leads to the formation of a complex constituted by Robo, Abl and N-cadherin and to the subsequent tyrosine phosphorylation of the beta-catenin, mediated by Abl or by other active kinases. This phosphorylation results in a reduction of affinity for cadherin and consequently in a loss of N-cadherin-actin connections (Rhee et al., 2002).

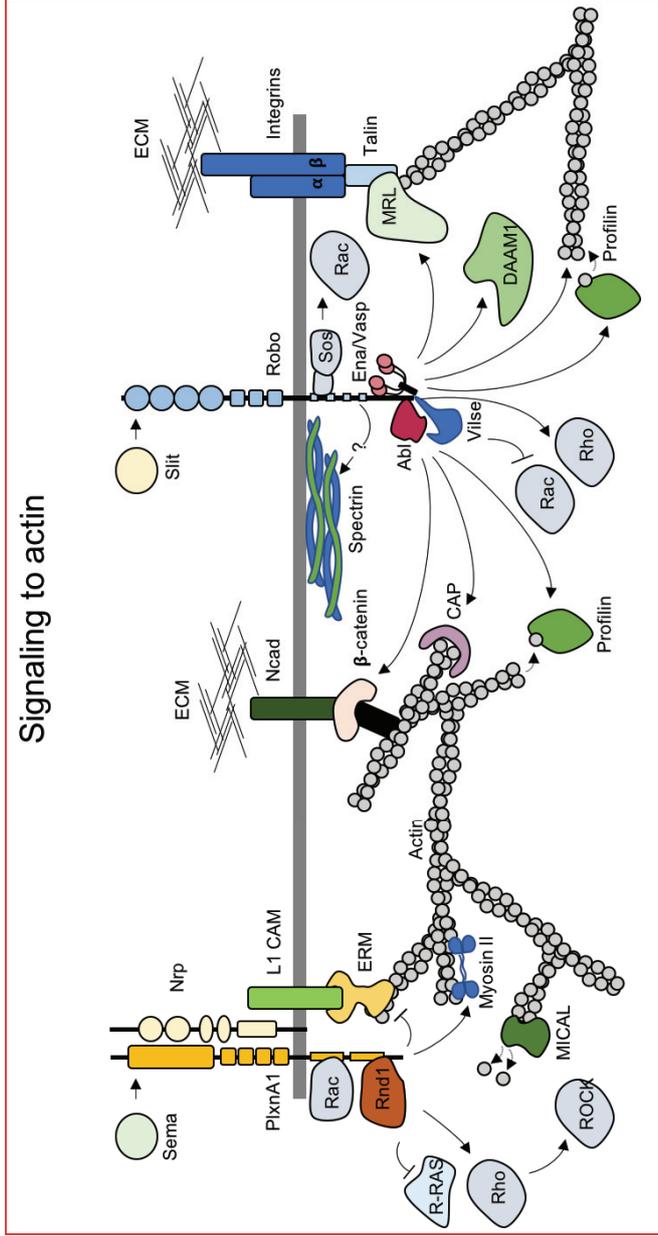
Compartmentalization of different Rho-family GTPase activities has been described occurring in migrating fibroblasts and neurons treated with epidermal and nerve growth factors (Kurokawa et al., 2005a). The development of FRET probes for Rho-family GTPases, allowed to measure the activity of these proteins in the protrusions formed by the aforesaid cell types. These experiments revealed a striking and differential spatial pattern of these proteins. In particular, in migrating cells stimulated with growth factors, Rac1 and Cdc42 activities were detected in a distal^{high} proximal^{low} gradient that was more expanded for Rac1 than for Cdc42 (Itoh et al., 2002)(Kurokawa et al., 2005b), whereas Rho1 activity was mostly detected at leading process tips and at the contractile tail (Kurokawa et al., 2005b). These activity patterns are consistent with reported involvement of Rac1 and Cdc42 in lamellipodia dynamics, and of RhoA at the tail of migrating cells in promoting actomyosin contractions and subsequent retraction of the cytoplasmic tail (Kurokawa et al., 2005b). In dorsal root ganglia neurons grown on laminin, the activities of small GTPases were shown to segregate within the growth

cone. RhoA and Cdc42 were detected exclusively at the periphery, with Cdc42 accumulating specifically in neurite tips, whereas Rac1 was detected mainly in the peripheral domain but also in the central one (Kurokawa et al., 2005b).

A summary of the compartmentalized signaling in the growth cone is presented in **Figure 13**.



Adhesion
Microtubule dynamics
Vesicle trafficking



Filopodia dynamics
Lamellipodia dynamics
Adhesion
Vesicle trafficking



Figure 13: Compartmentalized signaling in the growth cone. Summary of the molecular interactions and downstream cascade targeting actin and microtubules after PlxnA1 and Robo activation.

Receptor segregation in the axon shaft.

Compartmentalization of guidance receptors in the axon shaft is also a prominent feature of the navigating axon, particularly well described in the context of commissural projections, as illustrated in the chapter 1 of this introduction. Some receptors occupy the pre-crossing axon segment, others are restricted to the post-crossing one. The composition of pre-crossing and post-crossing receptors can differ between species. For example, L1CAM accumulates in the post-crossing axon segment in the mouse but in the pre-crossing one in the chick. Conversely, Tag-1 is in the pre-crossing segment in the mouse but in the post one in the chick.

How are built such molecular compartments? A study has pioneered this topic by reporting a cell intrinsic ability of *Drosophila* neurons to form, in culture, intra-neurites patterns of receptors in absence of any added extracellular cues (Katsuki et al., 2009). The authors showed that Robo2 and Robo3 receptors spontaneously localize in the distal segment of the axons. These experiments do not exclude the possibility that this patterning results from secretion of cues having autocrine mode of action. Contrarily to Robo2 and Robo3, Robo was found to distribute homogeneously in the axon, whereas it is patterned in the commissural axon *in vivo*. Thus, extrinsic information might lack in the culture condition, which instructs this patterning *in vivo*. The authors of this study also showed that the DRL receptor is specifically addressed to the proximal axon segment (Katsuki et al., 2009).

Mechanisms of molecular compartmentalization in the axon and the growth cone

Several mechanisms have been discovered which selectively address, remove or retain membrane proteins, thus generating heterogeneous composition in different axon domains.

Trafficking as a mechanism for generating asymmetric receptor distribution

Trafficking is ensured by an array of specialized vesicles that transport membrane molecules from their site of synthesis to their final destination, that redistribute them within the cell, ensure their turn-over and recycling (Tojima and Kamiguchi, 2015). This highly complex trafficking machinery can generate molecular compartments in different ways. First, after their maturation in the Golgi compartment, proteins can be directly delivered to different membrane domains of the neuron via specific membrane-targeting motifs (Mellman and Nelson, 2008). Second, membrane proteins can be selectively removed from the plasma membrane through endocytosis, for recycling or degradation. Many insights were brought from extensive studies of trafficking of L1CAM/NgCAM (chick) in the growth cone. L1 is first dephosphorylated and internalized from the plasma membrane in the central domain of the growth cone via clathrin-dependent pathway. Upon internalization, L1 is transported within sorting and recycling endosomes to the peripheral domain of the growth cone, a process which depends on dynamic ends of microtubules. Then, at the distal growth cone edge, L1 is inserted again within the plasma membrane and phosphorylated. From this site, L1 moves back to the central domain through coupling with retrograde movement of actin filaments *via* ankyrin and other linker molecules. Strong adhesion in the front and weak adhesion in the rear generates a dragging force to tract the growth cone forward. NrCAM is also coupled to the actin retrograde flow and display similar dynamics at the growth cone surface (Kamiguchi and Lemmon, 2000)(Falk et al., 2004). Endocytosis has also been shown important for the maintenance of compartmentalized distribution of DRL protein in *drosophila* neurons. DRL endocytosis is mediated by dynamin and reduction of dynamin activity was found to result in a homogeneous distribution of DRL protein within the axon. Contrarily, Robo3 is fully insensitive to this mechanism, suggesting that its segregation is achieved via another mechanism (Katsuki et al., 2009). Third, polarized distribution of proteins also arises from transcytosis, a process for which proteins are initially delivered to a compartment, then internalized and redirected towards another membrane domain. Such a mechanism of delivery-retrieval of proteins in the growth cone has been described for L1-CAM (Kamiguchi and Lemmon, 2000). It is initially sorted to the somatodendritic neuronal compartment, from which it is secondly internalized, and transported in vesicles for sorting in the axon (Tojima and Kamiguchi, 2015). Finally, but nevertheless very poorly documented yet in the context of axon guidance, proteins can also be uniformly delivered but selectively retained in some membrane domains (Winckler and Mellman, 2010). This might occur through interactions

with binding partners at the membrane, or extra-cellular matrix components and exogenous ligands, or still through modifications of proteins at the cell surface. As an example, L1 interaction *in trans* with Nrp1 at the membrane blocked the endocytosis of Nrp1 normally induced by Sema3A ligand (Castellani, 2002).

Microdomains of the membrane and diffusion barriers: additional level of protein partitioning

The plasma membrane composition in lipid-raft, enriched in specialized cholesterol/sphingolipid, and non-lipid-rafts microdomains provide a basis for segregating cell adhesion and guidance receptors and generating focal signaling in the growth cone (Kamiguchi, 2006). Lipid rafts containing several CAMs, such as the Ig Superfamily member NrCAM and N-cadherins, have been detected, *in vitro*, in the growth cone leading front. Actin-dependent retrograde movement of NrCAM was shown to depend on its link to the cytoskeleton at lipid raft sites (Falk et al., 2004). Several studies documenting partitioning of guidance receptors by lipid rafts. As an example, DCC was shown associated with lipid raft, this location appearing necessary for the growth cone response to Netrin (Herincs et al., 2005). Ephrins have also been found in lipid rafts. Experimental manipulations of these lipid platforms equally highlighted the importance that these micro-domains have in Sema3A-induced repulsion and collapse (Guirland et al., 2004)(Gauthier and Robbins, 2003). Upon ligand-receptor binding, several downstream GTPases as Rac1, Cdc42 and RhoA are recruited to lipid rafts, suggesting that these platforms are essential for the activation of the downstream effectors of these GTPases (Pozo et al., 2004). Therefore, lipid rafts can modulate specific adhesion and guidance responses within the growth cone, and provide a molecular frame for the generation of localized signals (Averaimo et al., 2016).

The properties of the plasma membrane also influence the dynamics of receptors. Barriers can be set at the plasma membrane, to restrict the diffusion of proteins. Such barrier forms in the axon initial segment (AIS) to segregate proteins from those of the somatodendritic domain (Winckler and Mellman, 2010)(Nakada et al., 2003). *In vitro* studies measuring the fluorescence recovery after FRAP experiments demonstrated that similar barrier mechanisms are active in other segments of the axon. For instance, in *drosophila* neurons, a boundary is set at the interface between intra-neurite compartments, in particular those segregating

Robo3 and DRL proteins (Katsuki et al., 2009). At this frontier, the mobility of several transmembrane proteins was found to be significantly reduced. By filtering the vesicles according to their size and the efficiency of their molecular motors, cytoplasmic barriers contribute as well to the segregation of membrane proteins in the axon. Such as barrier implicating actin and ankyrin proteins was also reported in the AIS (Song et al., 2009).

A summary of the mechanisms providing compartmentalization in axons and growth cones is presented in **Figure 14**.

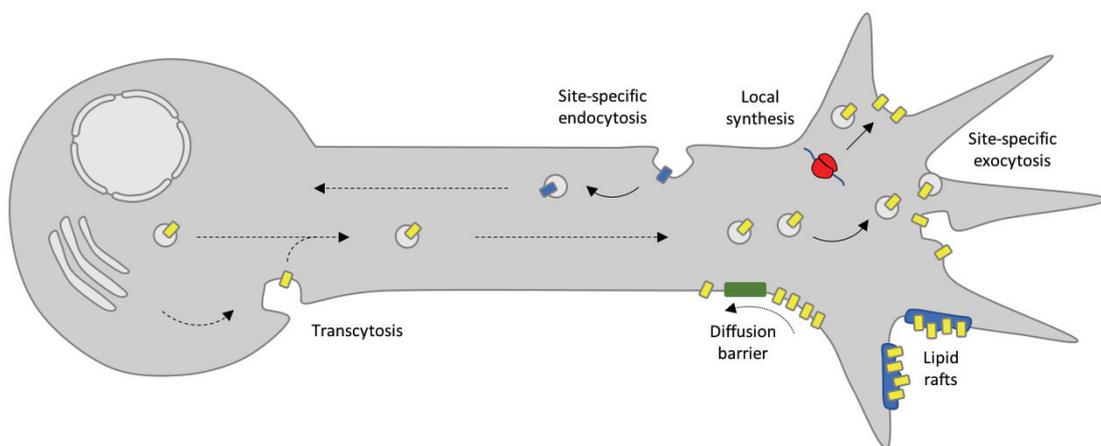


Figure 14: Mechanisms of molecular compartmentalization. A membrane molecule can be specifically retrieved from a compartment (site-specific endocytosis) or can be specifically targeted to a growth cone subdomain (site-specific-exocytosis). Molecules can also be first addressed to a first compartment and then, *via* transcytosis, be addressed elsewhere. Local synthesis can also participate in molecule compartmentalization. Plasma membrane can act as a boundary to limit or slow down the diffusion of molecules. Membrane molecules can also clusterize within lipid rafts.

THESIS PROJECT

Many studies have been carried out over the past years to investigate the mechanisms of commissural axon guidance in the spinal cord and have led to the identification of several guidepost territories expressing a panel of attractive and repulsive molecules. These molecules instruct and define, step by step, the pathway undertaken by the axons and also regulate their fasciculation state. Repulsive signals bring prominent and diverse contributions, propelling, channeling and confining the axons along their path. Several receptor-ligand couples have been described which mediate repulsive signaling responses in the growth cones, in correspondence with strategic guidepost sites. The roles exerted by these couples have been mostly analyzed using mouse genetics and experimental manipulations of the chick and zebrafish models as well.

In the developing mouse spinal cord, three main interactions were found essential for midline crossing: Robo1-2/Slits, Plxns/Nrps/Semas and PlxnA1/SlitC. When assessed using dedicated *in vitro* assays, each of these signaling induces similar responses of commissural growth cones that are collapse and turning away from the source of repulsive cue. However, analysis of mouse models supports some functional specificities of these signaling. Indeed, mice mutants for all three Slits display a severe phenotype characterized by axons stalling within the FP with some of them re-crossing the midline. Mice mutants for Robo receptors also display a severe commissural phenotype mainly represented by axon stalling within the FP. Robo1 mutant mice display much more aberrant axon behaviors within the FP than Robo2 ones. However, in none of them was observed the re-crossing phenotype of Slit1-3 mutants. In contrast, PlxnA1 loss results in re-crossing, and combinations of Slits and PlxnA1 allelic deletions support these genes function in a common pathway. Loss of PlxnA1 also induces crossing and post-crossing defects consisting mainly in stalling and in aberrant turning. These defects were found in Sema3B-null mutant mice. All of these results support that despite their common repulsive properties, these different signaling might play specific contributions during FP navigation. From these mouse model analyses, we can postulate that Robo1/SlitN (and possibly Robo1/Slit full length), signaling together with PlxnA1/Nrp2/Sema3B would mainly mediate axon expulsion from the FP whereas PlxnA1/SlitC signaling would prevent midline re-crossing.

How such functional specificities could be achieved? The expression of repulsive molecules and their receptors is assumed to be highly dynamic, varying all over axon navigation. Axons are attracted and repelled by the same guidance cues depending on the context, and they can fasciculate together or de-fasciculate at specific choice points, changing quickly their orientation and constantly adapting to new environments and new combinations of guidance cues.

Therefore, on one hand it is likely that some specificities arise from mechanisms that define how topographic cues are distributed and deposited in the tissues. On the other hand, at the growth cone level, among multiple possibilities of regulations, what accounts is the availability of the receptor at the growth cone surface, for binding the guidance cue and eliciting an intracellular signaling.

My thesis objectives were to address the possibility that, in commissural growth cones, the cell surface dynamics of the receptors for the different repulsive ligands present some differences that would enable the generation of specific functional outcomes. Such biological questions could not be addressed by loss-of-function or gain-of-function experiments. Rather they required the setting of experimental strategies allowing the monitoring, in real time, of receptor dynamics in axon navigating in their native environment. My work has been subdivided into first, the development of a set-up appropriate for achieving these objectives and the second the exploitation of this model for assessing differences of receptor dynamics.

RESULTS

Functionalization of midline repellents by spatiotemporal profiling of receptor dynamics during commissural axon navigation

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Abstract

Accurate perception of guidance cues is crucial for axonal pathfinding. During their initial navigation in the spinal cord, commissural axons are kept insensitive to midline repellents. Through yet unclear mechanisms acting during midline crossing in the floor plate, they switch on responsiveness to various repulsive signals, that establish a permanent midline barrier and propel the axons for exit. Whether these gains of response are coupled to occur in synchrony or rather are independently activated through signaling-specific programs is fully unknown. We set-up a paradigm for live imaging and super resolution analysis of guidance receptor dynamics during commissural growth cone navigation in chick and mouse embryos. We uncovered a remarkable program of delivery and allocation of receptors at the growth cone surface, generating receptor-specific spatial and temporal profiles. This reveals a mechanism whereby commissural growth cones can discriminate coincident repulsive signals that they functionalize at different time points of their navigation.

Introduction

In a broad range of biological contexts, cells are exposed to a complex array of environmental cues from which they receive specific instructions. This is well exemplified by the model of axon responses to guidance cues during the formation of neuronal circuits. Axons navigate highly diverse environments to reach their targets. Unique trajectories emerge from the perception by axon tips, the growth cones, of combinations of extracellular cues exposed in choice points along the paths. A typical case is provided by commissural neurons, which must project their axons across the midline to build with contralateral target cells circuits integrating left and right neuronal activities (Evans and Bashaw, 2010; Pignata *et al.*, 2016; Stoeckli, 2018). Midline crossing of commissural axons in the floor plate (FP) of the developing spinal cord has been extensively worked out to explore axon guidance mechanisms, especially those regulating growth cone sensitivity to guidance cues. Various repulsive forces provided by proteins of the Slit and Semaphorin families are needed to prevent midline re-crossing and expel the axons away towards their next step. They are also thought to control the lateral position relative to the FP that commissural axons take to navigate their rostrally-oriented longitudinal path after the crossing (Long *et al.*, 2004; Jaworski *et al.*, 2010). Semaphorin3B (Sema3B) acting via Neuropilin2 (Nrp2)-PlxnA1 receptor complex, N-terminal and C-terminal Slit fragments resulting from Slit processing acting respectively via Roundabout (Robo)1/2 and PlexinA1 (PlxnA1) receptors are guidance cues all found expressed in the FP and playing instructive roles during commissural axon navigation (Zou *et al.*, 2000; Long *et al.*, 2004; Jaworski *et al.*, 2010; Nawabi *et al.*, 2010; Delloye-Bourgeois *et al.*, 2015).

Manipulations of Semaphorin and Slit repulsive signaling in mouse and chicken embryo models brought the view that the sensitivity of commissural axons to midline repellents must be silenced in a first step, prior to the crossing, and switched on in a second step to allow repulsive forces to set a midline barrier and expel the growth cones away. Consistently, manipulations presumably inducing premature sensitization or preventing it resulted in failure of FP crossing, with axons arrested before or within the FP, turning back or longitudinally before reaching the contralateral side (Chen *et al.*, 2008; Nawabi *et al.*, 2010). Noteworthy, the FP navigation is not a synchronous process. For example, it extends over several days in the mouse embryo, from the first axon wave of earliest-born commissural neurons at E9.5 to the latest one at E12.5 (Wilson *et al.*, 2008; Pignata *et al.*, 2016). The repellents are expressed in the FP over the entire period of commissural navigation (Pignata *et al.*, 2016). Thus, independently from the ligand

expression profiles, the switch towards sensitivity upon crossing has to be set at individual growth cone level. Whether the sensitization of commissural axons to the various repellents they encounter in the FP occurs in synchrony, or rather presents signaling-specific features is unknown. Also, whether repulsive guidance receptors distribute homogeneously or present spatial specificities at the growth cone surface is undetermined. Insights are still scarce essentially due to the deficit of experimental paradigms giving access to molecular events in single living commissural axons navigating in their native context.

We postulated that functional engagement of midline repellents could arise from peculiar dynamics of guidance receptors at the surface of navigating growth cones. To address this hypothesis, we investigated the cell surface dynamics of four receptors mediating repulsion by midline cues, Nrp2, PlxnA1, Robo1 and Robo2, in chick and mouse embryo models. Surprisingly, we found striking differences between Robo1 and Robo2 temporal patterns, which excludes Robo2 as a mediator of Slit repulsion during FP crossing but places it as a major player of the lateral funiculus navigation. Our study also revealed exquisite specificities of PlxnA1 and Robo1 dynamics. Both receptors are not only sorted at different timing of FP navigation but also are distributed in distinct domains of the growth cones. This spatial and temporal compartmentalization is achieved at post-translational and post-intra-axonal trafficking levels, specifically at the step of membrane delivery in the growth cones. Analysis of the dynamics of PlxnA1-Robo1 chimeric receptors demonstrated that the intracellular domain of PlxnA1 but not that of Robo1, is sufficient for coding the receptor-specific temporal pattern. Finally, FRAP analysis in growth cones navigating the FP further confirmed dynamics specificities of these two receptors. Our study thus revealed remarkably unique spatial and temporal sequences of cell surface sorting of guidance receptors during the navigation of spinal commissural axons. This mechanism enables the growth cones to discriminate in time and space coincident guidance signals and provides a basis for these cues to exert non-redundant and concerted actions.

Results

Development of an experimental paradigm to visualize cell surface receptor dynamics in navigating commissural axons

We setup time lapse imaging to monitor the cell surface dynamics of Semaphorin and Slit receptors in commissural axons navigating the FP in native spinal cords of chicken embryos. Nrp2, PlxnA1, Robo1 and Robo2 receptors were fused to the pH-sensitive GFP, pHLuorin (pHLuo), whose fluorescence at neutral pH enables to report membrane protein pools and cloned in vectors with ires-mb-tomato (**Fig. 1a**) (Jacob *et al.*, 2005; Nawabi *et al.*, 2010; Delloye-Bourgeois *et al.*, 2014). The pH-dependency of receptor fluorescence was verified by *in vitro* cell-line transfections (**Supplementary Fig. 1a**) (Delloye-Bourgeois *et al.*, 2014). The vectors were then transferred to spinal cord commissural neurons using *in ovo* neural tube electroporation (**Fig. 1b**). Then, isolated spinal cords were dorsally opened and imaged over several hours for mapping the receptor cell surface sorting reported by pHLuo fluorescence. The FP entry and exit limits were delineated using DIC channel or based on the observation of some typical features of axon trajectory, such as the presence of wrinkles when axons enter the FP and the rostral turning when axons exit the FP (**Supplementary Fig. 1b**).

PlxnA1 and Robo1 are specifically and successively sorted to the growth cone surface during FP navigation

Using our setup, we analyzed individual growth cone trajectories from time-lapse sequences. We plotted the position of the growth cones that turned on the pHLuo fluorescence to build cartographies of receptor cell surface sorting positions along the navigation. First, we observed that Nrp2 is exposed at the commissural growth cone surface from the pre-crossing stage and remains over entire FP crossing (**Fig. 1c,f,h,i; Supplementary Movie 1,2**). In contrast, we found that the membrane sorting of both PlxnA1 and Robo1 specifically occurs during FP navigation. Interestingly, the timing of their sorting significantly differed. PlxnA1 was addressed to the surface when commissural growth cones navigate the first half of the FP, thus from the FP entry point to the midline (**Fig. 1d,g,h,i; Supplementary Movie 3,4**), while Robo1 was sorted during the navigation of the second FP half, from the midline to the FP exit point (**Fig. 1e,g,h,i; Supplementary Movie 5,6**).

Figure 1

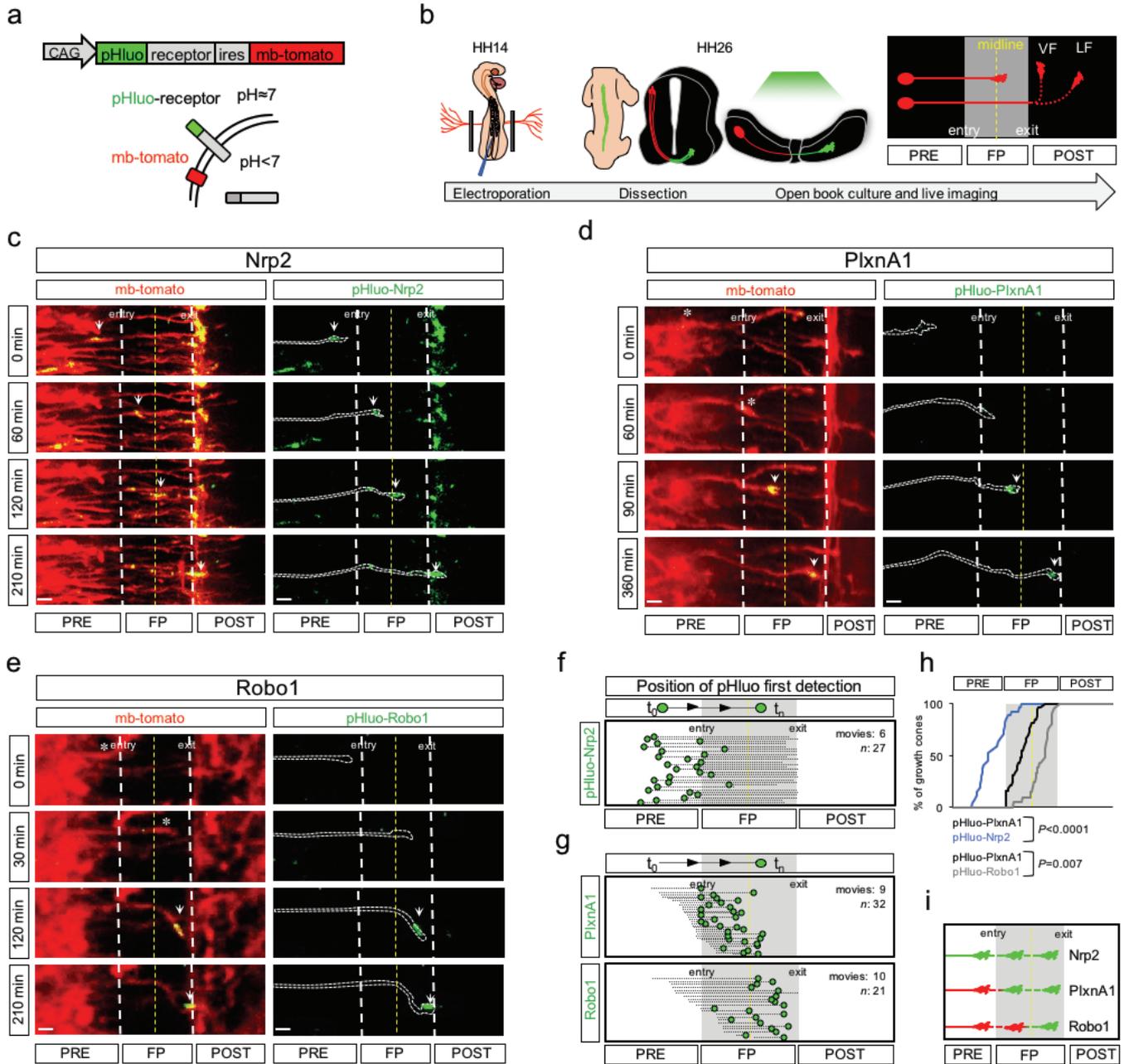


Fig. 1: PlxnA1 and Robo1 are successively sorted to the growth cone surface during FP navigation

(a) Schematic drawings of pHLuo vector and pH-dependent pHLuo fluorescence activity in cells reporting cell surface protein pool. pHLuorin-receptor and mb-tomato coding sequences were cloned in pCAG vector, spaced by an ires. (b) *In ovo* electroporation procedure. 48h after electroporation, spinal cords were dissected and mounted as open books for time lapse microscopy. pHLuo fluorescence was monitored in 3 compartments of the open-books: the pre-crossing, where the cell bodies of commissural neurons are located, the FP, in which commissural axons cross the midline, and the post-crossing, in which commissural axons chose between ventral and lateral longitudinal trajectories. VF: ventral funiculus, LF: lateral funiculus. (c-e) Time-lapse sequences illustrating the spatial and temporal dynamics of pHLuo-Nrp2, pHLuo-PlxnA1 and pHLuo-Robo1 during FP navigation. The asterisks point growth cone positions before pHLuo flashes and the white arrowheads those of pHLuo flashes and subsequent growth cone positions. (f) Cartography of pHLuo-Nrp2 dynamics from movie analysis. Dashed lines indicate the overall trajectory of single growth cones and green spots the first pHLuo detection. Nrp2 is exposed at the growth cone surface since the onset of spinal cord navigation (Nrp2: N=5 embryos, 6 movies, 27 growth cones). (g) Cartography of pHLuo-PlxnA1 and pHLuo-Robo1 dynamics, plotting position of pHLuo flashes. The upper panel illustrates pHLuo-PlxnA1 sorting in the first FP half, the lower panel that of pHLuo-Robo1 in the second FP half (PlxnA1: N=5 embryos, 9 movies, 32 growth cones; Robo1: N= 9 embryos, 10 movies, 21 growth cones). (h) Cumulative fractions showing differential pHLuo-Nrp2, pHLuo-PlxnA1 and pHLuo-Robo1 dynamics during FP navigation (*P* value is from the Kolmogorov-Smirnov (KS) test). (i) Summary of the temporal sequence of pHLuo-Nrp2, pHLuo-PlxnA1 and pHLuo-Robo1 membrane sorting during FP navigation. Scale bars in c-e, 10 μ m.

PlexinA1 and Robo1 temporal patterns are set by control of cell surface sorting

Next, we assessed whether these temporal patterns are profiled by control of receptor cell surface sorting or rather by control of protein availability within the axon. Spinal cord open books were fixed with paraformaldehyde (PFA) at neutral pH to detect the total pHLuo receptor pool. While in live axons pHLuo signal was found restricted to the growth cones during FP crossing, we observed in contrast for both receptors that the total pools had much broader distribution than the surface ones. In 90% of the cases for PlxnA1 and 75% for Robo1, the pre-crossing axon segment immediately adjacent to the FP entry of growth cones that were found to navigate within the FP, contained pHLuo-receptors (**Fig. 2a,b**). We also measured pHLuo⁺ pre-crossing segment length in the fixed samples and found significant difference between PlxnA1 and Robo1, the latter having more restricted length and punctate pattern than the first one (**Fig. 2c**). These observations are consistent with previous works that reported in cultured commissural neurons Robo1 intra-axonal vesicular trafficking (Philipp *et al.*, 2012) and PlxnA1 processed within axons to prevent its membrane expression (Nawabi *et al.*, 2010). Thus, PlxnA1 and Robo1 are available within commissural axons and their cell surface sorting is spatially and temporally controlled in a receptor-specific manner.

