MEETING REVIEW

Neural development and regeneration: it's all in your spinal cord

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ABSTRACT

The spinal cord constitutes an excellent model system for studying development and regeneration of a functional nervous system, from specification of its precursors to circuit formation. The latest advances in the field of spinal cord development and its regeneration following damage were discussed at a recent EMBO workshop 'Spinal cord development and regeneration' in Sitges, Spain (October, 2014), highlighting the use of direct visualization of cellular processes, genome-wide molecular techniques and the development of methods for directed stem cell differentiation and regeneration.

KEY WORDS: EMBO workshop, Spinal cord, Wings for Life

Introduction

Circuits in the spinal cord are essential for the performance and regulation of crucial functions such as locomotion, sensory processing or breathing. From the early ages of neuroscience, studies of the spinal cord have been key to our understanding of the basic rules for building the vertebrate nervous system; many of the principles learned from work on the spinal cord also apply to the brain. The recent EMBO workshop 'Spinal cord development and regeneration' held in Sitges, a pleasant Spanish Mediterranean town, highlighted the spinal cord as a complex and accessible model system in which to analyse neural circuit formation and repair. The meeting, organized by Elisa Martí (IBMB-CSIC, Barcelona, Spain), Jonas Muhr (Karolinska Institutet, Stockholm, Sweden) and Kate Storey (University of Dundee, UK), brought together international researchers interested in spinal cord development and those who are developing strategies to restore the function of the spinal cord in pathological situations using regeneration of the damaged tissue, including controlled cell replacement. In this Meeting Review, we discuss exciting new findings and highlight emerging themes in the field (Fig. 1).

Shaping the neural tube

Early in central nervous system (CNS) development, the neural tube forms from the neural plate. This requires the coordination of many cellular processes, disruptions of which can result in spina bifida, the most common developmental disease affecting the spinal cord.

Lee Niswander (University of Colorado, Denver, USA) showed beautiful time-lapse images of the closing neural tube to introduce how forward-genetics screens in the mouse have been invaluable in identifying important genes that are now being examined in the quest for causative factors for neural tube defects (NTDs) in humans (a database of mouse mutants can be found at https://ntdwiki. wikispaces.com/). Her work has also addressed the influence of environmental factors, unexpectedly revealing that folic acid

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supplements in NTD mouse mutants can worsen their defects (Marean et al., 2011). This raises questions about the general recommendation of folic acid supplementation during pregnancy. Additional work on the role of folic acid in preventing NTDs was presented by Nick Greene (University College London, UK), who emphasized the importance of the mitochondrial folate one-carbon system and, in particular, the glycine cleavage system in neural tube closure (Leung et al., 2013).

Andy Copp (University College London, UK) stressed that different mechanisms of closure operate depending on the rostrocaudal level, so their failure results in lesions at specific levels and thus determines the severity of the outcome. To explore these different mechanisms, his group is analysing various mouse mutants with altered neural tube closure, employing imaging approaches to visualize cell behaviours such as protrusions of cells at the edge of the closure point, as well as techniques such as atomic force microscopy to measure tissue elasticity.

Regulating cellular organization, cell cycle and neurogenesis

Formation of the neural tube also requires tight control of apico-basal cell polarity, which first allows the correct spatial organization of cells depending on the cell cycle phase and is subsequently responsible for the spatial separation of proliferating and differentiating cell populations during neurogenesis. Here again, the spinal cord is an ideal system for live visualization of the cellular and subcellular behaviours associated with cell polarity and division, and their impact on neurogenesis.

Jon Clarke (King's College, London, UK) presented work on the mechanisms that regulate apico-basal polarity of neuroepithelial cells in zebrafish. He uses live imaging and analysis of mutants to address whether extracellular signals might impose directionality to coordinate an intrinsic programme of epithelial polarity development in the neural tube (Girdler et al., 2013). Sebastian Pons (IBMB-CSIC, Barcelona, Spain) discussed a role for the Wnt/β-catenin/TCF pathway in the maintenance of apico-basal polarity and in formation of the apical complex in chick (Herrera et al., 2014).

Apico-basal polarity is crucial for the control of spindle orientation, which is predominantly planar in neuroepithelial cells. Xavier Morin (Ecole Normale Supérieure, Paris, France) presented a centrosome 3D tracking method to explore spindle orientation dynamics in the chick. His work indicates that a mechanism involving a direct interaction between the baso-lateral protein Discs Large (DLG1) and the spindle orientation regulator the Leu-Gly-Asn repeat-enriched protein (LGN) ensures mitotic spindle movements and planar orientation of division (Saadaoui et al., 2014).

