Construction and reconstruction of brain circuits: normal and pathological axon guidance.

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Construction and reconstruction of brain circuits: normal and pathological axon guidance

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Abstract:

Perception of our environment entirely depends on the close interaction between the central and peripheral nervous system. In order to communicate each other, both systems must develop in parallel and in coordination. During development, axonal projections from the central nervous system (CNS) as well as the peripheral nervous system (PNS) must extend over large distances to reach their appropriate target cells. To do so, they read and follow a series of axon guidance molecules. Interestingly, whilst these molecules play critical roles in guiding developing axons, they have also been shown to be critical in other major neurodevelopmental processes, such as the migration of cortical progenitors. Currently, a major hurdle for brain repair after injury or neurodegeneration is the absence of axonal regeneration in the mammalian CNS. By contrasts, PNS axons can regenerate. Many hypotheses have been put forward to explain this paradox but recent studies suggest that hacking neurodevelopmental mechanisms may be the key to promote CNS regeneration. Here, we provide a seminar report written by trainees attending the second Flagship school held in Alpbach, Austria in September 2018 organized by the International Society for Neurochemistry (ISN) together with the Journal of Neurochemistry (JCN). This advanced school has brought together leaders in the fields of neurodevelopment and regeneration in order to discuss major keystones and future challenges in these respective fields.

Introduction:

The first anatomical reference of the brain dates back to the ancient Egyptian mummifications. However, the tremendous complexity of this organ was revealed by the work of the world-renowned neuroscientist Santiago Ramón y Cajal. Nevertheless, his anatomical descriptions could not fully explain the cellular and molecular events at the origin of behavioral, motor or sensitive responses. Today, it is clear that the central nervous system (CNS) is the processing center for these events. Moreover, fine sensory perception and intricate motor control are orchestrated by a discrete and permanent

communication between the CNS and the peripheral nervous system (PNS). In the last century, neuroscientists have investigated the mechanisms involved in the development and plasticity of this structure. To address these fundamental questions, researchers made use of simple and accessible animal models. *Drosophila melanogaster* was one of the first organisms used due to several technical advantages: amenability to genetic manipulation, short lifespan and large number of offsprings. Studies emanating from this model system paved the path towards our understanding of major neurodevelopmental mechanisms involved in vertebrate behavior, neuronal migration and differentiation among many others (Bellen *et al.* 2010). *Danio rerio* (zebrafish), quickly emerged as an attractive more complex animal model. Like the *Drosophila melanogaster*, the zebrafish model also possessed a short lifespan and a large number of offsprings. However, it provided the advantage of studying neurodevelopmental mechanisms in vertebrates (d'Amora and Giordani 2018). While findings in these two models have led to major findings in the field of neuroscience, there are still significant gaps in our understanding of human development. Over the last 50 years, *Mus musculus* and *Rattus norvegicus* are classic models in neuroscience research due to their closer phylogenetic proximity to humans (Ellenbroek and Youn 2016).

In parallel to these findings, a large number of pathologies related to the CNS have emerged over the last century. This is mainly related to the aging population, encountering previously unknown neuro-degenerative diseases. The rising prevalence of these neurodegenerative diseases has urged the need for novel and more effective therapies (Gitler *et al.* 2017). Quickly, the idea emerged that developmental processes could be reinitiated to induce regeneration and brain repair. In an effort to target these fundamental questions, the Journal of Neurochemistry organized in September 2018 a workshop in Alpbach, Austria, gathering some of the most prominent researchers in the field of developmental neurobiology and regeneration in order to discuss the most significant findings and current challenges in these fields. Trainees attending this workshop have drafted a seminar report of this workshop listing the major advances and putting forward major questions in the field.

The Developing Nervous System

Metazoans all possess an axis of symmetry. In contrast to *radiata* (radial symmetry), *Bilateria* possess a two-fold symmetry. Thus, *bilateria* have a front and rear as well as left

and right sides. To connect its two lateral halves, the CNS of *bilateria* possesses commissural neurons. These neurons, which are born embryonically, project their axons contra-laterally to connect the left and right side of the organism. Together, these commissural networks not only allow for integration and coordination of left-right neuronal activities, but are essential for the correct processing and interpretation of various sensory information, the coordination of motor responses and other brain functions (Ducuing *et al.* 2019; Stoeckli 2018; Gaudet and Fonken 2018). Many commissural tracts exist in the CNS (Chédotal 2014). Here, we will discuss the three major systems: the corpus callosum, the ventral commissure of the spinal cord, and the optic chiasm.

Forebrain

The forebrain possesses two main cortical projection neurons: cortico-cortical, that form the corpus callossum, and cortico-fugal, further subdivided into corticothalamic and coroticospinal tracts (Leyva-Díaz and López-Bendito 2013).

The corpus callosum (CC) is the largest brain commissure and develops alongside neocortex expansion. Interestingly, this structure is unique to eutherian mammals and relays information between left and right hemispheres via the midline (Suárez *et al.* 2014; Gazzaniga 2000). Corpus Callosum dysgenesis affects ~1:4000 live births that result in either partial or complete absence (agenesis) of the CC (Edwards *et al.* 2014). Initially the two hemispheres are separated, at the midline, by the interhemispheric fissure (IHF)(Rakic and Yakovlev 1968). This region is lined by specialized astroglial and neuronal cells that are required for proper CC tract formation (Gobius *et al.* 2016; Silver *et al.* 1982; Gobius *et al.* 2017; Niquille *et al.* 2009). In addition to providing a permissive substrate for callosal growth cones to grow across the midline, midline cells also secrete guidance cues. Pre-crossing CC axons are sensitive to Slit2, expressed by these astroglia, which acts as a repulsive cue to constrain callosal axons expressing the Roundabout (Robo) receptors 1/2 (Unni *et al.* 2012). In contrast, netrin-1, expressed by the cingulate cortex, counteracts the Slit2 repulsive signal by attracting callosal axons expressing the transmembrane receptor Deleted in Colorectal Cancer (Dcc) (Fothergill *et al.* 2014). Indeed, loss of Dcc or netrin-1 leads to CC agenesis (Serafini *et al.* 1996; Fothergill *et al.* 2014). In addition to netrin-1, semaphorin (Sema3C) is secreted at the midline and attracts callosal axons expressing the neuropilin 1 receptor (Nrp1, figure 1A)

(Niquille *et al.* 2009). Once CC axons have reached and crossed the midline, this attractive signal is switched off (Mire *et al.* 2018). This coincides with an upregulation of the transmembrane protein ephrin-B1 in post-crossing CC axons. Interestingly, ephrin-B1 possesses a unique Asparagin residue (N-139), not shared by other ephrins, which once glycosylated can allow ephrin-B1 interaction with Nrp1 and silence Sema3C/Nrp1 attraction (Mire *et al.* 2018)(figure 1B). These findings identify a novel mechanism involving interaction between Sema3c/Nrp1 and Ephrinb1 during midline crossing in the corpus callosum (figure 1).

Optic Chiasm

Another critical component of the CNS is visual perception. The functional unit of the eye is the retina (figure 2A), which is a highly organized structure. Photoreceptor cells are photosensitive cells that transform photons of light into an electrical impulse that is transmitted to bipolar cells and subsequently to Retinal ganglion cells (RGCs). RGCs relay this electrical signal to the brain along their axons that form the optic nerve. Importantly, whilst other retinal cell types can modulate the electrical signal mediated by photoreceptor cells, such as amacrine and horizontal cells, RGCs are the only output neuron from the retina and connecting it to the brain. During visual system development, retinal ganglion cells (RGCs) extend axons towards a specialized structure at the midline, named the optic chiasm (OC). At this point, RGCs will either project to the same hemisphere (ipsi-lateral) or cross the midline to project to the opposite hemisphere (contra-lateral). Therefore, two types of RGCs, ipsilateral RGCs (iRGCs) and contralateral RGCs (cRGCs) can be defined by the laterality of their projections (Williams *et al.* 2004).

This process is critical for depth perception, stereopsis. Indeed, since both eyes will obtain a “picture” of our environment, by combining these pictures we will generate a three-dimensional (3D) representation of the picture. Interestingly, the amount of overlap between each eye is directly proportional to the amount of ipsi-lateral projections. For instance, species with laterally positioned eyes, such as mice, possess only 3-5% of ipsi-laterally projecting RGCs. However, humans and primates, with more frontally positioned eyes, possess approximately 50% of ipsi-laterally projecting RGCs (Guillery *et al.* 1995; Herrera *et al.* 2019; Jeffery and Erskine 2005). Mouse iRGCs and cRGCs are

characterized by specific transcriptional patterns and, in part, spatial localization, with iRGCs residing in the ventro-temporal retina, and cRGCs being dispersed across the retina (García-Frigola *et al.* 2008; Herrera *et al.* 2003; Pak *et al.* 2004; Williams *et al.* 2006; Kuwajima *et al.* 2017)(figure 2B-C).

In order to control the crossing of RGC axons at the OC, two processes take place: repulsion of axons with an ipsi-lateral fate, and the crossing of contralateral axons across the midline (figure 2D). EphB1/ephrin-B2 signaling pathway is a key component of ipsi-lateral axon repulsion. Expression of the EphB1 tyrosine kinase receptor is restricted to axons of iRGCs, while its ligand, the repulsive axon guidance molecule ephrin-B2, is expressed at the OC (Williams *et al.* 2003). When the axons reach the proximity of the OC, a chemo-repulsive gradient of ephrin-B2 leads to growth cone collapse and pausing of axonal outgrowth, eventually causing changes of trajectory and driving the axon towards ipsi-lateral visual nuclei (Petros *et al.* 2010). It was further shown that RGC axon laterality is transcriptionally regulated. The transcription factor Zic family member 2 (Zic2) was identified as a key regulator of iRGCs identity (Herrera *et al.* 2003; Wang *et al.* 2016). Furthermore, Zic2 is sufficient to induce the expression of EphB1 receptor in iRGCs (Lee *et al.* 2008; García-Frigola *et al.* 2008)(figure 2C). In addition, the transcription factor Forkhead box D1 (Foxd1) was shown to be critical in maintaining iRGCs fate by promoting the expression of Zic2 (Herrera 2004). In addition to the EphB1/ephrin-B2 repulsion pathway, another pathway also controls ipsilateral RGC repulsion: Shh is expressed by contralateral RGCs and transported axonally and anterogradely to the optic chiasm (Peng *et al.* 2018). At the optic chiasm, ipsilateral RGCs, which express the Shh receptor Boc, are repelled by Shh and therefore do not cross the optic chiasm, remaining ipsilateral (Peng *et al.* 2018; Fabre *et al.* 2010) (figure 2D).

In contrast, cRGC axons express the L1 cell adhesion molecule (L1CAM), the neuronal cell adhesion molecule (NrCAM), and the semaphorin receptor Plexin-A1. Together, these molecules provide a permissive substrate for cRGCs to invade and cross the OC (Williams *et al.* 2006; Kuwajima *et al.* 2012). Transcriptionally, the Sox C family of transcription factors (Sox4, Sox11, Sox12) was identified as key regulators for cRGC fate by regulating NrCAM and PlexinA1 expression (Kuwajima *et al.* 2017)(figure 2C,D). In

addition, the transcription factor Islet2 is expressed by ~30% of cRGCs, mainly expressed by late-born cRGCs (Pak *et al.* 2004; Kuwajima *et al.* 2017). Furthermore, the leucine-rich repeat (LRR) receptor Islr2 has been shown to be expressed on cRGCs and its deletion leads to aberrant ipsi-lateral projections in *Danio Rerio* (Panza *et al.* 2015).