We found in previous work that medium conditioned by cultured isolated FP tissues (FP^{cm}) could trigger PlxnA1 cell surface expression (Nawabi *et al.*, 2010). Such medium was also reported to induce in commissural growth cones Robo3.2, the Robo3 isoform expressed in post-crossing axons (Colak *et al.*, 2013), providing the evidence that local signals emanating from the FP are implicated in synchronizing the sorting of these receptors with midline crossing. How is triggered the sorting of Robo1 is yet unknown. We thus examined whether it could also be under local FP control. We treated dorsal spinal cord explants electroporated with pHLuo-Robo1-ires-mb-tomato with FP^{cm} and ctrl medium and recorded Robo1 dynamics by measuring pHLuo fluorescence in the growth cones at T0 and T1, 20 min later (**Fig. 2d**). We observed a significant increase of pHLuo fluorescence at T1 compared to T0 for the FP^{cm} but not the control condition (**Fig. 2e,f**), thus indicating that FP cells release cues triggering Robo1 at the growth cone surface.

Next, we assessed whether disturbing the temporal pattern of receptor sorting impacts on growth cone behaviors. Open-books were electroporated with high concentration of vectors (3µg/µl and 4µg/µl) to overcome the internal control of PlxnA1 and Robo1 surface sorting in commissural neurons and create premature surface expressions. We monitored individual growth cones and found that commissural growth cones having premature cell surface receptor

exposure failed to cross the FP, rather turning or stalling before or within the FP (**Supplementary Fig. 2; Supplementary Movie 7,8**). These findings indicated that pHLuo-receptors are functional and confirmed that the temporal pattern of receptor sorting is critical for proper FP navigation.

Next, we asked whether the gain of PlxnA1 and Robo1 at the surface could be correlated with acquisition of novel behavioral properties of the navigating growth cones. To address this question, we analyzed in time-lapse movies growth cone trajectories at time-points preceding and succeeding the pHLuo flashes, by measuring the deviation angles of growth cone direction from the trajectory baseline (**Fig. 2g**). Interestingly for Robo1, we observed that acquisition of surface receptor was coincident with a significant increase of exploratory behavior along the rostro-caudal axis as if the growth cones were starting sensing cues that will direct their longitudinal turning at the FP exit. In contrast, we found no difference of exploration for PlxnA1 (**Fig. 2g,h,i**).

Figure 2

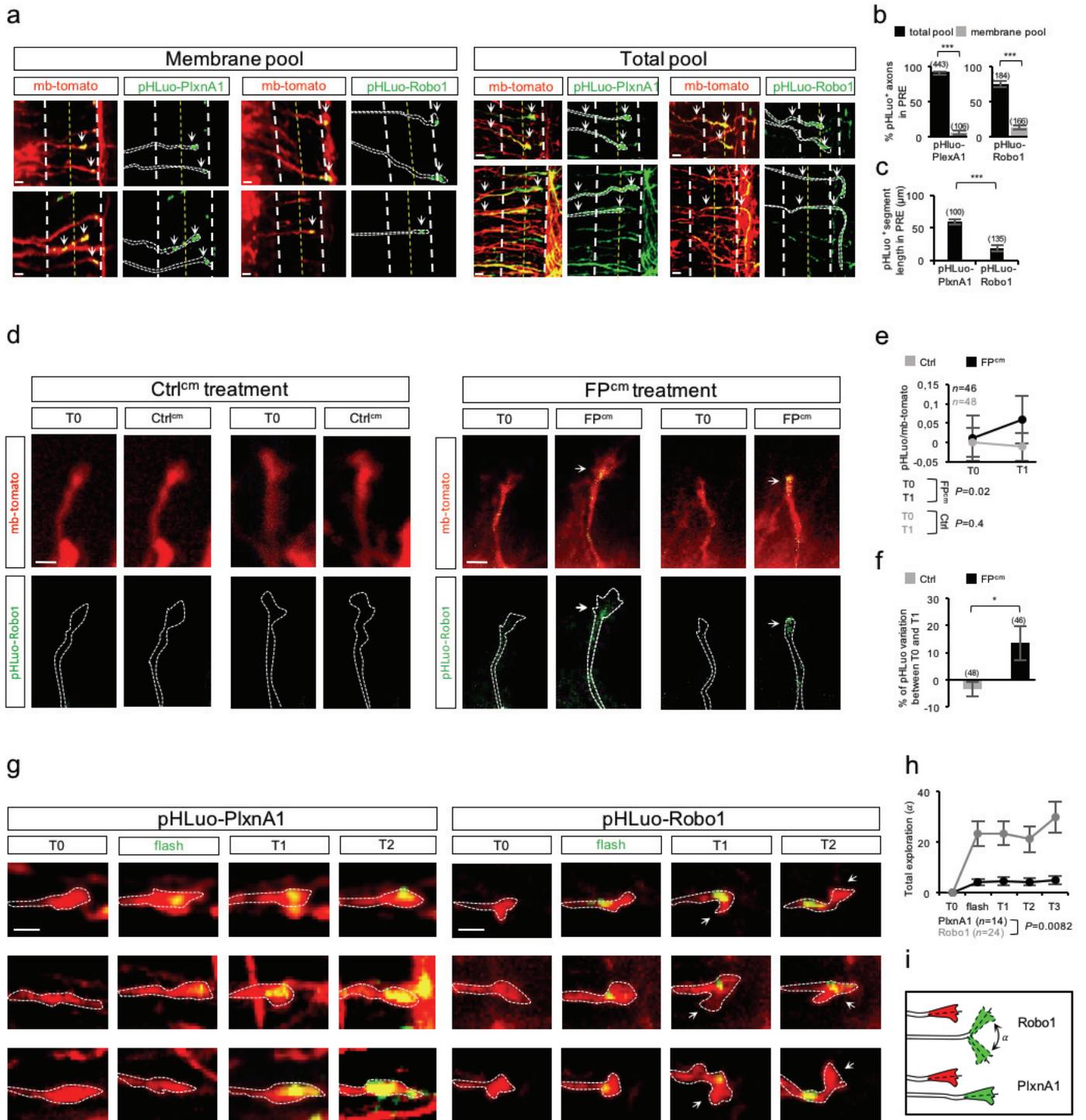


Fig. 2: Cell surface sorting of PlxnA1 and Robo1 is temporally controlled and coincident with changes of exploratory behavior for Robo1

(a) Microphotographs of open books illustrating pHLuo-PlxnA1 and pHLuo-Robo1 membrane pool (left panel) and total (intracellular + membrane) receptor pool (right panel). Arrowheads point discrete pHLuo⁺ growth cones and axon segments. (b) Quantification of the % of growth cone population observed to navigate within the FP and containing pHLuo-receptor in the pre-crossing segment immediately adjacent to the FP entry. Histograms show much broader total than surface fluorescence in this segment (total PlxnA1: N=4 embryos, 443 growth cones; membrane PlxnA1: N=5 embryos, 106 growth cones; total Robo1: N=4 embryos, 184 growth cones; membrane Robo1: N=17 embryos, 166 growth cones. Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *P* values are from Mann-Whitney test). (c) Histograms of measured lengths of pre-crossing segment containing total pHLuo-PlxnA1 or pHLuo-Robo1 in growth cones observed to navigate the FP, showing a more restricted pHLuo-Robo1 pattern, compared to that of pHLuo-PlxnA1. (d) Electroporated dorsal explant cultures treated with Ctrl^{cm} (left panel) or FP^{cm} (right panel) showing pHLuo-Robo1 increase at the growth cone membrane after FP^{cm} application. (e) Quantitative analysis showing the increase of pHLuo-Robo1 at the growth cone surface after 20 min (T1) of FP^{cm} treatment. For each growth cone, pHLuo is normalized to mb-tomato signal (3 independent experiments; Ctrl: N=19 explants, 48 growth cones; FP^{cm}: N=18 explants, 46 growth cones. Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *P* values are from paired Student t test). (f) Quantification of pHLuo-Robo1 signal variation between T0 and T1 in Ctrl^{cm} and FP^{cm} conditions, showing the increase of surface Robo1 after FP^{cm} application (Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *P* values are from unpaired Student t test). (g) Photomicrographs of pHLuo-PlxnA1 (left panel) and pHLuo-Robo1 (right panel) sorting at the growth cone membrane. Arrowheads in pHLuo-Robo1 condition point the exploratory behavior of growth cones after pHLuo sorting. (h) Quantitative analysis of the total angle explored by growth cones from the time point just preceding the flash (T0) to 1,5 hours after the flash (T3) (PlxnA1: N= 3 embryos, 32 growth cones; Robo1: N=10 embryos, 21 growth cones. Error bars indicate mean \pm SEM. *P* value is from the Kolmogorov-Smirnov (KS) test). (i) Graphic summary of the exploratory behavior of the growth cone following pHLuo-Robo1 surface sorting. Scale bars in a and d, 10 μ m; scale bars in g, 5 μ m.

Robo2 is sorted not in the FP but in the post-crossing lateral funiculus

Next, we investigated the dynamics of Robo2. In sharp contrast with Robo1, we found that Robo2 was absent from the surface of commissural growth cones navigating the FP and turning longitudinally at medial position in the ventral funiculus (VF). Instead, we observed that it was specifically sorted in post-crossing axons that chose to navigate in the lateral funiculus and turned longitudinally to the FP (LF) (**Fig. 3a,b,c; Supplementary Movie 9,10**). To assess if Robo2 cell surface sorting correlates with this change of trajectory, we measured the angle formed by a vector aligned along the axon tip and the FP axis at the two time points framing Robo2 pHluo flash. Interestingly, we found that the angle was significantly more pronounced at post-flash time points compared with pre-flash ones, supporting that Robo2 sorting contributes to directional growth cone changes along the longitudinal axis (**Fig. 3d,e**). Thus interestingly, signaling by Robo1 and Robo2 appear to have similar outcome on growth cone behaviors, that they control at two different time points of commissural navigation.

Figure 3

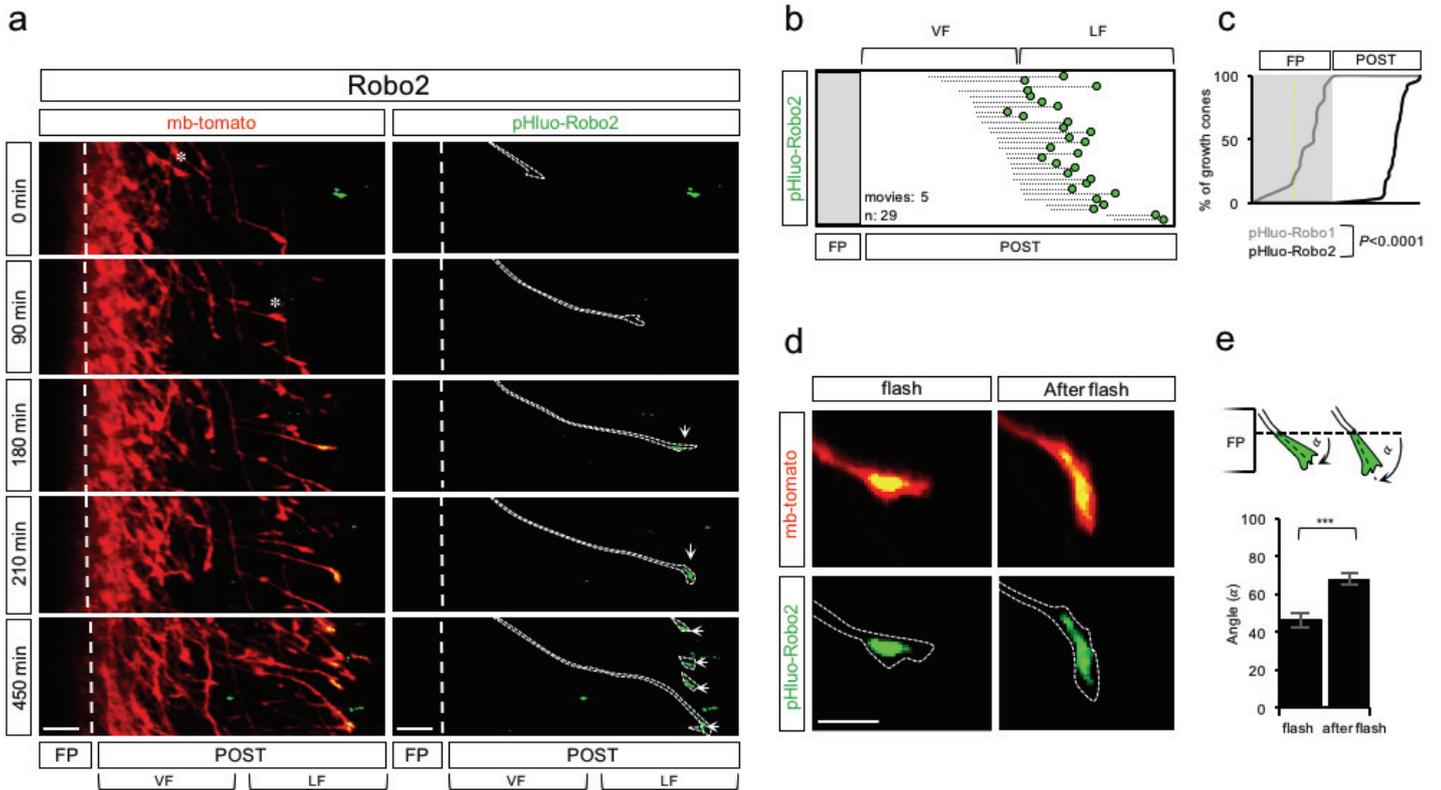


Fig. 3: Robo2 is not sorted in growth cones navigating the FP but during post-crossing pathfinding of the lateral funiculus

(a) Time-lapse sequences of open-books illustrating pHLuo-Robo2 spatio-temporal dynamics in commissural growth cones. The asterisks point growth cone positions before pHLuo flashes and the white arrowheads those of pHLuo flashes and subsequent growth cone positions. (b) Cartography of pHLuo-Robo2 flashes. Dashed lines indicate the overall trajectory of individual growth cones, from imaging onset to the time point of flash occurrence (Robo2: N=4 embryos, 5 movies, 29 growth cones). (c) Cumulative fractions showing differential pHLuo-Robo1 and pHLuo-Robo2 dynamics during spinal cord navigation (P value is from the Kolmogorov-Smirnov (KS) test). (d) Representative images from time-lapse sequence illustrating a shift of growth cone orientation subsequent to pHLuo-Robo2 flash. (e) Schematic drawing and quantification of growth cone turning after pHLuo-Robo2 flashes (Robo2: N=3 embryos, 30 growth cones. Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. P value is from Mann-Whitney test). Scale bars in **a**, 50 μm , in **d**, 10 μm .

The temporal sequence of receptor sorting is conserved in the mouse

Next, we studied whether the temporal control of receptor sorting during FP navigation uncovered in the chicken embryo is conserved in the mouse and whether it also instructs growth cone guidance choices. We electroporated pHLuo-PlxnA1 and pHLuo-Robo1 constructs in the developing spinal cord of E12 wild-type mouse embryos. We plotted the position of fluorescent growth cones in living open-books at fixed time point, 48 hours post-electroporation, when many FP crossing are ongoing, as depicted by the distribution of mb-tomato⁺ growth cones (**Fig. 4c,d**). In pHLuo-PlxnA1 electroporated spinal cords, growth cones exposing the pHLuo were distributed almost homogenously in all FP and post-crossing compartments (**Fig. 4a,b,c**) whereas in the pHLuo-Robo1 electroporated littermates, most of the growth cones exposing Robo1 were situated between the midline and FP exit (**Fig. 4a,d,e**). Therefore, the spatial and temporal cell surface pattern of PlxnA1 and Robo1 observed in chick spinal cord is conserved in mice (**Fig. 4e**).

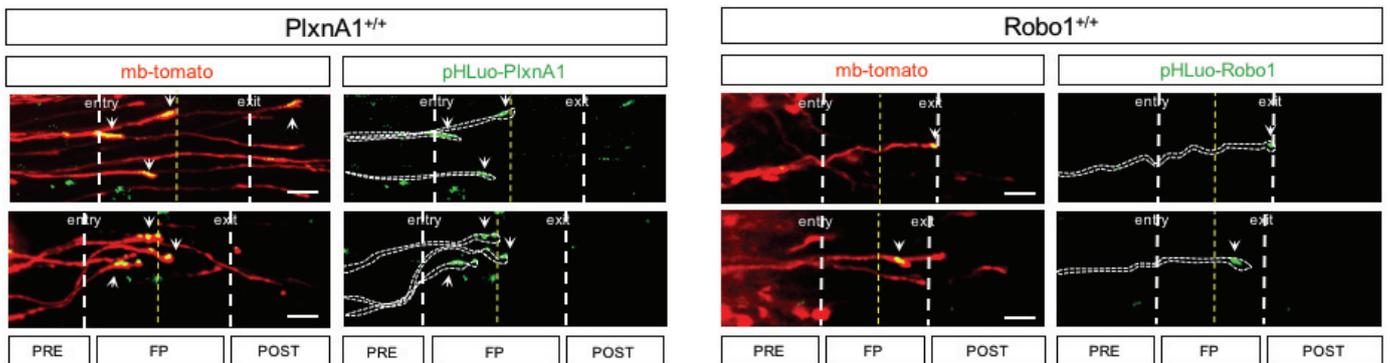
We then electroporated pHLuo-Robo1 construct in Robo1/2^{-/-} open books to determine whether reducing Robo1 dose by removal of the endogenous pool results in a modified Robo1 temporal pattern. We found that the profile of receptor sorting was identical to that observed after electroporation of Robo1/2^{+/+} embryos (**Fig. 4f,g,h**). Thus, our experimental conditions of expression are likely to model the dynamics of endogenous receptor. This result also established that Robo1 sorting at the plasma membrane is Robo2 independent.

Next, we investigated whether the re-expression of pHLuo-Robo1 in Robo1/2^{-/-} mice could rescue the previously reported stalling phenotypes resulting from Robo1/2 deletion (Long *et al.*, 2004; Delloye-Bourgeois *et al.*, 2015). We analyzed the distribution of mb-tomato⁺ growth cones over pre-to post-crossing steps, distinguishing growth cones that exposed Robo1 at their surface (mb-tomato⁺pHLuo⁺) from those that did not (mb-tomato⁺pHLuo⁻) in Robo1/2^{+/+} and Robo1/2^{-/-} embryos. We observed that Robo1/2 loss resulted in significantly shifted distribution of mb-tomato⁺pHLuo⁻ towards the first FP half (**Fig. 4i,j**). Interestingly, the expression of Robo1 at the growth cone surface was sufficient to rescue the distribution observed in the WT condition, as observed by the matching of the distribution of mb-tomato⁺pHLuo⁺ growth cones in Robo1/2^{-/-} and Robo1/2^{+/+} embryos (**Fig. 4k**). Thus, re-expression of Robo1 coding sequence in commissural neurons and subsequent cell surface exposure at a time when the growth cone navigates the second half of the FP is sufficient to rescue proper navigation. Moreover, and consistent with its observed temporal sorting profile, Robo2 is dispensable for FP crossing and

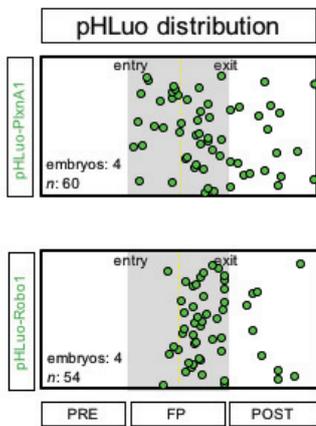
Robo1 surface exposure. Thus, this supports that this temporal profile properly reports the dynamics of endogenous Robo1 receptor.

Figure 4

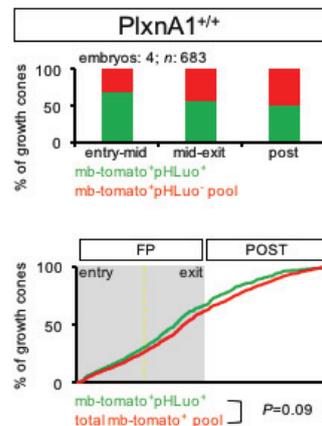
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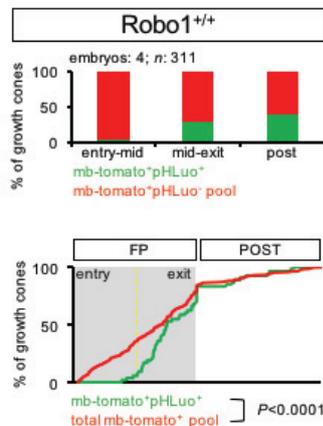
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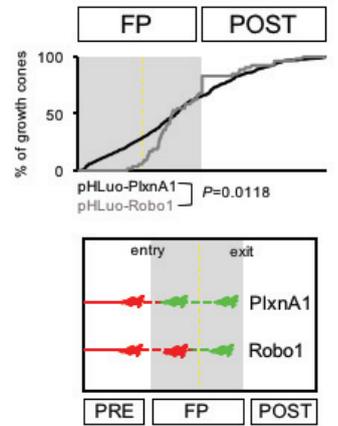
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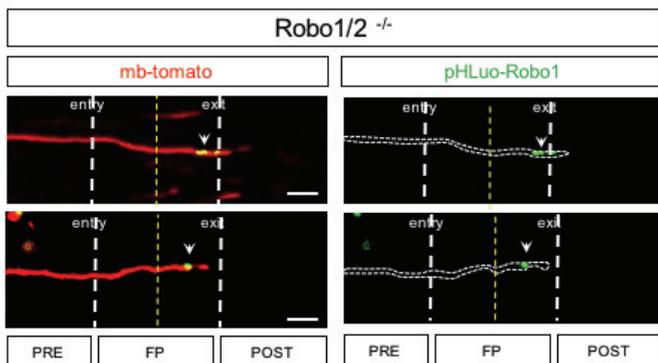
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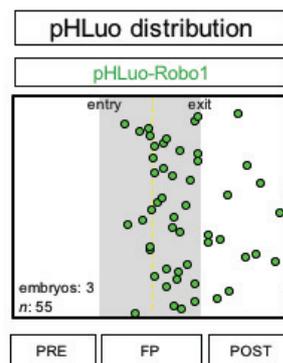
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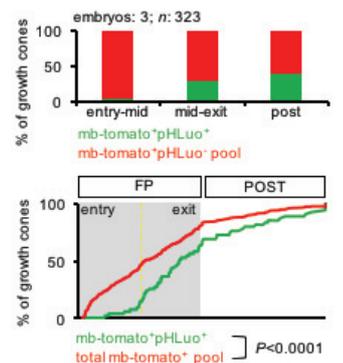
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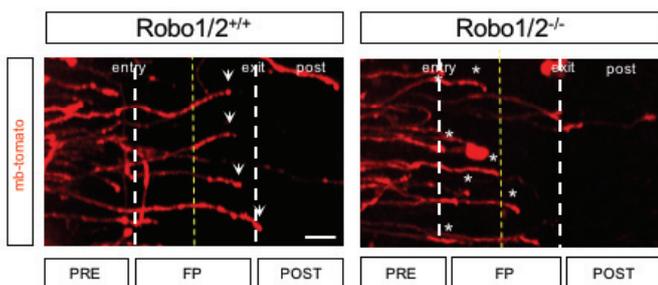
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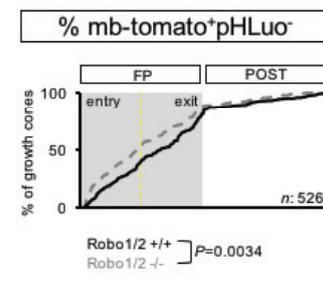
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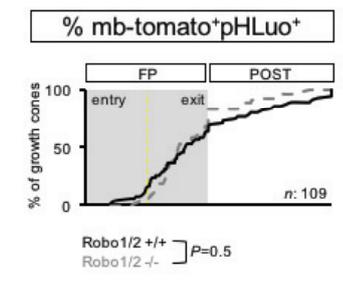


Fig. 4: chick PlxnA1 and Robo1 temporal sequences are conserved in the mouse

(a) Microphotographs of PlxnA1^{+/+} and Robo1^{+/+} open-books illustrating pHLuo-PlxnA1⁺ (left panel) and pHLuo-Robo1⁺ (right panel) growth cones pointed by white arrowheads. (b) Upper panel: cartography of pHLuo-PlxnA1⁺ growth cones (N=4 embryos, 60 growth cones); lower panel: cartography of pHLuo-Robo1⁺ growth cones (N=4 embryos, 54 growth cones). (c) Upper panel: distribution of mb-tomato⁺pHLuo⁺ and mb-tomato⁺pHLuo⁻ populations in PlxnA1^{+/+} open books. Lower panel: cumulative fraction of mb-tomato⁺pHLuo⁺ and total mb-tomato⁺ (composed of mb-tomato⁺pHLuo⁺ and mb-tomato⁺pHLuo⁻) populations showing that mb-tomato⁺pHLuo⁺ growth cones are detected from the onset of FP navigation (*P* value is from the Kolmogorov-Smirnov (KS) test). (d) Upper panel: distribution of mb-tomato⁺pHLuo⁺ and mb-tomato⁺pHLuo⁻ populations in the spinal cord of Robo1/2^{+/+} open books. Lower panel: cumulative fractions of mb-tomato⁺pHLuo⁺ and total mb-tomato⁺ (composed of mb-tomato⁺pHLuo⁺ and mb-tomato⁺pHLuo⁻) populations showing that whereas the total mb-tomato⁺ population distributes from the first FP half to the post-crossing compartment, mb-tomato⁺pHLuo⁺ growth cones are only detected from the second half of the FP (*P* value is from the Kolmogorov-Smirnov (KS) test). (e) Cumulative fraction of pHLuo-PlxnA1⁺ and pHLuo-Robo1⁺ growth cones, showing the differential timing of sorting of the receptors in the FP (*P* value is from the Kolmogorov-Smirnov (KS) test). (f) Microphotographs of Robo1/2^{-/-} open-books illustrating pHLuo-Robo1⁺ growth cones pointed by white arrowheads. (g) Cartography of pHLuo-Robo1⁺ growth cones (N=3 embryos, 55 growth cones). (h) Upper panel: distribution of mb-tomato⁺pHLuo⁺ and the mb-tomato⁺pHLuo⁻ populations in Robo1/2^{-/-} open books. Lower panel: cumulative fractions of mb-tomato⁺pHLuo⁺ and total mb-tomato⁺ (*P* value is from the Kolmogorov-Smirnov (KS) test). (i) Representative images of mb-tomato⁺ growth cones illustrating reduced number of growth cones (asterisks) on their way for FP exit in Robo1/2^{-/-} compared to Robo1/2^{+/+} open-books. (j) Cumulative fractions reporting the distribution of mb-tomato⁺pHLuo⁻ growth cones in Robo1/2^{+/+} and Robo1/2^{-/-} embryos, showing a significantly shifted distribution of tomato⁺pHLuo⁻ growth cones towards the first FP half in Robo1/2^{-/-} embryos. (k) Cumulative fractions reporting similar distribution of pHLuo-Robo1⁺ growth cones in open-books of Robo1^{+/+} and Robo1/2^{-/-} embryos (*P* value is from the Kolmogorov-Smirnov (KS) test). Scale bars in **a** and **f** 20 μm, in **i**, 50 μm.

PlexinA1 and Robo1 are partitioned at the growth cone surface

Next, we used super resolution microscopy to assess whether Robo1 and PlxnA1, on top of the different timing at which they are sorted during FP crossing, also differ in their spatial distribution at the growth cone surface. Living open-books were incubated with ATTO-647N-conjugated GFP nanobodies to label cell surface pHLuo. After fixation, receptor pools were imaged in commissural growth cones at different steps of their FP navigation, using STED microscopy (**Fig. 5a**). First, we measured the density of the fluorescent signal in individual growth cones. We found that PlxnA1 and Robo1 receptor clusters have differential cell surface distributions, PlxnA1 predominantly accumulating at the growth cone front, and Robo1 at the rear (**Fig. 5b,c,d**). This was also confirmed by determining the centers of mass of the signals, which segregated along the growth cone front-rear axis (**Fig. 5e**). Second, we studied whether the distribution patterns of PlxnA1 and Robo1 vary over FP navigation. According to their temporal sorting, we compared PlxnA1 distributions between FP entry and exit, and those of Robo1 between midline and exit. Analysis of the number and the size of labelled individual particles revealed modifications of Robo1 but not PlxnA1 patterns (**Fig. 5f,g**). Although not differing in their numbers, the size of pHLuo-Robo1⁺ particles increased from the midline to the exit, indicative of Robo1 diffusion at the surface (**Fig. 5g**).