As well as polarity, progenitor cells have to regulate cell cycle duration and exit. The groups of Elisa Martí (IBMB-CSIC, Barcelona, Spain) and Fabienne Pituello (Centre de Biologie du Development, Toulouse, France) have established *in vivo* marking of different modes of division and cell cycle phases with single cell
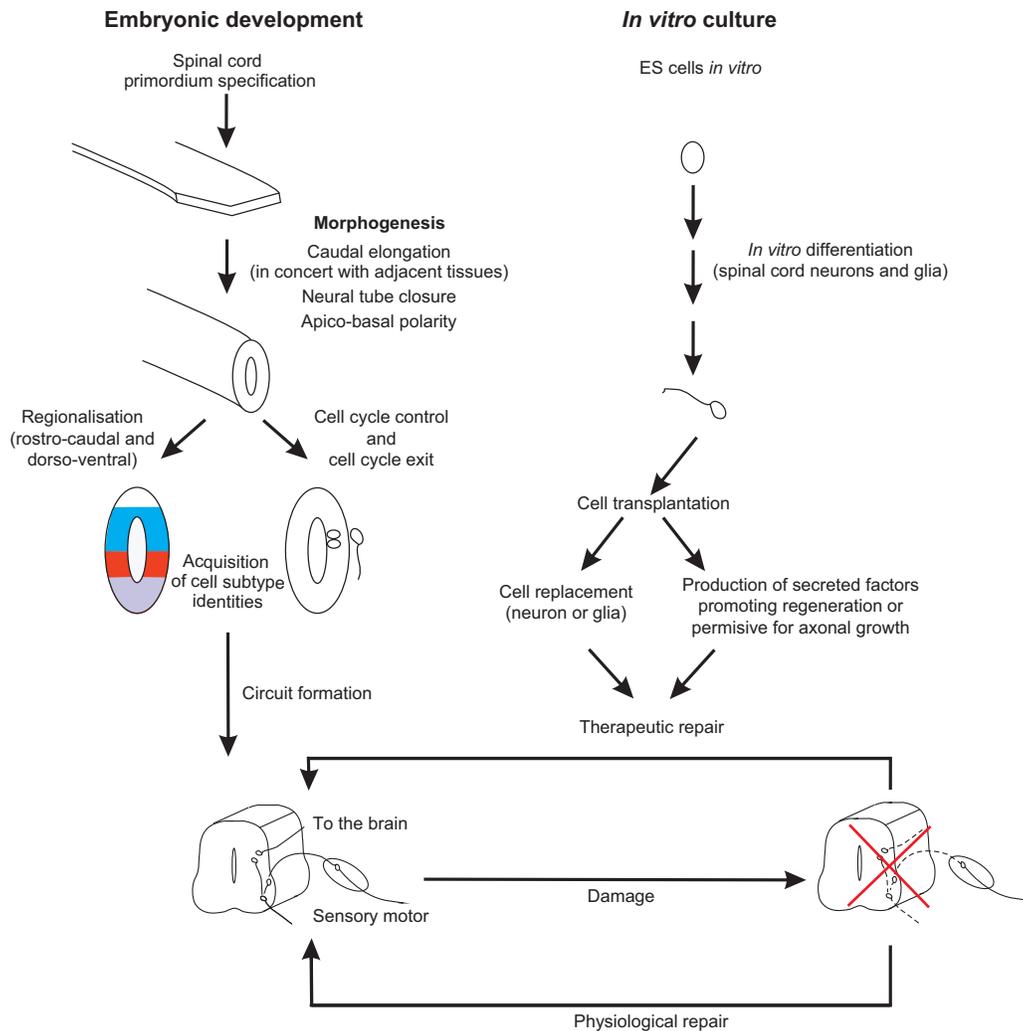
Fig. 1. Crucial steps in spinal cord development and repair strategies. (Left) Spinal cord precursors are located in the caudal-most region of the neural plate. Morphogenetic cell movements along the rostro-caudal and dorso-ventral axes (medio-lateral in fish) are responsible for the formation of the elongated tube that will connect the brain to organs and body muscles. At the cellular level, several processes are highly controlled, including the establishment and maintenance of apico-basal polarity, the control of the cell cycle and cell cycle exit, and the acquisition of cell subtype identities. This will allow the establishment of the appropriate neuronal connections to build functional circuits for motor and sensory processing. (Right) *In vitro* differentiation of subtype-specific spinal cord neurons from embryonic stem (ES) cells is becoming more successful and may become an important therapeutic strategy for restoration of a functional spinal cord after damage. Use of secreted factors that favour axonal regeneration and growth are currently under clinical trials.