Interestingly, binocular vision is impaired in patients with albinism (an absence of melanin production of the retinal pigmented epithelium). This led researchers to study the role of pigmentation on iRGCs. It was found that albino mice have less iRGCs, but a normal number of cRGCs (Rebsam *et al.* 2012). This appears to be linked to the timing of RGC differentiation: albino animals have a shorter time window during which iRGCs are born which is compensated by an increased number of cRGCs (Bhansali *et al.* 2014). Furthermore, the functional comparison of gene expression in albino and pigmented retinas, showed that the Wnt-pathway, which controls iRGC differentiation and cell proliferation, is dysregulated in albino animals (Iwai-Takekoshi *et al.* 2018). Rescue of ipsi-lateral deficit via blockage of Nr-CAM may improve visual capability in albino animals, thereby providing a paradigm for functionally investigating the consequences of natural ipsi-lateral depletion (Williams *et al.* 2006).

Interestingly, the existence of another population of RGCs has been described to project between the two retinas (retino-retinal projection) in various vertebrate species (Tóth and Strznicky 1989; Müller and Holländer 1988; Nadal-Nicolás *et al.* 2015). More recently, it was described that this population resides in the ventro-nasal retina and is transient (E16.5 to postnatal day 4) (Murcia-Belmonte *et al.* 2019). These late-born RGCs were shown to express Unc5c, a netrin-1 receptor. Upon reaching the optic chiasm, Unc5c-positive RGCs are repelled by netrin-1 and project into the contralateral optic nerve. Indeed, Unc5c is both sufficient and necessary for retino-retinal projections (Murcia-Belmonte *et al.* 2019). However, the precise connection and function of this projection remains to be characterized. Moreover, the implication of this projection in co-ordinating spontaneous activity remains to be studied.

Spinal Cord

In the developing spinal cord, midline crossing takes place ventrally through a structure named the floor plate (FP). The FP is a crucial patterning center composed of specialized

cells that contribute to the specification of the neuronal lineages of the neural tube and adjacent territories. Moreover, the FP is a source of both growth-promoting and growth-repulsive cues for commissural axons, such as netrin-1 and Slits (Chédotal 2019). In vertebrates, spinal commissural axons navigate first ventrally toward the floor plate (figure 3A), cross the midline and then turn rostrally or caudally (figure 3D). According to the current model, the sensitivity to midline repellents is silenced in pre-crossing commissural growth cones as they navigate toward the FP. However, during FP crossing, commissural growth cones gain responsiveness to FP repulsive cues. The post-crossing commissural neurons are thus expelled from the midline, and also prevented from re-crossing the FP. At later stages, they follow rostro-caudal gradients of guidance cues, turning rostrally or caudally in the ventral or lateral funiculi (Gaudet and Fonken 2018; Ducuing *et al.* 2019; Chédotal 2019).

Commissural axon guidance before midline crossing

The earliest born spinal commissural neurons will extend their axons towards the pial surface of the spinal cord and ventrally towards the FP (figure 3A). For many years it was thought that a long-range gradient of the secreted protein netrin-1 is generated by the FP and attracts commissural neurons ventrally upon binding the receptor Dcc (Finci *et al.* 2015; Hiramoto *et al.* 2000). However, recent studies have challenged this model and rather support a local and haptotactic function of netrin-1. Indeed, netrin-1 is not only expressed by FP cells but also by the neural progenitors of the ventricular zone of the spinal cord and brainstem. In support to this model, specific deletion of netrin-1 at the FP, does not perturb commissural axon crossing in the hindbrain (Dominici *et al.* 2017; Yamauchi *et al.* 2017). Interestingly, in the spinal cord, midline crossing appears slightly delayed (Moreno-Bravo *et al.* 2019) and some axons are misguided before crossing (Moreno-Bravo *et al.* 2019; Varadarajan *et al.* 2017; Wu *et al.* 2019). These results suggest that floor plate-derived netrin-1 is dispensable for commissural axon crossing, but also highlight different mechanism of action of netrin-1 between the hindbrain and the spinal cord. Importantly, ablating netrin-1 expression in ventricular zone progenitors severely perturbs midline crossing in the brainstem (Dominici *et al.* 2017; Yamauchi *et al.* 2017) but only mildly in the spinal cord (Moreno-Bravo *et al.* 2019). However, the simultaneous deletion of ventricular and FP derived netrin-1 prevents midline crossing

(Moreno-Bravo *et al.* 2019). Therefore, in the spinal cord, both sources of netrin-1 cooperate to guide commissural neuron at the midline. Other secreted proteins such as VEGF (Ruiz de Almodovar *et al.* 2011) and Shh (Bovolenta and Sanchez-Arrones 2012; Charron *et al.* 2003; Sloan *et al.* 2015; Wu *et al.* 2019) are expressed at the floor plate and act redundantly with netrin-1 to attract axons as they get close to the FP.

Robo3, a member of the Roundabout (Robo) family, plays a key role in midline guidance. This receptor is expressed transiently by commissural axons in mouse spinal cord, midbrain and hindbrain and then is rapidly down-regulated after the axons have crossed the FP (Belle *et al.* 2014; Zelina *et al.* 2014). It is expressed in human pontine neurons (Jen *et al.* 2004) and in hindbrain and spinal cord commissural axons of birds (Escalante *et al.* 2013; Friocourt and Chédotal 2017; Philipp *et al.* 2012) and other vertebrate species (Friocourt *et al.* 2019). The absence of Robo3 leads to a complete loss of several commissures in mice and in humans (Jen *et al.* 2004; Marillat *et al.* 2004; Renier *et al.* 2010; Sabatier *et al.* 2004; Michalski *et al.* 2013). The mechanism through which Robo3 controls commissure development is not completely understood. However, it was proposed that Robo3 expression in pre-crossing commissural neurons repress Slit/Robo repulsion (figure 3B), thus allowing commissural axons to reach, enter, and cross the ventral midline in response to netrin-1 attraction (Jaworski *et al.* 2010; Sabatier *et al.* 2004; Chédotal 2011). This mechanism has been validated in the spinal cord and lateral reticular nucleus. Interestingly, the inferior olivary nucleus does not seem to follow the same mechanism (Di Meglio *et al.* 2008). However, it was initially proposed that Robo3 may facilitate attraction of commissural neurons to the floor plate, independently of Slit/Robo signaling (Di Meglio *et al.* 2008; Jaworski *et al.* 2010; Sabatier *et al.* 2004). More recent studies support this notion. Indeed, whilst non-mammalian Robo3 retained its ability to bind Slits, the mammalian orthologue of Robo3 has lost key residues in the Slit/Robo binding domain (Zelina *et al.* 2014). Instead, it possesses the ability to bind to netrin-1, by creating a receptor complex between Dcc and Robo3 via Src kinases, on a conserved tyrosine residue and contributes to the attractive actions of netrin-1 (Zelina *et al.* 2014). Therefore, Robo3 might promote attraction to the ventral midline rather than counteract repulsion.

To date, several transcription factors have been associated with the most dorsal commissural population, dI1, which arises from the *Atoh1*$^{+}$ domain (Chédotal 2014). These interneurons are divided in two different subtypes depending on the location of their targets: ipsilateral (dI1i) or contralateral (dI1c) (Wilson *et al.* 2008). Interestingly, their projection pattern relies on the balance between the expression of two transcription factors *Lhx2* and *Lhx9* (Lim homeobox) and their upstream activation by the transcription factor *Barhl2* (Ding *et al.* 2012). Lhx2 is able to directly bind to the regulatory region of *Robo3* and modulate its expression in a dose-dependent manner. Moreover, in *Lhx2/9* knockouts, most of dI1 interneurons fail to cross the midline and project ipsilaterally (Wilson *et al.* 2008; Marcos-Mondejar *et al.* 2012).

Furthermore, the transcription factor Zic2 triggers an ipsilateral transcriptional program but also inactivates a contralateral one (Escalante *et al.* 2013). Indeed, downregulation of *Zic2* by *in utero* electroporation of siRNA induces an abnormal upregulation of Robo3 and a contralateral projection of dorsal horn neurons. On the other hand, a *Zic2* gain of function has the reverse effect, reducing *Robo3* expression and an increase of ipsilateral projections. In addition to modulating Robo3 expression, Zic2 is necessary and sufficient to induce EphA4 expression and commissural neuron repulsion in response to midline ephrinB's.

Commissural axon guidance after midline crossing

Upon FP crossing, commissural axons become sensitive to a myriad of repulsive guidance molecules expressed at the FP. However, prior to midline crossing, commissural axons do not express the receptors (at the surface) required to sense this repulsive environment. One such example is the repulsive receptor, PlexinA1, which is down-regulated at the surface of commissural neurons prior to midline crossing (figure 3C). PlexinA1 down-regulation at the growth cone involves the protease, Calpain-1 (Charoy *et al.* 2012; Nawabi *et al.* 2010). However upon FP entry, commissural neurons become exposed to the neuronal cell adhesion molecule (NrCAM) that inhibits calpain-1 activity (figure 3F). As a result, PlexinA1 can accumulate at the growth cone which becomes sensitive to the repulsive cue Sema3B (expressed at the FP) (Charoy *et al.* 2012; Nawabi *et al.* 2010). In addition to PlexinA1, the semaphorin receptor Neuropilin 2 (Nrp2) is also expressed at the growth cone following FP entry. Indeed, Sema3B and

Nrp2 double mutants display FP stalling as well as post-crossing misrouting (Nawabi *et al.* 2010; Parra and Zou 2010).

Slits are other repulsive cues expressed at the FP (Brose *et al.* 1999). As with PlexinA1 and Nrp2, commissural axon growth cones start expressing the Robo 1 and Robo2 receptors only after midline crossing, and become sensitive to Slit repulsion (figure 3E). Indeed, deletion of Robo receptors results in commissural axons stalling at the FP (Long *et al.* 2004; Garbe and Bashaw 2007; Blockus and Chédotal 2016). However, Silts can also function independently of Robo receptors. In vertebrates, Slits can be cleaved into two separate fragments (Brose *et al.* 1999; Wang *et al.* 1999). The shorter fragment (Slit-C) is able to bind to PlexinA1 in commissural neurons to induce growth cone collapse (Delloye-Bourgeois *et al.* 2015).

Once commissural axons have exited the FP, they are then guided by other cues to continue either rostrally or caudally. Little is known about the cues guiding postcrossing axons along the midline. However, Wnt signaling has been shown to be critical in this process (Onishi *et al.* 2014). An expression gradient of several Wnt family proteins controls the rostral turning of post-crossing commissural axons through an attractive mechanism involving the Frizzled3 (Fzd3) receptor (Lyuksyutova *et al.* 2003; Yoshikawa *et al.* 2003). The disruption of the Wnt gradient, results in a randomization of the growth of post-crossing commissural axons, which randomly turn towards the anterior or posterior part (Yoshikawa *et al.* 2003; Zou 2004). Recently, a mechanism orchestrating Wnt activation has been proposed. During FP crossing, commissural neurons expressing Smoothened (Smo) are exposed to the morphogen sonic hedgehog (Shh). This interaction leads to the reduction in mRNA translation of Shisa2, a well-known Wnt signaling inhibitor. Shisa2 inhibits the Wnt receptor Frizzled (Fzd3) trafficking to the cell surface by interfering with its glycosylation, inactivating Wnt signaling (Onishi and Zou 2017). Moreover, it has been shown that components of the planar cell polarity (PCP) signaling pathway mediate Wnt attraction and the anterior turning of commissural axons (Onishi *et al.* 2014; Zou 2012; Lyuksyutova *et al.* 2003). In addition to the PCP pathway, the canonical Wnt signaling pathway is critical in mediating post-crossing commissural neuron turning. Indeed, down regulation of both Lrp5 and Lrp6 (Low density lipoprotein receptor-related protein, co-receptors for Frizzled), which are required in the β-catenin-

mediated canonical Wnt pathway, lead to major defects in post-crossing commissural neurons (Avilés *et al.* 2016).