Figure 5

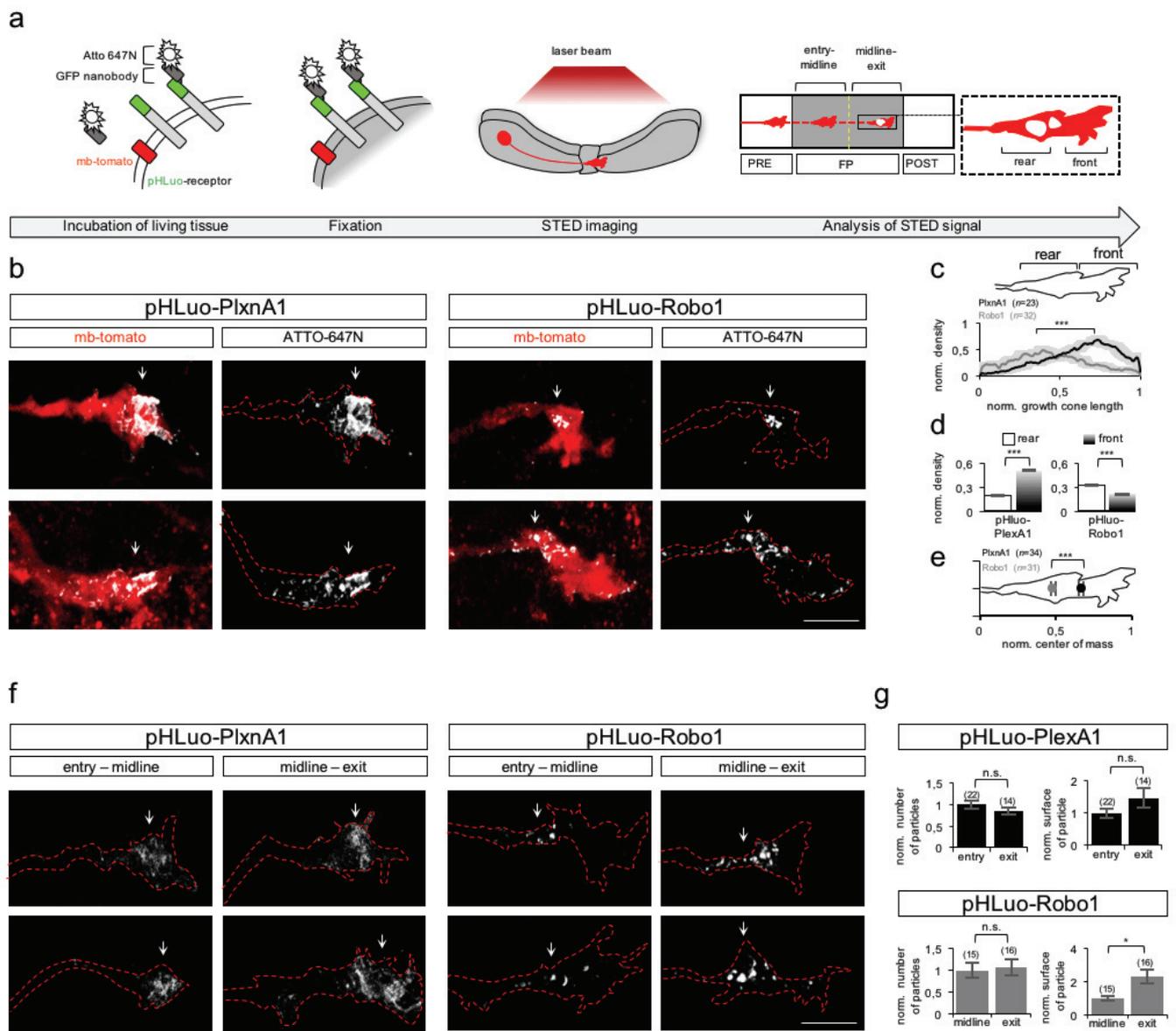


Fig. 5: PlxnA1 and Robo1 are partitioned at the cell surface of commissural growth cones navigating the FP.

(a) Super resolution imaging procedure. Open-books of embryos electroporated with pHLuo-vectors were live-labelled using ATTO-647N-conjugated GFP nanobodies and were fixed with PFA before STED imaging. Membrane pHLuo density and distribution were analyzed in the growth cones navigating the first (entry-midline) and the second (midline-exit) halves of the FP. The growth cone was segmented into front and rear sub-domains. (b) Microphotographs of representative commissural growth cones delineated with mb-tomato and labeled with ATTO-647N-conjugated GFP nanobodies. White arrowheads point ATTO-647N signal. (c) Densities of membrane pHLuo-PlxnA1 and pHLuo-Robo1 signals normalized to the growth cone length, showing their differential distribution along the growth cone rear-front axis (PlxnA1: N=8 embryos, 23 growth cones; Robo1: N=12 embryos, 32 growth cones. Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. P is from Kolmogorov-Smirnov (KS) test). (d) Histograms showing the comparison between normalized density of pHLuo signal in the front and the rear domain for both pHLuo-PlxnA1 and pHLuo-Robo1 (P value is from Mann-Whitney test). (e) Positions of pHLuo-PlxnA1 and -Robo1 center of mass normalized to growth cone lengths (N PlxnA1: 34 growth cones; N Robo1: 31 growth cones). (f) Microphotographs of mb-tomato⁺pHLuo⁺ commissural growth cones labeled with ATTO-647N-conjugated GFP nanobodies and navigating the entry-midline or the midline-exit compartments. (g) Upper panel: histograms showing normalized numbers and surfaces of pHLuo-PlxnA1 individual clusters detected in growth cones navigating the FP entry and exit. The number and the surface of pHLuo-PlxnA1 clusters were unchanged along FP navigation (PlxnA1: N=7 embryos, 22 growth cones in entry, 14 growth cones in exit (Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. P value is from Mann-Whitney test). Lower panel: histograms showing the normalized numbers and surfaces of pHLuo-Robo1 individual clusters detected in growth cones navigating the FP midline and exit. Note that the particle number remained unchanged whereas the particle surface increased between midline and exit (Robo1: N=5 embryos, 15 growth cones in midline, 16 cones in exit. Error bars indicate mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. P value is from Mann-Whitney test). Scale bars in **b,f**, 5 μm .

Discussion

During neural circuit wiring, focal and timed patterns of signaling are thought to be crucial for the specification of axonal trajectories but are yet highly challenging to visualize since this requires complex experimental paradigms preserving the topography of guidance molecules and their proper perception by the growth cones. Our live imaging and STED microscopy set-up thus paves the way to access the temporal and spatial resolution of signaling activities in neuronal growth cones facing guidance decisions in their physiological navigation environment. We report here that beyond their synthesis and trafficking to the axon, guidance receptors are exposed at the surface of the growth cones at precise timing and location. During commissural axon navigation, these receptor-specific cell surface codes can thus shape spatially and temporally distinct signaling from coincident midline cues, allowing their concerted action. Such a dynamic regulation of receptor equipment at the cell surface might be exploited in a variety of biological processes during which cells must adapt their perception of extracellular cues in a context-dependent manner. Particularly, accommodating fast and flexible perception of extracellular signals is indeed a prerequisite for cells which, as the axons do so, migrate along highly stereotyped and long-distance pathways, getting exposed to fluctuating combinations of guidance cues (Te Boekhorst *et al.*, 2016).

In the context of commissural axon navigation, our results bring new insights and directions for further investigation of the functional outcome resulting from the different receptor-mediated signaling. Our data report a temporal sequence of Nrp2, PlxnA1, Robo1 and Robo2 surface sorting, which equip commissural growth cones at successive steps of their navigation. PlxnA1/Nrp2-mediated Sema3B and PlxnA1-mediated SlitC activities can thus be switched on from the FP entry, while Robo1-mediated SlitN signaling starts after the midline, and Robo2-mediated signaling at a next post-crossing choice point. Our analysis from super resolution microscopy showing that Robo1 and PlxnA1 receptors have distinct distribution at the growth cone surface gives complementary information on the organization of the repulsive activities mediating FP crossing. It could be that Slits and Sema3B repellents have synergistic contributions to growth cone progression across the FP, receptor compartmentalization concentrating their signaling onto different sub-domains of the growth cone. Nevertheless, and also supported by the distinct temporal patterns of their receptors, the repellent activities

are likely uncoupled, regulating different steps of the growth cone navigation, such as midline crossing and FP exit, through distinct downstream signaling cascades. Whatever the case, the front-rear partitioning of PlxnA1 and Robo1 receptors appears interestingly to correlate with the organization of the growth cone cytoskeleton and could serve as a mechanism to focus the signaling onto these different cytoskeletal components. Indeed, growth cone responses to guidance cues rely on regulations of the dynamics of both microtubule and actin cytoskeleton, which physically mainly occupy the central and the filopodia-rich peripheral domains, respectively (Cammarata *et al.*, 2016; Kahn and Baas, 2016).

Finally, our findings that the membrane sorting of another receptor, Robo2, at the time when commissural axons operate another key choice of their navigation strengthen the view that temporally controlling the availability of the receptors at the growth cone surface is a general mechanism to accommodate the growth cones to novel guidance challenges. Despite evidence in several systems that Robo1 and Robo2 have distinct contributions (Kim *et al.*, 2011), which specific guidance decisions they control during commissural axon navigation is yet unclear. Both Robo1 and 2 receptors transduce Slit signals and are expressed by commissural neurons. Both have been reported expressed at low levels in pre-crossing/crossing commissural axons and enriched at the post-crossing stage (Long *et al.*, 2004).

In the mouse, Robo1 but not Robo2 deletion alters FP crossing. Nevertheless, double Robo deletion was reported to aggravate the impact of Robo1 invalidation (Jaworski *et al.*, 2010). In contrast, specifically in Robo2^{-/-} embryos, commissural axons failed to reach the lateral funiculus, consistent with reported Robo2 enrichment in post-crossing lateral axon segments (Long *et al.*, 2004). Dominant-negative based approach to abrogate Robo signaling in the chick embryo also resulted in alteration of the post-crossing lateral navigation (Reeber *et al.*, 2008). In the drosophila ventral cord, post-crossing commissural tracts segregate at distinct lateral positions from the midline, as a result of tract-specific combinations of Robo receptors conferring them different sensitivity to midline Slits (Neuhaus-Follini and Bashaw, 2015). As a parallel, combinations of Robo receptors setting distinct responses to FP Slits are thought to control the position of post-crossing tracts navigating within the ventral and lateral funiculi in the mouse (Long *et al.*, 2004; Jaworski *et al.*, 2010). Our study enlightens drastic differences

between Robo1 and Robo2 receptor sorting that could not be anticipated from their general expression patterns. In contrast to Robo1, Robo2 is sorted only in growth cones navigating the VF and accomplishing their longitudinal turning. Such temporally accurate Robo2 is unlikely involved in the perception of FP Slits. It rather suggests that the growth cone acquire perception of a guidance cue precisely at the VF/LF transition, which calls to question the exact mechanisms controlling medio-lateral position choices of post-crossing axons.

Overall, we uncovered at different choice points strong and specific regulations of membrane sorting for three major guidance receptors known to contribute to the pathfinding of multiple neuronal tracts (Jongbloets and Pasterkamp, 2014; Blockus and Chédotal, 2016). Thus, dynamic control of cell surface expression might have general and crucial contribution to axon navigation. Mechanisms controlling the cell surface addressing of PlxnA1 and Robo1 in vertebrates have been proposed, implicating regulated exocytosis and protein processing (Nawabi *et al.*, 2010; Charoy *et al.*, 2012; Philip *et al.*, 2012). Our findings add another piece of information, showing that Robo1 sorting depends on local signals released by the FP. The identity of these signals remains to be determined. Whether they are receptor-specific or similar to those that trigger PlxnA1 and Robo3.2 is yet unknown. This latter possibility would imply that the temporal differences of Robo1 and PlxnA1 are dictated by receptor-specific mechanisms of addressing at the plasma membrane. Further investigations of the mechanisms that set accurate temporal sequences of guidance receptor sorting will be the next important step towards understanding these key regulations of growth cone navigation.

Acknowledgments

We thank A. Chédotal for sharing Robo mouse model, E. Derrington for input on the manuscript, O. Raineteau for scientific discussions and the BioImaging Center (BIC) of Bordeaux for advices. This work was conducted within the frame of the Labex CORTEX and DEWECAN of Université de Lyon, within the program “Investissements d’Avenir” (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR). The study was supported by an ANR funding to VC and OT, and the Association Française contre les Myopathies (AFM) to VC.

Author contributions

conceptualization: VC; methodology: AP, HD, JF, LB, VC, OT; investigation: AP, HD, KK, MB, TG, ST; writing: AP, VC; writing editing: HD, JF; formal analysis: AP, HD, JF, VC.

Competing interests

Authors declare no competing interests.

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Supplementary Materials

Figure S1

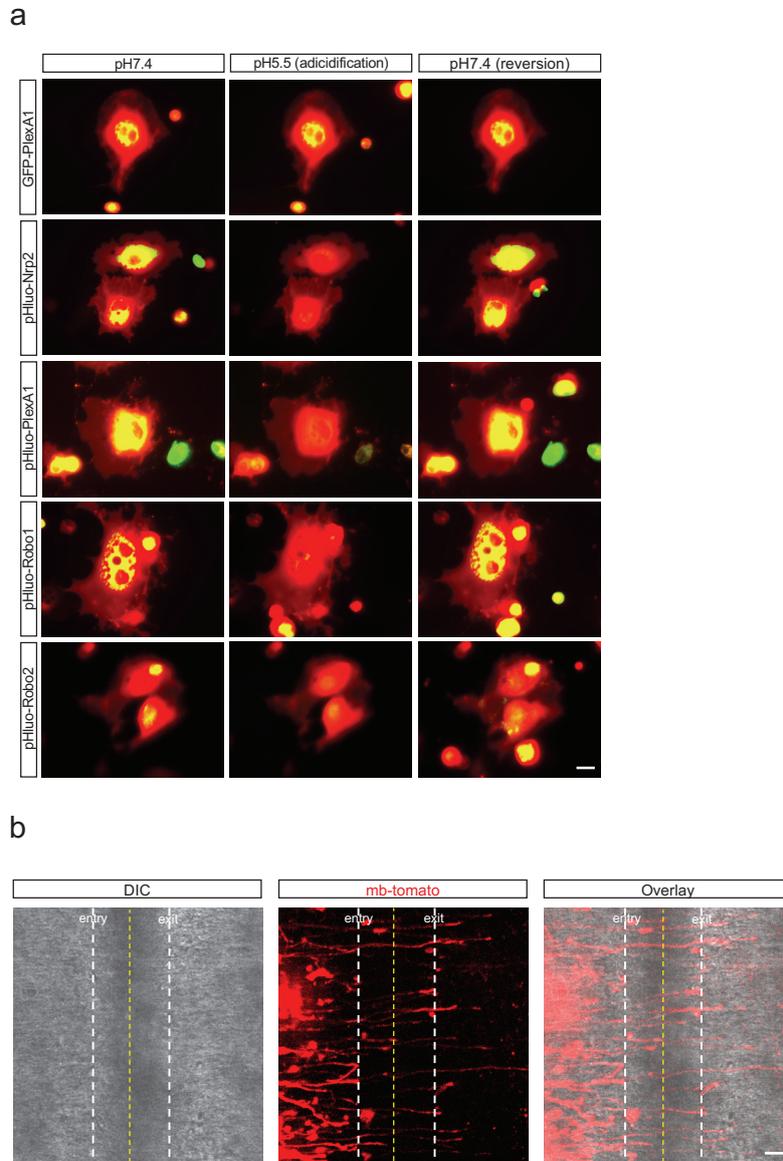


Figure S1: pH-dependency of receptor fluorescence and FP delimitation in open-books

(a) Cos7 cells transfected with pHLuo receptors and live monitored using confocal microscopy. pHLuo receptors fluoresce at the membrane when cells are cultivated in a pH7.4 culture medium. When the culture medium was acidified up to pH5.5, pHLuo lose its fluorescence. pH reversion to neutrality restored the cell surface fluorescence. These pH-dependent properties were not observed in control GFP-PlexA1 transfected COS cells. Scale bars, 5 μ m.

(b) Spinal cord morphology was revealed using DIC to delimit FP entry and exit. Scale bars in b, 50 μm .

Figure S2

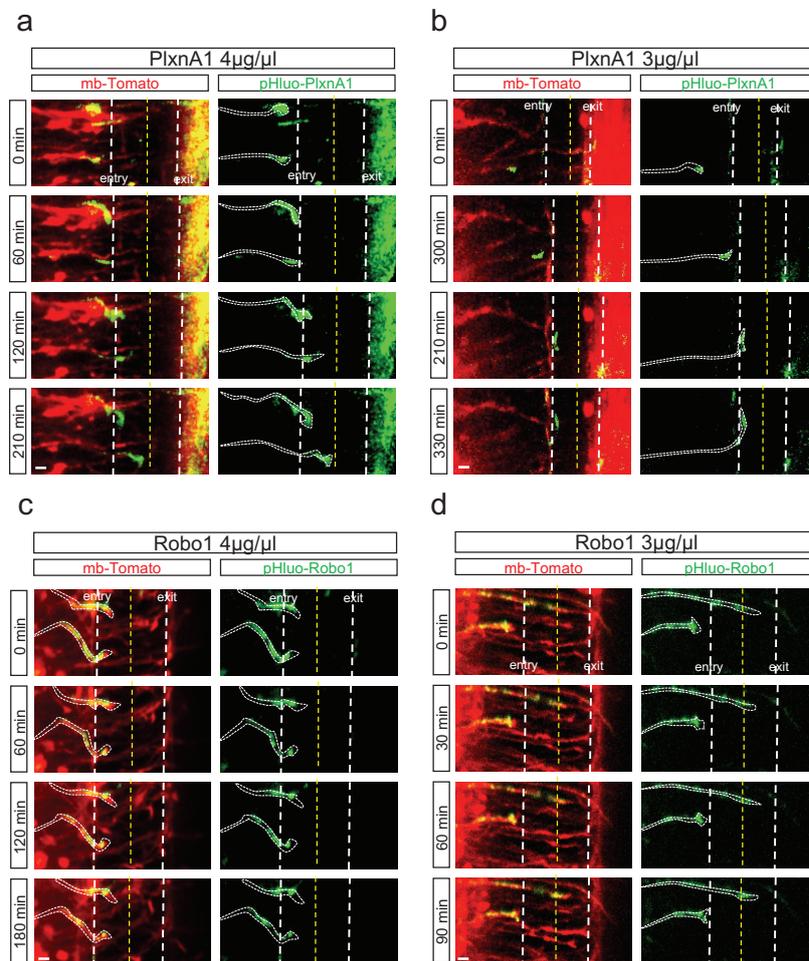


Figure S2: Over-loading commissural growth cones with receptors alters their FP navigation (a-d) Time-lapse sequences showing over-expression phenotypes induced by the electroporation of high doses of pHLuo-PlxnA1 or pHLuo-Robo1 plasmids (4 $\mu\text{g}/\mu\text{l}$ and 3 $\mu\text{g}/\mu\text{l}$). Harrow-heads point growth cones sorting prematurely the receptors, which stall or aberrantly turn at the FP entry. Scale bars in a-d, 10 μm .

Movies S1-S2: Dynamics of pHLuo-Nrp2

pHLuo-Nrp2 is exposed at the commissural growth cone surface from the pre-crossing stage and remains over entire FP crossing. White arrows point the growth cones during FP navigation. FP: floor plate.

Movies S3-S4: Dynamics of pHLuo-PlexA1

pHLuo-PlxnA1 is addressed to the surface when commissural growth cones navigate the first half of the FP, from the FP entry point to the midline. White arrows point the growth cones during FP navigation. FP: floor plate.

Movies S5-S6: Dynamics of pHLuo-Robo1

pHLuo-Robo1 is sorted to the cell surface when commissural growth cones navigate the second half of the FP, from the midline to the FP exit point. White arrows point the growth cones during FP navigation. FP: floor plate.

Movies S7: Over-loading commissural growth cones with pHLuo-PlexA1 alters their FP navigation.

Time-lapse sequences showing over-expression phenotypes induced by the electroporation of high doses of pHLuo-PlxnA1 plasmids (4 μ g/ μ l). White arrows point growth cones prematurely stalling at FP entry. FP: floor plate.

Movies S8: Over-loading commissural growth cones with pHLuo-Robo1 alters their FP navigation.

Time-lapse sequences showing over-expression phenotypes induced by the electroporation of high doses of pHLuo-Robo1 plasmids (4 μ g/ μ l). White arrows point commissural growth cones prematurely exposing pHLuo-Robo1 and stalling at the FP entry. FP: floor plate.

Movies S9-S10: Dynamics of pHLuo-Robo2

pHLuo-Robo2 is sorted to the cell surface in post-crossing growth cones that chose to navigate in the lateral funiculus, turning longitudinally to the FP (LF). White arrows point the growth cones during the navigation in post-crossing compartment. FP: floor plate; VF: ventral funiculus; LF: lateral funiculus.

Material and methods

Receptor molecular biology

FL mouse pHLuo-PlxnA1 was generated by introducing in Nter the coding sequence of the pHLuo cloned from a vector encoding GABA A pHLuo-GFP (Jacob et al., 2005). pHLuo derived from this vector was fused to FL rattus Robo1 and Robo2 sequences, kindly provided by A. Chedotal laboratory, to obtain pHLuo-Robo1 and pHLuo-Robo2 vectors. Using the same strategy, FL mouse Nrp2 kindly provided by Püschel laboratory, was fused to pHLuo to obtain pHLuo-Nrp2 vectors. pHLuo-receptors were finally cloned into a PCAGEN vectors with an ires-mb-tomato sequence.

***In vitro* test of pH fluorescence dependency**

Cos7 cells were plated in a glass-bottom dish 35mm in 2ml of complete Dulbecco's modified eagle medium (DMEM – 10% fetal bovine serum – 1 mM sodium pyruvate – 25 U/ml penicillin/streptomycin – 2,5 µg/ml Amphotericin B – pH7.4). 24h after, cells were transfected with 2 µg of plasmid encoding pHLuorin-tagged receptors and the transfection reagent was added. 48h later, live imaging of the cells was performed at 40X magnification. Cells were first imaged at pH 7.4 then, 1,25ml of pH3.5 complete DMEM was added to achieve a pH of 5.5 in the culture medium. Next, 1.2 of pH complete DMEM was injected to revert the pH of the medium to neutrality. Images were taken every 20 seconds for 10 minutes.

***In ovo* electroporation**

In ovo electroporation of HH14/HH15 chick embryos was performed as described previously (Delloye-Bourgeois *et al.*, 2014). Plasmids were diluted at the following concentration: 1.5 µg/µl pHLuo-Robo1-ires-tomato; 2.5 µg/µl pHLuo-Robo2-ires-tomato; 1 µg/µl pHLuo-Nrp2-ires-tomato; 2 µg/µl pHLuo-PlexA1 and 0.3 µg/µl mb-tomato. Plasmids were diluted in UP H₂O and the solution was injected into the lumen of the neural tube using picopritzer III (Micro Control Instrument Ltd., UK). Electrodes (CUY611P7-4, Sonidell) were placed along the back of the embryo, at the thoracic level, and 3 pulses (25V, 500ms interpulse) were delivered by CUY-21 generator (Sonidell). Electroporated embryos were then incubated at 38.5°C.

Open book culture

48 hours after electroporation, embryos at HH25/HH26 were harvested in cold HBSS and the spinal cords were dissected. Spinal cords were mounted in 0.5% agarose diluted in F12 medium and placed on glass bottom dishes (P35G-1.5-14-C, MatTek). After agarose solidification, spinal cords were overlaid with 3ml of F12 medium supplemented with 10% FCS (F7524; Sigma-Aldrich), 1% Penicillin/Streptomycin (Sigma-Aldrich) and 20mM HEPES buffer (15630-049, ThermoFischer Scientific).

Mouse spinal cord electroporation and culture

E12 mice embryos were collected and fixed on a SYLGARD (Dow Corning) culture plate in Leibovitzs 15 medium (ThermoFisher) supplemented with Glucose 1M (Sigma-Aldrich). Plasmids were injected into the lumen of the neural tube using picopritzer III (Micro Control Instrument Ltd., UK). Electrodes (CUY611P7-4, Sonidel) were placed along the back of the embryo, at the thoracic level, and 3 pulses (25V, 500ms interpulse) were delivered by CUY-21 generator (Sonidell). Spinal cords were dissected from the embryos and cultured on Nucleopore Track-Etch membrane (Whatman) for 48 hours in Slice Culture Medium (*Polleux and Ghosh., 2002*).

Live imaging and data analysis

Live imaging was performed with an Olympus IX81 microscope equipped with a spinning disk (CSU-X1 5000 rpm, Yokogawa) and Okolab environmental chamber maintained at 37°C. Image were acquired with a 20X objective by EMCCD camera (iXon3 DU-885, Andor technology). Usually for spinal cord culture, 15-30 planes spaced of 0,5-3µm were imaged for each spinal cord at 30-minute interval for 10 hours approximatively. To reduce exposure time and laser intensity, acquisitions were done using binning 2x2. Images were acquired using IQ3 software using multi-position and Z stack protocols. Z stack projections of the movies were analyzed in ImageJ software. The analysis of pHluo-flashes was performed from time-lapse acquisitions *in vivo*. For cartography representation, the lengths of PRE-crossing and POST-crossing compartment were normalized on FP length.

Detection of the total pool of pHluorin

48h after electroporation, at HH25/HH26, the embryos were harvested in cold HBSS and the spinal cords were dissected and fixed for 2 hours with PBS supplemented with 4%

paraformaldehyde (PFA). The % of the commissural population expressing total pool of pHLuo in PRE-crossing was calculated by qualitative analysis of Z stack projections. The length of the PRE-crossing segments expressing total pHLuo was measured with Image J software.

Explant cultures

FPs were isolated from HH25/HH26 chick embryos and cultured in tridimensional plasma clots in B27-supplemented Neurobasal medium (GIBCO). The supernatant (FP^{cm}) was collected after 48h. Electroporated spinal cord were dissected, cut into explants and left retrieve for 30min at 37°C. Then explants were placed on glass bottom dishes, previously coated with 10µg/ml Laminin and 50µg/ml polylysine and cultured for approximately 30h at 37°C in F12 medium supplemented with 0.4 Methylcellulose, 1X B27, 100ng/ml Netrin, 1/1000 Penystreptomycin. Explants were imaged at T0, then FP^{cm} or Ctrl medium were used for the treatment and 20min after a second time point was recorded.

Atto647N staining

Spinal cords were incubated at 38°C for 20 minutes with F12 medium supplemented with 5% FCS (F7524; Sigma-Aldrich), 20mM HEPES buffer (15630-049, ThermoFischer Scientific) and 1/100 GFP-nanobodies Atto647N. Spinal cords were then rinsed 4 times with the same medium (not containing the GFP-nanobodies) and were fixed at room temperature for 2 hours with PBS supplemented with 4% paraformaldehyde (PFA) and 1% BSA (A7638 Sigma-Aldrich).

STED imaging and data analysis

The staining was observed with a STED microscope (TCS SP8, Leica). STED illumination of ATTO 647N was performed using a 633-nm pulsed laser providing excitation, and a pulsed bi-photon laser (Mai Tai; Spectra-Physics) turned to 765 nm and going through a 100-m optical fiber to enlarge pulse width (100ps) used for depletion. A doughnut-shaped laser beam was achieved through two lambda plates. Fluorescence light between 650 and 740 nm was collected using a photomultiplier, using a HCX PL-APO CS 100/1.40 NA oil objective and a pinhole open to one time the Airy disk (60mm). Images were acquired with using Leica microsystem software and a Z stack protocol. Usually 10-20 planes spaced of 0,5µm where imaged for each growth cone. The growth cone perimeter was outlined basing on the mb-tomato signal. Average density of pHLuo-receptors in the growth cones was calculated from Z stack projections with Matlab

software. Particle numbers and surfaces were calculated from Z stack projections with image J.

Statistics

All embryos which normally developed and expressing pHLuo-vectors at the thoracic level were included in the analysis. Sample size and statistical significances are represented in each figure

and figure legend. For each set of data, normality was tested and Student t or Mann-Whitney tests were performed when the distribution was normal or not, respectively. Statistical tests were performed using Biosta-TGV (CNRS) and Prism 6 software.

SUPPLEMENTARY RESULTS

SUPPLEMENTARY RESULTS 1

The spatio-temporal pattern of PlxnA1 but not Robo1 is encoded by the intracellular domain of the receptor

We hypothesized that upon exposure of commissural growth cones to FP cues, the cell surface sorting of PlxnA1 and Robo1 at different timing of the FP navigation could result from structural differences of the receptors.

To address this possibility, we generated vectors encoding for chimeric receptors in which the extracellular (ECD) and intracellular domains (ICD) were swapped (**Fig. 1a**). We then examined whether swapping PlxnA1 and Robo1 intra- and extra-cellular domains results in a reversed or altered timing of receptor sorting. pHLuo-PlxnA1^{ECD}/Robo1^{ICD} and pHLuo-Robo1^{ECD}/PlxnA1^{ICD} vectors were electroporated in the neural tube of chicken embryos, for time-lapse imaging of pHLuo dynamics in spinal cord open books (**Fig.1 b,c**). In the pHLuo-PlxnA1^{ECD}/Robo1^{ICD} condition, we detected pHLuo flashes in commissural growth cones at the FP entry, as previously observed with the PlxnA1 native receptor (**Fig. 1b upper panels and Fig.1d left panel**). Nevertheless, we also found cases with growth cones that sorted the chimeric receptor prior to the FP entry, and maintained it over the entire period of FP navigation. Such a pattern was observed neither for pHLuo-PlxA1 nor for pHLuo-Robo1 receptors (**Fig. 1b lower panels**). In the pHLuo-Robo1^{ECD}/PlxnA1^{ICD} condition, the chimeric receptor was sorted at the FP entry, as observed with the PlxnA1 native receptor (**Fig. 1c upper and lower panels and Fig. 1d right panel**).

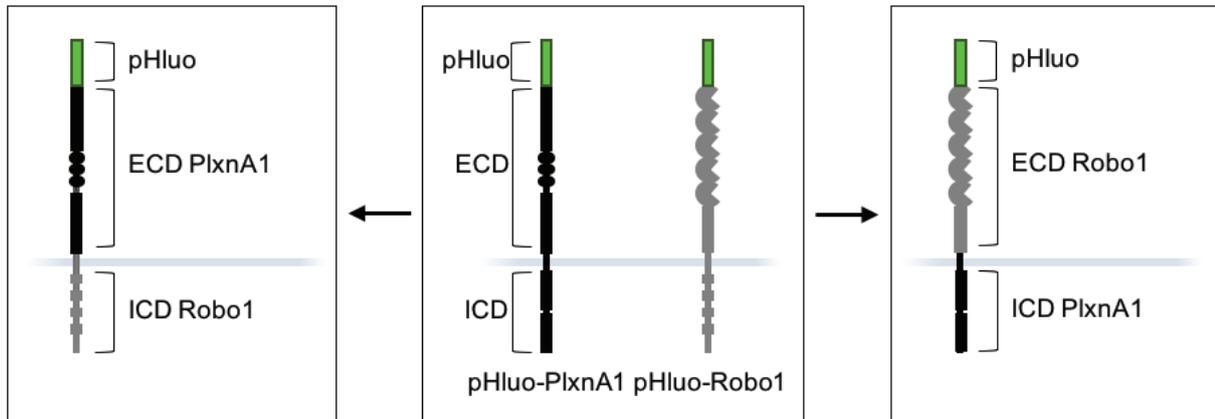
These experiments are currently repeated to increase the number of analyzed growth cones. They suggest that the Robo1 ICD is not sufficient for conferring to the receptor a proper timing of cell surface sorting. In contrast, the PlxnA1 ICD seems sufficient to encode the temporal pattern of receptor sorting. Possibly, the timing of Robo1 sorting in the second step of the FP navigation reflects the setting of a functional machinery for delivering the receptor to the cell membrane. This would be consistent with a previous report showing that Robo1 is trafficked in vesicles and exocytosed via upregulation of RABGDI. By conditioning medium with FP

cultures, we found that FP cues trigger Robo1 cell surface sorting. It is thus possible that these signals target not the receptor itself but Robo1 sorting machinery. In contrast for PlxnA1, our findings support that the ICD of PlxnA1 is sufficient to encode its timing of sorting. This is fully consistent with previous report of our laboratory showing that PlxnA1 sorting is achieved by suppression of receptor processing, triggered by FP signals.

