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Successful neural regeneration in amniotes: the developing chick spinal cord

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Abstract. Although early after birth the central nervous system is more plastic than in the adult, it already displays limited regenerative capability. This becomes severely impaired at specific stages of embryonic development; however, the precise cellular and molecular basis of this loss is not fully understood. The chick embryo provides an ideal model for direct comparisons of regenerating and non-regenerating spinal cord within the same species because of its accessibility *in ovo*, the extensive knowledge of chick neural development and the molecular tools now available. Regenerative ability in the chick is lost at around E13, a relatively advanced stage of spinal cord development. This is most likely due to a complex series of events: there is evidence to suggest that developmentally regulated changes in the early response to injury, expression of inhibitory molecules and neurogenesis may contribute to loss of regenerative capacity in the chick spinal cord. (Part of a Multi-author Review)

Keywords. Axon, apoptosis, chick, neural stem cell, spinal cord, regeneration, secondary injury.

Spinal cord regeneration in embryonic higher vertebrates

Significant spinal cord regeneration following injury is not observed in any adult higher vertebrates (amniotes), but can occur in some lower vertebrates (anamniotes) such as tailed amphibians and fish. Certain higher vertebrates, including avian and mammalian species, however, display a high degree of regenerative capacity in their spinal cord during embryonic development, which is lost as development progresses [1]. These systems can therefore provide information about factors affecting loss of regenerative capacity in amniotes. The most useful embryonic systems for experimental manipulation are those that do not have to be accessed *in utero*, allowing complex manipulations to be carried out which would be poorly tolerated by most mammalian embryos. These models include the embryonic chick, which can be accessed while in the egg, and marsupials such as the opossum, which are born early during development and undergo a significant portion of their spinal cord development postnatally, while attached externally to the mother. In this review we will focus on the chick, which though a non-mammalian model, is an amniote, and provides some advantages over marsupials. The chick is a very well established developmental model, its nervous system has been extensively studied and many tools, including the sequence of its genome and gene arrays for differential screening, are now available. We will briefly summarize the key events in spinal cord development and then describe the response to spinal injury at permissive and non-permissive stages for regeneration and discuss what is known about the mechanisms that contribute to spinal cord regeneration and its loss with the progression of development.

Development of the chick spinal cord

As in all vertebrates, the walls of the chick neural tube initially consist of a pseudostratified layer of highly proliferative multipotent progenitor cells, the neuro-

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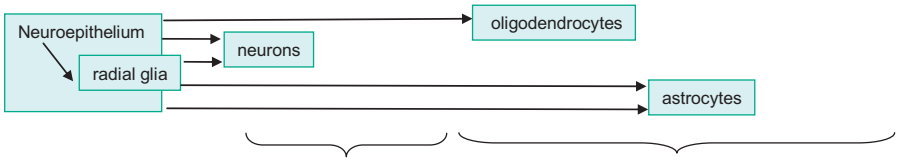

Radial glia born	Neurogenesis	Programmed cell death	Gliogenesis		
			Oligodendrocytes	Astrocytes	Myelination
Unknown	E3 – E9 (Fujita, 1964)	E6 – E7 (Oppenheim, 1991)	E5 – E6 (Ono et al., 1995)	E8 -E19 (Pringle et al., 2003)	E13 (Ono et al., 1995)

Figure 1. Temporal birth of neurons and glia in the developing spinal cord. Figure at top shows the approximate sequential order of birth of glia and neurons in the developing spinal cord. Table shows studies that have recorded the approximate dates at which each cell type/ event is observed in the chick spinal cord.

epithelium, from which all CNS (central nervous system) neurons and glia are ultimately derived. The differentiation of neural stem cells into neurons and glia and establishment of neuronal identity involves a series of decision-making steps in which cells first become postmitotic and then commit to a specific lineage [2–4]. The mechanisms underlying regulation of these decisions are complex and not yet fully determined. However, it is known that neurogenesis and gliogenesis are initiated in progenitor domains in a specific temporal and spatial pattern and that this pattern is determined early. Radial glia, defined as cells surrounding the central canal that maintain contact with the pial surface via processes spanning the width of the developing spinal cord, can be first distinguished from neuroepithelia cells around the onset of neurogenesis [5–7]. Therefore, they could be considered the earliest cell type to differentiate, followed by neurons, oligodendrocytes and astrocytes [7]. Figure 1 illustrates the relative timing of these events in the chick spinal cord.