Shh also guides post-crossing commissural axons (Bourikas *et al.* 2005; Yam *et al.* 2012). After crossing, commissural axons become repelled by Shh and project anteriorly along a posterior-high Shh and anterior-high Wnt4 gradients. However, instead of mediating its action through Patched or smoothened, Shh acts through the Hedgehog Interacting Protein (Hip). Further experiments showed that this switch in Shh responsiveness depended on the levels of 14-3-3 proteins, which are low in pre-crossing and high in post-crossing commissural neurons, and modulate Protein Kinase A activity (Yam *et al.* 2012).

Peripheral nervous system development

The bilaterian nervous system is subdivided in two main components: the central and the peripheral nervous systems (CNS and PNS). Permanent cross-talk between the CNS and PNS is critical for integration of sensory inputs. In the 4th century BC, *Alcmaeon of Croton* (Goddard *et al.* 1996)(Zolog 1994) proposed the first theory about channels ("poroi" in ancient greek) that would connect the senses and the brain, this last one being the center of human perception. Later, it became clear that all sensory perception being mechanical, auditory, gustatory and olfactory were relayed to the CNS through the "nerves" (Mazengenya and Bhikha 2017). Indeed, the precise interplay between these two networks develop in parallel during embryonic development (Ben-Arie *et al.* 2000). Additionally, it has been demonstrated that both axon guidance and neuronal activity can strongly modulate connections between the PNS and the CNS (Wang and Bergles 2015; Bonanomi and Pfaff 2010). Nevertheless, the PNS is itself formed by different components, each specialized in the transmission of a specific signal to the CNS. These signals are transmitted by mechanosensory, chemical or thermal receptors projecting to the mammalian spinal cord via nociceptive afferents.

Drosophila bristles are sensory organs that are tightly distributed and contain one single mechanosensory neuron that specifically projects to the CNS. These axons can be guided by cell adhesion molecules, such as Neuroglian or Flamingo (Martin *et al.* 2008; Steinel and Whitington 2009), but also by other guidance molecules, such as Plexins or semaphorins (Wu *et al.* 2011). Down syndrome cell adhesion molecule (DSCAM) is a

transmembrane receptor of the immunoglobulin-superfamily (Chen *et al.* 2006). DSCAM has since been described to regulate cell targeting, axon branch specification, and dendrite patterning (Schmucker *et al.* 2000; Wang *et al.* 2002a; Dascenco *et al.* 2015). The repulsive molecule, Slit, has been shown to bind and signal through DSCAM1 independently of Robo receptors (Chen *et al.* 2006). Indeed, local binding to Slit drives spatial specificity of axon collateral formation. Furthermore, Chen *et al.* report that many DSCAM isoforms exist and particular DSCAM isoform mosaicism in a specific growth cone appears to dictate local guidance decisions, such as the formation of axon collateral projections (Chen *et al.* 2006).

The inner ear is essential for the transmission of sounds and their integration by the CNS. This complex sensory organ is composed of bipolar spiral ganglion neurons (SGN) that connect the ipsilateral cochlear nucleus and the mechanosensory inner and outer hair cells located in the organ of Corti (Nayagam *et al.* 2011). SGNs project to both the inner (IHC) and the outer hair cells (OHC). During the course of development, both type I and II project to the OHC but type I SGNs appear to refine in later stages and only project to the IHC (Huang *et al.* 2012; Safieddine *et al.* 2012; Druckenbrod and Goodrich 2015). The use of molecular markers to target single spiral ganglia has revealed key morphological differences between type I and type II SGNs as well as their specific projection patterns to the IHC or outer hair cells OHC (Druckenbrod and Goodrich 2015; Coate *et al.* 2015). Indeed, type I and II SGNs were shown to be molecularly different. Type I SGNs express the semaphorin receptor Nrp2 and its co-receptor PlexinA3 (Coate *et al.* 2015). Upon binding Sema3F, secreted by the OHC, type I SGNs are repulsed and restrict their projections to the IHC (Coate *et al.* 2015). More recently, the use of single sequencing has allowed a more in depth characterization of SGNs. In this study, Shrestha *et al.* identified that type I SGNs can be further classified into three different subtypes (Shrestha *et al.* 2018). These data suggest a growing complexity of the auditory system formation and integration of external signals.

These examples underline the complexity of PNS development. With the aim of understanding the surrounding environment, each of these systems seems to have its own guidance mechanisms, which through a tight and orchestrated regulation, establish an essential pathway between sensory neurons and superior brain areas. Novel genetic

and technical approaches also highlight the cellular heterogeneity in these systems, most of them considered quite homogeneous until recently. The understanding of the molecular differences between cell types in a determined structure is a key element in establishing therapeutic approaches such as stem cell therapy. Moreover, these molecular differences can also help to understand the possible effects of different known guidance mechanisms. Lastly, several groups have tried to understand the role of spontaneous activity in these structures. Neural activity has been observed to happen randomly in most structures, since early development (Shrestha *et al.* 2018). This neuronal activity could be essential to pattern and reinforce synapses, as was shown in the visual system (Ackman and Crair 2014).

Non-traditional roles of axon guidance molecules

Axon guidance molecules have been extensively studied during axonal development but have also been shown to be critical in many diverse biological processes such as angiogenesis and cell migration (Castets and Mehlen 2010; Aberle 2019). Undeniably, cortical development is dependent on cellular migration. A fundamental question for the past decades has been the emergence of the neocortex, a specific feature of the mammalian brain (Northcutt 2006; Finlay and Darlington 1995). Cortical development begins with the division of radial glial progenitor cells (RGPCs), which gives rise to all cortical neurons and glia. RGPCs are aligned at the cortical ventricular zone and undergo mitosis to either self-renew (symmetric division = indirect neurogenesis), or differentiate into cortical neurons (asymmetric division = direct neurogenesis). However, in mammals RGPCs can also divide symmetrically to give rise to an intermediate progenitor cell (IPCs) (Haubensak *et al.* 2004; Noctor *et al.* 2004; Miyata *et al.* 2004). IPCs can either self renew or differentiate into cortical neurons. Thus, IPCs have been proposed to serve as the main determinant for cortex expansion in mammals (Malatesta *et al.* 2000; Noctor *et al.* 2001; Noctor *et al.* 2004; Kriegstein *et al.* 2006; Hansen *et al.* 2010; Smart 2002). Recently, Cardenas *et al.* have identified a novel role for Robo 1/2 in regulating radial glia cortical migration (Cárdenas *et al.* 2018). By comparing the mouse olfactory bulb (OB) (reminiscent of the reptilian paleocortex) to the cortex (Cx), Cardenas *et al.* observed that the OB solely developed by direct neurogenesis whereas the Cx was developed mostly by indirect neurogenesis (Cárdenas *et al.* 2018). Interestingly, Robo1/2 were expressed in a gradient, with a high-low expression in the OB compared to the Cx. Furthermore, it

was shown that Robo1/2 can regulate the notch canonical signaling pathway via Delta-like 1 (Dll1) as well as the ligands *jagged* 1 (*Jag1*) and *Jag2*. The mechanism put forward is that high Robo1/2 expression in the OB reduces Dll1 expression and increases *Jag1* and *Jag2* expression, resulting in asymmetric division and direct neurogenesis. Indeed, gain of function experiments showed that high Robo1/2 expression is sufficient to induce direct neurogenesis in the mouse Cx (Cárdenas *et al.* 2018). Interestingly, amniotes deprived of a neocortex, such as birds and reptiles, show high Robo1/2 expression in the cortex (Cárdenas *et al.* 2018). Thus, the authors propose silencing of Robo1/2 as an evolutionary switch giving rise to indirect neurogenesis in mammals.

The Fibronectin Leucine Rich-repeat Transmembrane (FLRTs) proteins have also been identified as axon guidance molecules. For instance, thalamocortical axons expressing Dcc are not sensitive to the netrin-1 gradient present in the thalamus due to Robo1 silencing (Leyva-Díaz *et al.* 2014). However, FLRT3 can sequester Robo1 to allow for Dcc expression at the surface of thalamocortical neurons, thereby activating netrin-1 responsiveness (Leyva-Díaz *et al.* 2014). More recently, FLRTs have also been implicated with cortical progenitor migration (del Toro *et al.* 2017). In mammals the cortex initially forms as a laminar sheet. Whilst some mammals (mice and rats) will retain this smooth cortical development (lissencephaly, figure 4A), other mammals (primates and ferrets) develop cortical folds (gyrencephaly, figure 4B). FLRT1/3 have recently been shown to be critical players in this process (del Toro *et al.* 2017). Interestingly, genetic ablation in mice of FLRT1/3 promotes cortical folding (del Toro *et al.* 2017). Whilst the proliferation rate of radial glia cells was unchanged, their migratory patterns were significantly perturbed. Indeed, loss of FLRT1/3 increased neuronal clustering and radial migration rate. This creates columns of migrating progenitors, inducing an asymmetric proliferation across the surface of the cortex and as a result creating sulci. Of note, FLRT1/3 expression is reduced in gyrencephalic species, suggesting that the abundance of FLRT1/3 during evolution promoted cortical smoothing (lissencephaly).

Whilst typical axon guidance proteins such as Slits and ephrins have been largely discussed, a growing body of literature has shown that some lipids could be atypical guidance molecules. Phospholipids are considered the major components of cell membranes and they have the ability to form amphipathic lipid bilayers. Their role in axon guidance was first proposed by some in vitro experiments in which Lysophosphatidic acid

(LPA), an intermediate substance of lipid synthesis, was shown to be able to induce growth cone collapse, neurite retraction and cell rounding in neuroblastoma-derived neuronal cell cultures (Jalink *et al.* 1993). Later on, further evidence highlighted their role on primary cultured chick embryo neurons and on isolated retinal growth cones (Saito 1997; Campbell and Holt 2001). Additional *in vivo* evidence of the axon guidance role of lysophospholypids were obtained in the *Xenopus* visual system. In absence of sphingosine 1-phosphate (S1P), retinal projections were misguided and invaded abnormal areas (Strochlic *et al.* 2007). Recently, a novel role for phospholipids on axon guidance was brought to light: Phosphatidyl-B-D-Glucoside (PtdGlc) is localized in radial glia and nascent astrocytes *in vivo* (Nagatsuka *et al.* 2006; Kinoshita *et al.* 2009). PtdGlc can be hydrolysed in *lyso*PtdGlc and released into the extracellular environment (Guy *et al.* 2015). In the embryonic chick and mouse spinal cord, TrkA and TrkC dorsal root ganglion axons enter the CNS through the dorsal root entry zone (DREZ). Only TrkC axons get into the primordial dorsal funiculus (PDF) where *Lyso*PtdGlc is found (Guy *et al.* 2015), suggesting a possible repulsive role for nociceptive afferences (TrkA). TrkA enriched DRG explants showed chemorepulsion *in vitro* in presence of a *lyso*PtdGlc gradient. Furthermore, blocking antibodies for *lyso*PtdGlc used *in ovo*, showed a misprojection of TrkA axons in the PDF. Finally, a receptor screening proposed GPR55 as putative receptor for this extracellular cue. GPR55 *knockout* mice phenocopy the DRG axon misprojections induced by *lyso*PtdGlc blocking antibodies, confirming the role of this receptor-sensing glia released *lyso*PtdGlc in this system (Guy *et al.* 2015).