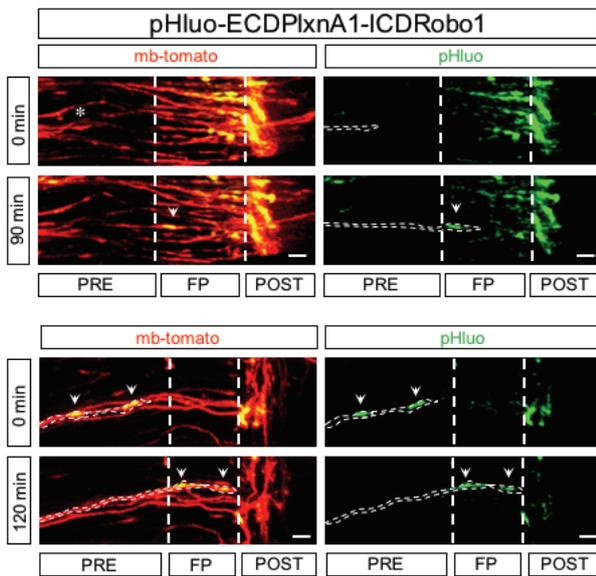
Overall, the mechanisms sorting PlxnA1 and Robo1 are likely to be very different, which provides a basis for receptor-specific control of their spatio-temporal pattern of addressing at the growth cone surface. Given the striking differences observed in the temporal pattern of Robo1 and Robo2, it would be highly interesting to swap their domains and examine whether it reverses the timing of their sorting.

Figure 1

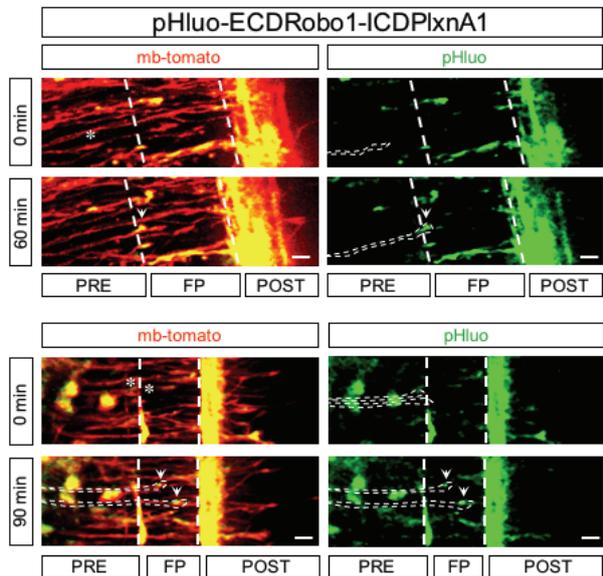
a



b



c



d

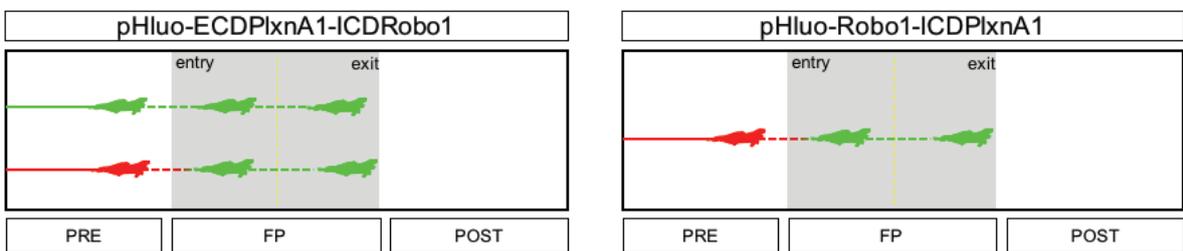


Fig. 1: The spatio-temporal pattern of PlxnA1 is encoded by its ICD-domain. (a) representation of PlxnA1 and Robo1 receptor structures. The PlxnA1-Robo1 chimera (left panel) consists of the PlxnA1 ECD fused to Robo1 transmembrane (TM) and ICD. The Robo1-PlxnA1 chimera consists of the Robo1 ECD fused to PlxnA1 TM and ICD. (b) Time-lapse sequences illustrating the dynamics of PlxnA1-Robo1 chimera. Arrowheads point pHluo detected in growth cones at the FP entry (upper panel) and in growth cones prior to their arrival to the FP g (lower panel). (c) Time-lapses sequences showing the dynamics of Robo1-PlxnA1 chimera. The flashes are detected at the FP entry. (d) Summary of the temporal sequences of PlxnA1-Robo1 and Robo1-PlxnA1 chimeric receptors membrane sorting during FP navigation. Scale bars in **b** and **c**, 10 μm .

SUPPLEMENTARY RESULTS 2

Background of the study

Recently, a novel mechanistic model underlying the gain of response to Sema3B has been proposed, which challenges the model reported by our laboratory (Hernandez-Enriquez et al., 2015). Four essential aspects ground this model:

- (i) Commissural growth cones are sensitive to Sema3B from the pre-crossing stage
- (ii) Sema3B in the FP is chelated by Nrp2, which prevents its diffusion and thus its perception by commissural growth cones before they reach the FP.
- (iii) Nrp2 expression in the FP, which is high from E10 to E13, is transcriptionally downregulated at E13 which allows the commissural axons to proceed for FP navigation.
- (iv) Commissural axons express PlxnA1 from the onset of their navigation.

I conducted a series of several experiments to test these different aspects.

Results

1) Responsiveness of commissural growth cones to Sema3B

Hernandez-Enriquez and collaborators cultured spinal cord explants in three-dimensional substrates and applied to the culture medium increased concentrations of recombinant Sema3B. They observed a reduction of axon outgrowth whose amplitude correlates with the dose of Sema3B.

We repeated these experiments under the same conditions, with increasing doses of recombinant Sema3B (0µg/ml, 0.5µg/ml, 1 µg/ml, 2 µg/ml) for 30 hours, and analyzed axon growth from the explants by quantification of neurofilament immunostaining with Neurite-J ImageJ plugin. Contrary to Hernandez-Enriquez and collaborators that even reported total loss of outgrowth at the highest Sema3B dose, we failed to see any difference between the treated and control conditions, and at the highest dose observed a consistent outgrowth as in the other conditions.

Work by three different labs including ours made use of collapse assay to evaluate the sensitivity of commissural growth cones to Sema3B. We repeated these experiments with neurons dissociated from mouse dorsal spinal cords. We also included a condition where the neurons were treated with GDNF, that our laboratory reports as being the FP cue that trigger the sensitization of commissural growth cones to sema3B. This condition was also used as a control of the efficiency of the recombinant Sema3B, since we had obtained no effect of its application in the explant assays. As a result, a growth cone collapse response was observed only in the GDNF+Sema3B treated condition, consistent with previous work of the lab (Charoy et al., 2012) (Delloye-Bourgeois et al, 2015).

Next, we investigated how Sema3B distributes in the FP using a Sema3B-gfp conditional mouse model which our lab generated in previous work (Arbeille et al., 2015). To assess whether Nrp2 in the FP could chelate Sema3B, we crossed Sema3B gfp and Robo1/2 mouse lines. The distribution of Sema3B was assessed by immunolabeling of GFP in embryonic sections. We measured total and average Sema3B within the FP and established a plot profile of Sema3B distribution within the FP. The analysis revealed no significant differences between the condition WT for the Robo receptors and the condition mutant for the receptors.

Finally, using home-made antibody recognizing intracellular epitope of PlxnA1 Hernandez-Enriquez and collaborators reported that PlxnA1 is present at the cell surface of commissural axons. This conclusion appeared erroneous to us. First, immunohistochemistry on tissue sections cannot discriminate surface and intracellular pools, which was even obvious in this case given that the antibody is directed to an intracellular epitope. We thus concluded that the labeling reported in the sections might reflect PlxnA1 processed fragments that our laboratory reported to be present in commissural axons prior to the crossing.

Figure2

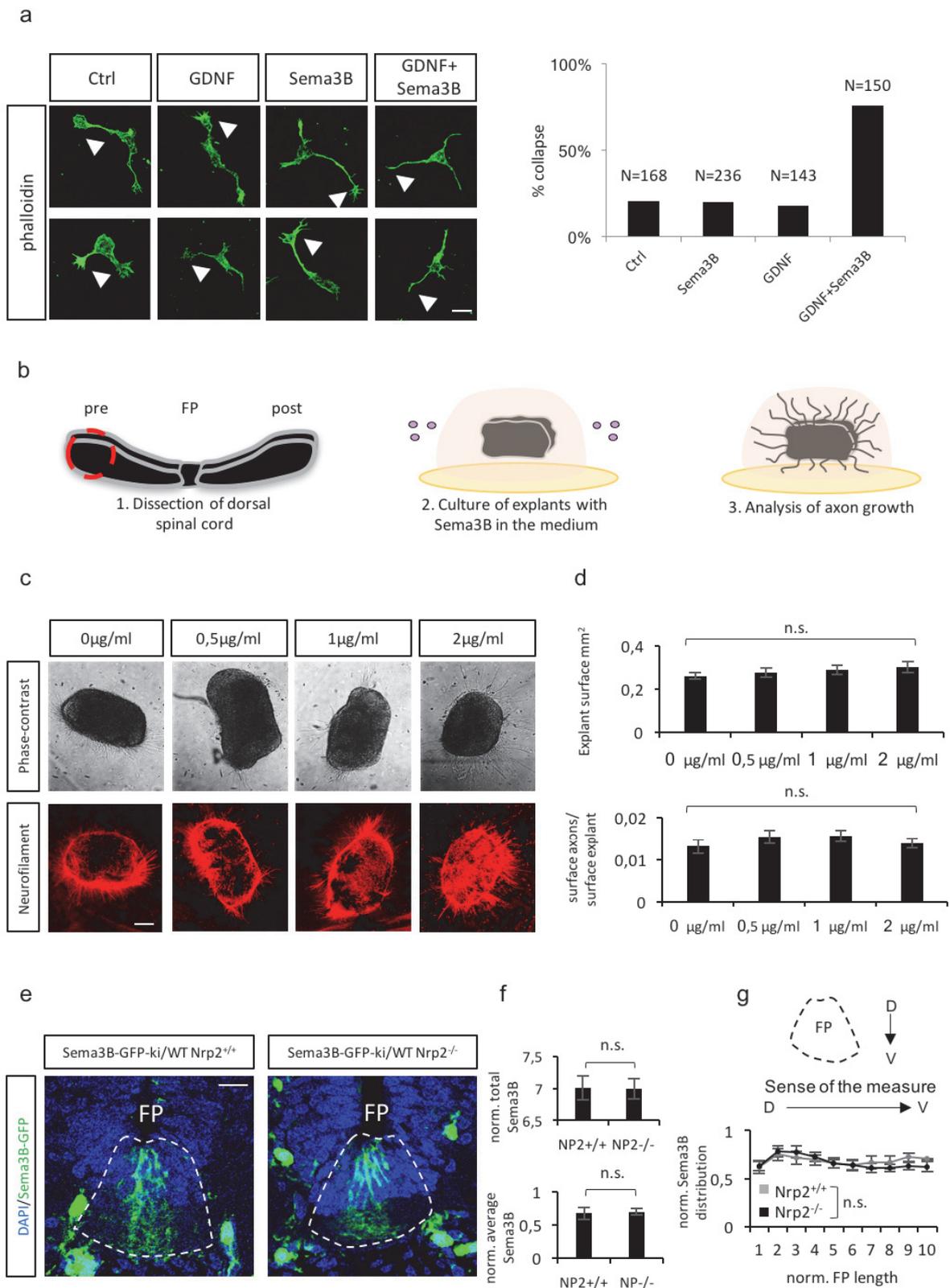


Figure2: Pre-crossing axon are insensitive to Sema3B and Nrp2 does not chelate this cue.

(a) Growth cone collapse assay performed on cultures of dissociated commissural neurons. Left panel: Photomicrographs of collapsed and non-collapsed growth cones. Right panel: quantification of the collapse **(b)** Spinal cord explant assay procedure. Dorsal spinal cord explants are dissected from E12.5 mouse spinal cord and cultivated in medium containing Sema3B. After 30h of culture, explants are fixed, immunostained and processed for analysis. **(c)** Photomicrographs illustrating explants cultivated with increased doses of Sema3B. **(d)** Upper panel: Quantification of the surface of the explants in the different conditions (3 independent experiments. 0 μ g/ml: N= 31 explants; 0,5 μ g/ml: N= 23 explants; 1 μ g/ml: N= 26 explants; 2 μ g/ml: N= 21. Error bars indicate mean \pm SEM. $P= 0,5132$ is from ANOVA one way test). Lower panel: Quantification of the surface of axon outgrowth in the different conditions. (Error bars indicate mean \pm SEM; $P= 0,4883$ is from ANOVA one way test). **(e)** Transverse sections of E11.5 Sema3B-gfp/Nrp2^{+/+} and Sema3B-gfp/Nrp2^{-/-} embryos immunolabeled with anti-GFP showing the pattern of Sema3B distribution. Nuclear staining distinguishes the region of the FP soma (dorsal FP) from the region of commissural axon path (ventral FP). **(f)** Upper panel: quantification of the total Sema3B-gfp signal within the FP (Error bars indicate mean \pm SEM; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. $P>0,9$. P value is from Mann-Whitney test). Lower panel: quantification of the average Sema3B signal within the FP (Error bars indicate mean \pm SEM; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. $P>0,9$. P value is from Mann-Whitney test). Both quantifications revealed no significance between the conditions (Nrp2^{+/+}= 5 embryos, 69 stacks; Nrp2^{+/+}= 7 embryos, 67 stacks). **(g)** Upper panel: Schematic representation of the D-V axis along which the Sema3B distribution was appreciated with GFP staining. Lower panel: quantification of the Sema3B distribution along the D-V axis showing no significance between the conditions (Nrp2^{+/+}= 5 embryos, 69 stacks; Nrp2^{+/+}= 7 embryos, 67 stacks. Error bars indicate mean \pm SEM. $P= 0.771$ is from Kolmogorov-Smirnov (KS) test). Scale bars in **c**:200 μ m; in **e**:20 μ m.

Conclusions

Overall, our data make it unlikely that the commissural growth cone response is silenced through the mechanism proposed by Hernandez-Enriquez and collaborators. FP crossing is not a synchronous process, but proceeds in waves reflecting the successive generation of the neurons. How this developmental sequence can be conciliated with the mechanism of

Sema3B trapping by Nrp2 and its release at E13 proposed by Hernandez-Enriquez and collaborators remain puzzling. Despite we could not find any evidence for an effect of Sema3B in the explant assays, it could be that axon growth is regulated by Sema3B. Whether such an effect exists, and if so whether it is mediated by PlxnA1 rather than another receptor was not demonstrated by Hernandez-Enriquez and collaborators. It could also be possible that locally in the FP, Sema3B interacts with Nrp2 and that this interaction accounts for the FP navigation, but experimental evidences for such an interaction still lack.

CONCLUSIONS AND PERSPECTIVES

My thesis study has been first dedicated to the setting of an imaging set-up to investigate the dynamics of guidance receptors during spinal commissural axon navigation. Second, the use of this set-up allowed us to uncover novel specificities distinguishing the receptors mediating the repulsive functions of guidance cues in the FP. We found that the receptors are sorted successively at the growth cone surface, which might generate a sequence of successive sensitization to the repellents at different points of the commissural navigation. In addition, we found for two receptors sorted during FP crossing a distribution pattern in different compartments of the growth cone, which also bring a basis for the generation of localized and distinctive signaling. To avoid redundancy, I discuss into details here aspects that were not addressed in the article.

1) A paradigm to explore the dynamics of guidance receptors in commissural growth cones

Several studies in the past have revealed the mRNA and protein expression patterns of Robos and PlxnA1 receptors in chick and mouse spinal cord. During the time-window of FP and post-crossing navigation, mRNAs for Robo1 and Robo2 have been detected in distinct while overlapping populations of commissural neurons, Robo2 expressed by commissural neurons having their cellular bodies located at more lateral positions, compared to those expressing Robo1 (Brose et al., 1999). PlxnA1 and Nrp2 transcripts were also found expressed in the dorsal spinal cord over the time of commissural axon navigation (Zou et al., 2000) (Nawabi et al., 2010)(Mauti et al., 2006). At protein levels, the data are quite discordant among studies, which probably reflect differences and efficacies of the antibodies that were used. In the mouse, Robo1/2 were seen present in pre-crossing axons in some reports, while in others the signal was extremely weak and restricted to the FP and the post-crossing compartments. The data are also conflictual for PlxnA1 protein, reporting its expression since the pre-crossing stage or not (Hernandez-Enriquez et al., 2015)(Delloye-Bourgeois et al., 2015)(Nawabi et al., 2010). Beyond the observations that these receptors are compartmentalized in the commissural axon, with the midline delineating a pre-crossing and a post-crossing segment, it has remained ignored whether these different receptors have different dynamics and if so whether this accounts for growth cone navigation.

An important innovation of our set-up resides in the possibility to monitor the surface expression dynamics of guidance receptors *ex vivo*, in axons experiencing midline navigation and achieving proper guidance decisions, while preserving the topography of guidance molecules in the tissue. In particular, the use of pHluorin enabled to discriminate the intracellular pool of guidance receptors from the membrane pool, this latter representing the fraction that is competent for conferring sensitivity to guidance cues.

An obvious weakness of the model relies on the ectopic expression of receptors in neurons already expressing their endogenous counterparts. Thus, are observations of the ectopically-expressed receptors indeed reflecting the dynamics of the native receptors? Having mouse models in which the receptor is substituted to a pHLuo-tagged version would be ideal. Nevertheless, several aspects can be highlighted which support that our strategy is relevant. First, it is noticeable that most mb-tomato⁺ commissural axons navigating the FP have intracellular pool of pHLuo-tagged receptors (assessed by antibody directed against pHLuo), but only a limited amount of them sorted the receptors at their growth cone surface. This attests that commissural growth cones successfully achieved robust control of “ectopically-expressed” receptor cell surface sorting. A second argument is that cases where receptors are produced at too high levels are eliminated because when it occurs, commissural growth cones are unable to proceed for FP entry. These over-expression cases could easily be detected and neglected in the analysis. In most of them, it resulted in premature turning and stalling behavior of the growth cones. This means that we considered only the growth cones that could manage maintenance of receptors at sufficiently low levels for succeeding in FP entry. This advantage of the system, to eliminate over-expression cases, also limited the pertinence of experiments combining knock-down of endogenous receptor and expression of the pHLuo-tagged version. Third, expressing the pHLuo-tagged receptors in the mouse embryo gave comparable dynamics to those established in the chicken embryo. Interestingly, no difference was observed when the tagged receptors were introduced in wild-type and embryos mutant for the corresponding receptor, thus supporting that the temporal profile that we established for Robo1 and PlxnA1 were likely not impacted by the overall levels of receptors in commissural growth cones.

Use of functionally-inactive pHLuo-tagged receptors (unable to elicit downstream response) could be implemented in the paradigm. In theory, this would enable the monitoring

of receptor dynamics with no interference with the physiological signaling. However, designing such an inactive receptor form is all except trivial. Affecting the binding site to the ligand could alter receptor dynamics, at least from the time when the receptor is assumed to have interacted with its ligand. Affecting the intracellular domain in order to abrogate the initiation of downstream signaling would be risky too since it could affect the interactions with key effectors of the trafficking and sorting. Insights from structural biology still lack for going into this direction. Finally, our experiments with chimeric receptors also indicate that the temporal profiles which we observed are not coupled to receptor levels but rather are dictated the receptor structure, at least for PlxnA1. Finally, these temporal profiles are also consistent with immunohistochemical labeling of the receptors in embryonic sections, showing that Nrp2 is expressed from the pre-crossing step whereas Robo1/2 and PlxnA1 are upregulated during FP crossing. Thus overall, these arguments support that our paradigm enable accessing the dynamics of endogenous receptors.

A second limitation of our set-up is the lack of specific targeting of the pHLuo receptors in the commissural neuron population, which prevented us to distinguish ipsilateral and commissural axons. To overcome this limitation, we generated vectors encoding for pHLuo-receptors under the control of Math1 promoter, to specifically target dl1c neurons. First, the electroporation of Math1-GFP vectors allowed the detection of dl1c population, located in the dorsal horn of the spinal cord, in close vicinity to the RP. The targeted neurons extended their axons ventrally towards the FP and, after crossing, projects both in the VF and LF, as expected from previous work (Wilson and Stoeckli, 2012). Second, we constructed Math1-pHLuoRobo2-ires-mbTomato and Math1-pHLuoPlxnA1-ires-mbTomato vectors and monitored the expression of these constructs in chick open-books. We observed that the expression rate in the dl1c population, reported by the mb Tomato was significant for both constructs. In contrast, the pHLuo signal was extremely faint, slightly above the background noise. Although the few cases of pHLuo-PlxnA1⁺ growth cones that we could observe were located in the FP as expected, any strong conclusion could be drawn from this analysis. Similarly, we observed some rare examples of Math1-pHLuo-Robo2⁺ growth cones in the post-crossing LF, thus as expected from our analysis with pCAGEN-pHLuo-Robo2 flashes. The very low probability of observing pHLuo flashes in Math1-pHLuo-Robo2 electroporated spinal cords discouraged us from persisting with additional time lapse recordings using these constructs.

2) Mechanisms underlying the temporal pattern of PlxnA1 and Robo receptor sorting

Our data support that the timing at which the receptor reaches the growth cone surface is set by the exocytosis event, and not by the availability of the receptor within the growth cones. Our analysis of the total protein pool revealed that the domain of PlxnA1 and Robo1 expression within the axons and growth cones is much spreader than the cell-surface pool, strictly restricted to the growth cone during FP crossing. This analysis was conducted on axons whose growth cones were seen navigating within the FP, in order to eliminate potential over-expression cases. Thus, the receptors might be stored in intracellular compartments before exocytosis.

Our observations also suggest that the mechanisms trafficking and sorting PlxnA1 and Robo1 differ. pHLuo-PlxnA1 was detected within fairly long segment of the pre-crossing axon shaft. Whether the pHLuo detected within the axon reflect the integral protein, the processed forms or both remain to be determined. This could be achieved by western blot performed on lysates of electroporated spinal cords. Our laboratory reported that PlxnA1 is processed prior to the crossing, which result in two fragments (Delloye-Bourgeois et al., 2015)(Nawabi et al., 2010)(Charoy et al., 2012). The antibody that was used likely recognizes the integral form only, since it labeled PlxnA1 only in the crossing and post-crossing compartments. A recent study reported the presence of PlxnA1 in pre-crossing commissural axons, using a home-made antibody directed to an epitope of PlxnA1 intracellular domain (Hernandez-Enriquez et al., 2015), with a pattern reminiscent to that of the pHLuo total pool distribution, thus likely recognizing integral and processed PlxnA forms. Intriguingly, the intra-axonal pattern of PlxnA1 does not resemble vesicular. It is rather uniform, appearing to localize below the plasma membrane. Increasing examples are provided showing that calpains regulate both the functions and distribution of their processed targets (Araujo et al., 2018). For example, N-terminal talin fragment has been shown to concentrate beneath the plasma membrane, similar to full-length talin, while C-terminal fragments were observed diffusely distributed in cell cytoplasm (Araujo et al., 2018). Further characterization is needed to better define the outcome of PlxnA1 cleavage by calpains.

Concerning Robo1, although detected within the axons prior to the cell surface sorting, protein levels were low and the spread of pHLuo⁺ signal within the axon was much restricted

than for PlxnA1. Moreover, the signal was punctuated, compatible with expression of the receptor in trafficking vesicles. Experiments conducted in the chicken embryo suggested that Robo1 insertion in the plasma membrane is regulated by the cooperation between RabGDI, a component of the vesicle fusion machinery and calyntenin 1, which acts as a linker between vesicular cargoes and the molecular motor kinesin 1. RabGDI and Calsyntenin 1 shuttle Rab11⁺ vesicles at the plasma membrane (Alther et al., 2016)(Philipp et al., 2012). The authors also studied the trafficking of another guidance receptor, Fzd3, and found it is mediated by calyntenin and not by RabGDI. Consequently, Fzd3⁺ and Robo1⁺ vesicles barely overlapped. These results illustrate that guidance receptors segregate in different vesicles, which can provide a basis for setting differential patterns of their cell surface sorting. To get further insights, it would be interesting to monitor using our set-up the total receptor pool trafficking along the axon and in the growth cone. The transport of GFP-tagged receptors, reporting the total pool, could be recorded over time. Interesting insights could also come from FRAP experiments that would allow evaluating the recovery rate of fluorescence and the diffusion properties of the receptors within commissural growth cones. We are currently conducting these experiments, in collaboration with Olivier Thoumine lab.

The mRNAs of the pHluo-receptors lack the native 3'UTR and 5'UTR sequences. This prevents the transcripts to be packed in granules, transported in the axon towards the growth cones for putative local synthesis, since these processes rely on molecular interactions involving these regulatory sequences. mRNA 3' UTR also contains target sequences for specific complementary MicroRNAs (miRNAs), small noncoding regulatory RNAs which, post-transcriptionally, regulate the expression of specific target genes causing their translational repression or inducing their degradation (Ambros and Chen, 2007). As a consequence, neither local synthesis and likely nor silencing by microRNAs account for the temporal dynamics which we observed, in an experimental context in which these regulations were eliminated and proteins allowed to be transported to the growth cones.

Whether mechanisms of local synthesis are or not involved in the native context have to be assessed. Several years ago, local protein synthesis of the EphA2 receptor, reported using fluorescent timer GFP tag changing fluorescence color over-time, was proposed to occur in commissural growth cones upon their crossing (Brittis et al., 2002). More recently, *in vitro* studies, suggested that mir-92, a highly conserved miRNA, binds to Robo1 3' UTR causing its

translational repression, which, as proposed by the authors, might render pre-crossing axons insensitive to FP Slits (Yang et al., 2018). To our knowledge, the implication of such regulatory mechanisms has not been reported for Robo2 and PlxnA1, although a few studies report Plexins as targets of microRNAs (Wang et al., 2018)(López-González et al., 2018).

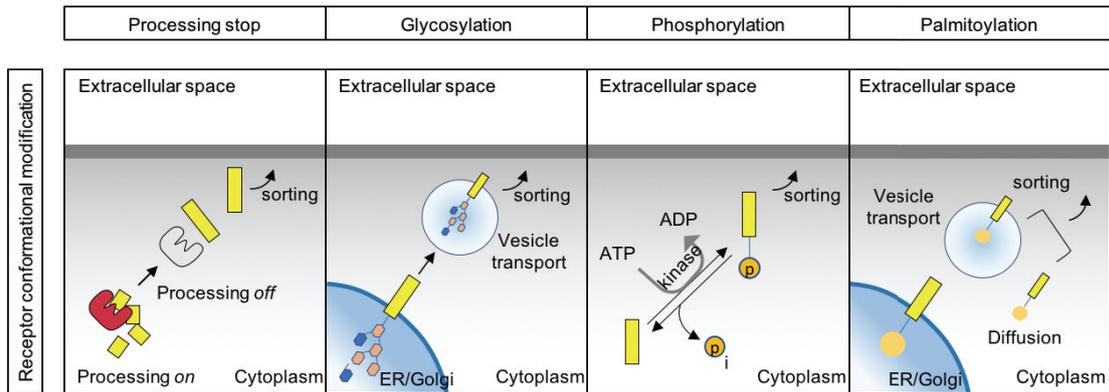
Nevertheless, local protein synthesis could well be implicated, bringing indirect contribution to receptor sorting. For example, certain membrane cargoes could be neosynthesized in the growth cones, very locally near the sites where they will be needed for sorting the receptors. These local synthesis events could be triggered by FP cues to which commissural growth cones are exposed when they reached the FP.

Our laboratory reported that PlxnA1 sorting is elicited by NrCAM and GDNF FP cues (Charoy et al., 2012)(Nawabi et al., 2010). We wondered whether FP cues could also control the sorting of Robo1. Analysis of pHLuo expression in individual growth cones extending from dorsal spinal cord explants exposed to acute application of medium conditioned by FP cultures validated this hypothesis. Additional experiments are needed to identify the signals mediating this effect and determine whether they are the same as those triggering PlxnA1 cell surface sorting.

Thus overall, whatever the nature of the mechanisms that enable PlxnA1 and Robo1 to become available upon FP crossing, the timing of their sorting might rely on post-translational mechanisms. We have postulated that it could be encoded in the protein structure. Receptor-specific motifs in the intracellular domain of the receptors would drive the receptors through distinct trafficking paths and post-translational modifications impacting their delivery to the cell surface (Winckler and Mellman, 2010)(Maeder et al, 2014). Alternatively, or in addition, amino-acid motifs in the extracellular domains could also drive specific interactions and pre-formed complexes conferring some specificities of membrane addressing (Winckler and Mellman, 2010)(Maeder et al, 2014). Our current investigations of the temporal patterns of chimeric receptors in which PlxnA1 and Robo1 intracellular (ICD) and extracellular domains (ECD) were swapped will enable us to obtain some clues on these aspects. Such strategy has been successfully used in previous studies to reveal the coding of divergent guidance behaviors in specific receptor domains (Bashaw and Goodman, 1999) (Spitzweck et al., 2010) (Stein and Tessier-Lavigne, 2001). Our data already indicate that the ICD of Robo1 is not

sufficient to confer to the chimeric receptor the temporal pattern of native Robo1. In contrast, although these findings need to be confirmed with additional cases, the ICD of PlxnA1 seems sufficient to confer the temporal pattern of PlxnA1. These experiments will also enable us to correlate receptor domains with guidance behaviors of commissural axons during FP navigation. We could report quite similar PlxnA1 and Robo1 surface expression profiles in chick and mice spinal cord, suggesting that the mechanisms controlling the spatio-temporal regulation of surface sorting might be conserved. Nevertheless, our characterization in mouse samples is not as precise of that conducted in the chick because it was conducted using static imaging at a single time point. Thus, we could only focus on FP and post-crossing compartments. pHLuo⁺ growth cones in the pre-crossing compartment were not analyzed since we could not infer from static imaging whether these growth cones are prone to cross the FP or not. It would be interesting to further confirm the dynamics of PlxnA1 and Robo1 in mice by time lapse imaging. A summary of the possible mechanisms underlying the temporal pattern of guidance receptors is available in **Figure 1**.