Radial glia cells are present transiently during neurogenesis in the spinal cord and have a role in the guidance of radially migrating neurons; an important role in axon guidance for radial glia-like cells has also been suggested in the developing and regenerating spinal cord of tailed amphibians [8]. At later developmental stages they can give rise to both neurons and astrocytes and become eventually restricted to the astrocytic lineage [9–11]. In the chick spinal cord, neuron birth starts around E2-E3 and is largely completed by E4.5 in the case of motor neurons [12], whereas generation of a few neurons in the dorsal horns has been reported as late as E8-E9 [13]. During the period of neurogenesis many more motor neurons are born than will ultimately be required, and the final number of neurons is refined by a wave of programmed cell death, which in the chick peaks at around embryonic day (E) 6–7 and is completed by E9 in the rostral lumbar segments [14, 15]. Interneurons in the chick spinal cord, in contrast, do not seem to undergo programmed cell death [16]. Gliogenesis is initiated later than neurogenesis. Oligodendrocytes begin to develop around E5–E5.5, after the birth of somatic motor neurons is completed: they arise first from a restricted ventral region of the CNS and then migrate out to cover all regions [17, 18]. Astrocytic development from intermediate glial precursors takes place in regions of the neuroepithelium distinct from those which give rise to oligodendrocytes [19]. Astrocytes begin to be detected towards the end of neurogenesis. A few scattered cells expressing Fgfr3, a putative astrocytic marker, are first observed in the spinal cord parenchyme around E8, and cells expressing the later astrocytic marker GFAP (glial fibrillary acidic protein) are detected from around E10 [20, 21].

Crucial to functional development is the establishment of appropriate neural networks connecting the brain and spinal cord, and forming local connections within the spinal cord itself. Axon guidance has a crucial role in neural development and is subject to a complex array of attractive and repulsive cues, such as secreted or membrane bound guidance molecules [22]. The establishment of descending, ascending and intersegmental axonal projections is observed as early as E4. Electrophysiological and retrograde labelling studies have shown that by E6–E7 there are functional descending connections from the brainstem to the lumbar cord and by E11–E12 the descending supraspinal projections are much like those of the adult spinal cord [23–26]. As in other vertebrates, myelination is a relatively late event in development, which begins once the neural networks are well established. In the chick spinal cord oligodendrocytes begin to surround neuronal axons with a myelin sheath at around E13, and myelination is completed at the time of hatching [27]. Whereas myelin is of key importance for the rapid conduction of nervous impulses, there is much evidence that several of its component proteins have inhibitory effects on axon elongation [28, 29].

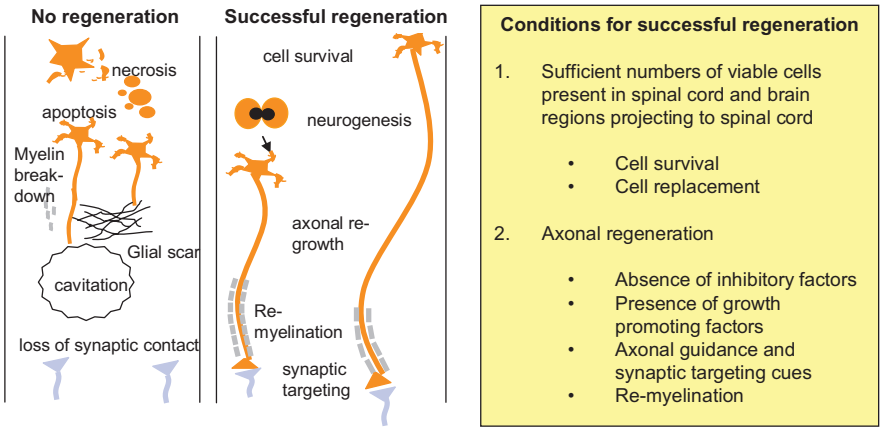

Figure 2. Conditions necessary for successful spinal cord regeneration. Figures illustrate the typical challenges to neuronal regeneration occurring in the non-regenerative spinal cord and the processes contributing to successful regeneration. The hypothesized conditions for successful regeneration are listed in the box on the right.