Axonal Regeneration

In the early 20th century, pioneering studies from Ramón y Cajal showed that the mammalian CNS is unable to regenerate following a lesion (Ramón y Cajal 1914). Neuroscientists have since delved on the idea that understanding CNS development could be the key to hi-jack regenerative mechanisms following CNS injury. Interestingly, similar lesion experiments carried out on dorsal root ganglia axons (which belong to the PNS) resulted in robust regeneration of their peripheral branch and functional recovery following the lesion, whereas their central branch projecting into the CNS did not regenerate. Therefore, something either intrinsic or extrinsic to CNS neurons is

responsible for their lack of regeneration. This fundamental question has sparked years of intensive research to understand the molecular mechanisms activated after axotomy and to develop strategies for inducing axonal regeneration. Here, we will discuss some of the current approaches and challenges in regeneration of CNS and PNS axons.

Central Nervous System regeneration

Visual system regeneration

Optic nerve (ON) transection or crush, have become a predominant models for studying CNS regeneration. The ON only contains axons which originate from RGC neurons in the retina. Directly following ON lesions, multiple inhibitory pathways are triggered. The Activator Protein 1 (AP1) and the transcription factor subunit c-Jun (Fos-binding protein p39) both act in synergy to trigger cell death in RGCs following an experimental axotomy (Hüll and Bähr 1994). In addition, interplay between c-Jun and the Activating Transcription Factor 2 (ATF2) dictates cell fate following ON crush. When both are upregulated, they promote cell survival. However, reduction in ATF2 expression induces apoptosis (Martin-Villalba *et al.* 1998). The activation of c-Jun is driven by calcium influx, since specific inhibition of calcium channels leads to reduction of c-Jun activity in ON crush models. Remarkably, the inhibition of calcium channels not only reduces acute axon neurodegeneration, but also improves axonal regeneration (Ribas *et al.* 2017). The RhoA/ROCK/LIMK pathway, which can be activated by a variety of cytokines and inflammatory mediators, is another critical inhibitory mechanism that mediates repulsive signals in the injured CNS. The knockdown of either Rho Associated Coiled-Coil Containing Protein Kinase 2 (ROCK2) or its downstream substrate LIM domain kinase 1 (LIMK1) promotes neuronal regeneration following ON crush. However, only ROCK2 knockdown was found to be neuroprotective in RGCs following ON axotomy (Koch *et al.* 2014). ROCK2 downregulation leads to reduced calpain and caspase3 activity and a concurrent increase in protein kinase B (Akt) activity (Koch *et al.* 2014). Pharmacological inhibition of RhoA/ROCK pathway through Y-27632 or Fasudil administration promotes neuronal regeneration in a dose-dependent manner, probably due to enhanced MAPK and Akt phosphorylation (Lingor *et al.* 2007; Lingor *et al.* 2008). Pharmacological modulation of this pathway, thus, could represent a therapeutic approach for CNS cell restoration.

Another inhibitory pathway exists in the surrounding environment of the lesion. The myelin inhibitory proteins: Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) suppress axonal growth in the optic nerve by acting through Nogo receptors (NgR) where they act in an orchestrated manner with other co-receptors, such as p75 neurotrophin receptor and epidermal growth factor receptor (EGFR) (Wang *et al.* 2002b; Wang *et al.* 2002c; Domeniconi *et al.* 2002; Koprivica 2005). Notably, deletion of its downstream signaling pathway, through protein kinase C (PKC) appears to restore axonal growth by blocking Rho activation (Sivasankaran *et al.* 2004). Furthermore, the over-expression of a NgR dominant negative in RGCs promoted axonal regeneration (Fischer 2004).

Alternatively to extrinsic factors inhibiting axon regeneration, many groups have shown that the intrinsic mechanisms prevent CNS axon regeneration (He and Jin 2016). One critical pathway, put forward by the group of Zhigang He, was the phosphoinositide 3-kinase (PI3K)/ mammalian target of rapamycin (mTOR) signaling pathway. Following ON crush, a dramatic decrease in PI3K/mTOR activity is observed (Park *et al.* 2008). Indeed, following ON crush, mTOR activity is suppressed by the phosphatase and tensin homolog (PTEN). Genetically deleting PTEN in RGCs, using an adeno-associated virus, induces robust and long-distance axon regeneration following ON crush (Park *et al.* 2008). Interestingly, PTEN deletion following ON crush only stimulates the regeneration of a subset of RGCs (Park *et al.* 2008). A large number of RGC types exists with distinct physiology and projection patterns (Sanes and Masland 2015; Martersteck *et al.* 2017). Therefore, it is not surprising that distinct RGCs types respond heterogeneously to injury. Further studies have shown that a specific subtype of RGCs, the α-RGCs, are able to survive following ON crush and express insulin-like growth factor receptor (IGF1) as well as osteopontin (OPN) (Duan *et al.* 2015). Reprogramming of RGCs after injury is accompanied by changes in mRNA expression profiles. The transcription factors that are expressed after injury appear to determine whether a specific sub-type of RGC will regrow. The Kruppel-like factors 4 and 9 (KLF4 and KLF9), for example, play a major role suppressing axon development (Qin *et al.* 2013; Apara *et al.* 2017). KLF4 interacts with Tyr705-phosphorylated signal transducer and activator of transcription 3 (STAT3) suppressing its activity and function as an intrinsic barrier for regeneration of damaged adult RGC axons (Qin *et al.* 2013). KLF9 functions as another intrinsic inhibitor for axon

regeneration as shRNA mediated knockdown of KLF9 promote RGC survival and axon regeneration following optic nerve injury *in vivo* (Apara *et al.* 2017). This KLF9-mediated inhibition is via interaction of the upstream kinase c-Jun N-terminal kinase 3 (JNK3) (Apara *et al.* 2017). Recent findings indicate that micro RNAs 135a and 135b (miRNA135s) could regulate KLF4 expression during axon development. Intravitreal administration of miRNA135 induced axon regeneration following ON injury, in part by suppressing KLF4 expression in RGCs (van Battum *et al.* 2018). SOX11, on the other hand, plays a dual role. When overexpressed, it can stimulate axon growth of non-α-RGCs while it induces cell death of α-RGCs following ON crush (Norsworthy *et al.* 2017). In addition to intrinsic growth metabolism, neuroinflammation has been shown to be critical for RGCs axonal regeneration (Smith *et al.* 2009). The simple intraocular injection of Zymosan (protein-carbohydrate complexes derived from yeast cell wall), which enhances macrophage infiltration in the injured ON, greatly stimulates the expression of GAP-43 in RGC axons, resulting in accelerated axonal regeneration (Leon *et al.* 2000).

During the past decades, major effort has been made towards the development of combinatorial therapeutic approaches, targeting two or more neuronal pathways. Over stimulating cell growth programs by deleting PTEN and suppressor of cytokine signaling 3 (SOCS3) promotes a well-sustained axon regeneration, by brake-releasing two independent pathways that converge into axon growth-related gene expression (Sun *et al.* 2011). In an alternative approach, the use of the pro-inflammatory Zymosan, in combination to PTEN inhibition and cAMP analogue administration promotes axonal regeneration, and allows the recovery of long distance axonal degeneration (Kurimoto *et al.* 2010). The critical step following regeneration and functional synapses formation is the restoration of the conductance and visual function following injury. Treatment with the voltage-gated potassium channel blocker 4-aminopyridine (4-AP) or its methyl derivative 4-AP-3-Me restores conduction and visual acuity following PTEN/SOCS3 co-deletion. Similar phenomena were observed when mice were treated with 4-AP following osteopontin (OPN) overexpression in the presence of insulin-like growth factor 1 (IGF1) and ciliary neurotrophic factor (CNTF). This highlights the importance of combination therapy for axon regeneration and improving visual conduction (Bei *et al.* 2016).

Spinal cord regeneration

Another attractive model for studying CNS regeneration is that of Spinal Cord Injury (SCI). In addition to its scientific interest, SCI has a dramatic clinical impact, with the world health organization approximating between 250,000 to 500,000 people suffer from SCI each year (Courtine and Sofroniew 2019). The Cortico spinal neurons (CSNs) located in the mammalian neocortex are the major output from the brain to the spinal cord (making up to 90% of projections), which mediate both motor and sensory functions (Wang *et al.* 2017). Indeed, Corticospinal Tract (CST) lesions, such as a bilateral pyramidotomy, lead to the complete loss of voluntary movement. Following a spinal cord lesion, extrinsic mechanisms such as growth inhibitors or glial scars, inhibit axonal regeneration (Gaudet and Fonken 2018). Several inhibitory signaling molecules have since been identified such as Nogo or myelin-associated glycoproteins (McKeon *et al.* 1991; Caroni and Schwab 1988; Afshari *et al.* 2009; Lang *et al.* 2015). Indeed silencing these inhibitory cues in mouse or rat models of spinal cord injury has shown some success (Schmandke *et al.* 2014). Another approach, led by Zhigang He's group, questioned whether the inability of CNS neurons to regenerate involved intrinsic factors. They identified that mTOR (mammalian target of rapamycin) activity and *de novo* protein synthesis are suppressed after CNS lesions (Park *et al.* 2008). Reactivation of the mTOR pathway by silencing of PTEN (phosphatase and tensin homolog) and TSC1 (tuberous sclerosis complex 1), leads to extensive CST axon regeneration (Park *et al.* 2008). Together, these findings proved that both intrinsic and extrinsic mechanisms were responsible for inhibiting CST axon regeneration after spinal cord injury. Accordingly, the combined genetic deletion of Nogo receptors and PTEN, led to a major increase in the regeneration and sprouting of lesioned CSNs (Geoffroy *et al.* 2015). However, there was little to no functional amelioration in lesioned animals.

The lack of functional rescue observed in double mutants of Nogo and PTEN led to the idea that a better understanding of the locomotor system was required. Indeed, very little was known about the localization, development, and function of the CSNs responsible for voluntary movement. Using retrograde viral tracing strategies, it was found that CSNs were localized in both the motor and somatosensory cortex. Two major nuclei were identified, the Rostral Forelimb Area (RFA) and the Caudal Forelimb Area (CFA) (Wang *et al.* 2017). To better understand the precise function of RFA and CFA CSNs in voluntary task, a food pellet retrieval task was used to tease apart which CSNs were

responsible for specific motor movements. Using AAV:Cre-driven GCamp6 expression in CSNs, it was shown that RFA CSNs fired prior to grasping the food-pellet, whereas CFA CSNs fired prior to reaching as well as post-grasping the food pellet (Wang *et al.* 2017). This data provide evidence supporting a parallel organization of motor tasks responsible for specific behavior organized in a topographic manner.

While much work has focused on spinal cord lesions, the majority of patients suffer from partial lesions. However, partial spinal cord injuries still result in complete loss of motor function below the site of injury. This hints towards the idea that unlesioned axons are unable to properly function despite being spared from injury. By carrying out a staggered hemisection of the thoracic spinal cord on opposing sides, Chen *et al*. took advantage of a partial spinal cord injury model to carry out a small compound screening approach (Chen *et al.* 2018). They identified that an agonist of the potassium/chloride transporter (KCC2) resulted in increased weight bearing strength in mice following lesion (Chen *et al.* 2018). Due to the complexity of spinal circuits, they questioned whether KCC2 activity was required by multiple or distinct neuronal subsets (excitatory, inhibitory, motor). Interestingly, only overexpression of KCC2 in inhibitory neurons (Vgat:Cre) resulted in an improved weight-bearing strength in injured mice. Moreover, inhibitory neurons below the staggered lesion were dispensable for this rescue, since overexpressing KCC2 only in inhibitory neurons between the lesions was sufficient to rescue the weight bearing strength. Overall, this identifies a crucial role for inhibitory interneurons in a closed-circuit to be critical in associating the excitation to inhibition ratio required to regulate functional recovery following a lesion (Chen *et al.* 2018).

The translation of these findings to the treatment of spinal cord injury, won't be simple as the genetic manipulation of tumor suppressor genes may not be suitable for the clinic. Attractive treatment alternatives could consist of applying growth factors (which occur endogenously in the CNS). However, adult CNS neurons lose their sensitivity to growth factors such as BDNF (Liu *et al.* 2017). Moreover, adult CSNs treated with OPN can be sensitized to the IGF1 by reactivating the mTOR pathway (Liu *et al.* 2017).