A



B

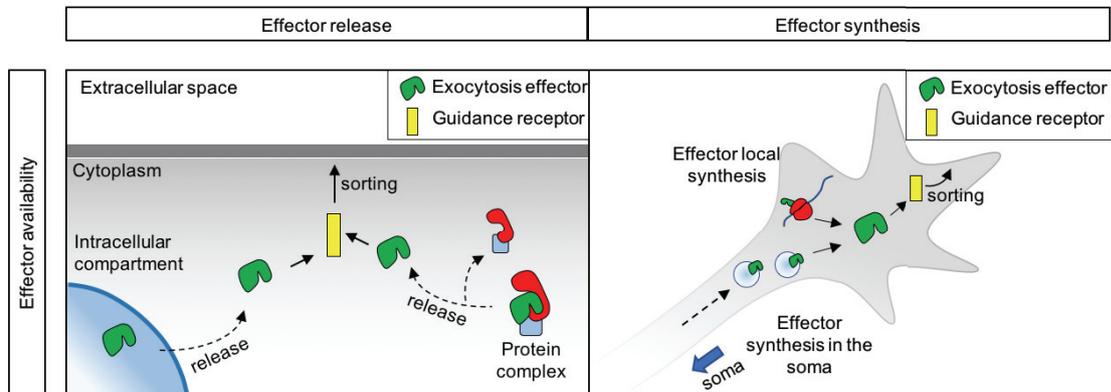


Figure 1: Potential mechanisms for setting specific spatio-temporal dynamics of guidance receptors. They rely on receptor conformational changes and effector availability. (A) The panel represents examples of mechanisms that might be involved in structural modifications of guidance receptors, such as receptor processing suppression, receptor glycosylation, phosphorylation and palmitoylation. (B) The panel represents examples of how an effector might become available in the growth cone: it can be released from an intracellular compartment or from a protein complex (left panel) or it can be synthesized locally or in the soma, and then transported to the growth cone (right panel).

3) From temporal patterns of receptor sorting to sequential activation of repulsive signaling during commissural growth cone navigation

Mapping the location of pHLuo flashes reporting the timing by which the receptors reach the growth cone surface reveals differences between receptors. Are these timing reflected in a sequential activation of the repulsive signaling? We do not have evidence from

observations of receptor activation or initiation of downstream transduction cascade. Nevertheless, we could notice changing of growth cone behavior following the sorting of receptors. For Robo2, analysis of the trajectory before and after the pHLuo flashes indicated that receptor sorting is correlated with an accentuating longitudinal turning. Interestingly for Robo1, we observed that after receptor sorting, commissural growth cones start exploring directions in the rostro-caudal axis. This suggests that, already when navigating the contralateral FP half, the growth cones start gaining responses to the rostro-caudal guidance cues which will drive their longitudinal turning after the crossing.

For PlxnA1, we failed to detect a “read-out” of receptor activity, although the presence of crossing phenotypes in context of over-expression suggests that pHLuo-tagged PlxnA1 receptor is an active receptor form. PlxnA1 receptor is sorted from the FP entry, thus at a time when the FP navigation is just starting. It is therefore more difficult to conceive a classical repulsive response to be mediated by this receptor. Indeed, would it be the case, one would expect the growth cones to be totally arrested, or to turn back by exiting the FP from the entry side, a behavior that was never observed in the movies. On the contrary, such re-crossing of commissural axons was observed in open-books of PlxnA1^{-/-} embryos, in accordance with previous studies (Delloye-Bourgeois et al., 2015). Thus, commissural growth cones need PlxnA1 to travel through the FP. Moreover, when PlxnA1 was expressed at high concentration, resulting in receptor sorting before FP entry, then commissural growth cones mostly failed to enter the FP and turn ipsilaterally. These are behaviors classically elicited by repulsive guidance cues. The actions of the guidance cues mediated by PlxnA1 thus remains to be understood.

Several scenarios can be proposed. First, despite the presence of PlxnA1 from the FP entry, the receptor is active only after midline crossing. This could be due to the amount of PlxnA1 reaching the cell surface in the first half of the FP, which could not reach the threshold of receptor activation. Alternatively, the receptor could be docked by a cell surface partner until midline crossing is achieved, and the interaction would be released to enable receptor activation in the second part of the navigation. Such a mechanism has been described in *Drosophila* for commissural Robo1, which is engaged in a *trans* interaction with midline Robo2 resulting in inhibition of Slit signaling (Evans et al., 2015). A mirror mechanism would be that Nrp2 or PlxnA1, both reported to be expressed by FP cells, interact in *trans* with PlxnA1 to

silence repulsive signaling by Slit-C and Sema3B. Finally, PlxnA1 could initially be maintained in an auto-inhibited conformation through the head-to-neck-folding of its ectodomain (Kong et al., 2016).

The second view is that PlxnA1 is active from the onset of its sorting, to transduce repulsive forces from the surrounding ligands in a “non-classical” way. Possibly, the compartmentalization of PlxnA1 in the growth cone could generate a gradient of activity following ligand binding, thus establishing a polarity within the growth cone from an initial uniform source of ligand, that would focalize growth direction straight forwards. Interestingly, our super-resolution analysis revealed a rear-low to front-reach distribution of PlxnA1 in commissural growth cones, from their entry in the FP to the exit.

Alternatively, particular arrangements of the ligands in the FP create the conditions for repulsion. This brings to light the general gap of knowledge on the topography of secreted guidance cues and in the present context, the need for better characterizing the distributions of Sema3B, SlitC and SlitN within the FP. Our laboratory is developing genetically-encoded fluorescent tools to visualize ligand-receptor interactions in living samples, to detect Slit cleavage and Slit N- and C-fragment distributions within the FP. What are the mechanisms that could generate specific patterns of ligands in the FP? Several studies reported that heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) can regulate the distribution and the activity of secreted and transmembrane proteins involved in axon guidance in the developing spinal cord (de Wit and Verhaagen, 2007). HSPGs are attached to the cell surface of glia and axons but can also be released into the extracellular matrix, after enzymatic processing. The carbohydrate polymer heparan sulfate (HS) is a highly negatively charged polysaccharide carried by HSPGs which interacts for example with the basic C-ter of Semaphorins (De Wit et al., 2005). Moreover, the same study showed, *in vitro*, a physical interaction between Sema3A and HSPGs. Binding between Sema3A and CSPGs has been suggested to occur in the optic tract in the *Xenopus* model and in the cortical plate in the mouse (Walz et al., 2002)(Emerling and Lander, 1996). Biochemical studies also demonstrated Slit binding to HSPGs (Wang et al., 1999). For instance, Slit2 at the cell surface can bind to the GPI-anchored HSPG glypican-1 *in vitro* (Brose et al., 1999) and an *in vivo* role of HSPG syndecan in regulating Slit distribution at the midline has been described in *Drosophila* (De Wit et al., 2005). Binding of Slit-C to PlxnA1 also requires HSPGs (Delloye-

Bourgeois et al, 2015).

Proteoglycans could not only be involved in ligand distribution but could also act as modulators of the guidance response (de Wit and Verhaagen, 2007). For instance, Sema3A binding to HS was shown to potentiate Sema3A signaling (de Wit and Verhaagen, 2007)(De Wit et al., 2005). *In vivo* studies also showed that the HSPG perlecan is required for Sema1A-PlxnA-mediated repulsive guidance (Cho et al., 2012). Another evidence of the modulation exerted by proteoglycans on the Semaphorin signaling was shown for transmembrane Sema5A. Sema5A has a bifunctional role, generating both attractive or repulsive effects on developing axons of the fasciculus retroflexus, depending on the physical interaction that it establishes with proteoglycans (Kantor et al., 2004). Proteoglycans can also modulate the guidance response to Slit molecules. *In vitro*, enzymatic removal of heparan sulfate from the axon surface through heparinase treatment, decreases the affinity between Slit2 and Robo, and this abolishes the repulsive activity of Slit2 on olfactory axons (Hu, 2001). Genetic evidences of HS role in modulating Slit response has been brought *in vivo* in zebrafish, worm, fly and mouse models. Both in mice and zebrafish, loss of enzymes involved in HS-synthesis induces defects in the formation of major commissural tracts, which are reminiscent of those occurring in Slit1/Slit2 double KO mice (Inatani, 2003)(Plump et al., 2002)(Lee et al., 2004). Interestingly, syndecan binds both Slit and Robo, acting as an essential coreceptor, and its loss in axons induce guidance errors at the midline that are also observed in Slit and Robo mutants (De Wit et al., 2005)(Steigemann et al., 2004). However, additional levels of complexity are provided by the diverse variations that the HS chains can undergo, which generate modifications in the sulfation and epimerization patterns (Turnbull et al., 2001). How such variations impact on the different contexts of axon guidance to which proteoglycans contribute is fully unknown and represents an interesting aspect to explore.

Whatever these possibilities, characterizing when and where is PlxnA1 receptor becoming active and interacting with its ligands would significantly increase our understanding of its mode of action during FP navigation. It would be highly interesting to develop fluorescent sensors of PlxnA1-mediated downstream signaling and reporters of conformation changes and ligand binding. Various FRET-based biosensors have been developed in the past to monitor the spatial and temporal dynamics of signaling events in living cells, for instance, FRET-based biosensors detecting second messenger dynamics (Herbst et al., 2009). *In vitro*

application of chemorepellents such as Semas and Slit, induces a rapid hyperpolarization and a local elevation of intracellular calcium in the growth cones. Upon PlxnA activation, fast increase of cGMP levels occurs, which in turn activates cyclic nucleotide gated (CNG) calcium channels at the plasma membrane, allowing calcium uptake from the extracellular space and growth cone repulsion (Togashi et al., 2008)(Nishiyama et al., 2008). Calcium influx and cyclic nucleotides production are therefore very early events occurring after PlxnA activation. Many FRET-based second messenger biosensors have been developed to detect cellular fluctuation in calcium and cGMP (Miyawaki et al., 1997)(Nicholls et al., 2011), even at specific subcellular region of interest, such as the plasma membrane, *via* specific amino acid targeting sequences (Herbst et al., 2009). Thus, simultaneous monitoring of PlxnA1 dynamics and downstream second messengers might allow mapping the spatio-temporal correlation between receptor sorting at the plasma membrane and receptor activation. FRET-based kinase activity biosensors reporting spatio-temporal kinase dynamics are also highly interesting tools to work with (Herbst et al., 2009). Several kinases could be interesting to look at, such as FAK, and Src family kinases, known to be recruited in proximal-membrane signaling, sometime even for receptor activation (Li, 2006). The possible models correlating PlxnA1 sorting pattern to its activity are summarized in **Figure 2**.

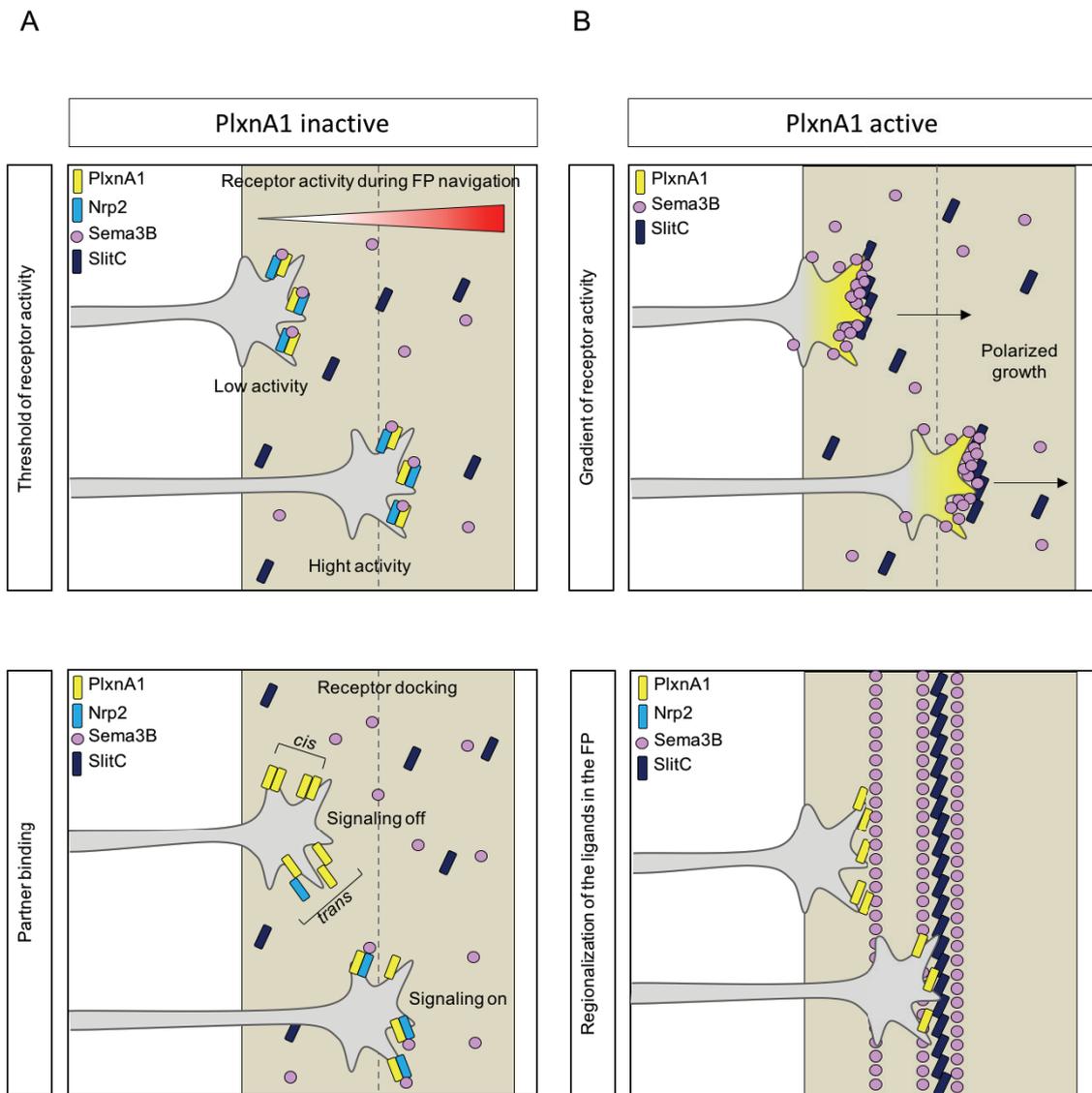


Figure 2: Possible models to correlate PlxnA1 sorting pattern and activity. PlxnA1 reaching the growth cone surface at the FP entry might be active from the onset or inactive. (A) Upper panel: PlxnA1 at the FP entry has weak activity. Such activity might increase as the growth cone navigates the FP. Lower panel: PlxnA1 at the FP entry cannot interact with the ligands because it is docked by *cis* and *trans* interactions. (B) Upper panel: PlxnA1 is active and its compartmentalization at the growth cone surface generates a gradient of activity that allow to polarize the navigation response despite a uniform ligand environment. Lower panel: Ligand compartmentalization within the FP favors stabilization/activity of PlxnA1 at the growth cone front, generating a polarized response.

4) Correlating PlxnA1 and Robo1 differential spatial distributions with functional specificity

Our results bring, for the first time, *in vivo* evidences of guidance receptor compartmentalization at the growth cone surface, during FP navigation. Our super-resolution microscopy revealed a striking partitioning of PlxnA1 and Robo1, with PlxnA1 receptor distributing more at the front of the growth cone and Robo1 at the rear. Interestingly, PlxnA1 receptor was observed not only present at the front of the growth cone central domain but also at the surface of filopodia where Robo1 is completely absent. Moreover, we found that Robo1 pattern is more dynamic than that of PlxnA. This conclusion was drawn from the observation that the surface of Robo1⁺ particles increases as the growth cones progress from the midline to the FP exit.

What could be the outcome of this differential distribution at the growth cone surface?

Spatially-restricted signaling from the two receptors onto different intracellular targets (Kurokawa et al., 2005) (Itoh et al., 2002) (Kurokawa et al., 2005) could regulate filopodial dynamics and adhesion along the front-rear axis of the growth cone. This could participate to adapt growth cone shapes to the constraints of FP navigation. Our video-time lapse observations of the growth cones showed that they have quite a simple form, lacking complex filopodia networks typical of *in vitro* neuronal growth cones. Comparable morphologies to those we observed have already described by pioneer studies of commissural axon navigation in the chick embryo (Yaginuma et al., 1991). It would be interesting to address whether manipulations of PlxnA1 and Robo1 affect growth cone morphologies during FP navigation.

How this front-rear partitioning specifically connects to the guidance growth cone responses to Sema3B, SlitC and Slit-N FP cues remains to be understood. Indeed, directional growth require both adhesions and cytoskeletal dynamics to be polarized (Barberis et al., 2004)(Zhou et al., 2008). Signaling cascades downstream of guidance cues integrate regulation of adhesion. For example, upon receptor activation, a complex constituted by Robo, Abl and N-cadherin was shown to form at the plasma membrane, resulting in tyrosine phosphorylation of beta-catenin mediated by Abl leading to loss of N-cadherin-actin connection (Rhee et al., 2002). Semaphorin-Plxn interactions regulate integrin and focal adhesion kinase dynamics (Tran et al., 2007). The front-rear partitioning could also regulate growth cone adhesion by

impacting onto endocytosis and recycling of cell adhesion molecules, both of which interestingly following polarized trafficking paths in the growth cone (Winckler and Mellman, 2010). This has been particularly exemplified for L1CAM (Kamiguchi and Lemmon, 2000), whose expression profile is in addition also temporally and spatially controlled in commissural growth cones (Long et al., 2004). Robo1 could transduce a propelling force from its surrounding ligands needed to exit the FP, by setting particular adhesion properties at the growth cone basis. PlxnA1 would play comparable role in the complementary growth cone front compartment.

The front-rear gradients of PlxnA1 and Robo1 receptor could as well serve for the generation of differential actin and microtubule dynamics. PlxnA signaling impacts on actin cytoskeleton primarily through the modulation of Rho-GTPases and *in vitro*, the activation of Rho promotes process retraction (Tran et al., 2007). Interestingly, it has been described that the activity of RhoA, in growing dorsal root ganglion cell growth cones is higher at the motile tips of protrusions (Kurokawa et al., 2005). Thus, as an example, receptor compartmentalization in commissural growth cones could confer PlxnA1-specific access to RhoA regulation in filopodia.

As described above, pHLuo-Robo1 flashes interestingly correlate with increase in the exploratory behavior of the growth cone along the rostro-caudal axis. This could for example reflect Robo1-mediated decreased adhesion, increase of focal adhesion turnover, or increase of cytoskeletal dynamics, all of these events conferring sufficient growth cone flexibility for exploration.

5) Differences of spatio-temporal dynamics generate Robo1 and Robo2 functional specificities

Despite their close molecular proximity, several previous studies suggested that Robo1, Robo2 and Robo3 have specific contributions during the formation of spinal commissures. Their deletion in mice produces different post-crossing phenotypes, suggesting that they guide guiding post-crossing axons to different pathways in the ventral and lateral funiculi (Long et al., 2004). These differences are intuitively thought to result from different expression patterns, documented by only partial overlapping of the transcripts revealed by *in situ*

hybridization and immunohistochemical labeling (Long et al., 2004).

Our data show that, beyond expression profiles, receptor-specific structural features drive differences in their temporal sorting, that echo some previous observations. In *Drosophila*, functional diversity of Robo receptors was reported to rely on differences in their ectodomains, as Robo cytoplasmic domains were found functionally interchangeable for longitudinal pathway selection (Evans and Bashaw, 2010). Robo1 and Robo2 were reported to bind Slits with similar affinities and also to share very close ectodomain structures (Brose et al., 1999)(Evans and Bashaw, 2010). However, their cytoplasmic domains are more divergent. For example, Robo2 lacks CC2 and CC3 cytoplasmic domains. Present in Robo1, these domain mediate interactions with downstream effectors such as Enabled, Dock/Nck adaptors, GAPs, Abelson (Bashaw et al., 2000)(Dickson and Gilestro, 2006). Further work is required for identifying the structural differences that trigger sorting of the receptors at different timing.

The lack of Robo2 sorting during FP navigation was rather unexpected. By antibody labeling, we also failed to detect significant pHLuo signal within the axon segment lying in the FP. Rather, a fluorescent signal was only seen from the time axons navigate the ventral funiculus. Thus, if we consider this signal reports the profile of the native receptor, it indicates that the protein is not present before commissural axons navigate the post-crossing step. The mechanisms regulating Robo2 surface sorting have not been identified yet. Whether they rely on exogenous signals, as found for PlxnA1 and Robo1, are currently investigated in our laboratory. Whether our conclusions can be extended to the mouse also remain to be tested.

Thus, Robo2 might be involved neither in FP crossing nor in positioning axons in the ventral funiculus, at least in the chicken embryo. Rather Robo2 sorting at the interface between the medio-lateral (ML) and the dorso-lateral (DL) funiculi appeared strikingly correlated with longitudinal turning of the growth cones. This reveals a FP-like boundary at this medio-lateral/dorso-lateral interface, where Robo2 could expel the growth cones out of the ventral funiculus and allow them responding to rostro-caudal guidance cues, exactly as Robo1 could do it earlier in the FP.

One cannot exclude that Robos more directly participate in guiding the growth cones along the rostro-caudal axis. Up to now, this guidance step is considered under the control of Wnt

and SHH morphogen gradients. Whether Slit proteins form a rostro-caudal gradient could be investigated. It would also be interesting to address the existence of molecular cross-talks linking Robos to Wnt and SHH receptors. Such as drosophila Fasciclin II (Hivert, 2002), Robos could also mediate selective axon-axon recognition important for tract formation and stabilization in the VF and LF. This would enable commissural axons to assemble in bundle. A pre-existing scaffold could also be provided by ipsilateral fibers, which establish their tracts before the arrival of the corresponding contralateral population as proposed in (Hivert, 2002). Autocrine Slit-Robo signaling has already been shown to organize the navigation of the phrenic nerve (Charoy et al., 2017).

The possible implication of PlxnA1 and Robos in the regulation of axon bundle is echoed by our observations. Indeed, in contrast to its profile restricted to the growth cone during FP crossing, the pHLuo signal was observed distributed along the axon shaft of commissural axons navigating their post-crossing longitudinal, at substantially increased levels in addition. These observations also showed that the pre- and post-crossing compartmentalization observed for native receptors was recapitulated in our paradigm, indicating that it relies on mechanisms that do not require more than the protein coding sequence. How is set the barrier that delineates these two main compartments is not known. Our laboratory made some attempts but failed so far to identify some significantly instructive mechanisms.

APPENDIX

Commissural axon navigation: Control of midline crossing in the vertebrate spinal cord by the semaphorin 3B signaling

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ABSTRACT

The mechanisms governing the navigation of commissural axons during embryonic development have been extensively investigated in the past years, often using the *drosophila* ventral nerve cord and the spinal cord as model systems. Similarities but also specificities in the general strategies, the molecular signals as well as in the regulatory pathways controlling the response of commissural axons to the guidance cues have been found between species. Whether the semaphorin signaling contributes to midline crossing in the fly nervous system remains unknown, while in contrast, it does play a prominent contribution in vertebrates. In this review we discuss the functions of the semaphorins during commissural axon guidance in the developing spinal cord, focusing on the family member semaphorin 3B (Sema3B) in the context of midline crossing in the spinal cord.

ARTICLE HISTORY

Received 11 May 2016
Revised 8 July 2016
Accepted 11 July 2016

KEYWORDS

axon guidance; commissural neurons; midline; semaphorins; spinal cord development

Introduction to the semaphorin family

The semaphorins form a large family of secreted and membrane-associated molecules present from virus to human.³² By activating a range of holoreceptors and downstream intracellular cascades, the semaphorins regulate the molecular machinery controlling actin and microtubule dynamics, thus contributing to a variety of processes implicating cell movements, from cell migration and axon migration in the nervous system, organ morphogenesis, to immunology and tumor metastasis.^{16,29,32} Many contributions of the semaphorins have been discovered in the developing and adult spinal cord, both in physiological and pathological contexts such as spinal cord injury, which exemplify the diversity of functional properties that these cues exert (O'Malley et al., 2014). In this review, we concentrate on the role of the secreted semaphorin 3B (Sema3B) during the formation of the spinal commissures.

Crossing the midline of the central nervous system: An obligatory step for all commissural axons

In bilateral organisms, multiple reciprocal neuronal projections interconnect the 2 halves of the central nervous system (CNS), forming a dense network of commissures that allow integration and coordination of left-right neuronal

activities.^{9,36} During embryonic and early postnatal developmental periods, commissural axons navigate through the central midline at all axial levels, crossing from one side of the CNS to the other one at specific time points and positions.⁹ Commissural axons can be surrounded by or mixed with ipsilateral axons that are committed to build circuits between neurons of the same side, and that never cross the midline. For instance, in the visual circuits of organisms with binocular vision, ipsilateral and contralateral ganglion cell axons exit the retina and navigate together, segregating at the optic chiasm when the contralateral axon tract achieves midline crossing.¹⁸ Similarly, tracts of ipsilateral and contralateral axons navigate in close proximity in the developing spinal cord⁴¹ (Fig. 1A).

Specialized groups of local cells lying at the CNS midline are instrumental in segregating the ipsilateral and contralateral axon populations, allowing only the latter to cross the midline (Evans and Bashaw, 2009^{9,36}). In the developing spinal cord, midline crossing takes place ventrally through the floor plate (FP), a crucial patterning center composed of glial cells, which contribute to the specification of the neuronal lineages of the neural tube and adjacent territories by secreting the morphogen SHH.^{17,24} The *drosophila* midline glia plays equivalent roles, secreting a TGF homolog to direct ventral cell fates

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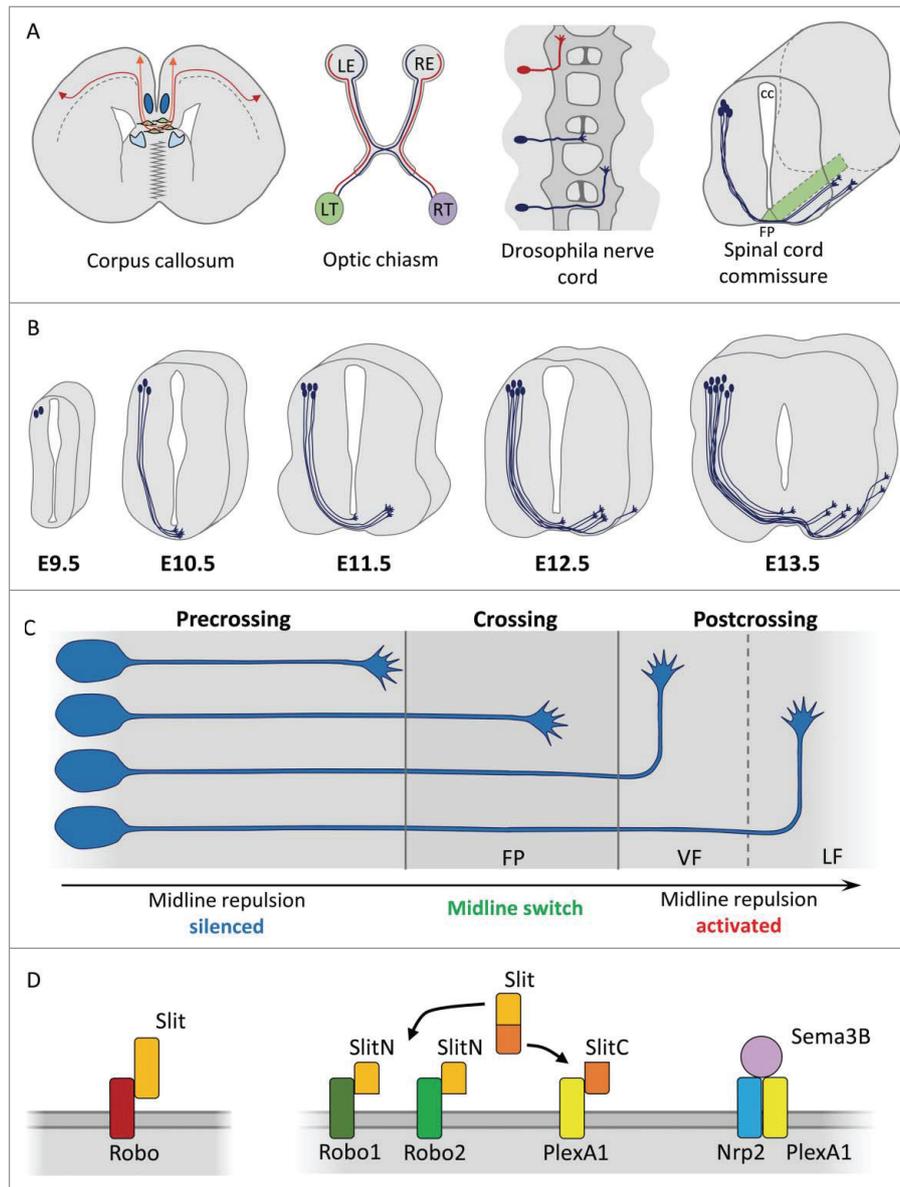


Figure 1. (A) Schematic representations of various commissural projections. In mammals, neocortical callosal axons (in red) turn medially and navigate toward the midline. They are guided by cues emanating from various sources: the midline zipper glia (in purple), the glial wedges (in light blue), the indusium griseum glia (in dark blue), and migrating neurons (in green). Ganglion cell axons exiting the retina connect both sides of the brain, forming ipsilateral (in red) and contralateral (in blue) tracts, the latter crossing the midline at the optic chiasm (LE: left eye; RE: right eye; LT: left thalamus; RT: right thalamus). In the *Drosophila* ventral nerve cord, ipsilateral (in red) and contralateral (in blue) neurons extend their axons medially toward the midline, but ipsilateral axons turn before crossing while contralateral ones turn after the crossing. In vertebrates, spinal commissural axons navigate first ventrally toward the floor plate (FP) lying at the ventral edge of the central canal (cc), cross the midline and then turn rostrally. (B) Temporal sequence of d11 commissural interneuron generation and axon navigation. The first spinal commissural neurons are born from a dorsal territory around E9.5. Some of them already extend their axons across the midline at E10.5. By E12.5, most of them have completed midline crossing and all navigate post-crossing longitudinal routes by E13.5. (C) Temporal sequence of guidance signaling controlling the navigation of the midline. As they navigate toward the FP, the sensitivity to midline repellents is silenced in spinal commissural growth cones. During FP crossing, commissural growth cones gain responsiveness to these FP repulsive cues, which prevent them from re-crossing and drive them out of the FP. At the exit, they follow rostro-caudal gradients of guidance cues, turning rostrally in the ventral (VF) or lateral (LF) funiculus. (D) Representation of the principal signaling implicated in the midline repulsion. Slit/Robo in the *Drosophila*, Robo1-2/SlitN, PlexA1/SlitC, and Sema3B/Nrp2/PlexA1 in the mouse.

of the CNS, the ectoderm and the mesoderm.⁵ The FP also starts expressing guidance molecules at these early stages. It was recently discovered that semaphorin 3B (Sema3B) already has an instructive role prior to the stage of axon navigation. Sema3B is already detected at E9 and is secreted in the cerebrospinal fluid. Collected by receptors of neuroepithelial progenitors that undergo mitosis at the apical border lining the central canal, Sema3B triggers an intracellular signaling which regulates microtubule stability and promotes planar orientation of the cell divisions at the onset of the neuro-genesis.² Beyond this function, the FP source of Sema3B has been well characterized for its contribution to commissural axon guidance, a role that we will discuss in detail below.