resolution. The outcome of a division – giving rise to two progenitors, two neurons or to a progenitor and a neuron – is labelled with reporters based on the *Sox2* and *Tis21* (*Btg2* – Mouse Genome Informatics) genes (Saade et al., 2013), and cell cycle progression is monitored using a combination of *PCNA*- and FUCCI-based reporters (F. Pituello, unpublished). Interestingly, increasing the levels of the cyclin regulator CDC25 shorten the duration of the G2 phase (Peco et al., 2012) and promotes a neurogenic mode of division. Murielle Saade (Martí lab, IBMB-CSIC, Barcelona, Spain) found that sonic hedgehog (Shh) promotes non-neurogenic divisions by controlling the localization of the catalytic subunit of PKA.

The control of cell attachment or detachment from the apical surface following cell division is pivotal for ordered neurogenesis. Paula Alexandre (University College London, UK) provided evidence that reattachment of non-differentiating daughter cells to the apical surface in the zebrafish neural tube depends on the connection between the two daughter cells. Meanwhile, Raman Das (Storey lab, University of Dundee, UK) showed that cells about to

differentiate in the chick neuroepithelium abruptly lose their apical polarity (which is important for maintenance of a progenitor state) by the regulated process of apical abscission that mediates loss of the apical membrane and disassembly of the primary cilium (Das and Storey, 2014).

Specification of neural cell types and subtypes: the regionalizing signals

The generation of the multiple cellular subtypes of the spinal cord is a highly ordered process that initiates before cells exit the cell cycle. Extracellular signals provided by signalling centres are crucial for the regionalisation of progenitor cells along the rostro-caudal and dorso-ventral axes. Throughout the nervous system, ventral patterning is directed by Shh produced from the notochord and floor plate, and the understanding of the mechanisms regulating this pathway are still a strong focus. Several talks stressed the exquisite modulation of Shh signalling. Kim Dale (University of Dundee, UK) and Ben Novitch (UCLA, Los Angeles, USA) independently reported unexpected roles of Notch

signalling in modulating the Shh cascade in the specification of ventral neural progenitor domains. Ruth Diez del Corral (Cajal Institute-CSIC, Madrid, Spain) showed the interaction of the FGF and the Shh pathways, which is important for proper initiation of ventral patterning during caudal elongation of the spinal cord. Cathy Danesin (Soula lab, University of Toulouse, France) discussed a role in zebrafish for the secreted enzyme sulfatase 1 in controlling Shh signal strength in time (Al Oustah et al., 2014), and Alex Holtz (Allen lab, University of Michigan, Ann Arbor, USA) provided evidence in mice for a functional secreted form of the Shh inhibitor hedgehog interacting protein (Hhip1), previously considered to be exclusively membrane bound (Holtz et al., 2013). Douglas Epstein (University of Pennsylvania, Philadelphia, USA) presented his work elucidating the complex gene regulatory architecture responsible for Shh expression in different regions of the CNS and establishing the ancient origin of one of the Shh enhancers, predating the chordate phylum.

Sean Megason (Harvard Medical School, Boston, USA) described another layer of complexity required in fish to compensate for the molecular noise inherent to interpretation of Shh concentration. By means of elegant single cell measurement of gene expression, his team has found that expression of regionalizing genes is initially poorly defined and that subsequent cell repositioning, which is dependent on gene expression levels, is required to achieve clear domain borders (Xiong et al., 2013).

A novel mechanism of temporal control of signal production in the hindbrain was presented by Johan Ericson (Karolinska Institutet, Stockholm, Sweden). He showed that the expansion of a TGFβ domain, via a positive-feedback loop, is responsible for the switch in gene expression underlying the sequential specification of motor neurons (MNs) and serotonergic neurons from the same ventral domain (Dias et al., 2014).

Together, these results stressed the importance of the timing of the exposure of the spinal cord to different signals, which may contribute to the diversification of cell fates and to the coordination of the development of adjacent structures.

Specification of neural cell types and subtypes: the transcription factor effectors

Cell identities are determined by specific transcription factors expressed in restricted patterns that will drive the final fate of neurons and glia. A wealth of information is now being accumulated using global techniques, such as ChiP-Seq, to search for the binding sites of specific transcription factors or to assess chromatin states, and RNA-Seq, to determine transcription factor targets. However, all this information needs to be assimilated in the context of the embryo in order to understand the regulatory logic.

