Neural circuit reorganisation after spinal cord injury in Zebrafish

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Authors: François El-Daher[1], Catherina G. Becker[1*]

1 Centre for Discovery Brain Sciences, Edinburgh Medical School:Biomedical Sciences, University of Edinburgh EH16 4SB
*** corresponding author catherina.becker@ed.ac.uk**

Abstract

Spinal cord injuries disrupt signalling from the brain leading to loss of limb, locomotion, sexual and bladder function, usually irreversible in humans. In zebrafish, recovery of function occurs in a few days for larvae or a few weeks for adults due to regrowth of axons and de novo neurogenesis. Together with its genetic amenability and optical clarity, this makes zebrafish a powerful animal model to study circuit reorganisation after spinal cord injuries. With the fast evolution of techniques, we can forecast significative improvements of our knowledge of the mechanisms leading to successful or failed recovery of spinal cord function. We review here the present knowledge on the subject, the new technological approaches and we propose future directions of research.

Introduction: establishment of neural circuits in zebrafish spinal cord

During the development of the spinal cord of zebrafish, a well-defined order of cell integration and connection occurs leading to fully functional circuits. Getting a better knowledge of these regulated processes allow us to study how they might be de novo used to repair the damaged spinal cord after an injury or if new mechanisms are involved, or both.

During embryogenesis, spontaneous activity appears at the 30-somite stage and displays a characteristic pattern of connectivity showing ipsilateral connection and rostro-caudal action potential propagation [1]. The control of locomotion then matures through sequential changes in the operation of motor circuit in the spinal cord [2][3]. Motoneuron ensembles emerge, first forming small local networks that coalesce and grow, leading to a global synchronization of their activity, before recruiting interneurons [4].
The chronology of establishment of the circuits of descending V2a interneurons has been determined from behaviour analysis: the early-born interneurons project first into the spinal cord to connect with early-born motoneurons to achieve fast dynamics movements, whereas later-born V2a neurons connect more laterally in a parallel layer with late-born motoneurons to allow slow locomotion [5]. Indeed, V2a interneurons operate as a single interconnected layer without spatially distinct arrangement, but ensure a correct chronology of connections with target motoneurons through individual differences in weights and probabilities of interaction [6]. Motoneurons are then selectively recruited by a microcircuit formed by Rohon-Beard and V2a neurons directly interacting through monosynaptic connections to achieve fast locomotion during the escape response [7]. Down to the molecular scale, the escape response requires an asymmetry of electrical synapses between Mauthner neuron axons and commissural local interneurons, based on two distinct pre and post-synaptic Connexins [8].

In contrast to the brain [9], a full atlas at cellular resolution of the spinal circuit in unlesioned fish and after regeneration is still missing.

Towards a "new" spinal cord: circuit reorganisation

Cells

After a spinal cord injury, neurons die and they, or their function need to be replaced. In zebrafish, regeneration occurs through complex interactions between different cell types and is orchestrated by a range of signals, as reviewed in [10].

Amongst the cells involved in this process, immune cells are key players. Neutrophils are recruited early after an injury and transiently contribute to inflammation [11][12]. Contrary to the situation in the adult brain [13], microglia do not appear to play a major role in larval spinal cord repair, where peripheral macrophages are controlling axonal regeneration through the production of the tumour necrosis factor (TNF) effecting a decrease of the levels of interleukin-1 beta released from neutrophils.

Other cells contribute to the structural reorganisation of the injury site, like glial cells, whose proliferation and bridging activity is partly promoted by Ctgfa in adult fish [14]. In larvae, Wnt/Beta-catenin signalling controls the production of extracellular matrix components from fibroblasts, in particular Collagen XII, which in turn promotes axonal regeneration [15] (Figure 1A).

Lesion induces regenerative neurogenesis from resident progenitors under the influence of developmental and regeneration-specific signals. Serotonin [16] and dopamine [17] can promote spinal motor neuron regeneration by acting on the proliferation of pMN-like progenitors. Bioinformatic analyses of gene expression after injury can identify gene regulatory programmes associated with successful regeneration. One such analysis [18] has recently confirmed the regulation of Wnt [15] and Notch signalling [19], as well as providing evidence for the importance of other pathways in repair that need to be followed up in functional experiments.