Commissural axon navigation in the spinal cord: The prototypical dl1 tract

The dorsal interneuron lineages in the developing spinal cord are specified by transcriptional programs, according to their position, birthdate and pattern of connections. Various classes of commissural interneurons have been identified among which the commissural component of the dl1 population, born from a Math1⁺ progenitor pool that lies close to the roof plate and which also generates an ipsilateral component.²² dl1 commissural neurons, specified by Lhx2/9 transcription factors,⁴⁴ elaborate a typical pattern of axonal projections, whose navigation has been widely investigated. The axons extend ventrally toward the FP, cross the midline, exit the FP and turn rostrally to ascend toward supraspinal levels, to convey proprioceptive information to the cerebellum.¹ The generation of transgenic mice expressing LacZ or GFP under the control of the Math1 promoter has allowed a precise spatial and temporal mapping of commissural dl1 axon navigation in the mouse embryo. Born from around E10, dl1 commissural neurons extend waves of axons toward the FP starting at this stage. Some of them already cross the midline as early as E10.5. It is widely accepted that most of them have crossed the midline by E12.5, and are already navigating distant longitudinal routes at E13^{11,25,26,27,30,44} (Fig. 1B).

Repulsive signaling controlling midline crossing in the vertebrate spinal cord and invertebrate nerve cord

The FP and midline glia are sources of both attractive and repulsive cues for commissural axons. A temporal sequence has been proposed which orchestrates the different steps of commissural axon navigation by controlling axon responsiveness to the FP attractive and

repulsive cues, thus preventing conflict of guidance information. First, the commissural growth cones perceive chemoattractants, including Netrin, which orient their trajectory toward the midline. Commissural growth cones then interact with local cells to navigate the FP/midline glia. Next, upon crossing, they gain responsiveness to FP/midline glia-derived repulsive cues, which prevent them from turning back and re-crossing the midline, and also push them out of the FP toward the contralateral side. Finally, after FP exit, rostro-caudal gradients of guidance cues elicit a longitudinal turning of commissural axons, accompanied by a sorting of the axons into the ventral and lateral funiculi in which they navigate rostrally (Fig. 1C).

Such a temporal sequence of guidance programs relies on a tight control of growth cone sensitivity to the guidance cues. In particular, the sensitivity to the midline repellents must be first silenced before crossing to be triggered only after the crossing. The premature action of the repellents would prevent the axons from entering the FP.

The repulsive signaling found to regulate midline crossing in vertebrates and invertebrates present some degrees of conservation, although vertebrates evolved significant differences.³⁶ In *Drosophila*, midline repulsion was shown to be ensured by SLIT, a gene encoding a protein which acts through binding to Roundabout (Robo) Robo 1 and Robo2 receptors on commissural axons. Similarly, in vertebrates, 3 SLIT genes (Slit1,2,3) were shown to act in synergy to control midline crossing, mediating their effects through Robo1 and Robo2 receptors.⁶

The N-terminal (140 kDa) and C-terminal products (55-60 kDa) resulting from Slit protein cleavage were recently found to both contribute to the FP navigation in vertebrates. Until this recent work, Slit-Ns, which contain the binding sequence for Robo receptors were logically considered as the bioactive protein fragments. In contrast, the Slit-C fragments, for which no receptor was identified, were thought to be inactive. From initial analysis of mutant mouse models, deletion of Slit1-3 or Robo1/2 genes in mice were both known to disturb commissural navigation. However unexpectedly, phenotypic differences were noted between the 2 deletion contexts. Midline re-crossing was observed after Slit1-3 but not Robo1/2 deletion.^{27,33} These studies raised the idea that Slits exhibit some Robo-independent functions that might be mediated by an as yet-unknown receptor. This receptor turned out to be PlexinA1,¹⁵ a receptor shared by members of another prominent axon guidance family, the semaphorins.³² Indeed, PlexinA1 deletion was found to induce the re-crossing phenotype, and also to confer the re-crossing phenotype to Robo1/2 mutants. Various combinations of PlexinA1 and Slit1/2/3 allelic deletions also confirmed that Slits and PlexinA1 both participate

in preventing midline re-crossing during commissural axon guidance. Moreover, biochemical analysis revealed that PlexinA1 binds Slit full-length and Slit-Cs, but not Slit-Ns, and mediates a repulsive action of the Slit-C fragments.¹⁵ Slit processing also occurs in invertebrates. However, in transgenic rescue assays in *Drosophila*, expression of a Slit mutant that resists proteolytic cleavage in the midline glia lineage was shown to rescue midline crossing defects resulting from general Slit loss equally as well as the wild type Slit, thus supporting that Slit processing is dispensable for midline navigation.¹³

PlexinA1 was identified in previous work as the Plexin A member which associates with Neuropilin2 (Nrp2). Nrp2/PlexinA1 complex forms a functional commissural receptor for Semaphorin 3B, a semaphorin that was demonstrated to act as a FP repellent for post-crossing commissural axons in the mouse.^{34,38} Mouse embryos lacking Nrp2 were shown to present FP crossing defects consistent with a role of semaphorin ligands as repellents for commissural axons after midline crossing. Among the class 3 semaphorins, Semaphorin 3B expressed by FP cells, exhibits the expected profile for exerting this role. In contrast, Semaphorin 3F, another prominent Nrp2 ligand, instead of being expressed by the FP, localizes in a domain adjacent to the FP.^{34,38} Consistently, Semaphorin 3B deletion was reported to result in alterations of FP crossing and these defects were phenocopied in PlexinA1 null mutant embryos.³⁴ Implication of semaphorins in the FP navigation of spinal commissural axons also came from studies in the chick model. Over-expression of a dominant negative PlexinA, abolishing the signaling by all PlexinAs, resulted in strong alterations of crossing and post-crossing axon trajectories.^{15,34} Similarly, specific knock-down of individual PlexinAs such as PlexinA1, PlexinA2 and PlexinA4, all induced stalling at the FP exit and failure of rostral turning.¹

Altogether, these studies suggest that several repulsive signaling mechanisms operate during FP crossing in the mouse, such as Slit-N/Robo, Slit-C/PlexinA1 and Semaphorin 3B/PlexinA1-Nrp2 (Fig. 1D). How these signals are acting and which specific aspects of commissural growth cone behavior they control remains unclear. Mouse models allowing the investigation of the specific contributions of Slit-Cs and Slit-Ns still lack, in part because the nature of the protease(s) responsible for Slit cleavage remains unknown.

Silencing of the semaphorin signaling during FP navigation

Based on a variety of experimental set-ups, several studies provided evidence that the commissural axon responsiveness to Slits and Semaphorin 3B-derived FP repellents is

silenced before the crossing. In early work, spinal cord open books were co-cultured with COS cell aggregates secreting Slits or Semaphorin 3B.⁴⁷ The behavior of commissural axons emerging from the explant border facing the cell aggregates was examined. In these spinal cord preparations, the endogenous FP was removed to mimic a pre-crossing context, so that commissural axons exiting the ventral side of the open-book had not experienced FP crossing in their native tissue before growing out of the explant. Under these conditions, similar growth toward control, Semaphorin 3B or Slit2-secreting cell aggregates was observed, indicating a lack of sensitivity of commissural axons to Semaphorin 3B and Slit2. These experiments were reproduced for Semaphorin 3B and the data were confirmed in more recent works^{10,15,38} (Fig. 2A).

A second assay was used to demonstrate the lack of sensitivity to FP repellents at the pre-crossing stage which consisted in grafting FP and roof plate (RP) tissues as well as cell aggregates along the lateral side of an intact spinal cord. This assay challenges the ability of cues released by the ectopic tissue or the cell aggregate to re-route the trajectory of commissural axons toward or away from their natural path to the endogenous FP.⁴⁷ Such a model had been developed to show that the RP releases a repellent for pre-crossing commissural axons, whose action is to orient their initial trajectory toward the ventral side of the spinal cord.³ Using this assay, commissural axons were found re-routed toward FP tissue, and Netrin1 expressing cell aggregates, reflecting that they are subjected to attractive cues. In contrast to ectopic RP, both Slit2 and Semaphorin 3B-secreting cell aggregates failed to deflect commissural axons away, confirming their lack of sensitivity to the FP repellents prior to crossing (Fig. 2B).

Finally, a last paradigm was employed in several studies, to assess the individual response of commissural axons to Semaphorin 3B.^{15,34,43} Growth cone behavior to Semaphorin 3B application was examined in dissociated commissural neuron cultures collected from E11 to E13, which revealed their inability to undergo the collapse response, normally observed when growth cones perceive repulsive cues (Fig. 2C). Collapse assays were also conducted on dorsal spinal cords (lacking endogenous FP) collected from *Atoh1*-tauGFP and *NeuroG2*-tauGFP, transgenic mouse embryos, GFP identifying the dl1 and dl4 populations of commissural interneurons respectively. Both populations were found unresponsive to Semaphorin 3B.⁴³

Activation of the semaphorin signaling after midline crossing

Spinal cord open-books and commissural neuron cultures were used to establish that commissural axons acquire sensitivity to the FP repellents after the crossing.

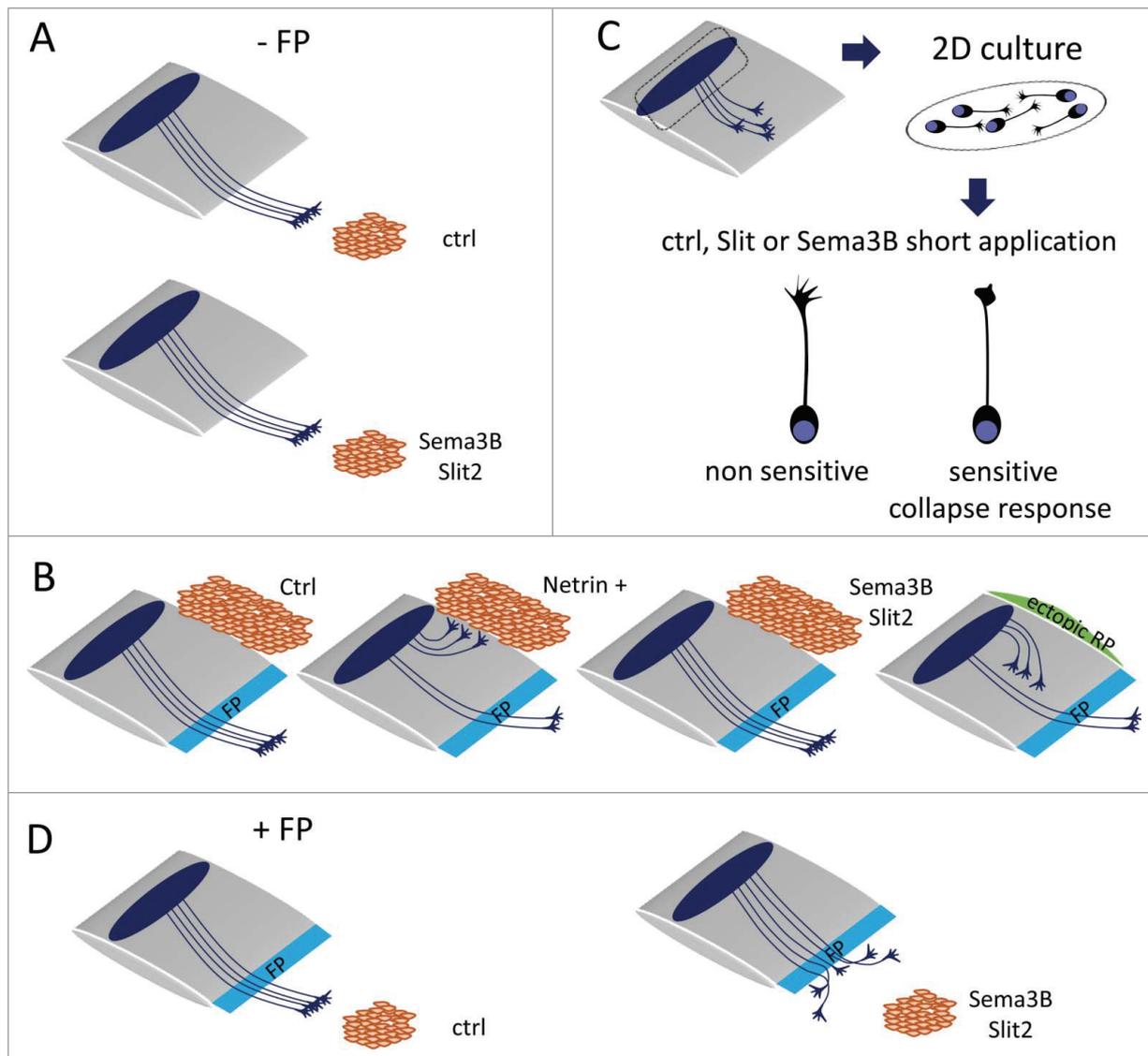


Figure 2. Experimental paradigms to assess commissural axon sensitivity to FP repellents. (A) Spinal cord open-book co-cultured with control (ctrl) COS cells or COS cells secreting Sema3B or Slit2. In the absence of endogenous FP, comparable axonal growth is observed toward ctrl and Sema3B or Slit2-secreting aggregates. (B) Ectopic graft of COS cells along the lateral side of the spinal cord open-book. Netrin1-secreting aggregate induces a re-routing of pre-crossing commissural fibers toward the ectopic cells. Sema3B and Slit2-secreting ectopic aggregates do not deflect pre-crossing commissural axons. Lateral grafting of roof-plate tissue (RP) re-routes commissural axons away from the ectopic tissue. (C) Collapse assay on dissociated neurons dissected from the dorsal spinal cord and grown in cultures. Growth cones responsive to repulsive cues collapse after short-term application of the cues, whereas unresponsive growth cones remain intact. (D) In the presence of the endogenous FP in the open-book, the growth of commissural axons emerging from the tissue toward the Sema3B or Slit2 sources is inhibited.

First, the behavior of commissural axons emerging in front of a Sema3B or Slit-secreting cell aggregate was examined in open-books in which the endogenous FP was left intact. In contrast to what was observed when the FP had been removed, the growth of commissural axons that experienced FP crossing was strongly prevented by Slit2 and Sema3B released from the cell aggregate⁴⁷ (Fig. 2D). Second in collapse assays, commissural growth cones insensitive to Sema3B at basal condition

acquired a strong collapse response induced by application of FP conditioned medium (FP^{cm}). The sensitization was obtained in condition of co-application but also when FP^{cm} was applied first and Sema3B treatment was applied after washing. This indicated that some FP signals prime commissural growth cones for Sema3B repulsion.^{10,34} In collapse assays conducted on intact open-books from Atoh1-tauGFP and Neurog-tauGFP transgenic mouse embryos (having endogenous FP

through which the axons navigate), dl1 but not dl4 commissural growth cones were reported to collapse in response to Sema3B. Thus, some cell-type specificities might exist in the responsiveness of post-crossing commissural axons to the FP repellents.⁴³

Mechanisms mediating pre-crossing silencing of repulsive signaling

The control of the sensitivity of commissural axons to midline repellents has been the topic of extensive investigations over the years, and pioneered by studies of midline crossing in the drosophila ventral nerve cord. This work uncovered key molecular mechanisms acting within commissural neurons to control guidance receptor trafficking and consequently responsiveness to midline repellents. Thereafter, in vertebrates, a panel of molecular mechanisms which regulate guidance receptors has been discovered, showing that, similar to the control of midline crossing in drosophila, strict control of receptor distribution and function is required for setting the temporal sequence of responsiveness to midline repellents.³⁶

Silencing by receptor degradation

In drosophila, the silencing of pre-crossing commissural axons to Slit repulsion was found to be achieved by active degradation of Robo receptors. The endosomal protein Comm plays a key role in preventing the presence of Robo at the growth cone surface, acting essentially but not exclusively by sorting the receptor to lysosomal degradation. After crossing, Comm is down-regulated, and as a consequence, Robos become available in commissural growth cones to transduce the Slit repulsive signal.³⁶ A key aspect of this regulatory pathway thus resides in the spatio-temporal control of Comm expression to restrict its action to the period of axon crossing. It was recently discovered that cleavage of Frazzled/DCC in commissural neurons, releases an intracellular fragment that acts as a transcriptional activator of Comm.³⁶

Silencing by receptor-receptor trans interactions

A second mechanism has been recently reported, which complements the Comm action by blocking the Robo receptors that start reaching the commissural growth cone surface before the crossing is completed. Such a situation is likely to occur, during crossing, after the onset of Comm down-regulation. This mechanism is mediated by trans interactions of this cell surface pool of Robo with Robo2 expressed by midline glial cells, which result in preventing Slit binding or activity. Such coupled

mechanisms might ensure the robustness of the spatial and temporal control of Robo receptor availability during midline crossing¹⁹ (Fig. 3A).

Notably, although the pre-crossing silencing of the midline repellents is conserved in vertebrates, the underlying mechanisms appears to differ sharply from those identified in the fly. First, the vertebrate genome was found to lack Comm. Second, active degradation of Robo receptors at the pre-crossing stage has not been reported yet. However, in the chick embryo, Robo1 was reported to distribute in intracellular vesicles, which might be the mechanism that maintains cell surface Robo at low levels before the crossing³⁹ (Fig. 3B). In addition, the silencing of Slit signaling was proposed to be achieved by Robo3, a divergent Robo family member having several binding partners including DCC and neural epidermal growth factor-like-like 2 (NELL2).^{28,46} An isoform of Robo3 gene, Robo3.1 was reported to have a distribution restricted to the pre-crossing commissural axons. *In vivo* manipulations in chick and mouse embryos resulted in alterations of FP navigation, consistent with a function in blocking Robo/Slit activity before the crossing.¹¹ How the silencing of Slit-Robo signaling by Robo3.1 is achieved remains to be understood.

Silencing by prevention of receptor cell surface sorting

Although distinct from degradation, the sensitivity of spinal commissural axons to Sema3B also appears to be controlled through regulation of the Sema3B receptor. The signaling moiety of the complex, PlexinA1, was found to be a target of calpains, proteases known to process rather than degrade targets, capable of modulating both their functions and binding partner interactions.^{8,45} Active calpain was shown to cleave PlexinA1, as well as other PlexinAs, generating 2 distinct PlexinA1 fragments. Both PlexinA1 integral and cleaved fragments could be detected by immunoblotting of lysates of dorsal spinal cord tissue. Treatment of fresh dorsal spinal cord tissue with calpain inhibitor prior to immunoblotting induced an increase of full-length PlexinA1 at the expense of the cleaved forms.³⁴ In the developing spinal cord, Nrp2 and PlexinA1 transcripts are both detected in commissural neurons at stages of pre-crossing navigation. Nevertheless, at protein levels, differences between the 2 receptor distributions were observed in embryonic immunolabelled sections. Using an anti-PlexinA1 antibody directed against an extracellular epitope and likely recognizing the integral protein, very low labeling was found in pre-crossing axon segments in stark contrast labeling was very strong on crossing and post-crossing axon segments.³⁴ Interestingly, Nrp2 was detected in both pre-crossing and post crossing commissural axon

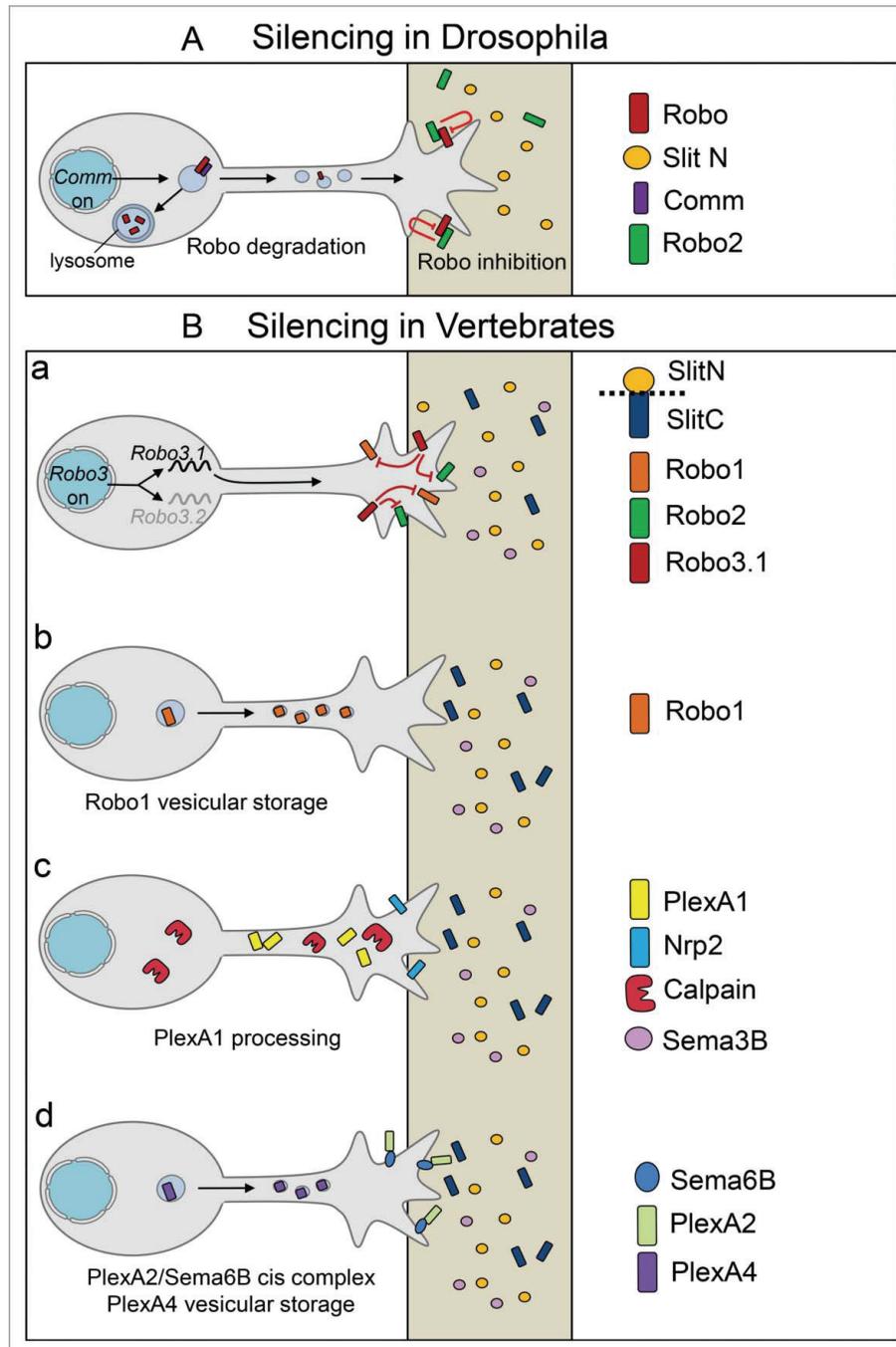


Figure 3. Mechanisms reported to mediate pre-crossing silencing to midline repellents. (A) In drosophila, endosomal protein Comm silences Slit responsiveness in pre-crossing axons, by sorting the majority of Robo receptors to the lysosomal degradation. Robos that escape the degradation and reach the growth cone surface are inhibited via a trans-interaction by Robo2 expressed by midline glial cells. (B) (a) In the mouse, the isoform Robo3.1 of the Robo3 gene is expressed in the pre-crossing axons and the resulting protein antagonizes Slit-Robo signaling. (b) In the chick, Robo1 is trafficked to vesicles to maintain it at low levels at the pre-crossing stage. (c) In the mouse, pre-crossing commissural axons express Nrp2 at their surface but only low levels of PlexinA1, whose cell surface expression is prevented through processing by Calpain proteases. (d) In the chick, PlexinA2 and Sema6B form *cis* complex in pre-crossing commissural axons and PlexinA4 traffics in vesicles and is excluded from the growth cones.

segments. *Ex vivo*, immunolabeling of PlexinA1 in DCC⁺ commissural axons emerging from spinal cord open-books with and without endogenous FP revealed that integral PlexinA1 labeling was only observed in

commissural axons that experienced FP crossing. Finally, *in vivo* experiments consisting of expressing PlexinA1 fused to the pH sensitive GFP pHluo, a selective reporter of the cell surface protein pool, in commissural neurons

of the chick embryo showed that in a very large majority of the cases, the green fluorescence was detected in commissural growth cones undergoing FP crossing.³⁴ The link between calpain-mediated PlexinA1 processing and commissural axon sensitivity to Sema3B was further investigated using *in vitro* and *in vivo* approaches. Inhibiting calpains was sufficient to confer a growth cone collapse response of commissural neurons to Sema3B. Administration of a pharmacological calpain inhibitor to pregnant mice resulted in strong alteration of FP crossing in the embryos. Commissural axons stalled at the FP entry, consistent with the acquisition of a premature sensitivity to Sema3B preventing them from entering the FP due to increased PlexinA1 cell surface levels. Similarly, overexpression of PlexinA1 leading to increased PlexinA1 levels at the pre-crossing stage also resulted in stalling at the FP entry.³⁴ Thus, post-translational regulation of PlexinA1 levels appears to be a first mechanism to prevent pre-crossing commissural axons from responding to Sema3B (Fig. 3B).

Silencing by ligand-receptor cis and trans complex

A variety of additional mechanisms have been characterized to control the semaphorin signaling during midline crossing. First, macro-complexes of receptors were shown to orchestrate midline crossing in the optic chiasm of vertebrates. NrCAM, which participates in the semaphorin signaling, and PlexinA1 from midline glia cells were found to interact with NrCAM and PlexinA1 from retinal commissural axons, temporarily switching repulsive effects of Sema6B at the midline into attraction, to allow the crossing.³¹

Second in the chick embryo, a recent study investigated the PlexinA/Semaphorin signaling during spinal commissural axon guidance.¹ PlexinAs were noted to have dynamic spatio-temporal expression patterns, with some members being expressed by commissural axons and FP cells such as PlexinA1 and PlexinA2, and others expressed only by commissural axons such as PlexinA4. Knock-down of individual Plexin A1, -A2, and -A4 in commissural neurons were all found to result in commissural axon stalling at the FP exit and failure of post-crossing rostral turning. Specific knockdown of FP-PlexinA2 also resulted in stalling, as did so the specific knock-down of Sema6B, which is endogenously expressed in commissural neurons. Thus, this identified a first signaling for crossing and post-crossing commissural axons arising from Sema6B acting as a commissural receptor for FP-PlexinA2, acting non-cell autonomously as a ligand.¹ In addition, PlexinA2 over-expression in pre-crossing commissural neurons strongly altered their ability to reach the FP, a phenotype that was interpreted

as resulting from oversensitivity to ventral spinal cord/FP repellents. The observation in cultured commissural neurons that Sema6B co-localized with PlexinA2 led the authors to propose a model whereby prior to the crossing, the Sema6B/PlexinA2 cis complex prevents commissural axons from sensing the FP repellents. The mechanism that silences the PlexinA4/Semaphorin signaling might be different. In cultured commissural neurons, while PlexinA2 was detected along axon shafts and growth cones together with Sema6B, PlexinA4 was reported to have in contrast a vesicular punctate pattern, and was excluded from the growth cones.¹

Overall, these mechanisms illustrate in various contexts that the silencing of semaphorin repellents is achieved through cell autonomous mechanisms desensitizing pre-crossing commissural axons, by controlling the cell surface sorting or the signaling activity of PlexinAs (Fig. 3B).

Silencing by ligand trapping

Recently, long-term (24h) application of Sema3B to dorsal spinal cord explant cultures was reported to result in reduced axon growth, with strong inhibition at high dose.²³ These data suggested that commissural axons at the pre-crossing stage might be able to perceive Sema3B. Indeed, since several previous studies failed to detect any Sema3B repulsion and collapse on pre-crossing commissural axons using a panoply of different paradigms^{15,34,38,43,47} it could be that Sema3B exerts 2 distinct and independent effects on commissural axons, acting as a growth regulator at the pre-crossing stage and a repulsive cue at the post-crossing stage.

How the growth-inhibition effect is achieved and whether it is mediated by PlexinA1, as suggested by the authors, remains to be determined. It could be that the pre-crossing sensitivity of commissural axons to Sema3B reported by the authors is mediated by other PlexinAs, several being expressed by commissural growth cones³⁴ This would be consistent with previous findings that the repulsive post-crossing response is conferred by a dual mechanism which first prevents PlexinA1 to be available at the growth cone surface before the crossing and second triggers cell surface expression when commissural axons navigate the FP. It could also be that low levels of PlexinA1 present in pre-crossing commissural growth cones are sufficient for Sema3B to elicit a long-term growth response, but not to produce a repulsive effect. The implication of PlexinA1 was suggested by immunohistochemistry with home-made antibody directed against an C-terminal epitope of PlexinA1, which was observed to label pre-crossing commissural axons,²³ while a commercial antibody directed against the extracellular PlexinA1 domain only revealed substantial

PlexinA1 levels in crossing and post-crossing axons.¹⁰ Since PlexinA1 cleaved fragments were found present in lysates from dorsal commissural tissue,³⁴ it could be that these PlexinA1 forms are those recognized in the pre-crossing commissural axons. Whatever the case, PlexinA1 contribution could be addressed by blocking PlexinA1 in commissural explants to examine whether long term Sema3B exposure still elicits growth-inhibition.

