DEVELOPMENTAL REGULATION OF REGENERATIVE POTENTIAL IN THE SPINAL CORD

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ABSTRACT

Injury to the adult central nervous system cannot be effectively repaired, leading to chronic disability. In contrast, embryonic neurons possess extensive regenerative capabilities, permitting functional recovery after axonal damage. The embryonic chick can functionally recover from spinal injury at developmental stages prior to embryonic day 13, at which point the CNS undergoes transition from a permissive to restrictive environment for neuronal regeneration. The developmentally regulated molecular changes responsible for this transition remain to be fully characterised: the principle aim of this study was to advance understanding in this area.

A number of markers have been investigated, using immunohistochemistry, RT-PCR, and Western Blotting to establish the contribution of neuronal and glial populations to the failure of regeneration in the embryonic chick and human. In addition, the relative contributions of primary and secondary tissue damage following spinal injury have been addressed by measuring the extent and duration to which apoptosis occurs during permissive or restrictive stages of development.

The major findings of this research are that: 1.) Cell death following injury, particularly due to secondary injury mechanisms, plays a crucial, and perhaps principal role in establishing a non-permissive CNS environment during the restrictive period for regeneration; 2.) The myelin-associated inhibitor of neurite outgrowth, Nogo-A, and its receptor, do not contribute majorly to the transition from permissive to restrictive states during development, and seems to play an important, previously un-described, role during embryogenesis; 3.) Elevated levels of chondroitin sulphate proteoglycans through development, in addition to altered cellular localisation, are likely to contribute to the overall non-permissiveness of the mature spinal cord.

To conclude, this study has clarified some of the issues concerning the generation of a non-permissive environment during development in the embryonic chick and human, and has laid foundation for further research concerning the novel role of Nogo proteins during embryogenesis.
ACKNOWLEDGEMENTS

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Finally I wish to say thanks, and buy an imaginary beer, to all my friends from the lab who have helped keep me relatively sane, especially to (in no particular order); Sarah Reid, Adam Rutherford, Iain McKinnell, Louisa Dunlevy, Ailish Murray, Gemma Brindley, Caroline Paternotte, John Chilton, Carles Gaston, Jason Neil-Dwyer, Fang Zhang, James Holt, Sally Walder, Karen Willis, and to all other members of DBU and NDU - past and present. I salute you all.
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<tr>
<td>ABC</td>
<td>avidin-biotin peroxidase complex</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulphate proteoglycan</td>
</tr>
<tr>
<td>CST</td>
<td>corticospinal tract</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochlorohydrate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylprocarbonate</td>
</tr>
<tr>
<td>dg</td>
<td>days gestation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DOC</td>
<td>deoxycholate</td>
</tr>
<tr>
<td>DPX</td>
<td>dextropropoxyphene</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EAA</td>
<td>excitatory amino acids</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FGF2</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAP-43</td>
<td>growth associated protein-43</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulphate proteoglycan</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>ICH</td>
<td>Institute of Child Health</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L-15</td>
<td>Leibovitz’s medium</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>M-phase</td>
<td>mitosis phase</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukaemia virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium hydrogen carbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<td>Nogo Receptor</td>
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<td>polymerase chain reaction</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PUAF</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RAG</td>
<td>regeneration associated gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA electrophoresis buffer</td>
</tr>
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</table>
1.1 THE SPINAL CORD

The adult spinal cord is an important neural structure that plays a crucial role in the transmission of nerve impulses to and from the brain. Damage to the spinal cord can result in complete ablation of motor and sensory functions. Here I shall describe the normal anatomy and physiology of the spinal cord before elaborating on how traumatic insult can disrupt the system.