Jonas Muhr discussed how Sox2, which is expressed in progenitor cells throughout the CNS, can activate different sets of target genes in the spinal cord and the brain. His work showed that, despite a similar 'open state' of chromatin in both tissues, binding of Sox2 to gene regulatory regions is tissue specific and depends on the presence of tissue-restricted proteins such as Hox factors.

Several talks focussed on the roles of transcription factors in defining specific progenitor domains. Aixa Morales (Cajal Institute-CSIC, Madrid, Spain) proposed that Sox5 is instrumental in the establishment of the dp2 and dp3 dorsal interneuron progenitor domains by antagonizing the Wnt pathway (Quiroga et al., 2015), and thus plays a dual role regulating both cell cycle exit and patterning. Vanessa Ribes (UMR 947, Paris, France) described a complex regulation of the transcriptional activity of the partially redundant factors Pax3 and Pax7, both expressed in the dorsal spinal cord

progenitor cells (Moore et al., 2013), whereby each can work as either activator or repressor, depending on the progenitor domain context.

Like neurons, glial cells also rely on specific transcription factors for acquisition of their identity and subsequent development. Michael Wegner (University of Erlangen-Nürnberg, Germany) presented a gene regulatory network involving SoxE factors that regulates multiple stages of oligodendrocyte development (Hornig et al., 2013). These Sox proteins participate in feed-forward loops to induce the proteins with which they cooperate. However, a different set of factors is induced in each cell type. Finally, David Rowitch (University of California, San Francisco, USA) discussed his work on the importance of astrocyte diversification for the maintenance of sensory motor circuit integrity and the survival of α MNs (Molofsky et al., 2014). He also presented data showing how astrocyte diversification depends on dorso-ventral regional origin and involves both intrinsic cues, as well as neuron-derived signals, for refinement.

Assembly of functional circuits

Precise wiring of the spinal circuitry, which relies on appropriate cell differentiation, is essential for sensory and motor functions. Advances in molecular, genetic and imaging techniques have enabled fundamental discoveries on spinal circuitry. Notably, trans-synaptic tracing using a modified rabies virus has allowed detailed analysis of neuronal connections. Targeted or naturally occurring genetic mutations have revealed specific functional roles for transcription factors controlling the assembly of functional circuits in the spinal cord.

One of those factors, Maf, has been examined by Carmen Birchmeier (MDC for Molecular Medicine, Berlin, Germany) for its role in controlling the development of primary and secondary mechanonsensory neurons in mouse. In dorsal root ganglia (DRGs), Maf controls Ret, MafA and Kcnq4, which may influence the firing frequency of DRGs (Wende et al., 2012). Interestingly, Maf is also expressed in cells of the dorsal spinal cord and its deletion leads to scratching, illustrating the central importance of one transcription factor in two different aspects of sensory processing.

In beautiful unpublished work, Martyn Goulding (Salk Institute, La Jolla, USA) has characterized and mapped a novel circuit in the dorsal spinal cord that plays a key role in sensing light touch. Meanwhile, Klas Kullander (Uppsala University, Sweden) described mutations in the transcription factor Dmrt3 that cause striking divergent gait patterns in horses (e.g. Icelandic ponies' 'Tölt') and mice (Andersson et al., 2012). Labelling Dmrt3-expressing interneurons allowed definition of the variety of activity frequencies during fictive locomotion. Kullander proposes a spinal cord 'gear box' in which neurons adapt to different levels of input, generating the variations in spinal locomotor output.

Despite over a century of spinal cord studies, a novel subpopulation of V2 interneurons was reported by Frederic Clotman (UC Louvain, Belgium). These are characterised by the expression of the transcription factor Vsx1 (RINX) and develop independently of Notch signalling in the developing mouse spinal cord, but their final fate is still unknown.

Another important factor is Hoxa5, investigated by Polyxeni Philippidou (Dasen lab, NYU Medical Center, USA) to understand the control of phrenic MN (PMN) identity in the development of respiratory circuits (Philippidou et al., 2012). Hoxa5 conditional knockout mice, which have fewer PMNs and thinner phrenic nerves, show dramatic changes in the dendritic patterns of PMNs and increased frequency of respiratory bursts, pointing to defects in the integration of PMNs into respiratory circuitry.