Requirements for successful regeneration

Injury to the central nervous system in the adult mammal invokes a series of pathophysiological events, which culminate in functional impairments. Characteristic features of spinal cord injury include neuronal and glial death, axonal degeneration and demyelination, the formation of large fluid-filled cavities within the spinal cord and the generation of a glial scar consisting mainly of reactive astrocytes that creates a barrier to axonal growth [30]. Many of these processes also occur, to an equal or lesser extent, in lower vertebrate and avian species, as we will discuss later. It is useful to begin by considering what conditions must be met for successful spinal cord regeneration to take place. Figure 2 summarizes a simplified view of these conditions, which can be divided into two broad complementary requirements for regeneration. First, in order for functional regeneration to occur, there must be enough viable cells present within the spinal cord. This includes both neurons that send projections long distances up and down the cord, and interneurons, which make the local connections that are responsible for the fine control of neuronal activity and glial cells. There is evidence that only a fraction of the original neuronal cell count needs to survive an injury in order for significant functional recovery to take place [31]. The number of viable cells can be influenced both by the degree of cell loss after the injury and by the ability of the spinal cord to generate replacement cells to take over from those lost.

Second, the neurons must be able to regrow disrupted axons or grow new axons to restore neuronal pathways. This can be controlled both by the intrinsic capacity of the cell for axonal regrowth and by the permissiveness of the spinal cord environment to axonal regrowth, which is influenced by the presence or absence of growth-promoting or inhibitory molecules. Regenerating axons must also be remyelinated, requiring the presence of surviving oligodendrocytes. Furthermore, limited benefit is likely to be gained by axon regrowth unless the connections established are functional and do not lead to abnormal neuronal activity. Axonal path finding and synapse formation must therefore be adequately controlled. As for other vertebrates, much research on neural repair in chick spinal cord has tended to concentrate on the second of these two conditions, whereas the first of the conditions outlined above has been relatively neglected. Cell survival and cell replacement after injury, which act to ensure the presence of sufficient numbers of viable cells in the spinal cord, is most likely crucial in determining regenerative capacity, as suggested by the regenerative process in the urodele amphibian spinal cord [32].

Regeneration versus regulation

An important issue when considering the response to injury in developing systems is the extent to which morphological and functional recovery is due to regulation of cells that have not yet become fully committed to a specific fate, as is the case in the frog early limb bud or the chick neural tube, rather then to regeneration, which is defined as replacement of an already highly specified structure. For example, following ablation of the dorsal third of the neural tube in 1.5-old-embryos (Hamburger and Hamilton stage 9, H&H9) the missing tissue is rapidly replaced and morphogenesis appears to progress normally [33–35]. This is most likely due to high proliferative activity and plasticity within the neural tube at those early developmental stages, which allow efficient reprogramming of neural progenitors to replace the ablated tissue. Longitudinal lesions spanning three somites, which induce spina bifida-like defects in 3–3.5-day chick neural tube (H&H18–19), can also be repaired, and by 10 days post-surgery 70% of the operated embryos do not display open neural tube

defects [36]. This ability to reclose the neural tube, however, is probably governed by forces different from those which govern tissue replacement, as relatively little cellular damage is induced by separating the dorsal halves of the neural tube at the midline, unlike the effects of dorsal ablations or spinal crush/transection.

Regeneration of the chick spinal cord

The occurrence of spinal cord regeneration in the chick following transection was first extensively reported in the early 1950s [37], but it was not until the early 1990s that this model was looked at again in more detail. Clearwaters (1954) carried out a histological analysis to compare the effects of spinal injury in 3- and 5-day-old embryos (E3 and E5) with that in chicks a few days post-hatching. She reported regeneration in the embryonic spinal cord, but lack of regeneration and extensive scarring in the postnatal spinal cord. Studies on E2 and E5, spinal cord transection from Oppenheim's group [38] were consistent with Clearwaters observations on effective morphological and functional regeneration in young embryos. They also showed that creation of a gap spanning two to three segments in E2 spinal cords, unlike transection, does not result in functional recovery [38, 39]. At E3–E5, though neurogenesis is well advanced, the cord is still rather immature, programmed cell death is high and cortico-spinal connections are not fully established. Therefore, it is difficult to rule out the possibility that at these stages of development, regulation of the developing cord plays a major role in the observed repair.