1.1.1 Structure and Function of the adult spinal cord

The adult spinal cord is a narrow strand of nervous tissue extending from the brainstem to the base of the spine. It functions as a conduction highway for both sensory information and motor commands. In transverse section, the spinal cord is seen to have two main structural components: white and grey matter (Tortora and Grabowski, 1996). White matter is primarily comprised of axonal tracts ascending to, or descending from the brain. In adulthood, these tracts are coated with myelin, a fatty insulating layer that aids rapid electrical conductance. The presence of myelin gives the white matter its distinctive ‘white’ colouring. The white matter is found surrounding a central ‘H’ shaped core of grey matter, and is divided into distinct funiculi. Sensory afferents are primarily channelled through the dorsal funiculus en route to the brain, whilst motor signals are chiefly routed via the ventral funiculus and lateral funiculi (Tortora and Grabowski, 1996). 31 pairs of spinal nerves branch from the spinal cord along the rostro-caudal axis. Although not physically segmented, the 8 pairs of cranial, 12 pairs of thoracic, 5 pairs of lumbar and 5 pairs of sacral spinal nerves control movement and receive sensory input from distinct levels of the body. This is illustrated in Figure 1.1.

The various ascending and descending tracts of the white matter are found in approximately consistent locations in the spinal cord, although overlap of tracts seems
to be considerable (Snell, 2001). The primary concentrations of the major nerve fibre tracts are depicted in Figure 1.2.

The **corticospinal tract (CST)** is vital for fine movement controls, especially those concerned with rapid, skilled voluntary motion of extremities. CST fibres arise from the primary motor cortex, secondary motor cortex, and from the parietal lobe. The axons then pass through the pons, before grouping in the medulla oblongata to form the swelling known as the pyramid (the CST is also referred to as the pyramidal tract). The tracts then cross the midline and descend the length of the spinal cord in lateral and ventral white matter. **Rubrospinal tracts** are closely associated with corticospinal tracts, and are thought to play a regulatory role, potentiating flexor muscle extension, whilst dampening extensor movements. These axons originate in the red nucleus, a well vascularised region of the midbrain with unclear function. The **Vestibulospinal tract** is vital in muscle movements associated with posture and balance. It arises from the vestibular nuclei where it receives input from the ear and cerebellum. **Tectospinal tracts** originate from the superior colliculus of the midbrain. They project medially through the ventral white matter. The function of the tectospinal tracts is believed to be involved in reflex movements in response to visual stimulation. The **olivospinal tract** originates in the inferior olivary nucleus from where it descends ventrally in the spinal cord. Its function is largely unknown, but may involve modulating other motor tracts.

In addition to facilitating motion, the spinal cord is the means by which sensory information is relayed to higher levels of the brain. Sensory information is detected by neurons of the dorsal root ganglia (DRG). These cells have 2 processes, a peripheral axon, which possesses sensory receptors, and a central axon, projecting to cells within the spinal cord. The cells upon which sensory neurons form synapses extend processes which decussate, and then ascend the spinal cord toward the brain, or can mediate local reflex actions (Snell, 2001).
The principal ascending pathways are also summarised in Figure 1.2. As with descending tracts, considerable overlap is believed to exist between the following pathways:

Sensory axons responsible for sensations of pain and temperature form synapses upon cell bodies within the most dorsal regions of the grey matter – the substantia gelatinosa. From here, fibres extend along the lateral spinothalamic tract before finally forming synapses in the thalamus. Further connections lead to the cerebral cortex, permitting conscious interpretation of the sensory input. The ventral spinothalamic tract arises in similar fashion; however, these axonal pathways transmit light touch and pressure sensations. The dorsal funiculus of the spinal cord is responsible for conducting information concerning discriminative touch and positional information from joints and muscles. This information is relayed through the fasciculus cuneatus and the fasciculus gracilis. Sensory neurons with long central processes extending from the DRGs to the medulla oblongata form these tracts. From here, second order neurons link to the thalamus and cortex. Information is relayed to the cerebellum from the skin, muscles, and joints, by way of the spinocerebellar tract. These sensory neurons form connections on Clarke’s nucleus within the dorsal horns, which then project to the cerebellum via the pons. This input is processed without the need of conscious intervention. Axons of the spino-olivary tract are similar, but project via the inferior olive.

Other projection pathways exist in the spinal cord and the system is made more complex by interactions between different systems. This permits intricate modulation of voluntary movements and reflexes, which may be influenced by numerous combinations of input from the descending pathways described above.