Furthermore, the identification that spared axons following partial spinal cord injury could be re-synchronized to induce functional recovery opens many questions, one of which, is the importance of inhibitory synapses conserved amongst other species. Indeed, the

complexity in human spinal cord circuitry may pose a major challenge towards this clinical implication.

For instance, how does our current understanding on excitatory/inhibitory ratios in the rodent spinal cord translate to the human spinal cord? This is a daunting question since inhibitory feedback loops in human spinal cord may be more complex and thus more challenging to re-stimulate. Furthermore, more research should focus on understanding the coordinated action between neurons and glial cells. Synaptic activity, axonal and dendritic growth and regeneration are fine-tuned by glial cells (Liu *et al.* 2017). There are a few studies investigating how glial scar-induced disruption may be overcome by improving the communication between neurons and glial cells. Of note, exosomes released by glial cells in the PNS have shown to promote robust axonal regeneration and survival (Lopez-Leal and Court 2016). A similar result was obtained from mesenchymal stem cells releasing exosomes following spinal cord injury (Liu *et al.* 2019; Li *et al.* 2018).

Peripheral Nerve Regeneration

Insults such as physical trauma, chemotherapy or metabolic disorders can lead to peripheral nerve damage (Scholz *et al.* 2009). As previously mentioned, PNS axons have retained considerable capacity to regenerate following injury and to form functional connections with their original targets (Bremer *et al.* 2017). This seems in part due to a cell-intrinsic growth-promoting response of PNS neurons and to a favorable environment for axonal regeneration (Bremer *et al.* 2017). However, many aspects of this process, including how regenerating axons navigate across the lesion site and select their original trajectory at branch choice points, are not well understood *in vivo*. Whilst many animal models have been used to study PNS regeneration, the zebrafish has been heavily studied due to its transparency (allowing for *in vivo* live imaging) as well as the ability for genetic manipulation. Adult and larval fish both have well-defined PNS circuits and stereotyped behaviors, facilitating cell biology studies underlying PNS axon regrowth and synapse re-establishment (Rasmussen and Sagasti 2017; He and Jin 2016).

In vertebrates, spinal motor nerve degeneration after transection occurs through morphological hallmarks characteristic of Wallerian degeneration (figure 6A), a stereotyped form of degeneration (Waller 1851). Live imaging on zebrafish showed that following nerve transection, degradation of the distal axon happens within 120-240

minutes (Rosenberg *et al.* 2012). Moreover, it highlighted that individual axons within the transected nerves initiate fragmentation at different times independent of myelination and thickness of the axons. Once initiated, fragmentation occurs along the entire length of the axon within minutes. Recruitment of macrophages to the injury site starts between 60-120 minutes before nerve fragmentation, and with the onset of axonal fragmentation, macrophages enter the nerve and begin to phagocytose nerve debris. Experimental elimination of Schwann cells through genetic ablation does not change the recruitment and behavior of macrophages to the injury site suggesting that this process is independent on Schwann cells (Dutton *et al.* 2001; Rosenberg *et al.* 2012).

In zebrafish, 80% of regenerating axons retain the ability to select their original branch-specific trajectory in both ventral and dorsal nerve branches following complete nerve transection (Isaacman-Beck *et al.* 2015). After complete nerve transection, usually a single axon emerges from the proximal nerve stump to pioneer a regenerative path across the injury site. At later stages, multiple emerging axons join the pioneering axon and extend with about twice the speed of the pioneering axon across the injury gap. The synaptic low-density lipoprotein receptor-related protein 4 (lrp4) is critical for the regrowth of follower axons across the injury gap and towards their original targets (Gribble *et al.* 2018)(figure 6C). Live-cell imaging shows that Schwann cells provide directionality to axons, by crossing the injury site and navigating to their original trajectory. An interaction between Schwann cells and motor axons is triggered following motor nerve transection leading to highly coordinated changes in axonal and Schwann cell morphology during both degeneration and regeneration (figure 6B). For instance, once axons start to fragment, Schwann cell membranes, localized distal to the lesion site, undergo dramatic morphological changes returning to a more immature state (Rosenberg *et al.* 2014). Lrp4 promotes the morphological changes associated with Schwann cells re-differentiation after injury-induced de-differentiation. *In vivo* evidence suggests that lrp4 promotes peripheral nerve regeneration through a non-canonical, Agrin/MuSK independent signalling pathway that is critical for neuromuscular synapse development in mammals and zebrafish (Gribble *et al.* 2018). The importance of this process in promoting vertebrate nerve regeneration is also confirmed by the impairment of peripheral nerve regeneration by Topoisomerase I inhibitor, identified through a fin removal assay

performed by Bremer and colleagues, which hypothesizes that Topoisomerase I promotes peripheral nerve regeneration by regulating gene transcription specifically in de-differentiated Schwann cells (Bremer *et al.* 2017).

In zebrafish mutants lacking Schwann cells, 50–80% of transected nerves show a failure in regenerating axons along their original trajectory through the ventral myotome compared with 20% in wild-type (Rosenberg *et al.* 2014). Netrin1 and its receptor DCC have been implicated in promoting the extent of axon regeneration (figure 6C): *in vivo Netrin1b* mRNA is expressed in Schwann cells before and after motor nerve transection, and *dcc* mRNA is detectable in motor neurons during initial axonal regrowth. In $dcc^{zm130198}$ mutants, characterized by a 90% reduction of *dcc* mRNA, 40% of regenerating motor axons extended not only along their original path but also along ectopic lateral trajectories. This suggests that DCC is required to guide regenerating ventral motor axons across the injury gap toward their original trajectory *in vivo* (Rosenberg *et al.* 2014).

Early after transection (7-11 hours post transection), dorsal axons sprout growth cones that explore the environment with multi-directional extensions and retractions. In the following 2-4 hours, only the growth cones extending along the correct dorsal path are stabilized and quickly extend, supporting the existence of extrinsic cues that drive the growth cones through the branch point (Isaacman-Beck *et al.* 2015). Among these cues, the collagen-modifying glycosyltransferase lysyl hydroxylase 3 (*lh3*), expressed by Schwann cells, has a crucial role in promoting target selectivity of regenerating dorsal, but not ventral nerve axons (figure 6B). For this process, equally fundamental is Collagen4a5 (col4a5), the *lh3* substrate, which is over expressed in a small group of Schwann cells located ventral and ventrolaterally to the transection gap. Col4a5 destabilizes mistargeted axons, directing regenerating axons toward their original targets. Following the nerve transection there is an upregulation of the canonical axon guidance repellent *slit1a* in cells expressing col4a5. Hypothetically, in response to injury, Schwann cells ventral to the transection site, secrete col4a5, which binds and accumulates Slit, thereby forming a repulsive barrier to direct dorsal axons onto their original dorsal path (Isaacman-Beck *et al.* 2015)(figure 6C).

Real-time imaging on live zebrafish has allowed deciphering some of the intrinsic and extrinsic mechanisms responsible for peripheral nerve regeneration, but the molecular mechanisms and the specific cellular interactions which are vital for axon regeneration are not completely understood. Furthermore, the identification of other regeneration-promoting molecules could allow the development of new neuronal repair strategies. Based on that premise a fin removal assay was developed that enabled to perform the first whole organism small molecule screen to identify pathways that promote vertebrate nerve regeneration (Bremer *et al.* 2017). The approach utilized the regeneration of the ring-like nerve of zebrafish larvae pectoral fin after removal as readout of axonal regrowth. 480 bioactive compounds with known biological targets were screened to identify molecular pathways promoting nerve regrowth. After excluding 134 compounds which affected larvae health, the remaining compounds were combined in 69 distinct pools and added each of these pool to larvae immediately following fin amputation to evaluate the re-formation of regenerating axons of a ring-like nerve network at the fin base that normally occurs in 24 hours. In larvae exposed to 15 pools regenerating axons failed to form the characteristic ring-like network. Next, each of the compounds was tested within a given pool individually. This failed to identify a singly effective compound in 20% of pools which reduced nerve regrowth in the first pass of the screen. Despite the fin removal assay is a powerful screening method to study nerve regeneration, the high false positive rate indicates the importance of a second method to confirm the results.

Other strategies for CNS/PNS repair

Many strategies have shown incredible potential for regeneration of the CNS following an injury, but a major concern remains the significant death of neurons (Ling *et al.* 2015; Mckee and Daneshvar 2015). Moreover, complex diseases such as stroke, amyloid-β plaque accumulation and inflammatory-mediated neurodegeneration lead to broad defects such as defective communication, glial scar formation and ultimately neuronal loss, which make it challenging to repair (Albrecht *et al.* 2015; Chauhan 2014). In such cases, the development of cell therapy has shown some promising results (Gates *et al.* 2000). Stem cell manipulation is a powerful tool to understand neurodevelopment and its integration into developing tissue can recapitulate neurogenesis. In vivo strategies based on stem cell replacement such as human and mice-derived embryonic stem cells (ESCs)

for brain repair in models of neurological diseases, have made significant progress in pre-clinical trials (Barker *et al.* 2015; Péron *et al.* 2017). It is known that the lack of endogenous repair in the brain leads to a condition of life-long disease and disability after a neuronal damage. Thus, understanding how well the transplanted neurons are able to mature and integrate into damaged circuits is a challenging task.

Due to the complexity of the organization of the cerebral cortex in terms of highly specific topography and connectivity (Guillemot *et al.* 2006; Tiberi *et al.* 2012), the replacement of lost neurons is a daunting challenge. A notable initial attempt at regenerative cortical cell therapy showed that transplanting embryonic cortical tissue from transgenic mice to lesioned cortex in an adult brain could regenerate neurons and establish neuronal projections and synaptic connections (Gaillard *et al.* 2007; Wernig 2004). These findings have been corroborated by data from other studies which have shown that not only ESCs have intrinsic mechanisms of corticogenesis (Gaspard *et al.* 2008) but induced pluripotent stem cell (iPSC)-derived neurons also can regenerate functional cortical neurons (Espuny-Camacho *et al.* 2018; Falkner *et al.* 2016).

There are certain prerequisites for the ESCs to successfully integrate the malfunctioning adult cortex. Re-establishment of neuronal connectivity and function requires identity match between the damaged brain area and the transplanted material (Michelsen *et al.* 2015). Human embryonic stem cell (hESC)-derived visual cortical neurons, once grafted into the lesioned adult murine cortex, are able to mature and integrate into the corresponding cortical layers, acquiring a visual-like identity and rewiring the circuit with appropriate inputs and outputs (Espuny-Camacho *et al.* 2018). Interestingly, hES-derived cells transplanted into motor cortical areas showed a reduced maturation and a lower capacity to send long-range projections, suggesting that the areal identity match represents an important factor for successful cortical transplantation.

Another key factor accompanying identify match, is the adaptation to the extrinsic *in vivo* environment to establish polarity, such as microenvironment generated by blood vessels (Javaherian and Kriegstein 2009) or composition of extracellular matrix (Fietz *et al.* 2012). Context-wise, junctional complexes block the incorporation of grafted cells at the apical surface (Espuny-Camacho *et al.* 2013). For this reason, a new grafting method called TETCaD (transplantation to epithelial tissue with calcium depletion) has been

developed, using moderate concentrations of EGTA (ethylene glycol tetra-acetic acid) to chelate calcium, increasing efficiency of transplantation and dissociating adherents junctions in the epithelial tissue (Nagashima *et al.* 2014). Lastly in the molecular level, lesion site-specific cues and signalling pathways such as the WNT signalling and NOTCH2NL genes have been implicated in human corticogenesis (Fiddes *et al.* 2018; Raitano *et al.* 2015; Suzuki *et al.* 2018). *In vitro* and *in vivo* use of iPSCs in animal models simulating human diseases such as stroke (Tornero *et al.* 2013), Alzheimer's disease (AD) (Espuny-Camacho *et al.* 2017) and multiple sclerosis (MS) (Theotokis *et al.* 2015), highlight the hallmarks of synaptic activity, connectivity and cortical neuronal maturation (Suzuki and Vanderhaeghen 2015). Specifically for AD, the significant expression of non- coding RNA sequences in grafted human neurons further opens up an entirely new avenue for investigating the involvement of non-coding RNAs in AD-induced neurodegeneration.