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Molecular markers not only define spinal neuron subtypes, but also complex rostro-caudal networks. Samuel Pfaff (Salk Institute, La Jolla, USA) described the motor circuitry that coordinates common movement patterns, i.e. the motor synergy encoder (MSE), which is defined by the presence of the transcription factors Tcafp2β and Satb1/2 (Levine et al., 2014). The MSE interneurons coordinate distal and proximal limb MNs. Stimulation of the MSE activates distal and proximal muscles in different patterns. Elegantly, Pfaff and colleagues can visualise the coordination of MN activity using expression of the Ca^{2+} indicator GCaMP.

Stem cells and *in vitro* generation of spinal cord

Spinal cell types are distinguished by morphology, position, synaptic inputs, electrophysiological properties, trophic requirements and axon trajectories. For some time, there has been a drive to differentiate stem cells towards specific neuronal fates for potential therapeutic purposes. The use of the large body of knowledge from developmental studies to define combinations of transcription factors and signalling molecules that determine particular cell identities is now bearing fruit.

Hynek Wichterle (Columbia University, New York, USA) showed that human embryonic stem cells (hESCs) can be differentiated into MNs by overexpression of Ngn2, Isl1 and Lhx3, an accelerated process that bypasses expression of the progenitor identity factor Olig2 (Mazzoni et al., 2013). Through ChIP-Seq and ChIA-PET experiments, Wichterle and colleagues have revealed thousands of Isl/Lhx3-binding sites, a subset of which become active enhancers in differentiating MNs.

Stéphane Nedelec (I-STEM, UMR 861, Paris, France) has developed a high-throughput assay to screen for factors modulating hESC conversion into different classes of MNs. He found that early graded WNT signalling cooperates with retinoic acid to determine rostro-caudal identity of spinal or cranial MN progenitors. These progenitors can be converted with an unprecedented efficiency and rapidity (14 days) upon inhibition of Notch signalling (Maury et al., 2014).

Targeted generation of caudal spinal cord in culture has been a challenge as neural precursor stem cells induced from ESCs default to rostral fates. James Briscoe (NIMR, London, UK) and Alfonso Martínez-Arias (University of Cambridge, UK) have independently identified a Wnt/FGF factor cocktail that drives stem cells towards caudal fates. They confirmed that the specification of a neuro-mesodermal precursor population precedes the generation of the caudal spinal cord precursors, similar to the *in vivo* situation (Tzouanacou et al., 2009). Moreover, the ability of Wnt to promote caudal spinal identities can be uncoupled from its ability to promote mesodermal fates (Gouti et al., 2014). Martínez-Arias showed that under appropriate 3D culture conditions, these ESC derivatives can display self-organizing and axial elongation properties reminiscent of the extending spinal cord and adjacent mesoderm (Turner et al., 2014).

Bench-to-bedside in spinal cord injury and pathology

Fundamental knowledge about the development of the spinal cord is essential to devise approaches for functional spinal cord repair, comprising neuroprotection after injury, replacement of lost neurons and glia, and axonal regeneration.

Pablo Villoslada (IDIBAPS, Barcelona, Spain) presented regenerative therapies for spinal cord injury and multiple sclerosis (MS) in which axonal damage, demyelination and clinical signs are closely correlated, including MESEMS (https://clinicaltrials.gov/ct2/show/NCT01854957), which is currently recruiting participants for a trial of neuroprotection by mesenchymal stem cell (MSC) administration. Similarly, Dearbhaile Dooley (Hendrix lab, Hasselt University, Belgium) has transplanted MSCs, virally transduced to express the TH2 anti-inflammatory cytokine interleukin 13, rostral to a spinal lesion site in the mouse. Dooley observed a reduction in lesion size and demyelination, potentially mediated by a decrease in the number of microglia/macrophages and increased T-cell numbers in the lesion (Dooley et al., 2014).

In MS and traumatic CNS injury, axons are demyelinated and remyelination is often inefficient. Remyelination usually involves the generation of new oligodendrocytes, but can also be carried out by Schwann cells, the myelinating cell of the PNS. Robin Franklin (University of Cambridge, UK) used lineage tracing to show that CNS-remyelinating oligodendrocytes and Schwann cells both derive from adult mouse CNS progenitor cells. Those Schwann cells derived from dorsally located progenitors had a greater propensity to engage in remyelination than those of ventral origin. *In vitro* assays of cell migration and differentiation have revealed intrinsic differences in the properties of progenitors of different developmental origin.