At the most basic level, a reflex arc consists of a circuit comprising of just 2 neurons. Sensory input from spindle fibres within muscles detect muscle stretch. This is rapidly countered by the initiation contraction within the same muscle (Figure 1.3). Other
reflexes are mediated by sensory information from the skin, joints, or ligaments; this being transmitted to the grey matter via the DRG. These axons form synapses with interneurons, which signals directly to motoneurons, initiating contraction of muscle fibres. Such circuits result in rapid responses as they contain only two synapses. Reflex reactions are used in the withdrawal response following a painful stimulus for example. Reflex responses are significantly influenced by input from the descending axonal tracts described above. The motoneuron’s response to stimulation by sensory nerves can be either dampened or enhanced, primarily by modulation of interneuron sensitivity.

It is also of interest that the local circuitry within the spinal cord can initiate complex rhythmic movements such as stepping. In these cases, moderately simple signals from the brain can instigate co-ordinated contractions in several muscles, permitting stereotypical repetitive movements to be made. Such intrinsic circuits are known as central pattern generators. They are of particular relevance in the interpretation of spinal cord recovery in patients and experimental animals. The spinal cord is capable of ‘learning’ to a limited extent and commonly extensive training can result in considerable locomotion following complete spinal transection (Rossignol et al., 2000).

Crucially, these pattern generators remain intact following spinal injury in regions caudal to the site of trauma. Thus effective functional recovery could potentially be achieved by re-establishment of central axons with these local networks. Regenerating entire motor circuits would be a far more daunting task.
Figure 1.1 Cartoon illustrating the spinal nerves and the organs they innervate. Injury to the spinal cord can potentially affect all musculature and sensation innervated by more caudal levels. From www.makoa.org.
Figure 1.2 Schematic representation of the main descending and ascending tracts of the human spinal cord. In these transverse sections, the dorsal aspect of the spinal cord is towards the top of the image.
Figure 1.3 A simple reflex arc. At its most basic level, the reflex arc consists of only 2 neurons, allowing rapid transduction of sensory input into muscle contraction. Small arrows indicate direction of nerve impulses. In this monosynaptic reflex, muscle stretch is detected via the muscle spindles and rapidly counteracted by instigating a contractile reaction.
1.1.2 Development of the spinal cord

All neural structures of the body are derived from the embryonic ectoderm. Thickening of this structure during early development results in the formation of the neural plate. This tissue then folds to form a tube in a process known as neurulation (Figure 1.4). Progressive proliferation of the neural plate, accompanied by invagination along the rostro-caudal axis produces neural folds. These folds go on to bend at a dorso-lateral hinge-point, leading to the apposition of their edges and internalisation of a neural 'tube'. This process does not occur simultaneously at all levels of the embryo. Rather, it is initiated at 3 distinct closure points, which then close together, in a zipper-like fashion (Shum and Copp, 1996). The final point of the neural tube to close entirely is the most posterior part – the posterior neuropore. Neural tube defects are caused by the failure of any of these processes. For example, craniorachischisis results from failure of neural tube closure between hindbrain and posterior neuropore. Spina bifida however is caused by failure of only the posterior neuropore to completely close (Copp and Henderson, 2000). Neural crest cells are formed at the boundary between neural and surface ectoderm (Figure 1.4d), this migratory population of cells gives rise to a range of tissues, including the majority of the peripheral nervous system, the craniofacial bones of the skull, Schwann cells, and melanocytes (Christiansen et al., 2000).

Following closure, the neural tube is composed of a single cell layer known as the germinal neuroepithelium. This layer is highly proliferative and gives rise to all cell types of the mature spinal cord. The nuclei of the neuroepithelial cells migrate as the cell cycle progresses; those undergoing mitosis are located at the luminal surface. Each cell division within the germinal zone gives rise to a daughter cell that migrates radially and can differentiate into various neural precursors. The migration of cells from the neuroepithelial layer results in the formation of a second cellular layer around the original neural tube. This second layer is known as the mantle zone, which itself is
surrounded by the subsequent formation of a third layer – the marginal zone. This tri-layered arrangement (see Figure 1.5) is maintained throughout the subsequent development of the spinal cord, the mantle zone forming the grey matter, the marginal zone forming the white matter, and the germinal neuroepithelium becoming the ependymal canal (Gilbert, 2000).