Altogether, establishing the molecular mechanisms regulating fate acquisition and plasticity after cell transplantation is of great importance in the light of preclinical studies. Determining which cues are involved in fate maintenance, area specificity and functional integration in the adult brain will be pivotal for successful outcomes in stem cell-based therapies. These signaling events may also play major roles in neurodegeneration so targeting pathways such as the Wnt pathway, may result in the establishment of novel therapeutic approaches. Nonetheless, potential adverse effects and host-transplant compatibility should be addressed before these approaches can be considered for clinical applications.

Conflict of interest disclosure

This Review was invited following the 2nd JNC-ISN Flagship School held in 2018 in Alpbach, Austria. Alain Chédotal was the Scientific Head of that Flagship School. The co-authors are students participating in the Flagship School.

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

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Conflicts of interest: This Review was invited following the 2nd JNC-ISN Flagship School held in 2018 in Alpbach, Austria. Alain Chédotal was the Scientific Head of the Flagship School. The co-authors are trainees participating in the Flagship School.
=> if 'none', insert "The authors have no conflict of interest to declare."
=> otherwise insert info unless it is already included

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Figure 1: Sema3C controls midline crossing in the developing *corpus callosum*

A – The role of semaphorin in midline crossing in the *corpus callosum* has been recently elucidated. Sema3C is expressed in a gradient across the callosal midline. It binds the Nrp1 receptor on callosal axon growth cones **(B)** acting as an attractive cue,. **C –** The Sema3C/Nrp1 complex is silenced by the presence of trans-membrane protein ephrinB1. This requires an N-Glycan post-traductional modification of ephrinB1. Ctx: cortex; CC: *corpus callosum*; AC: anterior commissure; CCA: *corpus callosum* axon.

Figure 2: Retinal ganglion cells development and their pathfinding at the optic chiasm

A – In the developing visual system, retinal ganglion cells (RGCs) project from the retina to the brain nuclei. During this process, some RGC axons cross the midline at the optic chiasm. **B –** RGCs projecting towards the contra or ipsilateral side are already specified in the retina by two sets of transcription factors: *SoxC* and *Islet2* in contralateral RGCs and *Zic2* in ipsilateral RGCs. The Sonic hedgehog (Shh) receptor Boc, is also expressed by ipsilateral RGCs. **C –** These two different combinations allow the expression of guidance effectors, regulating the pathfinding choices at the optic chiasm. Moreover, *Islet2* is also blocking the expression of *Zic2* and *Boc* expression in contralateral RGCs. **D –** The optic chiasm is the intermediate target where contralateral RGCs (green) project towards the contralateral side of the CNS whereas ipsilateral RGCs (red) follow the visual tract on their original side. Shh, transported by the contra-lateral RGCs (grey), is released at the optic chiasm. Ipsi-lateral RGCs expressing the transmembrane receptors Boc as well as EphB1 are repelled by Shh and ephrinB2 at the optic chiasm. An attraction of contralateral RGCs to the midline is mediated by the cell adhesion molecule NrCAM and transmembrane semaphorin Sema6D, through their interaction with NrCAM and the complex PlexinA1-Neuropilin2. RG: radial glia

Figure 3: Spinal cord commissural axons development: pre and post-crossing guidance mechanisms

A – Commissural axons arise from the dorsal portion of the spinal cord. To cross the midline, they first have to be guided to the floor plate. This first process involves several guidance receptors that trigger the axon outgrowth towards the ventral midline. **B –** Pre-crossing axons express the Roundabout 3 (Robo3) receptor. Robo3 interacts with deleted in colorectal cancer (Dcc) receptor and both promote axon extension to the floor plate in response to Netrin-1. Netrin-1 was first thought to act as long-range cue but recent studies suggest that it acts as short-range cue. Robo3 might also prevent Slit repulsion by interacting with the Robo1/2 receptors. **C –** Moreover, in pre-crossing commissural axons, the presence of calpain induces a cleavage of the PlexinA1 receptor, inactivating this repulsive signalling pathway. **D –** After midline crossing, commissural axons switch from midline attraction to repulsion. They become sensitive to repulsive cues secreted by floor plate cells which prevent midline re-crossing. Axon then start to

extend rostrally towards their final targets. **E –** At the floor plate, Robo3 is down-regulated, and Robo1/2 interaction with Slits blocks the Dcc-Netrin-1 attractive signalling. **F** – In addition, the expression of Gdnf by floor plate cells inhibits calpain activity on crossing fibres, allowing PlexinA1 to reach the membrane where it interacts with Neuropilin2, where this receptor complex triggers midline repulsion upon binding Sema3B.

Figure 4: Cortical folding relies on FLRTs expression

Cortical folding appears to be dependent on the presence of the cell adhesion proteins FLRT1 and FLRT3 during cortical expansion, where **(A)** higher expression in migrating cortical progenitors is associated with parallel migration, resulting in a lissencephalic cortex. Conversely, **(B)** lower FLRT1 and 3 expression favors lower migration rates, and promotes lateral adhesion resulting in a gyrencephalic cortex.

Figure 5: Molecular mechanisms for CNS regeneration

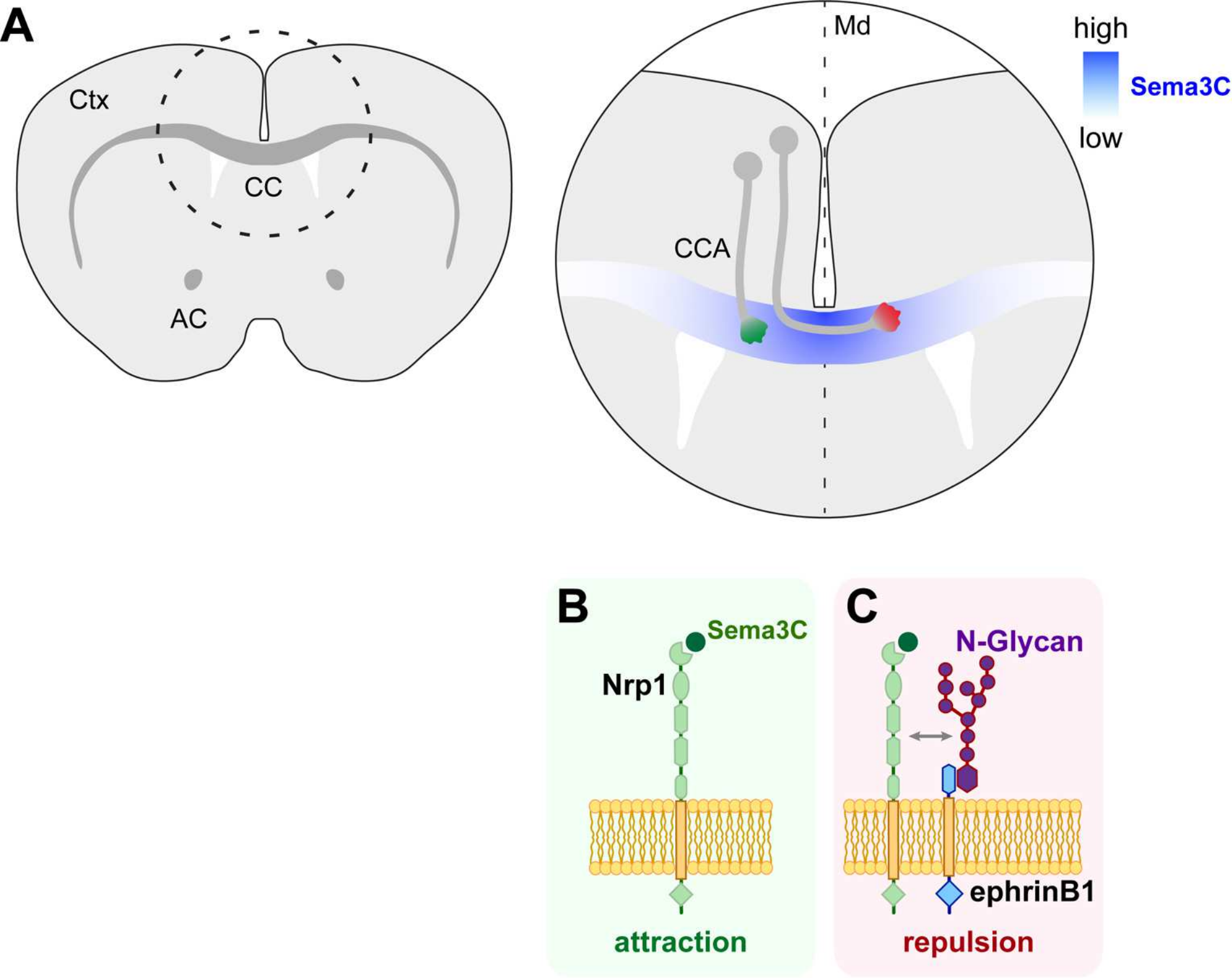
A – After CNS injury, extrinsic and intrinsic mechanisms impair axonal regeneration. These events occur both in the cell soma and the injure site and they are the main targets of strategies aiming at promoting regeneration. Most of the extrinsic inhibition comes from the recruitment of astrocytes and macrophages to the injury area and the "activation" of local oligodendrocytes. **B –** In injured neurons, Pten blocks the mTOR pathway that induces axon regeneration. The complete or conditional depletion of Pten in RGCs or CST neurons, promotes the regeneration of their axons after injury. **C –** Moreover, oligodendrocytes at the lesion site, start to express inhibitory signalling molecules such as Nogo and myelin associated glycoproteins that will block axonal regeneration. The simultaneous depletion of Pten and Nogo, significantly promotes axonal regeneration. **D –** Finally, The expression of cytokines by macrophages also promote axon outgrowth. SOCS3 expression in lesioned axons blocks STAT3, a downstream effector of this cascade. Together with Pten, the depletion of SOCS3 also increases the regeneration rate of RGC axons.