Beyond, is this growth inhibition acting at the pre-crossing stage or is it silenced as is Sema3B repulsion? In their study, the authors reported that deletion of Nrp2 in the FP affects the commissure formation. Measures of the ventral part of the pre-crossing tract and the crossing tract in embryonic transverse sections showed reduced thickness in the mutants compared with wild-types, suggesting that FP-Nrp2 has a non-cell autonomous role. Whether this defect results from loss of fibers, growth delay or increased fasciculation remains to be determined. The reduction of commissure thickness was no longer observed when FP-Nrp2 deletion was combined with a general loss of PlexinA1. In the scenario proposed by the authors, Nrp2 would trap Sema3B in the FP, thus silencing Sema3B by making it inaccessible to commissural axons. FP-Nrp2 deletion would then result in Sema3B release, inducing pre-crossing growth inhibitory effect and subsequently reduction of the ventral commissure (Fig. 4A). Additional ablation of PlexinA1 would then induce pre-crossing commissural axons to lose their sensitivity to Sema3B, thus rescuing the normal size of the commissure.

According to this model, Sema3B-mediated growth inhibition would not play an instructive role at the pre-crossing stage and needs to be suppressed. The transition toward sensitivity to Sema3B would not be triggered by changing of Sema3B responsiveness between the pre-crossing and the post-crossing stages. Rather it would be achieved through unmasking of Sema3B after the crossing, proposed by the authors to result from downregulation of Nrp2 transcripts. Nevertheless, Nrp2 is detected in the FP over the entire period of FP navigation (from E10 to E13.5 on the least;^{7,19,34} Hernandez-Enriques et al, 2015), during which asynchronous waves of commissural axons navigate the FP. Thus without any changing between pre-crossing and post-crossing stages, it is difficult to understand how this sole mechanism would be responsible for switching on Sema3B repulsion. Moreover, interpretations of the mouse phenotypes are particularly complex. Indeed, first, not only Nrp2 but also PlexinA1 is expressed by both commissural axons and FP glial cells. Second as shown in the chick embryo for PlexinA2,¹ FP-PlexinA1 could have non cell autonomous functions. Thus, a key experiment would be to test which of commissural- or FP-specific PlexinA1 deletion rescues the ventral commissure thickness in context of FP-Nrp2 deletion.

An alternative scenario can be proposed, which would fully mirror the mechanisms of Slit silencing by Robo/Robo2 interactions in the drosophila context.¹⁹ Rather than trapping Sema3B, FP-Nrp2 (complexed or not with PlexinA1) could interact *in trans* with Nrp2 on commissural axons approaching and entering the FP. This receptor trapping would silence Sema3B responsiveness until the crossing is accomplished by preventing axonal Nrp2 from forming *cis* complexes with the PlexinA1 receptor pool accumulating at the cell surface (Fig. 4B). FP-Nrp2 ablation would prevent this effect, resulting in premature action of Sema3B. Loss of PlexinA1 would desensitize commissural axons to Sema3B, thus rescuing the commissure. In this scenario, both cell autonomous (axonal PlexinA1 processing) and non-cell autonomous (via FP-Nrp2 and possibility FP-PlexinA1) would act in synergy to accurately control the silencing of pre-crossing/crossing commissural axon responsiveness to Sema3B.

Finally, the outcome of Sema3B trapping by Nrp2 might not be to mask Sema3B but rather to control its spatial distribution, restricting the cue to the FP, where it could be active to slow down commissural axon growth. Previous work already established that cues released by the FP regulate the outgrowth of commissural axons. Such a property was reported for the Stem Cell Factor (SCF), which promotes the growth of post-crossing commissural axons.²¹ Thus a balance of growth-promoting and growth-inhibitory effects could set a precise temporal pattern of FP navigation, adapting growth cone motility to the guidance decisions that have to be made.

Mechanisms controlling the transition from pre-crossing silencing to post-crossing sensitization to Slit and Sema3B repellents

The mechanisms controlling the switch of sensitivity to midline repellents after the crossing also appear to be highly diverse, depending on the signaling and the species (Fig. 5). In drosophila, down-regulation of COMM allows Robo to accumulate at the cell surface of commissural growth cones, resulting in the gain of sensitivity to Slit.²⁰ Studies conducted in the chick model revealed that Robo1 cell surface expression is up-regulated between the pre-crossing and post-crossing navigation, through transcriptional control of RabGDI, a key component of the exocytosis machinery.³⁹ Although different, these 2 mechanisms have in common that they control the temporal activity of Slit-Robo signaling at the midline by regulating guidance receptor cell surface levels. In the mouse, transition from Robo3.1 at the pre-crossing stage to Robo3.2 at the post-crossing stage was proposed to

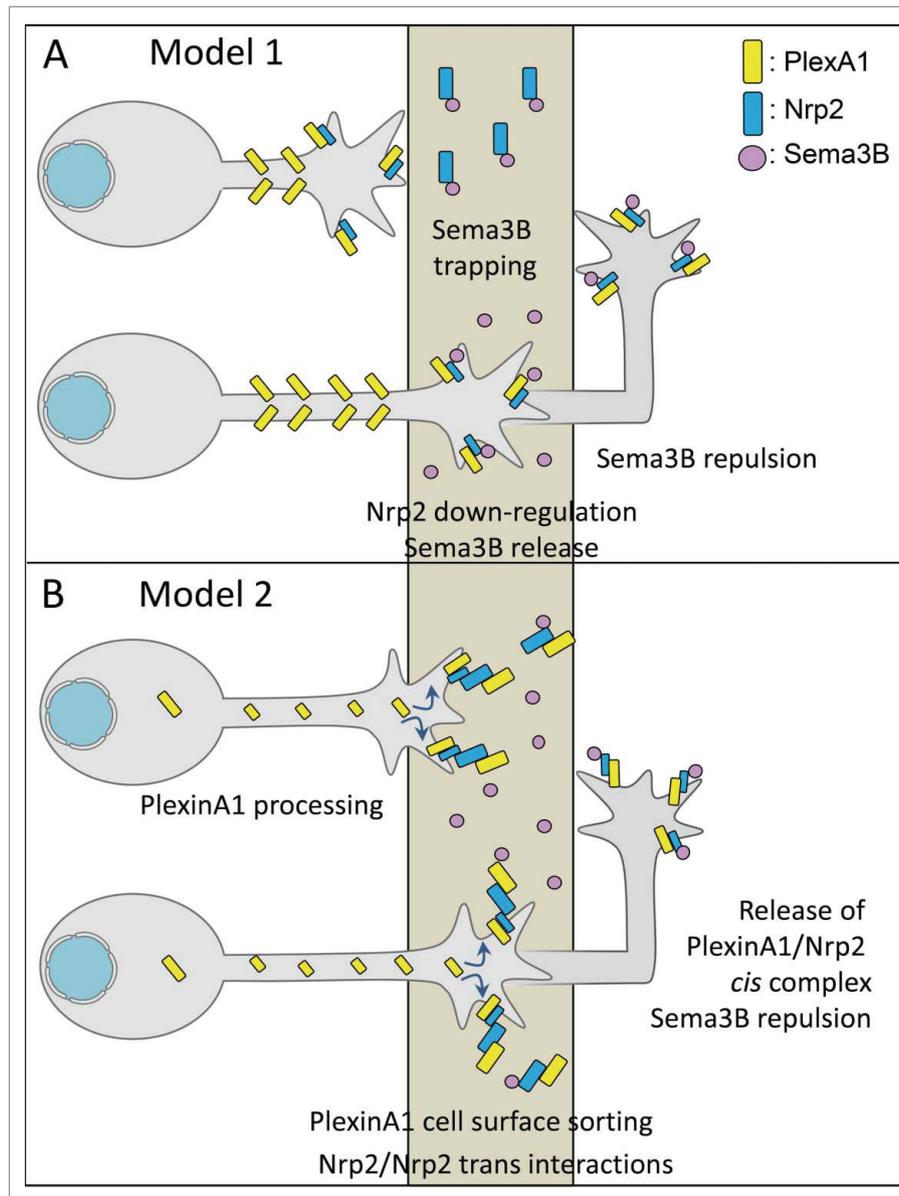


Figure 4. Models for the regulation of the semaphorin signaling from pre-crossing to post-crossing. (A) In this model, PlexinA1 and Nrp2 are both expressed at the growth cone surface of pre-crossing commissural axons. Their sensitivity to Sema3B is prevented by trapping of Sema3B by FP-Nrp2. After the crossing, Nrp2 is transcriptionally downregulated in the FP at E13, which releases Sema3B and allows repulsion. (B) In this model, cell surface PlexinA1 is kept at low levels in pre-crossing commissural axons, to desensitize them to Sema3B. Upon crossing, calpain activity is suppressed by FP GDNF, PlexinA1 reaches the growth cone surface and can associate with Nrp2. The receptor complex activity is blocked by FP Nrp2 and PlexinA1, until the crossing is achieved. After the crossing, the complex is functional for Sema3B repulsion.

switch on the sensitivity to Slit repellents.¹¹ How this transition is accomplished has been partially resolved by the findings that Robo3.2 mRNA is locally translated in crossing commissural axons, under the action of FP signals.¹²

The release of Sema3B silencing has been investigated in the mouse model, with the goal to identify cues present in the FP conditioned medium which conferred a collapse response of commissural growth cones to Sema3B. Two FP

cues were identified acting in synergy, the Ig SuperFamily Cell Adhesion Molecule NrCAM, probably released by ectodomain shedding, and the neurotrophic factor GDNF, which was found to provide the major contribution.¹⁰ Double GDNF/NrCAM deletion in mice resulted in strong alteration of PlexinA1 levels in crossing/post-crossing commissural axons, with synergistic effects compared with the single knockouts. Reductions of PlexinA1 levels were also correlated with FP crossing defects. In co-cultures of

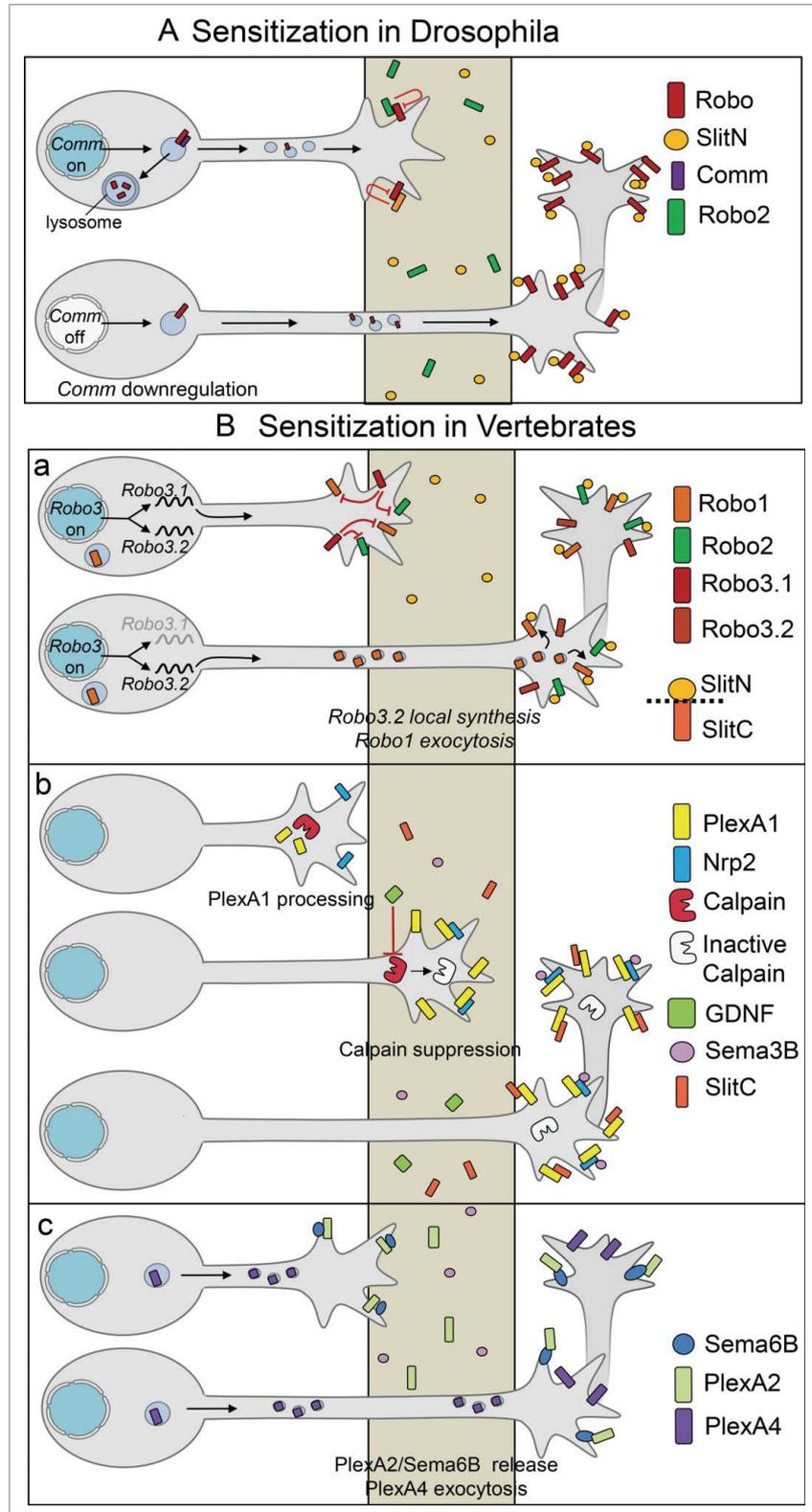


Figure 5. Mechanisms releasing the pre-crossing silencing and mediating sensitization to midline repellents. (A) In drosophila, after the crossing, Comm protein is down-regulated and Robo proteins are sorted at the growth cone surface to transduce the Slit repulsive signal. (B) (a) In the mouse, upon crossing, Robo3.1 is replaced by Robo3.2, locally synthesized under local FP triggers, which acts as an agonist of the Slit-Robo signaling. In the chick, exocytosis of Robo1 is activated by transcriptional up-regulation of RABgdi, and the receptor is sorted at the cell surface. (b) In the mouse, upon the crossing, GDNF secreted by FP glial cells inhibits Calpain activity and allows PlexinA1 to reach the surface, to associate with Nrp2 and to mediate Sema3B repulsive response. Gain of cell surface PlexinA1 also switches on repulsion by Slit-C fragments. (c) In the chick, the pre-crossing PlexinA2/Sema6B *cis* complex is released, and replaced by a Sema6B/PlexinA2 *trans* interaction, releasing commissural PlexinA2 which become available for semaphorin repulsion. PlexinA4 might be sorted at the cell surface to also mediate semaphorin repulsion.

dorsal spinal cord explants with COS cell aggregates, commissural axons were found to gain repulsion to *Sema3B* when the cell aggregate secreted both *Sema3B* and *GDNF*, compared to aggregate only secreting *Sema3B*. Both *GDNF* and *NrCAM* increased *PlexinA1* levels in the growth cones of cultured commissural neurons. *GDNF* acting independently from *RET* via the *NCAM* receptor and *GFR α 1*, both expressed by commissural axons, was able to inhibit calpain activity, switching off the mechanism ensuring *Sema3B* silencing. An additional FP cue, *SHH*, was reported to trigger gain of sensitivity to *Sema3B*, by down-regulating the activity of cAMP-dependent protein kinase A (PKA) in commissural growth cones.³⁸ Thus overall, these studies support that release from the pre-crossing *Sema3B* silencing is triggered by FP cues, through changes of the guidance machinery of commissural axons.^{10,34}

In the chick, the model proposed is that the *PlexinA2*/*Sema6B* cis complex formed in pre-crossing commissural axons would be released, allowing FP-*PlexinA2* to bind to *Sema6B* in trans, and commissural *PlexinA2* to bind to FP semaphorin repellents. In addition, as is the case for *PlexinA1* in the mouse, *PlexinA4* might be sorted to the growth cones during FP crossing to allow them to sense the semaphorin repellents.¹

Thus in conclusion, given the diversity of possibilities by which the semaphorin signaling can be modulated, significant issues remain unclear. In particular, it will be important to better characterize the dynamics of *Plexins*, *Neuropilin* receptors and their semaphorin ligands as well as their cell-autonomous and non-cell autonomous functions. Addressing these questions is required to obtain a clear picture of how the silencing of midline repellents is achieved and released during spinal commissural axon navigation of the FP.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Pr. Edmund Derrington, Dr. Servane Tauszig-delamasure and Dr. Céline Delloye-Bourgeois for proofreading the manuscript and helpful comments.

Funding

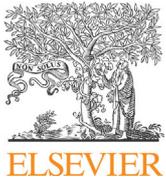
VC is supported by funding from the LABEX CORTEX and Labex DevWeCAN of Université de Lyon, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

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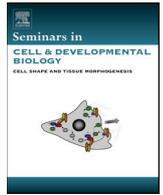
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Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

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Review

Commissural axon navigation in the spinal cord: A repertoire of repulsive forces is in command

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ARTICLE INFO

Article history:
Received 4 October 2017
Received in revised form
11 December 2017
Accepted 11 December 2017
Available online xxx

Keywords:
Axon guidance
Commissural axon
Midline crossing
Guidance signaling
Repulsion
Attraction
Growth cone

ABSTRACT

The navigation of commissural axons in the developing spinal cord has attracted multiple studies over the years. Many important concepts emerged from these studies which have enlighten the general mechanisms of axon guidance. The navigation of commissural axons is regulated by a series of cellular territories which provides the diverse guidance information necessary to ensure the successive steps of their pathfinding towards, across, and away from the ventral midline. In this review, we discuss how repulsive forces, by propelling, channelling, and confining commissural axon navigation, bring key contributions to the formation of this neuronal projection.

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1. Introduction

Early theories of chemotropism and chemoaffinity by Ramon Y Cajal and Sperry provided the basis for more than a century of research on axon guidance mechanisms [1,2]. These theories pos-

tulated the existence of molecules acting at long and short distances to attract the axon terminal, the growth cone. Their role was postulated to keep the axons along their proper path and to guide them towards their targets. Unanticipatedly from these theories, repulsive effects of axon guidance molecules turned out to provide major forces driving axon navigation. In 1984, Haydon and collaborators, using video-time lapse microscopy in neuronal cultures, reported that serotonin has a neuron-type specific inhibitory effect

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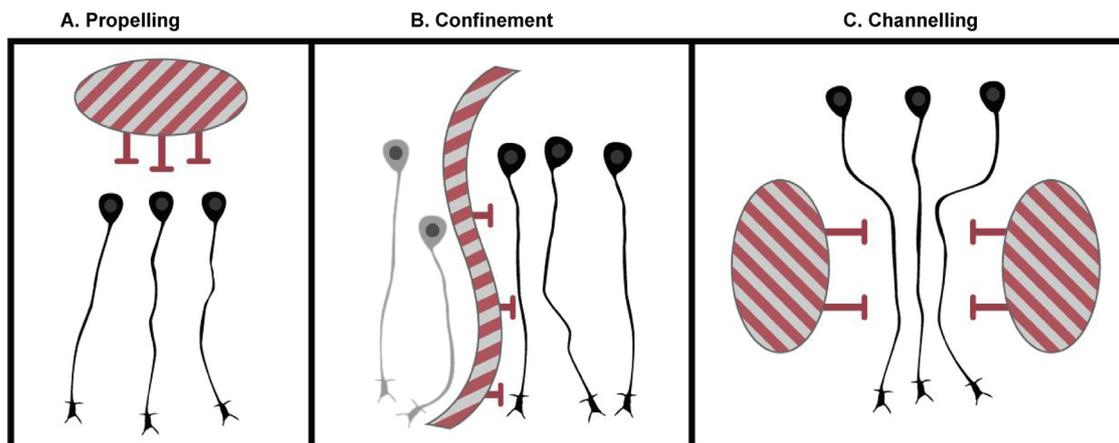


Fig. 1. Modes of action of repulsive forces in axon guidance.

As they grow, axons are oriented by a combination of attractive and repulsive forces. Among the repulsive ones, we can distinguish 3 main modes of action. A. Propelling. Axons perceive diffusible chemorepulsive cues emanating from a group of guidepost cells and turn away from this source. B. Confinement. A group of guidepost cells act as a barrier and confine the axons within a territory. They not necessarily deflect them away but prevent them from exiting it, partly by the emission of diffusible cues. C. Channelling. Several groups of physically separated guidepost cells constrain commissural axons within a narrow path, by releasing repulsive cues.

on growth cones [3]. The property of growth cone structures to retract after contact with some other axonal membrane surfaces was then discovered by Kapfhammer and Raper [4]. These observations echoed those of Verna, who wrote that dorsal root sensory neurons “interact differently with dermal than with epidermal cells. While nerve fibres readily extend over dermal cells, forming close membrane associations with some of them, they demonstrate a strong avoidance reaction with epidermal cells by changing their direction of extension” [5]. He postulated that molecules released by epidermal cells might deflect away nerve fibre growth trajectory, oppositely to those which were found to attract the axons, namely at that time, the neurotrophic factor NGF [6]. From these pioneer findings, repulsive forces have been demonstrated to play instrumental roles in a large range of developing neuronal circuits. As evidenced by numerous studies [7,8], repulsive forces can constrain axon navigation in various ways, channelling axonal bundles, deflecting away the growth cone trajectory, and creating sharp boundaries to delineate non-permissive territories (Fig. 1). As in a pinball, repulsive forces would act as launch pad, bumpers, and slingshots to propel and dynamically impact on the axon/ball trajectory. The development of commissural axons provides an appealing context to investigate how such repulsive forces can direct axon navigation. We review here the principal yet identified sources of repulsive cues, the nature of their influences and the molecular signals mediating their action during commissural axon navigation in the spinal cord.

1.1. Formation of commissural circuits

In Bilateria, commissural neurons form complex circuits that interconnect both sides of the central nervous system (CNS). They are essential for the correct processing and coordination of various sensory modalities, motor responses, and other brain functions [9]. These interneurons extend their axon across the midline at various axial levels of the CNS. For instance, the *corpus callosum* enables communication between the left and right cortical areas, the optic chiasm allows organisms with bilateral vision to correctly integrate visual cues, and spinal commissures ensure the correct coordination of various motor commands. These commissures are established during embryonic and early post-natal development in a highly specific spatial and temporal manner [10]. Defects in the correct wiring of commissural circuits have been observed in many neurodevelopmental disorders. However, if malformations of

the *corpus callosum* have been well correlated with various human disorders, little is known of the consequences of spinal commissures defects. Indeed, patients having mutations in *ROBO3* gene, affecting commissures of the hindbrain and the spinal cord, have no large sensorimotor deficit. Rather, they exhibit a very specific disease referred to as horizontal gaze palsy with progressive scoliosis (HGPPS) [11]. This suggests high degree of compensation of commissural defects with developmental origin.

1.1.1. Development of the dorsal commissural tract

The spinal commissural neurons are a heterogeneous population subdivided in several pools, differing in their location and timing of birth, each of them specified by various transcription factors [12,13]. Among them, d11 interneurons settle early in the most dorsal part of the spinal cord, close to the Roof Plate (RP). They arise from a MATH1-positive pool of progenitors, that generates both ipsilateral and commissural lineages and are specified by LHX2/LHX9 transcription factors [14,15]. d11 commissural (d11c) neurons trajectory is highly stereotyped and has been extensively studied in the mouse, notably by using MATH1::LacZ and MATH1::GFP transgenic mice [16]. d11c axons first extend ventrally, turning away from the RP and laying close to the pial surface (Fig. 2). At around mid-distance of the ventral border they break away from the lateral border to re-orient medially towards the central Floor Plate (FP) by running along the motoneuron domain. Such break of trajectory is also typical of chick commissural axons, apart from the pioneer ones which course with circumferential trajectory. In contrast, in xenopus and zebrafish embryos, the axons course by following the circumference of the tube until reaching the FP [17,18]. Next, commissural axons enter the FP, cross it and turn rostrally without ever crossing the midline again to connect their final targets. Commissural neurons arise around E9.5 in the mouse and navigate towards the FP from this stage. By E10.5, some of them have already crossed the midline and by E12.5, most of them have. By E13.5, they are navigating towards their final target following longitudinal routes [19–21].

1.2. Guidepost territories instructing commissural axon navigation in the spinal cord through repulsive action

Historically, the main intermediate target and crucial signaling hub for commissural axon navigation has been found to be the FP. It heavily influences the d11c guidance, and we can thus refer to

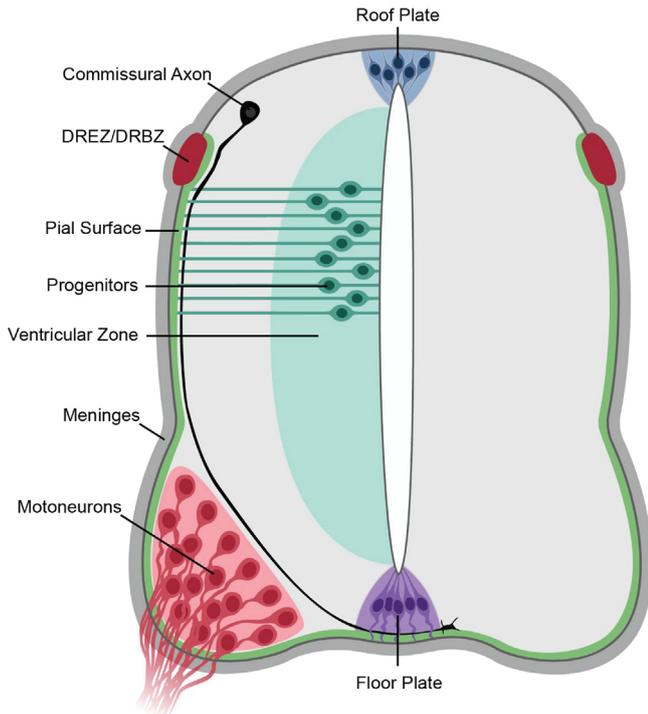


Fig. 2. Guidepost territories instructing commissural axons navigation in the spinal cord.

Spinal commissural axon trajectory is highly stereotyped. dl1c axons (in black) arise from a dorsal territory. They extend ventrally, cross the midline through the floor plate (FP), and then turn rostrally without ever crossing the midline again to connect their final target. This trajectory is influenced by various guidepost cells along the way. First, axons are pushed away by chemorepulsive cues emanating from the roof plate (RP) (in blue) and follow the pial surface (in green). Meninges (in grey) and the DREZ/DRBZ (in red) keep the axons away from the CNS/PNS boundary through diffusible cues. At around mid-distance from the ventral side, axons turn towards the FP (in purple), then run along the motoneurons domain (in red). Axons never enter the motoneuron domain nor the ventricular zone (in light green), these territories channeling the commissural tract towards the FP. As they reach the FP, axons interact with the basal lamina (in green) and navigate through the FP glial cells radial processes. Upon crossing, they gain sensitivity to repulsive cues emanating from the FP, that they did not perceive before and thus exit the FP, accomplish a sharp turning in the rostral direction and navigate longitudinally in bundles, guided by various gradients of guidance cues, including repulsive ones.

FP cells as guidepost cells. The FP has been extensively studied and many of its molecular mechanisms have been unveiled. However, a variety of other cells within the spinal cord bring contributions to the navigation of commissural axons, including glial cells, neurons and progenitors. These different cell-types contribute together to sharply delineate the path of commissural neurons.

1.2.1. Kick off repulsive forces to orient commissural axon navigation

1.2.1.1. The roof plate. The RP is probably the second most studied group of guidepost cells after the FP. It is composed of glial cells that lay on the dorsal midline of the spinal cord. These cells come from progenitors that are induced in the most lateral regions of the neural folds [22]. This induction relies heavily on BMP signalling, mediated by the transcription factors LMX1A/B [23]. Upon neural tube closure, they are not easily distinguishable from other cells, in particular neural crest cells, but as they differentiate they start to express specific markers, notably BMPs and WNTs [22]. The RP is the first dorsal structure to differentiate and then impacts all other dorsal populations differentiation. Electro-microscopy studies revealed that, when differentiated, RP cells have two small processes extending radially towards the pial surface and the central canal [24,25]. The RP acts as a barrier that no axon can cross

before E16.5, when a dorsal commissure is established [26]. Interestingly, the RP itself undergoes rather important morphological changes between E11.5 and E16.5, from an arch structure to a thin wall-like structure [25]. The RP is a crucial organizing centre of the different dorsal lineages. Notably through BMPs and WNTs, it specifies several classes of adjacent dorsal interneurons and regulates their proliferation, migration, and guidance [27].

Beyond this patterning function, RP cells provide the driving force which propels dl1c axons emerging from their soma away from the dorsal side. This effect was shown to be mediated by the morphogens BMPs, namely by GDF7:BMP7 heterodimers acting via the BMPRIIB receptor [28,29] (Fig. 3). Not only their direction but their growth rate also appears regulated by BMPs [30]. An additional repellent protein was found produced by the RP, a secreted factor named Draxin. Draxin mutant mice display commissural axonal migration and fasciculation defects consistent with a repulsive mode of action [31]. Draxin shares UNC5, DCC, and Neogenin receptors with Netrin-1, a secreted molecule initially discovered to act as a chemoattractant [32,33]. Although Draxin has been shown to bind UNC5 and Neogenin *in vitro* [34], its repellent role *in vivo* was reported to be triggered via its binding to DCC [35] (Fig. 3). It can also be noted that the repulsive factor Slit2 is highly expressed at the RP at E13 [36]. Most dorsal commissural axons are already on their way in the contralateral side at this stage, whether this source contributes to the kick off of commissural axons is therefore questionable.

1.2.2. Repulsive forces to confine commissural axon navigation in the central nervous system

Commissural axons are destined to connect neurons of the central nervous system (CNS) and must be consequently strictly confined within the spinal cord. This is not true for all spinal cord axons, since on the contrary, those of the motoneurons project out of the CNS through the Motor Exit Point (MET). Moreover, in this case, only the axons exit the CNS, the neuronal soma being confined within the CNS. Conversely, sensory axons of the dorsal root ganglia penetrate the spinal cord via the Dorsal Root Entry Zone (DREZ), while their soma remain outside. In contrast, some non-neuronal cells enter the CNS, such as endothelial cells which infiltrate the CNS tissue to build the blood vessels. Thus, cells and neurites trafficking across the CNS/PNS frontier is strictly controlled, from the onset and throughout life.