The temporal birthing of cells ultimately designates their fate; neurons are born first, followed by oligodendrocytes then astrocytes. Much of the work determining the temporal onset of neural development has been performed in the embryonic chick. In the chick embryo, which has a gestation period of 21 days, neurons are first evident at embryonic day (E) 2, and neurogenesis is largely complete by E5 (Hollyday and Hamburger, 1977). Oligodendrocytes, as detected by O4 immunostaining, are first evident around E5-E6, but the onset of myelination occurs several days later, around E12 (Ono et al., 1995).

Information regarding the appearance of astrocytes in the developing spinal cord is limited. Astrocytes are believed to originate from radial glia, and appear in the central nervous system (CNS) following completion of neurogenesis (Voigt, 1989; Misson et al., 1988). Studies in the chick are limited to post-hatching animals (Kalman et al., 1998).

Data from human embryogenesis regarding the precise timing of neural cell proliferation and maturation is more scarce, although the same trends are believed to apply. The neural folds of the human embryo are closed by 4 weeks gestation (England, 1996). Neurogenesis proceeds thereafter, the first synapses in the marginal zone being detectible by 8 weeks gestation (Wozniak et al., 1980). Oligodendrocytes are first detected in human spinal cords at around 7 weeks gestation (Hajihosseini et al., 1996; Aloisi et al., 1992). Myelination however does not commence until around 10-11 weeks gestation (Hajihosseini et al., 1996).
A crucial aspect of the maturation of the spinal cord is a transient ‘wave’ of programmed cell death. This is the mechanism through which the numbers of motoneurons produced are matched to the number of connections to muscle fibres required. Motoneurons within the spinal cord are dependent for survival on the actions of various neurotrophins, which are provided by targets of axonal innervation. Thus, only axons successfully reaching appropriate targets receive sufficient supplies of neurotrophins, and can survive. Unsuccessful neurons undergo apoptotic cell death.

The production of neurons during development is thus vastly in excess of the final number found in the adult. The time during development at which this programmed cell death occurs varies with the rostro-caudal location of the cells examined, and differs between species. For example in the chick, high levels of motoneuron cell death are found at cervical levels of the spinal cord at E6, while in thoracic and lumbar regions, levels are greatest at E7 (Yamamoto and Henderson, 1999). Elevated levels of cell death are present in the human spinal cord at around 7 weeks gestation (Wozniak et al., 1980).
Figure 1.4 The process of neurulation. The neuroectoderm thickens above the notochord, resulting in the formation of neural folds. These neural folds come together to form the internalised neural tube. The migratory neural crest cell population arises from cells located between the neural tube and surface ectoderm.
Proliferation in the neural tube occurs within the germinal neuroepithelium from which neural progenitor cells migrate laterally to form the mantle and marginal zones. Radial fibres extending from the ependymal zone to the pial surface guide the migration of these cells. Later in development, the mantle and marginal zones are defined as grey and white matter respectively (adapted from Gilbert, 2000).
1.2 SPINAL CORD INJURY

1.2.1 Consequences of spinal damage

The consequences of spinal injury are serious. Spinal cord integrity is vital for voluntary locomotion and sensation; damage to the spinal cord can result in complete paralysis affecting all regions below the damaged area. The severity of spinal injuries therefore depends not only on the extent to which the cord is affected, but also on the level at which the injury occurs, and thus which areas are deprived of innervation from the brain. Injuries to the upper cervical regions of the spinal cord are most debilitating; in these cases locomotor activity may be lost in all limbs, breathing may be compromised and injuries are commonly fatal. In other cases, where injuries are ‘incomplete’ or affect lower regions of the spine, only partial loss of motor functions may apply. Other consequences of spinal injury include incontinence and sexual dysfunction. Generally, patients with spinal injuries require extensive nursing and physiotherapy. Such care is expensive and may be required for the duration of the patient’s life, which is generally not shortened significantly by spinal injury.