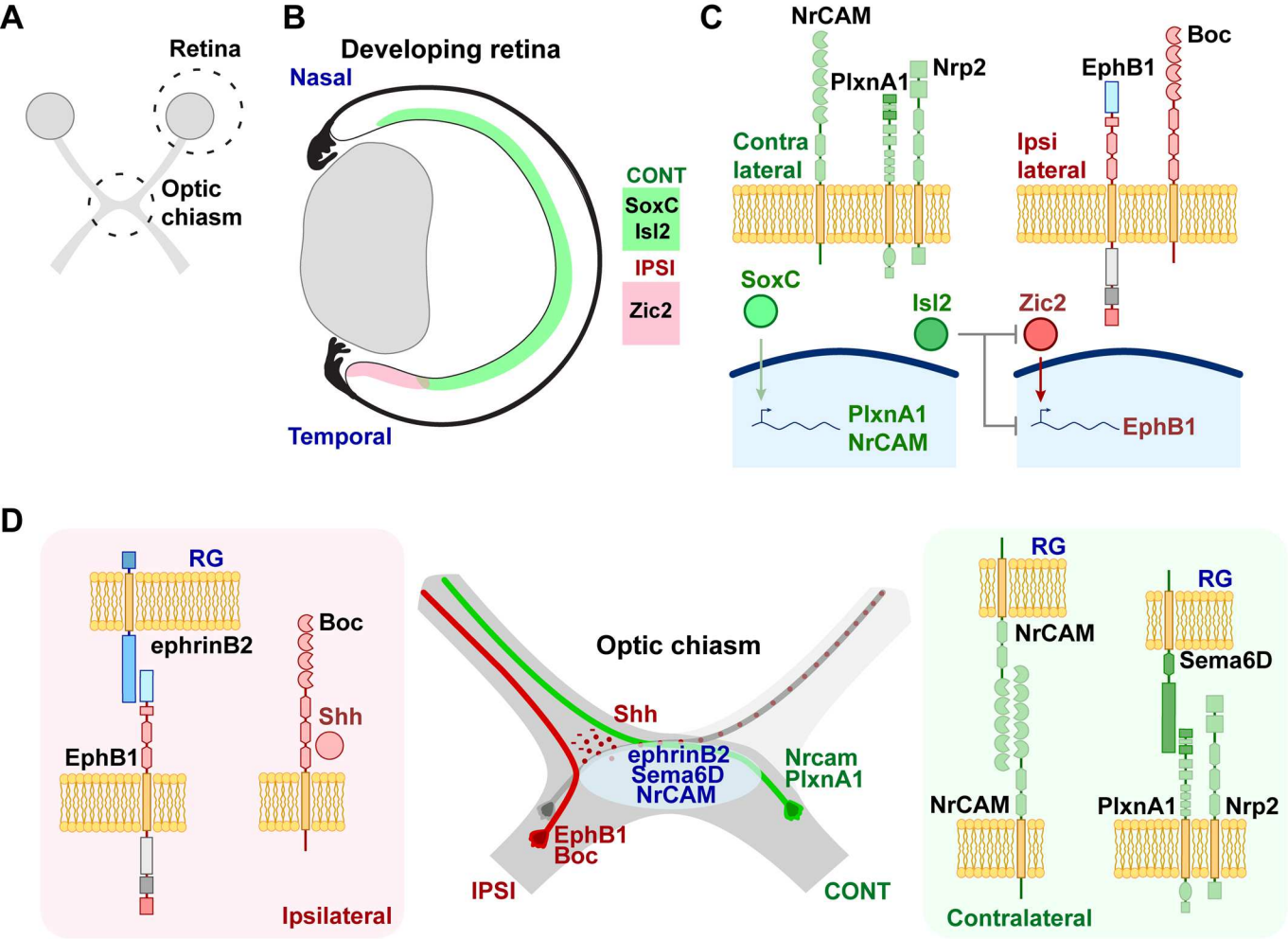
Figure 6: PNS axon regeneration in zebrafish spinal motor nerves

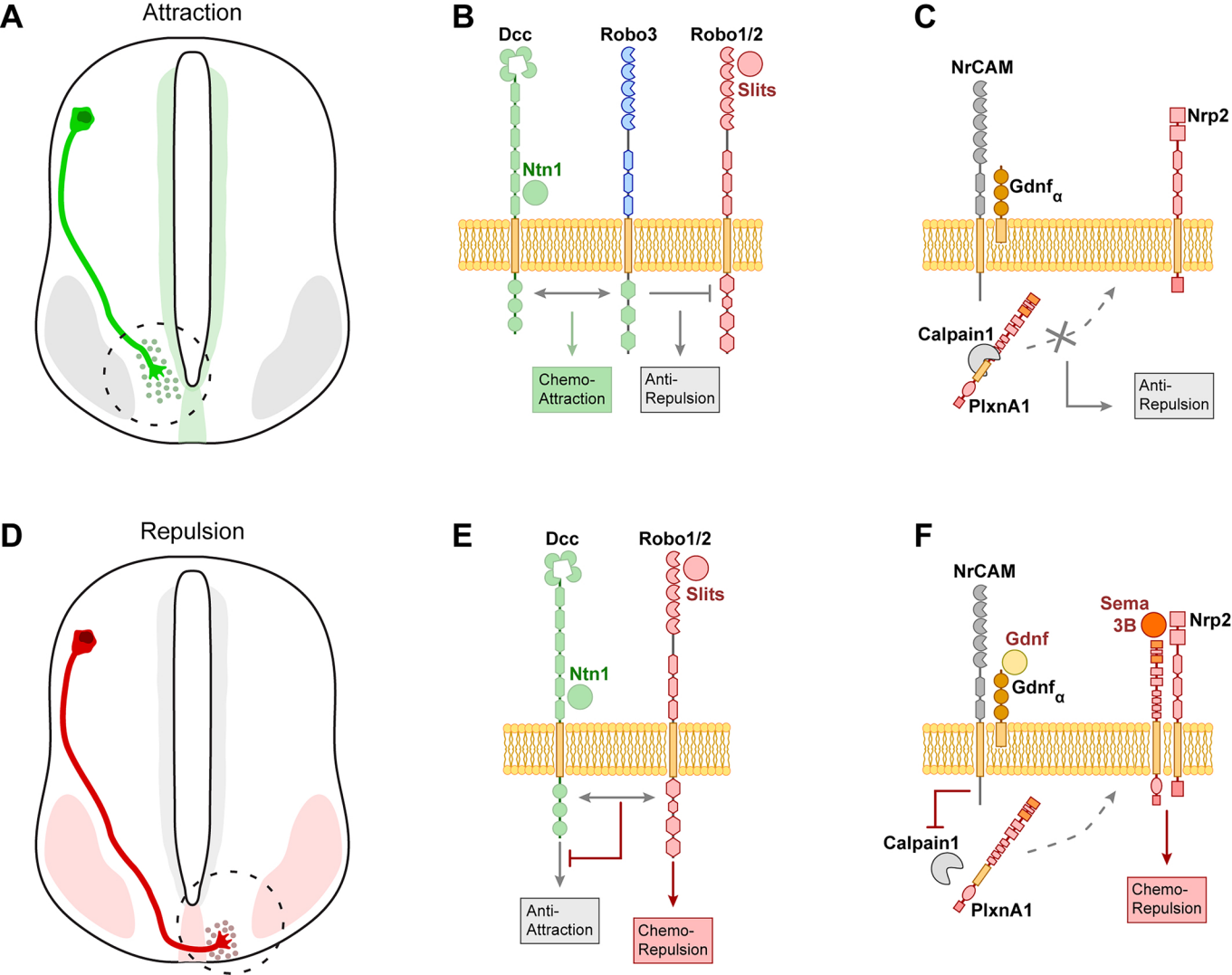
A – Zebrafish has extensively been used as PNS regeneration model. Its transparency allows live-imaging of axons and genetic manipulations remain simpler than in mammals. In this model, a transection of both, dorso-ventral spinal cord motor branches is performed. First, a Wallerian degeneration of the sectioned nerves occurs, dependent on macrophages activity. Then, spontaneous regeneration occurs, promoted by intrinsic and extrinsic signals. **B –** After injury, 80% of the lesioned axons retain the ability to regenerate. This relies on Schwann cell activation that will ensure the proper pathfinding of the regenerating axons through different mechanisms. This activation occurs by exposure to ΔLrp4 and triggers the expression of axon guidance molecules (Netrin-1 and Slit1a) and the remodelling of the extracellular matrix (ECM). **C –** There is evidence that nerve regrowth relies on the extension of a single pioneer axon that will be used as migration scaffold by follower axons. This process seems dependent on ΔLrp4 but no receptors have been identified yet. Axon guidance of dorsal or ventral projections differs. Netrin-1 and Dcc promotes the growth of ventral axons while Slit1a prevent dorsal axon from growing dorsally, through a yet unknown receptor.

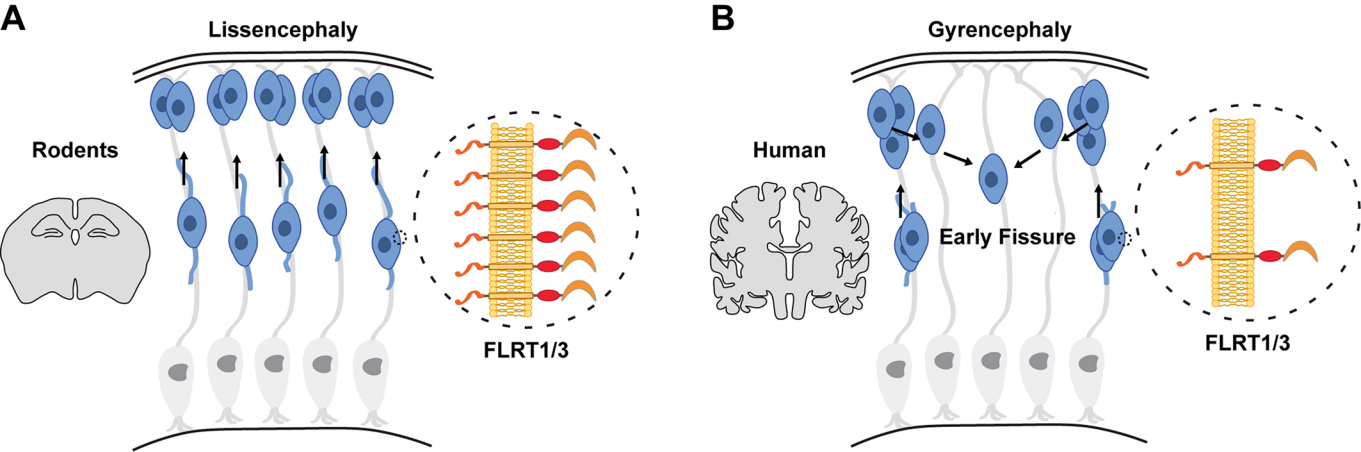
Figure 7: Cell therapy

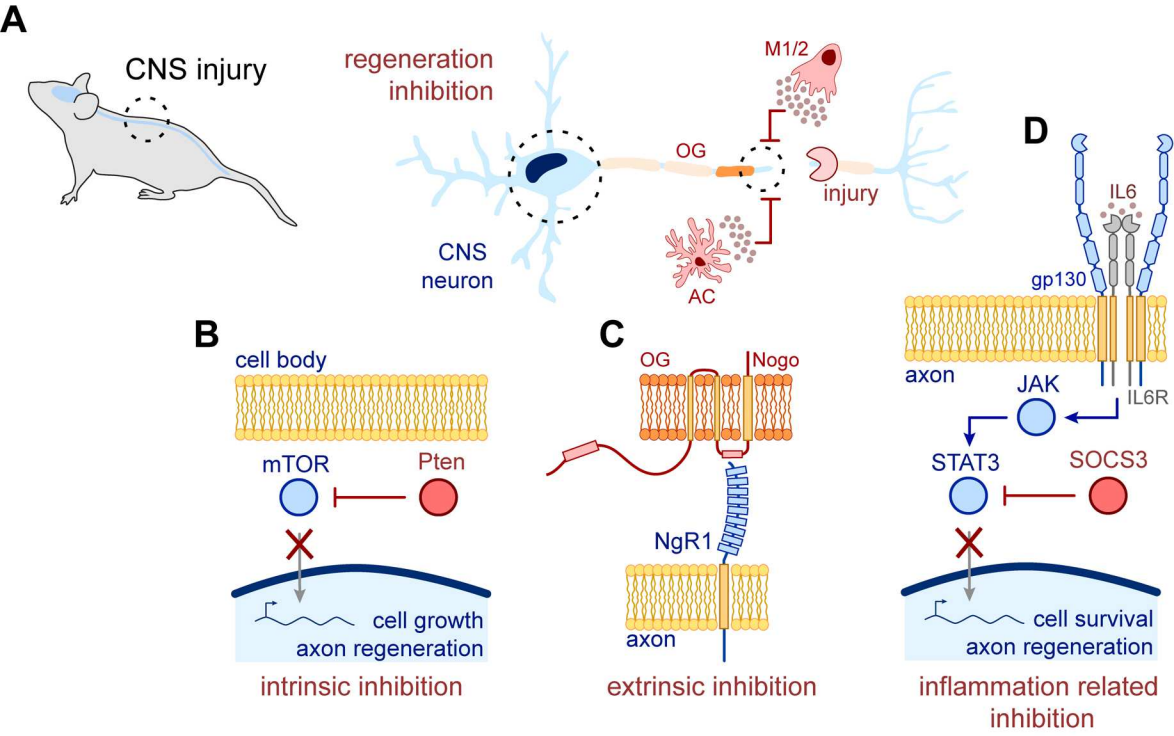
A – Strategies based on stem cell replacement such as human- and mouse-derived embryonic stem cells (ESCs) and induced pluripotent stem cell (iPSC)-derived neurons. Grafting iPSC-derived neurons in brain circuits requires **(B)** adaptation to the extrinsic in vivo microenvironment or composition of extracellular matrix, lesion site-specific cues and signalling pathways, and identity match between the damaged brain area and the transplanted material. **C –** Transplanted neurons could mature and integrate into damaged circuits; however, potential adverse effects as tumors or neuron death should be addressed before these strategies are transferred to the clinic.