Complete anatomical and functional repair after transection injury, however, is observed up until E12 [24, 38], when the spinal cord is at an advanced stage of maturation. By E9–E10 all cortico-spinal tracts have formed connections with their targets, and the spinal cord is well developed, with only myelination and progressive expansion of the astrocytic population still to occur. Retrograde labelling with horseradish peroxidase (HRP) at late developmental stages or post-hatching reveals HRP-positive neurons in the brainstem of E10, but not E15 transected chicks [24], indicating that regeneration of severed axons occurs only before E15. Further support to the occurrence of true regeneration as opposed to a continuation of development in E10–12 spinal cords comes from retrograde double-labelling experiments [40]. Analysis of brainstem-spinal neurons retrogradely labelled rostral to the injury site with two different fluorescent dyes before and after transection shows the presence of double-labelled neurons in the brainstem up to E12, demonstrating that neurites that grow across the injury site in embryos operated at E10 are derived from the regeneration of damaged axons. In addition to morphological analysis and retrograde tracking, re-establishment of neural connections in the spinal cords of chicks injured at E10–11, commonly used as a regeneration permissive stage, has also been demonstrated by electrophysiological recordings and behavioural measurements [40–42]. Indeed, following injury at either E5 or E10, chick leg movements recorded pre-hatching are comparable to those of controls, and after hatching chicks operated at these permissive times for regeneration are able to stand and walk. This is not the case for animals transected at E15.

Early response to injury

Cellular and tissue damage following injury to the mammalian CNS is divided into two broad stages, termed the primary and secondary injury [43]. The primary injury occurs immediately upon the traumatic disruption of spinal cord tissue, resulting in the immediate destruction of tissue and haemorrhage due to rupture of cell membranes and blood vessel walls. Damage tends to be focused in the delicate, highly vascularised grey matter at the cord centre due to the concentration of shearing forces in this region, and is localised to the injury site [44]. Although the primary injury results in significant disruption, overall damage is vastly expanded by a series of delayed injury processes, triggered by the initial insult, which collectively make up the secondary injury. These processes include vascular alterations, biochemical activity and cellular responses, and trigger cell death by both necrotic and apoptotic mechanisms [45].

In the chick spinal cord, several marked differences in response to injury at E11 and E15 are apparent (Fig. 3). Injury at E11 results in localised tissue damage and haemorrhage within the first day after injury. By 4 days after injury, the lesion site appears to have returned to normal and the regenerative process is well advanced [42]. By contrast, injuries at E15 result in extensive haemorrhage and much greater tissue damage is apparent within the first 24 h. No indication of improvement is observed within 4 days, and large cavities are present. Several of the observed morphological changes after injury at E15, including cavitation and haemorrhage, are characteristic of those observed in mammalian species, including human and rat, adding further support to the chick system as a useful model for human spinal cord injury [46]. Increased haemorrhage at non-permissive stages for regeneration coincides with a rapid increase in vascularization between E11 and E15 [42]. Further-