Axons

Spinal cord injuries induce a local calcium wave that is followed by a terminal wave that regulates axon fragmentation [13]. This axon fragmentation disrupts the signal transmission from brain to spinal cord. The re-establishment of axonal connections from proximal to distal of the lesion is thus essential for recovery of function. A recent study has shown, using live two-photon imaging and laser injuries, that axons of Mauthner neurons can regenerate almost completely in a few days, are remyelinated and re-establish synapses [21]. The regeneration is supported by an active transport of mitochondria along axons and is stimulated by cyclic adenosine monophosphate (cAMP) [22] in line with previous findings [23]. Axonal growth is also under control of the ubiquitin ligase PHR through cyfip2- and JNK-pathways. Furthermore, PHR is crucial for correcting mis-directed sprouts and thus controlling the robustness of circuit reorganisation in the zebrafish spinal cord [24]. Another protein, Semaphorin4D, involved in axon guidance, is overexpressed after spinal cord injury in young adult fish, enhancing axon regeneration and functional recovery [25] (Figure 1B).

Besides molecular signalling, mechanical properties of the injured spinal cord may play an important role for axonal regrowth. Indeed, in rodents, scar formation after spinal cord injuries, leading to a reduction in tissue stiffness [26], inhibits axonal regrowth, while the spinal cord tissue in adult fish transiently increases its stiffness (see Figure 1C), reaching a maximum when

regrowing axons cross the injury site [27]. This suggests that higher axonal regrowth occurs in stiffer tissue, as already observed in other models [28]. There may be a positive feedback loop, since previous findings have indicated that mechanical properties of the spinal cord are determined by cellular distribution and axon orientation [29], and that, in the context of morphogenesis, cell migration is triggered by stiffening of the tissue [30].

Therefore, after injury, neuronal and axonal regeneration create a new network with a motor output similar to the unlesioned fish but incorporating different and/or new neurons and likely a different wiring. This renewed circuitry needs thus to be analysed in detail to understand the differences from the initial one and the dynamics of its establishment, using recent technological advances described below (see Table 1 for an overview).

New approaches to study and understand SC neural circuit reorganisation

In order to decipher the role of the different cell types during spinal cord circuit reorganisation, it is necessary to be able to identify them by expression of specific reporters, e.g. for neurotransmitters, or to be able to manipulate them, by genetic ablation or optogenetics. A recent tool, CaSSA has been developed based on CRISPR/cas9 technology allowing to create genetic switches that can be used for instance to generate lineage-specific genetic mutations [31].

To reliably determine the effects of such mutations on the spinal network, observation and measurement must be performed on a cohort of animals. Using 3D printing, mounting single embryos can be standardized in a specifically designed microwell plate, optimised for imaging [32]. A similar technique has been developed, dedicated to spinal cord regeneration studies, by incorporating a millifluidic system to stabilize zebrafish larvae in homeostatic condition during long-term imaging sessions [33].

This long-term observation, combined with fast 3D imaging, is essential to capture the dynamics of cellular and axonal reorganisation. Amongst other techniques, light-sheet microscopy is particularly efficient for long-term fast 3D imaging by keeping phototoxicity much lower than other microscopy techniques. This allows for example to determine how neurons become mature during development by live lineage tracing and measurement of neural activity [4].

Extracting useful information from these observations relies on image processing tools, such as image registration, multimodal volumetric reconstructions, or 3D cell segmentation and tracking. Indeed, image drifts due the microscope instabilities or animal movements need to be compensated. A recent study obtained successfully registered volumes of fast (>120 Hz) beating zebrafish heart imaged over 24h allowing to study heart morphogenesis during development together with cell migration in intact and injured heart [34]. This might be then applied to images of the spinal cord during regeneration. Combining images from optical and electron microscopy, interneuron circuits could be analysed and showed a specific synaptic connectivity with large motoneurons from dorsally located interneurons, active only during fast locomotion [35]. Massive 3D cell tracking analysis, allowing single cell fate mapping in living fish, can be achieved using a new framework for interactive analysis on big datasets [36]. Cell morphology changes during tissue reorganisation can also be analysed, for instance using the software RACE which performs automated 3D cell segmentation and shape extraction [37].