1.2.2 Mechanisms of injury

Death of neurons, and severance of axonal tracts within the spinal cord is an inevitable consequence of spinal injury. In fact it could be said to define spinal injury. Further analysis however reveals a complexity in the timing and mechanism of injury pathology that offers hope for therapeutic intervention.

The initial cause of disruption in the spinal cord is caused by the injury directly. That is, direct mechanical trauma to the cord results in disruption of tissue architecture and cellular lysis. The extent of this primary injury will vary with the degree of trauma; a bullet wound to the spine would be expected to rupture more cells than a knock or a
fall, for example. The overall consequence of each injury however may turn out to be identical. The reason for this is that the primary insult triggers several other secondary tissue damage pathways. These processes can result in spread of the injury site to distal locations and greatly increase the numbers of cells lost and extent of tissue damage (Figure 1.6). Secondary tissue damage mechanisms can be chemical or molecular in nature, although the precise extent and means by which primary injuries are extended are not fully understood. Several factors are however known to be of importance, some of which are summarised in Figure 1.7.

**Haemorrhage** is commonly seen following spinal cord injury and may contribute to secondary damage processes. Spinal trauma will frequently cause compromise of the blood-spinal cord barrier, resulting in exposure of blood components to the nervous system. Blood contains many neurotoxic elements, and may also be a medium for transporting other potentially toxic factors to the injury site, such as cells of the immune system. Many blood proteins, including those involved in clotting cascades, are known to be neurotoxic. Thrombin for example, can promote apoptosis of neuronal cells (Ahmad et al., 2000; Donovan et al., 1997). Breakdown of blood supply to the spinal cord can also result in hypoxia, which is likely to result in necrotic death of cells, and may be particularly relevant to cavity formation.

Release of neurotransmitters into the injury site following spinal damage, both from the primary injury, and during the process of axonal degeneration can result in **excitotoxicity**. Excitatory neurotransmitters such as glutamate trigger calcium ion influx into neurons. Only cells possessing glutamate receptors such as N-methyl-D-aspartate (NMDA), or Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are affected by these toxic mechanisms. Sustained receptor activation elevates intracellular calcium concentrations, resulting in induction of various proteolytic enzymes, breakdown of membrane lipids, generation of free radical species, and ultimately cell death (Atlante et al., 2001).
Many other factors contribute to secondary injury processes. Frequently, these share a common effector: the production of reactive oxygen species (ROS). ROS are free radicals derived from oxygen molecules and are extremely reactive chemical moieties. The presence of unpaired electrons enables rapid oxidation of proteins, lipids and DNA. When unchecked within a cell, oxidative stress from ROS will lead to cell death as normal biochemistry and cellular integrity are compromised.
Primary Injury

Secondary Injury

Figure 1.6 The effects of secondary injury mechanisms on spinal cord pathology. The extent of the initial mechanical injury is greatly extended over time by secondary injury processes.

Figure 1.7 Components of the secondary injury response and the timeframe of their effects. Some aspects of the secondary reactions can persist in causing further deleterious effects for long periods of time. BSCB: blood spinal cord barrier, EAA: excitatory amino acids, PAF: platelet activated factor, PUFA: polyunsaturated fatty acids. From Velardo et al. (2000).
1.2.3 The Immune Response