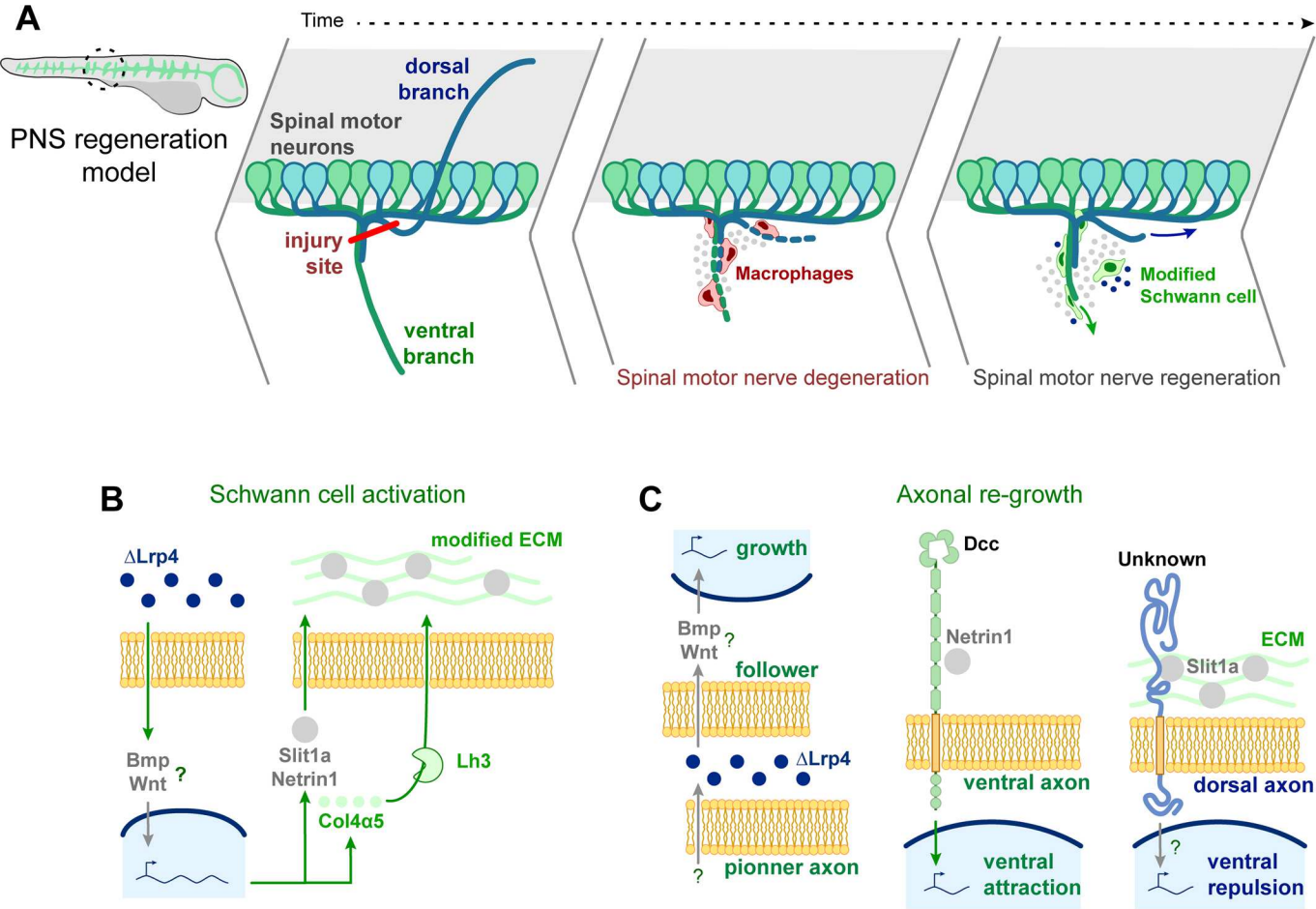

A
Ctx
CC
AC
Md
high
Sema3C
low
CCA
B
Sema3C
Nrp1
attraction
C
N-Glycan
ephrinB1
repulsion

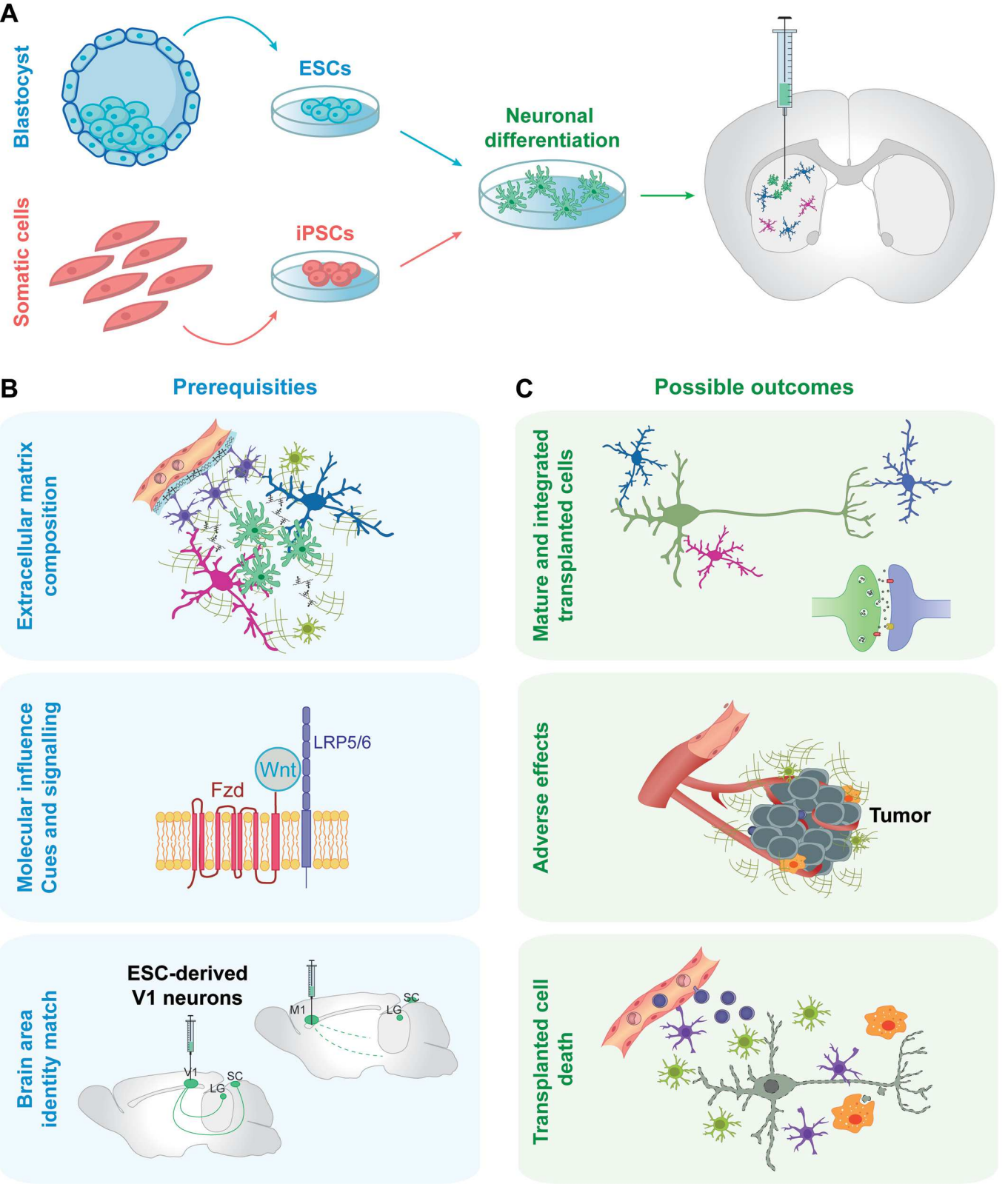

A
Retina
Optic chiasm
B
Developing retina
Nasal
Temporal
CONT
SoxC
Isl2
IPSI
Zic2
C
NrCAM
PlxnA1
Nrp2
Contra lateral
SoxC
Isl2
PlxnA1
NrCAM
Boc
EphB1
Ipsi lateral
Zic2
EphB1
D
RG
ephrinB2
EphB1
Boc
Shh
Ipsilateral
Optic chiasm
Shh
ephrinB2
Sema6D
NrCAM
Nrcam
PlxnA1
EphB1
Boc
IPSI
CONT
RG
NrCAM
NrCAM
Contralateral
RG
Sema6D
PlxnA1
Nrp2


A
Attraction
B
Dcc
Robo3
Robo1/2
Slits
Ntn1
Chemo-
Attraction
Anti-
Repulsion
C
NrCAM
Nrp2
Gdnf$_\alpha$
Calpain1
PlxnA1
Anti-
Repulsion
D
Repulsion
E
Dcc
Robo1/2
Slits
Ntn1
Anti-
Attraction
Chemo-
Repulsion
F
NrCAM
Sema
3B
Nrp2
Gdnf
Gdnf$_\alpha$
Calpain1
PlxnA1
Chemo-
Repulsion


A
Lissencephaly
Rodents
FLRT1/3
B
Gyrencephaly
Human
Early Fissure
FLRT1/3


A
CNS injury
regeneration inhibition
M1/2
OG
injury
CNS neuron
AC
B
cell body
mTOR
Pten
cell growth
axon regeneration
intrinsic inhibition
C
OG
Nogo
NgR1
axon
extrinsic inhibition
D
IL6
gp130
axon
JAK
IL6R
STAT3
SOCS3
cell survival
axon regeneration
inflammation related inhibition


A
Time
PNS regeneration model
dorsal branch
Spinal motor neurons
injury site
ventral branch
Macrophages
Modified Schwann cell
Spinal motor nerve degeneration
Spinal motor nerve regeneration
B
Schwann cell activation
ΔLrp4
modified ECM
Bmp
Wnt
?
Slit1a
Netrin1
Lh3
Col4α5
C
Axonal re-growth
growth
Bmp
Wnt
?
follower
ΔLrp4
pionner axon
?
Dcc
Netrin1
ventral axon
ventral attraction
Unknown
ECM
Slit1a
dorsal axon
?
ventral repulsion


A
Blastocyst
ESCs
Somatic cells
iPSCs
Neuronal differentiation
B
Prerequisities
Extracellular matrix composition
Molecular influence Cues and signalling
Wnt
LRP5/6
Fzd
Brain area identity match
ESC-derived V1 neurons
V1
SC
LG
M1
C
Possible outcomes
Mature and integrated transplanted cells
Adverse effects
Tumor
Transplanted cell death