At the tissue scale, as discussed earlier, mechanical forces play a role in reorganisation of the spinal cord. Brillouin imaging is being used to determine non-invasively mechanical properties of living larvae during regeneration, showing an immediate decrease of viscoelasticity properties just after the injury followed by a gradual increase [38].

Analyses of cell and tissue morphology and dynamics are not sufficient for understanding how the new spinal network is functionally organised. Functional imaging, mainly by recording fluctuations of calcium concentrations, thus holds a central tool in the exploration of neural circuits. Volumetric calcium imaging can now be more accessible by installing an additional module to a standard two-photon microscope, for example an axicon Bessel module allowing fast (50 volumes/s) calcium imaging of spinal projection neurons [39]. This can then be used to measure network dynamics of hindbrain reticulospinal neurons, with potential applications in the spinal cord [40]. Enabling faster action potential recordings, in vivo voltage imaging is now possible through the use of Voltron, an hybrid "chemigenetic" voltage indicator which allows recording of spikes of a dozen of neurons at the millisecond time scale simultaneously for several minutes while recording behaviour [41].

Neural activity measurements require efficient and reliable calcium signal analysis. Generic toolboxes have been made available in the few past years, proposing complete and modular pipelines [42] [43], or tools which are more specialized, like image registration used to reduce false spike detection [44]. However, results and in particular estimation of neural assemblies can strongly vary depending on the algorithm used [45]. As those tools have been dedicated to analysing signals in the brain, they may need some adaptation for use in spinal cord studies.

Beyond observing the spontaneous activity of neurons, larvae can be stimulated by optical, acoustic, mechanical or electrical stimuli eliciting various responses, such as fast escape behaviour or swimming speed regulation. Electrical stimulation has been used to record the response of multiple larvae simultaneously [46]. A local mechanical stimulation can be achieved by using optical trapping to directly apply forces on otoliths stimulating the vestibular system, which mimicks acceleration or roll movements independently of other neural systems [47]. Optogenetics, using channelrhodopsins for in vivo optical manipulations, make it possible to efficiently inhibit or activate neurons [48]. This has been applied to spinal V2b interneurons, showing that swimming speed is modulated by their activity [49].

The integration of different imaging modalities and photo-stimulation on the same microscope permit to simultaneously record neuron activity and behavioural analysis on freely moving zebrafish larvae [50]. This was used for screening effects of neuroactive drugs and might be applied for screening pro-regenerative drugs in the spinal cord.

Beyond imaging technologies, machine learning approaches, in particular unsupervised clustering methods have been developed to detect the different categories of movements during swimming, showing that larvae rely on a specific and limited set of locomotion patterns [51].

Genetic approaches, also benefiting from advanced computational approaches, can provide large amount of information on tissue changes at single-cell level during development. In particular, single-cell RNA sequencing has been combined with transposon-based barcoding [52] or CRISPR-Cas9 barcoding [53] to map cell lineages in animals at different stages of development. These approaches might be successfully used on lesioned animals.

Conclusion and future directions

Understanding how neural circuits reorganise after a spinal cord injury has major potential clinical applications but remains a challenge today. However, we posit that elucidating the mechanisms underlying successful functional recovery in a vertebrate spinal cord can be achieved through the development and use of well-controlled and quantitative methods presented here (Figure 2).

Further progress can be foreseen by recent trends in standardisation and optimization of experimental conditions. First, inducing controlled lesions, like : laser-induced lesions [54] and electric neurectomy [12], will help standardise the procedures and the interpretation of the experiments. Sample preparation can be also standardized and optimized, in particular through the use of 3D printing or polymer stamping [32][33]. Indeed, the flexibility and very low cost of those techniques make them highly accessible to any laboratory.