1.2.2.1. Confinement by the meninges. Meninges are a protective multi-layered structure that envelops the brain and the spinal cord. They are mainly composed of fibroelastic cells and blood vessels. Meninges originate from somatic mesoderm that covers the neural tube shortly after neural tube closure, around E9 in the mouse embryo [37,38]. They act as barriers throughout life, controlling exchanges between the central nervous system and what lays outside. In the brain, meninges have been shown to initiate a morphogenic signaling cascade that regulates the development of a major dorsal commissure, the *corpus callosum*. The meninges inhibit callosal axon outgrowth through BMP7. WNT3, expressed by pioneer callosal axons, later counters this effect. WNT3 expression is regulated by another member of the BMP family, GDF5, expressed by adjacent Cajal-Retzius neurons, which in turn is regulated by a soluble inhibitor, DAN, expressed by the meninges [37]. In the spinal cord, *in vivo* studies lack to highlight the role of the meninges on commissural neuron development. However, a recent *in vitro* study showed that the meninges are able to produce secreted cues that can either attract or repulse different neuronal populations. Consistent with *in vivo* behaviours, these experiences showed that motoneurons and sensory neurons are attracted by meninges while ipsilateral and commissural neurons are repelled by them [38].

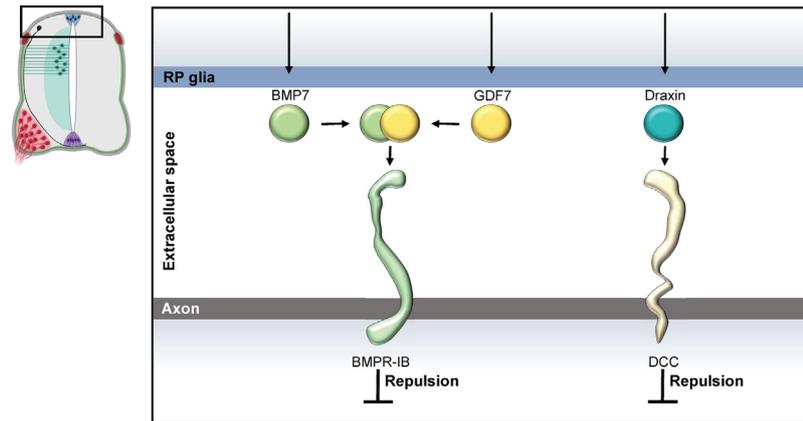


Fig. 3. Kick off repulsive forces at the roof plate. The BMP family members GDF7 and BMP7 are secreted by roof plate glia cells and bind to the axonal BMPR-IB receptor and repel axons toward the ventral part of the spinal cord. In parallel, the secreted factor Draxin propels axons via axonal DCC.

1.2.2.2. *Closing the CNS/PNS gate: confinement by the dorsal root entry zone (DREZ)/ dorsal root bifurcation zone (DRBZ).* Commissural axons navigate at close proximity of the pia. Early studies established that their growth cones rarely, if ever, contact the basal lamina (Holley & Silver 1987, Yaginuma et al 1991). The basal lamina is punctuated by the DREZ and the MET, which ensure the circulation between the central Nervous System (CNS) and the surrounding tissues. The DREZ consists of a break in the lamina and a cluster of specialized cells arising from the neural crest, the boundary cap cells, which prevent both cell bodies and their axons from leaving the CNS, and gaps between the glial end-feet [39]. Around E11, the peripheral sensory neurons send axons towards the spinal cord through the DREZ and start to form the dorsal root bifurcation zone (DRBZ), where they project in an anterior-posterior direction within the tract [40]. Therefore, the DRBZ is in direct apposition to the DREZ.

Some cues released from these gates to confine commissural axons have been identified. Netrin-1 is expressed at the border of the DRBZ between E11.5 and E12.5 [34]. In Netrin-1 mutant embryos, commissural axons invade the DREZ and DRBZ. The presence of ectopic axons can even be detected in the dorsal root ganglia (DRG) [34]. This study thus indicates that Netrin-1 participates in forming an inhibitory boundary at the border of the DRBZ and the DREZ [34]. The nature of the Netrin receptors mediating these effects has also been investigated. Ectopic axons were detected in the DRBZ of both DCC and UNC5C mutant embryos, while only in DCC mutant was their presence observed in the DREZ, suggesting a differential contribution of these receptors [34] (Fig. 4A). Other receptors could also potentially transduce a Netrin-1 signal. Down's syndrome Cell Adhesion Molecule (DSCAM), whose expression is high in the DREZ and in commissural axons, was indeed shown to modulate Netrin-1 attractive response with or without DCC [35] (Fig. 4A). In the chick which lacks DCC [41], a candidate could be Neogenin, a Netrin-1 receptor which is expressed in some commissural axons [42] (Fig. 4A). The mode of action of Netrin-1 remains unclear. A simple view would be that it acts as a repellent in this context. Moreover, it should be noted that Netrin-1 is not detected in the dorsolateral region of the spinal cord prior to E11.5 whereas ectopic axons are already observed at E10.5 in the DRBZ of Netrin-1 and DCC mutant embryos [34]. Thus, other Netrin sources might act to confine the axons at the early stages, whose release in the mutants result in their ectopic position in the DRBZ. Additional repulsive forces might also be involved in this confinement. Draxin, transducing repulsive signals via axonal DCC, is namely also expressed at the dorsal pial surface and in the DREZ [27] (Fig. 4A).

Furthermore, the role of extracellular matrix (ECM) components and glycoproteins in the confinement of axons along the pial surface might be essential. Laminin is present all along the pial surface in close contact with the commissural axons during their navigation [43]. Type IV Collagen and Heparan sulfate proteoglycans are also components of the basement membrane and their deposition is spatially and temporally controlled in coordination with morphogenesis [44]. Along this line, the glycoprotein Dystroglycan, an important scaffold for ECM proteins including Laminin, has been shown to interact with Slit and this interaction is detected all along the pial surface [43]. In the visual system, Laminin has been shown to modulate the attractive response of retinal axons to Netrin-1 by turning it into a repulsive signal [45]. Thus, co-expression of Netrin-1 and Laminin could, as well, contribute to set the pia as a repulsive barrier, explaining the observed lack of contacts between the basal lamina and commissural growth cones (Fig. 4A).

1.2.3. Repulsive forces to channel commissural path

Channelling of axon tracts can be achieved by dual lateral repulsive sources constraining their growth in between. Once reaching the half ventral half of the spinal cord towards the FP, commissural axons modify their initial circumferential trajectory and re-orient medially towards the FP, navigating at the border of the ventral motoneuron domain. A triad of three territories, the ventricular zone, the basal lamina, and the motoneuron domain, might thus act in synergy to channel the pre-crossing path of commissural axons in between these different territories.

1.2.3.1. *The ventricular zone.* The ventricular zone (VZ) is composed of the neuronal progenitors, laying the central canal. The different populations of progenitors are specified by a combinatorial code of transcriptional factors [12]. These factors are activated by opposing gradients of BMP/WNT and SHH emanating respectively from the RP and the FP [12]. Neural progenitors of the CNS have a bipolar morphology, extending two processes, one connecting the pial surface and the other connecting the central canal [46]. During the cell cycle, their nuclei oscillate between the apical and basal pole, a process referred to as the interkinetic nuclear migration. Post-mitotic neurons born from neurogenic divisions detach their apical anchor and migrate laterally to establish themselves in the mantle. Strikingly, dl1c axons navigate at the VZ/mantle interface but never enter the VZ. The mechanisms underlying this navigation choice are unclear. The Netrin-1 attractant produced by the progenitors and transported via their basal process for lateral deposition at the basal lamina was recently proposed to direct commissural axon growth out of the VZ and close to the pia [47]. Interestingly,

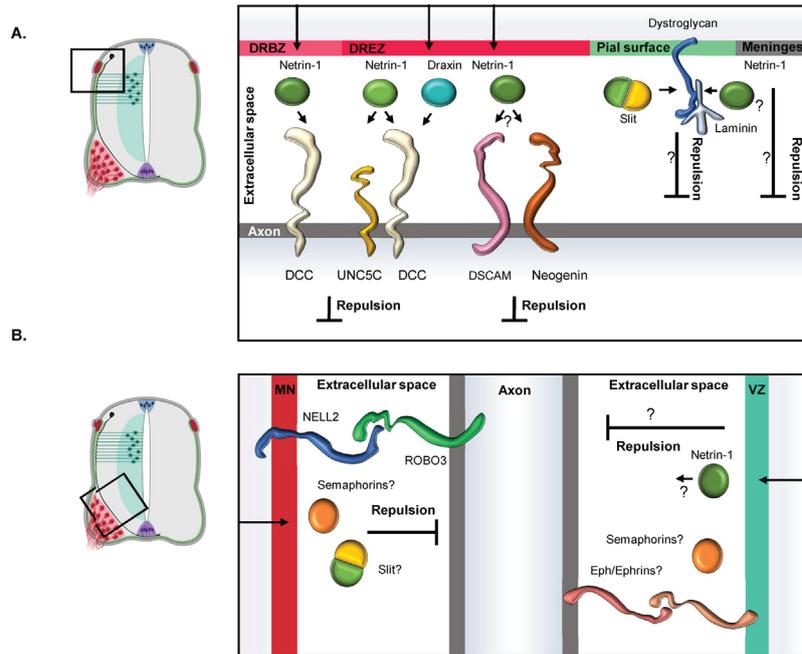


Fig. 4. Confinement and channeling of commissural axons. A. Repulsive forces confine axons within the spinal cord.

The dorsal root entry zone (DREZ) and the dorsal root bifurcation zone (DRBZ) confine axons in the spinal cord. Netrin-1 acts *via* DCC and DCC/UNC5 heterodimer in the DRBZ and in the DREZ respectively. DSCAM may modulate Netrin-1 attractive response with or without DCC. Neogenin, whose expression was reported in chick commissural axons may also transduce the signal initiated by Netrin-1. In addition, Draxin at the pial surface and in the DREZ has a repulsive effect on axons *via* DCC. Signals originating from the meninges and the pial surface are still largely unknown but may involve the glycoprotein Dystroglycan. Dystroglycan scaffolds Laminin, which has been shown in other context to switch Netrin-1 attraction into repulsion.

B. Repulsive forces channel axons between the ventricular zone (VZ) and the motoneuron domain.

The VZ is a territory non-permissive to the entry of axons. The mechanisms mediating this barrier are still unknown but might involve Semaphorins and Eph/Ephrins. Netrin-1 is produced by progenitors. The motoneuron domain expresses Slits, Semaphorins and ROBO3 ligand, NELL2. NELL2/ROBO3 signaling mediates repulsion on commissural axons.

releasing the lateral deposition of this cue by specific deletion of Netrin-1 in progenitors induced some commissural axons, identified by their ROBO3 expression, to invade the VZ [47]. Nevertheless, some others, expressing the commissural marker TAG1, were still constrained out of the VZ [47]. A tempting model is that some repulsive forces also directly emanate from the VZ (Fig. 4B). Indeed, such a mechanism was reported to prevent cortical and thalamic axons from invading the proliferative regions of the developing brain [48]. This would be consistent with reported expressions of transcripts encoding guidance molecules bearing repulsive activity, such as the Semaphorins and the Eph/Ephrins in mouse and chick spinal cord VZ [49–53].

1.2.3.2. The motoneuron domain. Motoneurons arise at around E9.5 in the mouse embryo, from a pool of ventral progenitors. They are specified by a set of homeodomain transcription factors (notably HB9, LHX3, ISL2, and ISL3) [54]. Post-mitotic motoneurons migrate out of the VZ in streams, and cluster at various medio-lateral levels of the mantle to form distinct and adjacent pools. It is noticeable that commissural axons break their circumferential path when they reach the emerging mass of motoneurons accumulating in the ventral horn. Nevertheless, whether this reflects an instructive role of this territory in the reorientation of commissural axons is unclear. Indeed, in mouse and chick embryos in which the FP has been genetically or experimentally ablated, commissural axons no longer break their circumferential trajectory, reaching the FP through a path that resembles that of xenopus and zebrafish embryos, all along the pial surface. However, ablating the FP and/or the notochord in these experimental contexts, simultaneously prevented the specification of motoneurons, which were thus also lacking [55,56]. Interestingly, motoneurons express

various guidance molecules that could define this territory as non-permissive for commissural axons, such as the Slits and the Semaphorins (Fig. 4B). A recent study features NELL2, expressed mainly in the motor columns, as mediating such a repulsive action of the motoneuron domain [57]. *In vitro*, NELL2 could repel commissural axons and this effect was found exerted *via* ROBO3. An *in vivo* contribution of signaling is suggested by the analysis of NELL2^{-/-}ROBO3^{-/-} embryos, in which many commissural axons were observed to defasciculate and invade the motor columns [57].

The Slit/ROBO signaling pathway has been shown to be essential for the maintenance of boundaries, compartmentalizing the visual centres in the *Drosophila* brain [58]. Slits are interesting candidates to consider in this channelling. Slit1/2 mRNAs are produced by both spinal progenitors and motoneurons [36]. The Semaphorin3F (SEMA3F) is also highly detected in the motoneuron domain and its repulsive action on spinal commissural axons has been evidenced *in vitro*, although it was reported to concern post-crossing rather than pre-crossing axons [59]. Several other members of the Semaphorin family are indeed expressed both by motoneurons and progenitors. In the chick embryo, this is for example the case of SEMA3C and SEMA3A [50,51]. Specific deletion of these candidates in progenitors and motoneurons would be highly informative to address their contribution to the channelling of commissural axons.

1.2.4. Travelling a repulsive field: navigation across the midline in the floor plate

Being a prototypical example of intermediate target for long distance connections, the FP has been, by far, the most studied group of guidepost cells. The FP is composed of glial cells that lay ventrally at the midline of the embryo. Though discrepancies exist regarding its exact developmental origin and the signaling pathways involved

in its specification between species, the FP influences neuronal differentiation and axon pathfinding in the spinal cord of all vertebrates. In the mouse, SHH through GLI2 and then FOXA2 is a key factor in FP induction [60–62]. FP cells display a morphology that resembles the one of RP cells. They possess 2 processes that extend radially, the shorter one towards the central canal and the longer one towards the basal lamina [24,63]. The FP is a unique group of guidepost cells for several reasons. First, up to now, it is the only one the commissural axons go through, instead of just passing by. Indeed, commissural axons enter the FP and then navigate through a meshing of FP cells basal processes, hugging the basal lamina [64]. FP cells and commissural axons are known to establish close contacts [65]. Interestingly, it is probably the territory in which the d11c axons form the most compact bundle during their navigation. Second, the FP is not only a group of guidepost cells but also an intermediate and not a final target. Thus, intermediate target must combine the properties of a target (a territory that the axons select to grow within) and a non-target (a territory in which the axons do not stop). Studies of FP crossing helped resolving some aspects of this apparent paradox, by bringing to the scheme the notion of temporality. Indeed, temporal regulation of axon sensitivity to the guidance cues emanating from the intermediate target is the key to endow it first, with the properties of a target and second, with those of a non-target.

Signals conferring to the FP the properties of a target tissue are the cues that are perceived first by commissural axons on their way for midline crossing. These signals are thought to combine both short-range and long range attractive/promoting effects. Cell adhesion molecules play a crucial role at a short-range level. Commissural axons and FP glial cells engage in dynamic and complex *cis* and *trans* interactions via various Cell Adhesion Molecules of the IgSuperfamily such as L1/NgCam, NrCam, and TAG1 [66–69]. Long range attraction by Netrins, and additionally SHH and VEGF, have been thought to provide major signals guiding commissural axons towards the FP [33,70–72]. This view has been recently revisited by studies demonstrating that Netrin-1 might indeed rather act much more locally and, as described in the above paragraphs, from spinal cord sources other than the FP, essentially neural progenitors [47,73]. Once the midline crossed, the FP must acquire the properties of non-target territory, to prevent axons to stall and terminate their navigation. This process, which has been the focus of recent reviews [74,75], appeared from several works to be achieved, not by expressing novel molecules with repulsive activity in the FP, but rather by sensitizing commissural axons to repulsive cues yet present but that had been ignored until midline crossing. The temporality of the target to non-target switch is crucial. Prematurely releasing the sensitivity to the repellents would be disastrous, as it would block commissural axons entry in the FP and midline crossing. The mechanisms controlling the switch have started to be decrypted in the recent years and turn out to be highly complex. A broad panoply of transcriptional and post-translational mechanisms is indeed deployed to first silence the perception of the repellents and second to release this silencing and set the repulsive commissural response. In parallel, the properties that made initially the FP as a target -i.e. attractive cues- have to be shut-down. Shirasaki and collaborators demonstrated twenty years ago that this is indeed occurring. In *ex vivo* assays, grafting an ectopic FP close to commissural axons coursing towards their endogenous FP induced their re-orientation towards the ectopic FP. In contrast, exposing commissural axons which have already crossed the endogenous FP to ectopic FP had no effect [76]. It was subsequently proposed that Slit signaling blunts Netrin-1 attraction. *cis* interaction between ROBO and DCC was reported to silence Netrin-1 attractive signaling [77]. In parallel, SHH has been proposed to be sequestered by its FP specific receptor

HHIP (Hedgehog Interacting Protein) to turn off its attractive function [78].

1.2.4.1. The molecular players mediating repulsive forces. Repulsion involves several couples of ligands/receptors (Fig. 5). The secreted Semaphorin 3B (SEMA3B) mediates repulsion at the midline by activating a complex formed of NEUROFILIN 2 (NP2) and PLEXINA1 (PLEXA1) receptors [79]. In addition, the membrane-bound SEMA6B is expressed by commissural axons when they cross the FP and was reported to interact with PLEXA2 expressed by FP glial cells [80]. Slit proteins are produced by FP cells and are submitted to proteolytic processing through yet unidentified protease(s). This cleavage releases N-terminal Slit fragment (SlitN), which binds to ROBO receptors, and C-terminal Slit (SlitC) which binds to PLEXA1 [81]. B-class Ephrins can function as ligands or receptors, mediating forward or reverse signaling respectively [82]. In the FP, midline glial ephrin-B3 interacts with commissural Eph-B3 receptors [83]. Inhibitory effects of Nogo are carried out through the interaction to the Nogo receptor complex [84,85]. Nogo is expressed by radial glia at the ventral midline of the spinal cord and Nogo receptors (NogoR) are detected in commissural axons extending through the FP [86]. Recently, blocking NogoR was found to result in axon stalling at the FP and therefore in a reduction of the number of commissural fibers properly reaching the contralateral side of the spinal cord. Furthermore, it has been reported that the ligand interacting with NogoR is a truncated form of Nogo released by glial cells [87].

1.2.4.2. Temporal regulation of commissural axon sensitivity to the repellents. Pioneer studies performed in *Drosophila* revealed that ROBO protein is degraded in pre-crossing commissural axons by the endosomal protein COMM [88,89]. Yet, ROBO mutant phenotype is rescued by a mutated version of ROBO that cannot be sorted by COMM. COMM thus probably regulates Slit/ROBO interaction through an additional, sorting-independent mechanism [90]. ROBO2 is also expressed by midline glial cells and has been shown to interact with ROBO1 in *trans*. This interaction would occur upon crossing, when ROBO1 receptors reach the cell surface consequent of COMM suppression and it would repress Slit repulsion until the crossing is completed [91].

How are ROBO distribution and activity regulated in time are therefore key questions to further understand the navigation of the midline. COMM expression was shown to be controlled at least in part by Frazzled/DCC in pre-crossing [92], while it remains unknown how it is repressed after midline crossing. The nature of mechanisms silencing Slit repulsion before the crossing has been thought to totally differ in vertebrates since no COMM homolog was found in their genomes. Nevertheless, vesicular trafficking also appears as an important process in vertebrates. Indeed, vesicles containing ROBO1 were observed in commissural axons. Calsyn-tenin1 and RabGDI were found to regulate their trafficking and to allow the pulse exposure of ROBO1 at the growth cone surface [93,94]. Interestingly too, a recent study identified PRRG4, a protein which displays structural similarities with COMM, as capable of re-localizing ROBO1 at the cell surface *in vitro* [95]. Another reported regulator of Slit/ROBO signaling is an isoform of ROBO3, a divergent member of the ROBO family, ROBO3.1. Its distribution is restricted to the pre-crossing commissural axons and it is thought to facilitate midline crossing by antagonizing Slit/ROBO1/2 mediated repulsion [21]. The underlying mechanism is not yet known. It might not involve ROBO3 as a Slit receptor since ROBO3 was shown to have lost its affinity to bind Slit ligands with evolution, instead rather acting as a DCC co-receptor for NETRIN-1 [96]. Thus, progress as yet to be accomplished to better understand how the regulators of ROBO receptors are temporally controlled and their activity synchronized with midline crossing.

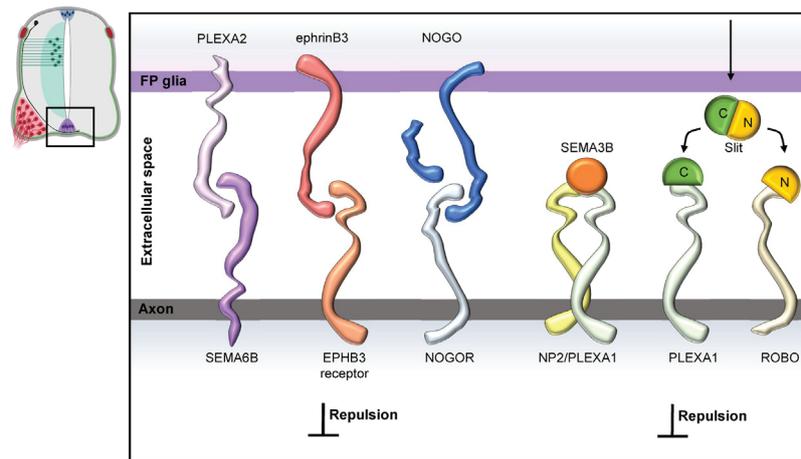


Fig. 5. Repulsive forces in the floor plate.

While crossing the floor plate (FP), axons gain sensitivity to repulsive cues they did not perceive at the pre-crossing stage. PlexinA2 (PLEXA2), EphrinB3 (EphrinB3) and NOGO, expressed at the membrane of FP glial cells, interact respectively with Semaphorin6B (SEMA6B), EphB3 receptor and NOGO receptor (NOGOR) expressed by commissural axons. In parallel, the FP glial cells also secrete repulsive cues. A truncated, diffusible version of Nogo can also bind to the axonal Nogo receptor. Sema3B binds to a heterodimer of Neuropilin2 (NP2) and PlexinA1 (PLEXA1) axonal receptors. Slit produced by FP glia is cleaved into a C-terminal (SLITC) and an N-terminal fragments (SLITN). SLITC binds to PLEXA1, while SLITN interacts with ROBO.

Similarly, axon sensitivity to Semaphorin repulsive signaling is tightly controlled and PLEXA1 sorting at the axon surface in the FP is thought to be a key event switching on the sensitivity of commissural axons to SEMA3B. We indeed found that PLEXA1 is processed by Calpains in pre-crossing axons. This processing prevents PLEXINA1 cell surface sorting, and thus the association with NP2 co-receptor needed to transduce SEMA3B signaling [79]. We also found that this silencing of SEMA3B responsiveness is released by local FP cues, GDNF and NrCAM, which trigger PLEXINA1 cell surface sorting by inhibiting Calpain activity [97]. In addition, SEMA6B and PLEXA2 were also shown to interact in *cis* at the growth cone surface and to silence the sensitivity of pre-crossing axons to midline-associated SEMA6B [80]. Thus, as it is the case for the ROBO/Slit pathway, post-translational mechanisms appear to be instrumental in regulating the timing of activity of the Semaphorin repellents.

1.3. After FP crossing: a new pinball game starts

A second pinball game starts when FP repulsive signals propel commissural axons out of the FP. Concomitantly, a drastic change of direction is accomplished by the dl1c axons which turn in the rostral direction to navigate longitudinally to the FP. Two opposite chemotropic gradients control this guidance choice: a caudal high to rostral low repulsive one shaped by SHH, and a caudal low to rostral high attractive one shaped by WNT. Thus, as during the pre-crossing navigation game, repulsive forces play an important role in propelling the axons and imprinting the direction of their longitudinal growth. A temporal control of these forces is also needed. SHH has a reported pre-crossing attractive activity, shown to be mediated by SMO and one of its receptors, BOC (Brother of CDO) [71,98,99]. Upon crossing, SHH attraction must be switched into repulsion, a process proposed in the chick to be achieved *via* the implication of another SHH receptor, HHIP (Hedgehog Interacting Protein), in addition to SMO in the mouse [78,100] (Fig. 6). The cytoplasmic adaptor protein, 14-3-3, increases in amount during the pre-crossing navigation to culminate at the post-crossing stage, during which it is required for SHH-dependent repulsion [100]. In parallel, the sensitivity of the complementary rostro-caudal chemoattractive gradient of WNT is also switched on upon the crossing. A mechanism was recently reported implicating a molecular cascade during which SHH/SMO downregulates transcript

levels of Shisa2 in commissural neurons. Down-regulation of Shisa2 allowed the WNT receptor Frizzled3 to be glycosylated and translocated to the surface of the commissural axon growth cones [101].

Once the rostrocaudal direction is given, commissural axons segregate in several tracts. Reorganizations of post-crossing commissural axons during this step likely implicate selective fasciculation. In xenopus, live monitoring of commissural axons in the spinal cord revealed striking changes in the behaviors of the growth cones during crossing process. In fact, during pre-crossing navigation, growth cones were observed to avoid each others whereas in the post-crossing stage (after their longitudinal turning), they accomplished a series of fasciculation choices which suggest a process of bundle selection [102]. Several cell adhesion molecules are up-regulated in post-crossing commissural axons, such as the IgSFCAM L1 in the mouse [69], which could contribute to this recognition process.

In *Drosophila*, commissural axons form three longitudinal tracts [103]. Their sorting and their position relative to the midline was shown to be controlled by a combination of ROBO receptors, differing between the tracts and thought to confer them different levels of sensitivity to midline Slit repellent [104]. A theoretical model predicts that a ROBO code based on quantitative differences of ROBO proteins could be on its own sufficient to generate different lateral tract position [105].

In vertebrates, post-crossing commissural axons are sorted into two main tracts, navigating the ventral and lateral funiculi. The mechanisms underlying this sorting are still elusive. An implication of the Slit-ROBO signaling was reported from the analysis of Slit and ROBO null embryos, in which this sorting is defective [106]. An interplay of Robo and N-cadherin was also found to contribute to the lateral sorting of post-crossing commissural axons [107]. The Ephrin signaling is also involved in the mediolateral positioning of the longitudinal tracts. Blocking EphB3/EphB signaling was reported to result in a lateral shift of commissural axons [83,108].

Even though the topography of post-crossing axons differs from that of pre-crossing ones, the longitudinal navigation after midline crossing also appears constrained and channeled by the FP, the lateral basal lamina and the motoneuron domain. Whether the underlying mechanisms and signaling pathways are similar to those operating at the pre-crossing stage remains to be determined.

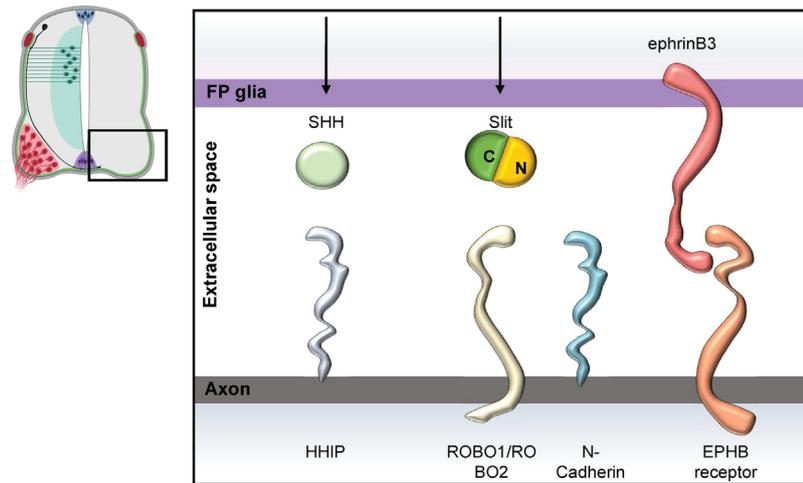


Fig. 6. Repulsive forces in command of post-crossing axon navigation.

After crossing the floor plate (FP), commissural axons turn rostrally in response to gradients of attractive and repulsive cues. Diffusible Shh from the FP glial cells interacts with the axonal receptor HHIP. In vertebrates, post-crossing commissural axons form two main tracts, the first turns in the ventral funiculus, while the second turns in the lateral funiculus. Slit contributes to this sorting through interaction with axonal ROBO1 and ROBO2. An interplay of ROBO and N-cadherin was also found to contribute to the lateral sorting of post-crossing commissural axons. Trans-interactions also occur between glial Ephrin-B3 (Eph-B3) and axonal Eph-B receptor to assign the medio-lateral position of post-crossing tracts.

1.4. Conclusion and perspectives

Important progress has been made over the years to identify components of the molecular machinery controlling axon pathfinding. The versatility of growth cone responsiveness to guidance cues endows the axon with multiple possibilities to interpret in a highly specific manner the topographic signals and the physical elements encountered in the environment during its navigation. Recent tissue-specific deletions of guidance molecules have revealed unexpected contributions of territories surrounding axonal pathways. This provides a better integrated picture, which would require to be further extended, on how axon guidance proceeds in an environment that is also subjected to continuous developmental constraints. In addition, a series of molecular mechanisms has been discovered, which brings to light the importance of spatial and temporal controls of growth cone sensitivity to guidance cues. Altogether, this raises the need of better understanding at protein levels how topographic cues are distributed and deposited in the tissues and how the activity of guidance receptors and downstream effectors in the growth cones is focused and fine-tuned.

In the context of commissural axon guidance, many types of repulsive forces which remain underestimated are likely to play key contributions in the robustness of commissural axon pathfinding. While it is rather straightforward to conceive how a repulsive cue repel away a growth cone, it is much less simple to conceive how commissural growth cone can navigate a field expressing repellents to which it progressively acquires sensitivity. Several theoretical models of axon guidance have been elaborated to explain the building of topographic maps. A recent one was based on the principle that growth cones undergo concentration-dependent alternated choices of repulsive and attractive response to guidance cues [109]. Interesting insights would come from such models of FP navigation that would integrate the different forces at play in time and space.

Acknowledgments

VC is supported by the LABEX CORTEX and DEVWECAN of the "Université de Lyon", within the programme 'Investissements d'Avenir' (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR), ANR-16-CE16-0023-01, and French Muscular Dystrophy Association (AFM).

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