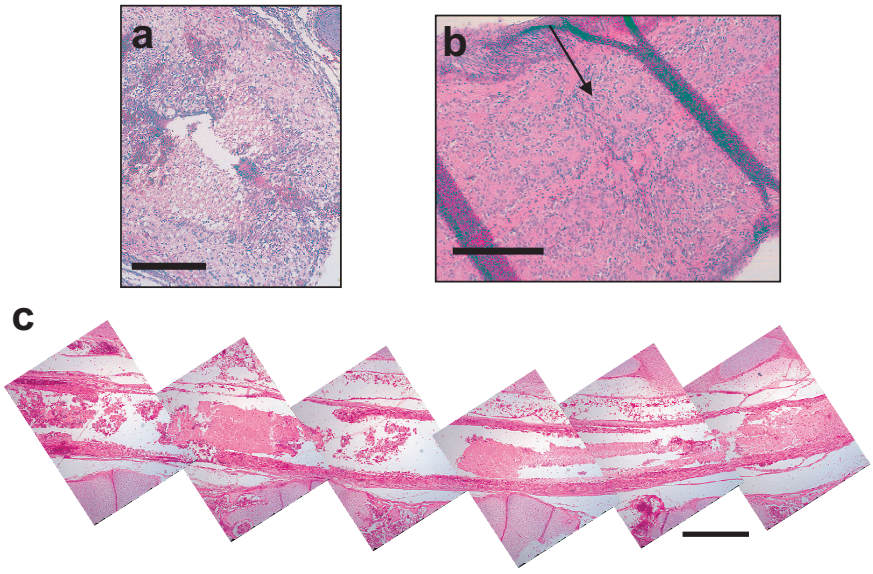

Figure 3. Analysis of morphological changes after injury at E11 and E15. Longitudinal sections of spinal cords at various time points after injury stained with haematoxylin and eosin. (*a*) Spinal cord crushed at E11 at 24 h after injury. (*b*) Spinal cord crushed at E11 4 days after injury; the barely visible injury site is indicated by the arrow. (*c*) Spinal cord crushed at E15, 24 h after injury. The precise injury site cannot be determined, due to the extensive nature of the injury. Scale bars, 1 mm.

more, this appears to be causally associated with extensive apoptosis and cavitation, which are observed after injury at E15 but not at E11. Reducing haemorrhage at E15 or increasing it at E11 by pharmacological manipulations results either in a decrease or an increase in apoptosis, respectively, following spinal cord injury [42].

A clear apoptotic response following spinal injury is observed at 24 hours but is basically undetectable 4 days after surgery in embryos operated at E11, whereas is still observed in embryos operated at later stages [42], where it is mainly found in white matter distal from the injury site [47]. Limited apoptosis observed in spinal cords at regeneration-permissive stages is also consistent with no significant change in motor neuron number observed in E10 transected spinal cords as compared to controls [38]. Analysis of cell death in the white matter demonstrates age-related changes in the apoptotic response of glia to injury, which may be linked to the onset of myelination [47, 48]. In embryos operated at E10, an increase in apoptosis is observed 3 days later. This may be due to oligodendrocyte fragility resulting from the reduced number of axons to be myelinated at that stage, rather than a direct response to the injury [47]. Caspase-dependent mechanisms are at least partially involved in the extensive apoptotic response following injury in the E15 chick spinal cord, as suggested by work from our laboratory (Fig. 4) and a study by Steeves and colleagues [47]. Inhibition of caspase-3 activity using the specific 3DEVD-fmk inhibitor can reduce apoptosis, and might reduce the secondary injury response. However, caspase activation represents a relatively late step in the progress of apoptosis, and direct inhibition of caspases might act too far down these pathways to save cells from damage. It might be of greater use for therapeutic purposes to identify factors acting upstream of caspases, which are responsible for triggering the apoptotic response. Given the link between haemorrhage and apoptosis, at least some of these factors are likely to be either blood-borne, or activated or induced by factors carried in the blood. Although a wide range of different factors and cell types could fit this description, the serine protease family of coagulation factors are interesting candidates for this role [49].

A reduction in the extent of cavitation and neuronal damage such as that observed in response to the anti-haemorrhagic drug desmopressin might be expected to improve the likelihood of successful axonal regeneration [42]. Although a detailed analysis of axonal regeneration was not within the remit of that study, in a desmopressin-treated embryo some axons appear to be crossing the injury site as indicated by neurofilament staining. However, it is not possible to determine from neurofilament staining alone whether this is the result of axonal regrowth or sprouting, and overall there is no evidence of robust axonal regeneration. In order to determine fully whether reduced haemorrhage alone improves axonal regeneration and consequent behavioural recovery, it would be necessary to look at post-hatching stages and to use a retrograde axonal tracing method such as that used to demonstrate regeneration in the E11 spinal cord [40, 41]. It seems most likely that other factors in addition to haemorrhage will require manipulation in order to promote axonal growth. Nevertheless, a reduction in cavitation and cell death, such as that produced by desmopressin treatment, would certainly make the process of axonal regeneration much easier to achieve.