Moreover, highly detailed and fast dynamics of spinal cord development or repair is now accessible thanks to a recent development in super-resolution imaging, based on advanced light-sheet microscopy [55]. This allowed observation of growth cone dynamics in the spinal cord in which newly differentiated neurons expressed random combinations of fluorophores and measurement of clathrin dynamics during endocytosis and organelle morphology (mitochondria, Golgi apparatus) in neural progenitor in the brain. This could certainly be applied to the spinal cord.

In complement with these techniques, artificial intelligence approaches, like deep learning or data mining, will become more accessible and widespread to improve data quality and deal with the increasing amount of information contained in datasets produced by high-resolution or high-throughput imaging and genetic techniques.

Lastly, in order to build a better conceptual view of re-establishment of neural circuits, mathematical modelling would be highly beneficial. Of growing use in other fields of biology, this modelling effort would ultimately bring us a unified understanding of the complex and highly dynamic cellular and molecular mechanisms underlying successful spinal cord regeneration.

Given the recent progresses in deciphering neural circuits in zebrafish and with these new technologies, it is now time to apply multidisciplinary efforts in understanding the reorganisation of spinal circuits after injury that allow return of function.

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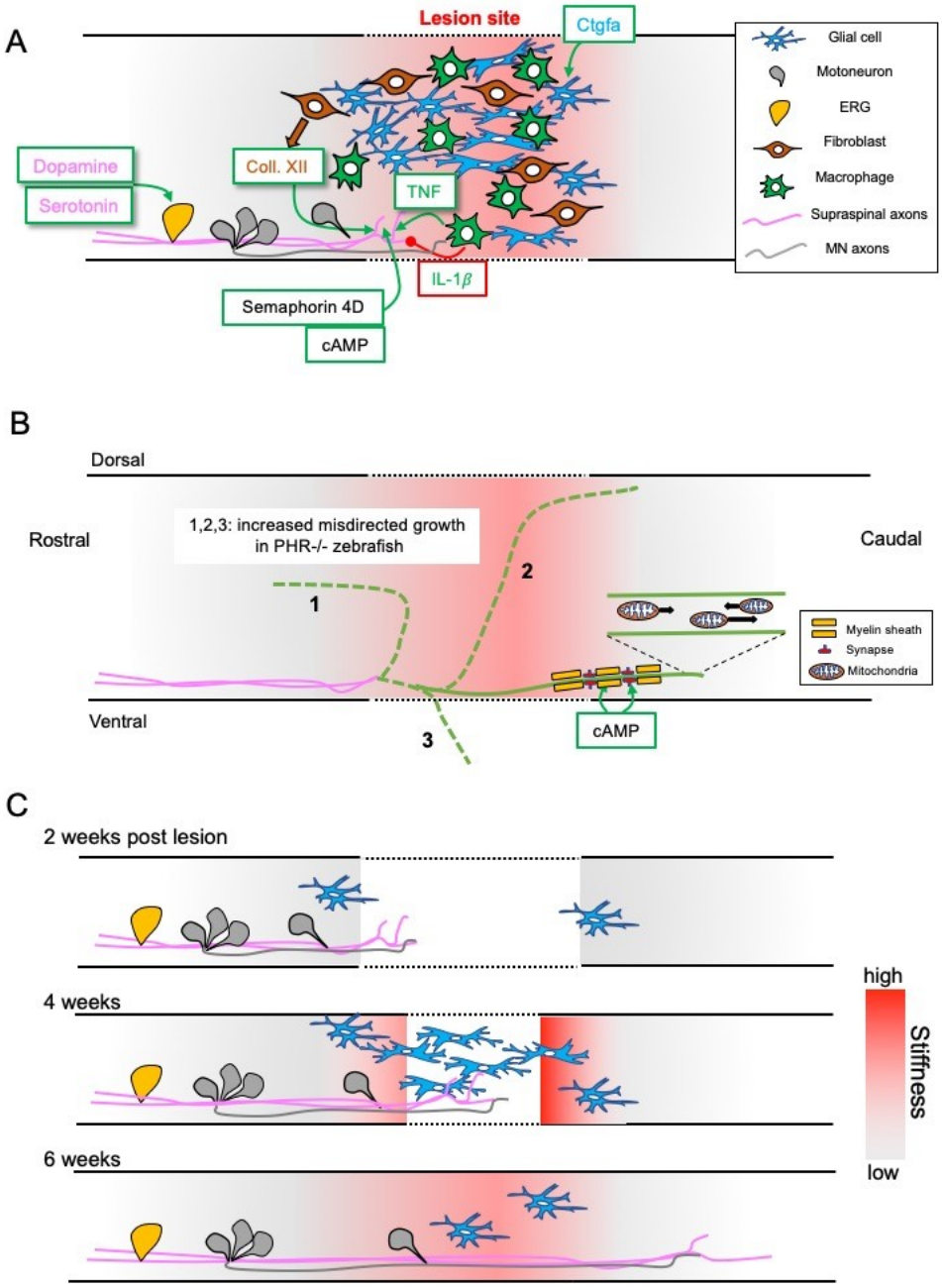