Components of the immune response can also trigger secondary damage mechanisms. Agents responsible for initiating inflammatory processes are released rapidly following damage to the CNS. These include; cytokines such as tumour necrosis factor alpha and interleukin-1 beta; platelet activating factor; prostaglandins; and leukotrienes (Velardo et al., 2000). The release of such factors results in activation and infiltration of inflammatory cells; commonly inflammatory cells both intrinsic and extrinsic to the CNS are recruited. Microglia are the CNS’s resident population of macrophage (Matyszak, 1998). They can respond rapidly to acute pathological events within the CNS, migrating to sites of trauma. Once present at the site of injury, microglia develop into activated phagocytes (Kreutzberg, 1996). When the blood-spinal cord barrier is damaged following injury, peripherally located macrophages and lymphocytes can also migrate to the injury site although this process takes over 48 hours to commence (Perry et al., 1995). Injury to peripheral nervous tissue results in rapid recruitment of monocytes that are believed to be important in clearance of cellular debris and initiation of repair processes. The delayed and understated monocyte response to CNS injury may be a crucial factor in the limited regenerative response seen in adult spinal cord and brain (Lazarov-Spiegler et al., 1998).

However it is also apparent that immune cell infiltration also causes deleterious effects and can negatively influence the extent of successful spinal cord repair. In studies where the influx of peripherally located macrophages and monocytes was inhibited by chemically depleting their numbers with the drug clodronate, experimental animals displayed signs of functional recovery from spinal cord injury (Popovich et al., 1999). Increased tissue sparing was also observed, suggesting that the inflammatory cells were extending the site of spinal cord injury. Other studies confirmed that macrophages synthesise nitric oxide at the injury site following spinal trauma, and this
leads to localised apoptotic cell death (Satake et al., 2000). Activated macrophages have also been shown to express molecules that inhibit re-growth of axons, thus hindering successful repair (Fitch and Silver, 1997b).

1.2.4 Axonal degeneration and cavitation

Spinal cord injuries, although diverse in nature, share some common pathological characteristics such as the degeneration of severed axons and the development of fluid-filled cavities within the injured cord. Wallerian degeneration is the process by which the distal portion of a severed axon is disassembled. The recent identification of a mouse mutant – *Wallerian degeneration slow* (*Wld*) in which this process is delayed has led to the belief that Wallerian degeneration is, at least to an extent, an active process (Ribchester et al., 1995). This active cellular deterioration is however distinct from apoptosis as no caspases are involved in Wallerian degeneration (Finn et al., 2000). Severance of axons leads to the development of terminal clubs at the cut ends. These are formed by the continuing axoplasmic flow of organelles and vesicles along the axon. The recurrent formation, and bursting of these clubs are believed to contribute to the processes of secondary tissue damage by releasing noxious molecules into the CNS, including lysosomal enzymes (Kao, 1980).

The formation of acellular cavities within the spinal cord is also a characteristic feature in both human and rat (Kakulas, 1984; Kakulas, 1999a; Taoka and Okajima, 1998). Cavitation is thought largely to occur due to progressive hypoxia and necrosis within the damaged cord (Kao, 1980). Recent studies have also implicated inflammatory responses in the development of cavities. Fitch et al. (1999) promoted cavitation in the rat spinal cord by treatment with a pro-inflammatory agent in the absence of physical injury. Their data indicate that persistent macrophage activity is sufficient to form cavities. Other studies suggest that nitric oxide released from macrophages may be a major mechanism responsible for this effect (Matsuyama et al., 1998).
1.2.5 Cell death

One way in which the outcome of spinal injury can be positively regulated is to prevent cell death. Although cell death is an inevitable consequence of spinal injury, much is caused by additional secondary tissue damage mechanisms (see above). Also of importance is the survival of cell bodies at sites distant from the injury. Numerous cells in the cortex and brainstem suffer axotomy following spinal transection. Survival of these cells is crucial and a prerequisite for regeneration. Generally, neurons are more likely to survive when their axon is severed at sites distant from the cell body. However, atrophy often occurs, although this can be prevented by treatment with appropriate neurotrophins. For example, BDNF can reverse atrophy of axotomised rubrospinal neurons when applied close to the cell body, and can enhance expression of regeneration associated genes (RAGs), promoting some re-growth of fibres (Kobayashi et al., 1997).