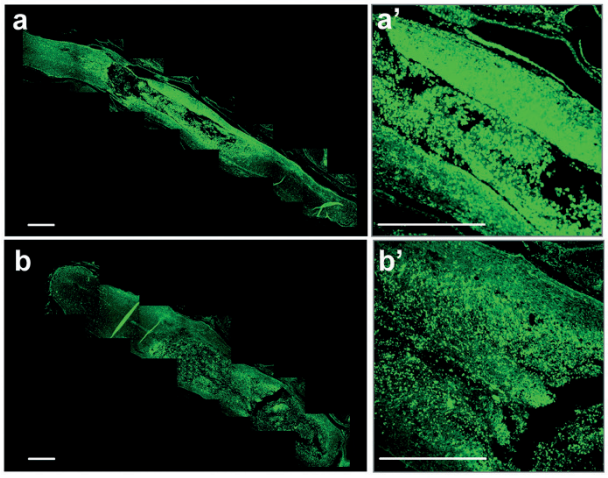

Figure 4. Analysis of the effects of a caspase inhibitor on apoptosis after injury at E15. Longitudinal sections of spinal cord stained by TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling) for apoptotic cells 18 h after injury at E15 and treatment with the caspase-3 inhibitor DEVD-Biotin-FMK. (*a*) Section from PBS-treated injured embryo. (*b*) Section from injured embryo treated with 2 mM DEVD-Biotin-FMK. (*a′*, *b′*) High magnification images of the injury site in each case. Inhibition of caspase-3 partially reduces the apoptotic response to injury. Scale bars, 0.5 mm.

Even a small increase in the number of axons spared after an injury has been shown to have significant effects on recovery, and as little as 7 % axonal survival below the injury site can result in significant motor function in humans [50].

Role of neurogenesis in spinal cord regeneration

In lower vertebrates, such as the urodele amphibians, cellular replacement is a key element of the regenerative response, but it has yet to be established whether this is the case in developing amniotes. Urodele spinal cord regeneration is associated with the proliferation of progenitor cells in the ependyma, and their subsequent differentiation into neurons and glia [51]. One major issue that remains unresolved is the relative contribution of such undifferentiated progenitor populations versus the dedifferentiation or transdifferentiation of already differentiated cell types. This has been addressed in cell lineage-tracing experiments with varying results [52–54]. Both transdifferentiation and dedifferentiation seem to occur during the regeneration of organs, including the limb and the eye [55], whereas analysis of markers expressed in the regenerating urodele spinal cord favours a process of dedifferentiation in this system [56]. At present, in a number of regenerative systems it has not yet been fully established whether transdifferentiation/dedifferentiation mechanisms predominate over stem cell recruitment, and it is likely that each individual situation may rely on different processes for regeneration [57]. In the adult mammalian spinal cord, neural stem cells have been suggested to be present in the ependymal region surrounding the central canal and in the parenchyma [58–60]. However, although these cells have been reported to proliferate in response to injury, and upregulate markers such as nestin, neurogenesis has not been observed and in most cases only glial cell types are generated [58–63].

It is not yet clear to which extent *de novo* neurogenesis plays a role in regeneration of the chick spinal cord at permissive stages, nor from which cells they may be generated and whether a transdifferentiation process may be involved at late stages. We have recently found that, unlike previously suggested, in the E11 chick spinal cord neurogenesis is still an ongoing process, whereas it is complete by E15 [64]. Therefore, there appears to be a correlation between the end of neurogenesis, which occurs somewhere between E11 and E15, and the loss of regenerative capacity. The presence of a pool of cells with neurogenic potential and a supportive environment for neurogenesis in the regeneration-competent chick spinal cord may be a key contributing factor to its favourable response to injury, either by simply allowing for better neuronal survival and axonal regrowth or by increasing the rate of neurogenesis following injury. Though an increase in proliferation in the spinal cord grey matter, together with expression of doublecortin and an increase in the nestin-like protein transitin in response to injury may be indicative of an upregulation of neurogenesis after injury at E11 [64], further analysis will have to be carried out to properly assess whether this is the case.

Axonal regrowth

Key to regenerative capability and functional recovery is the ability of surviving neurons to extend axons to the appropriate target. To recapitulate axon guidance signalling from scratch in the regenerating nervous system is a daunting task. Nevertheless, in systems that do regenerate, sufficient guidance cues are presumably either already present or upregulated in response to injury, whereas inhibitory molecules are either absent or downregulated. Formation of the glial scar is considered an important inhibitor of regeneration in adult mammals [30]. In the embryonic chick, a glia scar is not apparent either at permissive or non-permissive stages for regeneration; hence the chick provides a useful model for the identification of inhibitory mechanisms for axonal regrowth independent of the glial scar.