Figure 1

Figure1: **Circuit reorganisation after a spinal cord injury**.
A: Schematic view of recent findings on signals involved in regeneration of motoneurons and axons. (ERG: Ependymo-Radial Glial cell); **B:** Axons are regenerating upon control by ubiquitin ligase PHR and cAMP stimulate re-myelination and mitochondria transport (adapted from [22, 24]); **C:** Spinal cord stiffness increases after an injury in adult Zebrafish (adapted from [27})

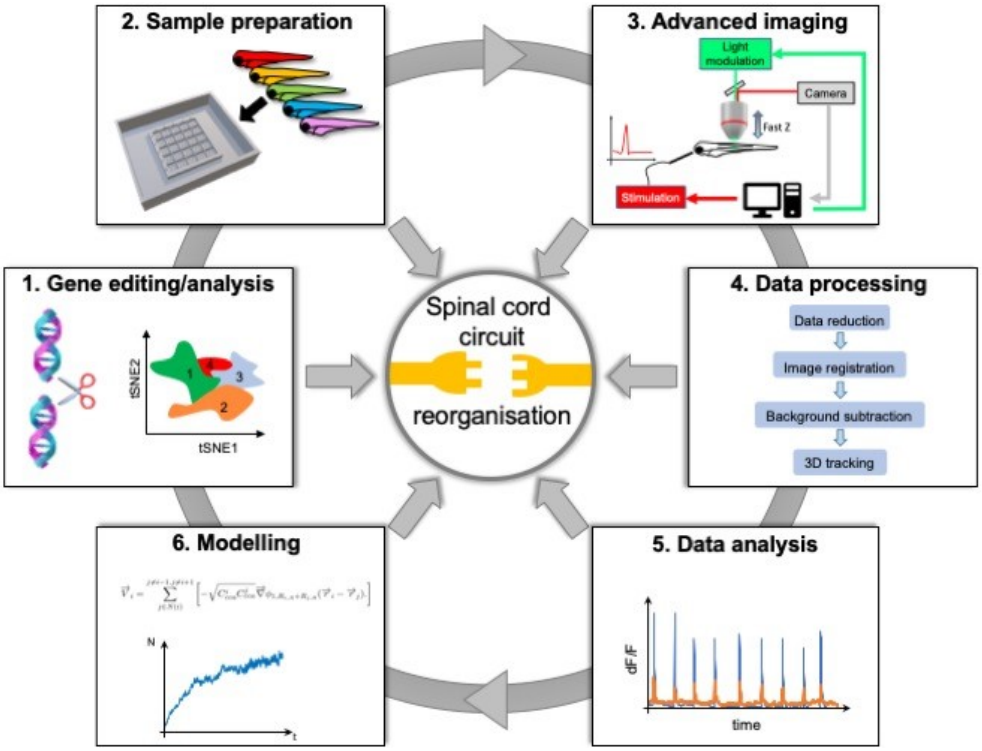

Figure 2

Figure2: **Integrated workflow of the use of new technologies for understand spinal cord circuit reorganisation**. Important progress is being made in the different techniques used for studying circuits in intact and injured spinal cord of zebrafish, from genetic manipulation and analysis to mathematical modelling. A full integration of these approaches as schematized here is the key for future research in this field.