Ependymal cells (ECs), endogenous progenitors found in several niches in the spinal cord, significantly contribute to lesion repair and may determine regenerative success. Jean-Philippe Hugnot (University of Montpellier, France) focused on the diversity of ependymal cells in the central canal niche and how these cells can be re-activated by a spinal lesion to generate cell types important for repair. He provided evidence that neurospheres generated from the central canal of human adult spinal cord can be used to explore the mechanisms of reactivation (Bauchet et al., 2013). Jonas Frisén (Karolinska Institutet, Stockholm, Sweden) showed that quiescent ECs are lesion-activated progenitors (Sabelstrom et al., 2013), whereas type A pericytes contribute to scar formation, depositing extracellular matrix and facilitating the invasion of endothelial cells. Frisén emphasized that an as yet unidentified signal in the spinal cord makes the environment non-conducive to neurogenesis from endogenous progenitor cells.

Unlike mammals, anamniotes such as zebrafish and axolotl show a strong capacity for regeneration: in zebrafish, lesion induces spinal progenitors to proliferate and to generate motor and interneurons. Catherina G. Becker (University of Edinburgh, UK) reported how different neurotransmitters derived from the spinal projections of supraspinal neurons act on these progenitors to control spinal neurogenesis (Reimer et al., 2013) and promote the generation of MNs after a complete spinal cord transection. Osvaldo Chara (ZIH, Dresden, Germany and IFLYSIB, La Plata, Argentina) presented a computational model based on previous ideas (Chara et al., 2014) and used *in vivo* experimental data on axolotl to show that both proliferation and an influx of ependymal cells are crucial for spinal cord repair.

Injury-induced lesion sites in mammals are generally not appropriately bridged by glia or axons. Instead, a glial scar forms, which inhibits axonal regeneration. Cátia Lopes (Pego lab, INEB, Universidade de Porto, Portugal) presented approaches to bridge the gap in spinal cord injury using a scaffold-driven cell-based regenerative therapy. She showed that engineered implants consecutively seeded with endothelial cells and neural stem cells enhance axonal regrowth and recovery, underscoring the importance of the composition of the spinal lesion site.

For successful regeneration, axons may need to re-extend before the damaged environment deteriorates. Samantha Butler (UCLA, Los Angeles, USA) finds that the balance between *Limk1* and cofilin activity regulates axon regeneration velocity. In *Limk1* mutant mice, MN axon growth into hindlimbs is 15%

faster, and axon regeneration and functional recovery are enhanced. Given these and similar advances made in enhancing axonal regrowth, e.g. by manipulating PTEN activity, it is now essential to define how limited axonal regrowth and reconnection to distal targets can support functional regeneration. Martin Schwab's (University of Zürich, Switzerland) investigations on spinal cord repair through neutralisation of myelin-associated inhibitors, in particular Nogo-A, are the basis of the clinical trials currently under way. Functional studies of rats treated with an inhibitor of Nogo-A revealed functional recovery without adverse effects of miswiring (e.g. spasms), while macaque monkeys regenerated corticospinal tracts and recovered precision grasp. Interestingly, exercise following inhibition of Nogo-A significantly improves further functional outcome in rats (Wahl and Schwab, 2014).

Conclusion and emerging themes

The neuroscience field has benefitted tremendously from recent technological advances. These methods, applied to the study of spinal cord development and repair in a number of model organisms, allow us to unravel the principles underlying formation of the spinal circuits and their connections to the brain. Overall, this stimulating workshop elucidated the processes involved from different viewpoints and at different levels, from the molecular to the organismal. Imaging approaches are providing new perspectives to the understanding of single cell and organelle behaviours within a whole tissue context. Unravelling the gene regulatory networks responsible for the acquisition of subtype specific properties is invaluable to devise protocols to direct stem cells to particular fates that ultimately contribute to cell replacement. In addition, characterisation of the environments permissive for endogenous neurogenesis and gliogenesis, as well as axon extension – processes all required for successful regeneration – raise real hopes for therapeutic strategies that are currently subject to the first clinical trials in MS, MN diseases and spinal cord injury.

Moreover, this successful meeting has strengthened a sense of community among spinal cord researchers, facilitating the sharing of reagents, methods and ideas. To this aim, Julio Barbas (Spanish Ministry of Economy and Competitiveness) introduced the ERANET-Neuron 2015 call for collaborative projects devoted to neurodevelopmental disorders (http://www.neuron-eranet.eu/). Attendees agreed that meetings like this, bringing together scientists working on all aspects of spinal cord development and regeneration, should be repeated at regular intervals to accelerate our fundamental understanding of spinal cord development and ultimately find therapeutic strategies for spinal cord injury and degenerative diseases.

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