The contribution of some known inhibitory factors, such as proteoaminoglycans and myelin-associated proteins, to regeneration has been investigated in the embryonic chick. The regeneration-competent period is associated with a low ratio of chondroitin sulphate proteoglycan (CSPG) expression to expression of the growth-promoting heparin sulphate proteoglycan (HSPG) in the spinal cord; this ratio was demonstrated to increase after E13 [65]. In mammals, CSPGs are key components of the glial scar [30]. Therefore, though a glial scar is not morphologically apparent in the injured chick spinal cord, the presence of high levels of CSPG at non-permissive stages for regeneration may contribute to lack of significant axonal regrowth. As already mentioned, the chick spinal cord is capable of regeneration only at stages prior to myelination, and myelin components have been shown to inhibit axonal regeneration in several systems. The onset of myelination can be delayed by about 4 days by injecting in the E9–E12 chick spinal cord an anti-galactocerebroside antibody with complement [66, 67]. Such experimental delay in myelination extends the permissive period for axonal regeneration, and both morphological and functional recovery is observed, as chicks are able to stand and walk after hatching [66, 68]. In contrast, when the same approach is used to alter myelin structure in chick spinal cord post-hatching, there is no locomotor recovery, though partial morphological recovery occurs, as indicated by retograde labelling of brainstem neurons post-injury, and peripheral nerve activity can be induced by brainstem electrical stimulation [68].

The most prominent of the inhibitory myelin proteins investigated over recent years has been Nogo-A, and targeting of the Nogo proteins has yielded therapeutic benefit in experimental models of CNS injury [28, 69]. Different regenerative phenotypes, however, have been observed in knockout mice investigated in three separate studies pointing to a complex role of Nogo proteins and their receptor(s) in modulating axonal growth [70–73]. In addition to inhibiting axon regrowth via the Nogo-66 receptor (NgR), Nogo proteins also interact with mitochondrial proteins such as Nogo-interacting mitochondria protein (NIMP), anti-apoptotic proteins such as Bcl-2 and axonal proteins such as Caspr, suggesting a variety of endogenous roles for these proteins [74]. In the adult, Nogo-A is expressed in oligodendrocytes, but not in Schwann cells, while NgR is expressed in CNS axons, consistent with a role in axonal outgrowth inhibition by Nogo-A, but not in regeneration-competent peripheral nerves [75–77]. Nogo-A, however, is also expressed by some CNS neurons, and both Nogo-A and NgR are detected in mammalian and chick CNS early during development, when extensive axon growth takes place [75, 78, 79]. This indicates that the presence of these factors in the spinal cord does not always prevent neurite extension. Indeed, both Nogo and NgR are expressed in the E11 chick spinal cord, and these proteins are not downregulated following injury at this stage of development, but regeneration does occur [78]. The fact that a change in Nogo cellular localization, from mainly neuronal to oligodendrocytic, occurs around the time regenerative ability is lost may be causally related to its inhibitory activity [78]. Furthermore, different receptors might mediate Nogo's different roles in development and following injury [80, 81].

In addition to environmental changes, the decreased ability of older spinal cord neurons to regenerate axons seems to involve some changes intrinsic to the neurons, as suggested by heterochronic grafting experiments [82]. Whereas E9 neurons can extend axons either within regeneration-competent or incompetent spinal cord, E15 neurons cannot grow axon in either, suggesting that the lack of regenerative ability is not due solely to environmental changes. Reduced expression of L1, ß1 integrin and N-cadherin in older neurons with development, or their disruption in younger neurons, correlates with limited axonal growth, suggesting that these molecules may play a role in controlling the regenerative ability in the spinal cord [83]. Furthermore, the ability of neurons to extend axons on various substrates decreases with age [82]. Overall, it appears that both intrinsic and extrinsic changes contribute to loss of neuronal regeneration in the chick spinal cord.

Conclusions

The chick spinal cord provides an ideal model for combining experimental embryology approaches with mechanistic analysis at the cellular and molecular level. This has yet to be fully exploited in the context of spinal cord regeneration. The tools now available to carry out genomics and proteomics analysis in the chick and the possibility of manipulating relatively easily expression of protein/gene of interest *in ovo* will allow identification of several molecules and cellular interactions which may contribute to loss of regenerative ability. This will not only help to answer some fundamental questions in biology but also to devise novel strategies to gain successful regeneration in the injured or diseased mammalian spinal cord.

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