Most cell death occurring at the site of injury in the spinal cord is thought to be necrotic in nature (Kao, 1980; Kakulas, 1984; Kakulas, 1999a). However, recent evidence has indicated that programmed cell death may play a role in the pathology of spinal cord injury (Lou et al., 1998; Liu et al., 1997; Li et al., 1999; Newcomb et al., 1999; Crowe et al., 1997). The revelation that some of the cell death observed following spinal cord injury is the result of an active process is an exciting one, as it offers the prospect of potential therapeutic intervention. Anti-apoptotic agents may form part of future treatments for acute spinal injury.

1.2.6 Current treatment regimens

Chronic spinal cord injuries are currently untreatable. Physiotherapy is commonly used to prevent muscle wastage; any medication administered to these patients is aimed at enhancing lifestyle and avoiding complications associated with spinal damage, such as urinary tract infection. Following acute injury, the synthetic corticosteroid
methylprednisolone is commonly administered. This treatment is believed to improve outcome by limiting the extent of secondary damage occurring post-injury. Clinical trials have confirmed its effect (Bracken et al., 1985; Bracken et al., 1990; Bracken et al., 1998), although the mechanism of action remains unclear, and recent studies using animal models have disputed its efficacy (Rabchevsky et al., 2002).

It is the current goal of regenerative research to aid the development of new treatment strategies, by both further reducing the consequences of secondary injury mechanisms, and actively promoting the re-growth of severed fibres.

1.3 SPINAL CORD REGENERATION

"In the adult centres the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."

The words of Ramon y Cajal in 1928 provided little in way of hope for the prospective repair of any injury to the brain or spinal cord. His pioneering research highlighted the difficulties in ever achieving effective repair in nervous tissue. However, science has gone some way in lifting the harsh decree imposed by Cajal. It is now accepted that the adult brain is not incapable of growth or cell division.

I will now discuss some of the evidence accumulated in recent years that supports the notion that nerve regeneration is an achievable therapeutic goal.

1.3.1 The Concept of 'Inhibitory Environment'

Observations concerning the different capabilities of central versus peripheral neurons to regenerate axons following injury have been made since the early 20th century.
(Ramon y Cajal, 1928). Progress in identifying the mechanisms responsible for such
differences has, however, been slow. Indeed not until 1980 was any real advancement
made in understanding why axons fail to re-grow in the CNS. For the first time, it was
noted that CNS axons could extend for considerable distances into peripheral nervous
system (PNS) tissue grafts: the context of a Schwann cell rich environment was
enough to overcome the abortive sprouting that is typically observed following CNS
injury (Richardson et al., 1980). In a continuation of these experiments, Aguayo et al.
(1981) transplanted PNS tissue grafts into the spinal cord of adult rats and mice.
Sections of sciatic nerve were used to create a PNS tissue ‘bridge’ between the
thoracic spinal cord and medulla. After up to 7 months, retrograde labelling techniques
were utilised to identify the source of any nerve fibres invading the tissue bridge. It was
discovered that many neurons from the grey matter of the spinal cord had innervated
the PNS grafts and extended processes for up to 3.5 mm.

These experiments resulted in the formation of a new hypothesis; that CNS axons are
intrinsically capable of regeneration, and that environmental factors within the CNS
prevent regeneration from occurring.

The experimental strategy of PNS transplantation was investigated further by a number
of researchers (Fernandez et al., 1985; Asada et al., 1998). However problems
remained in adapting such an approach for clinical use. Although CNS axons could be
persuaded to enter PNS grafts, extending axons rarely left the peripheral environment
to re-enter the CNS. Thus, even if grafting could effectively ‘bridge’ a region of spinal
damage, the regenerating axons would be highly unlikely to make any useful
connections. Although these early grafting experiments did not provide the quick
therapeutic answer hoped for, they did provide crucial clues and starting points for
subsequent research.
1.3.2 Evidence for regeneration

The evidence that nerve fibres can regenerate is now irrefutable. Numerous experiments have demonstrated the ability of neurons to re-grow damaged processes.

Following Aguayo's demonstration that CNS neurons can extend axons into peripheral tissue bridges (David and Aguayo, 1981), other investigators have injected DRG cells into the adult brain atraumatically and observed long-distance extension of axons across classically 'non-permissive' CNS environments (Davies, 1997). This study implicated the astrocytic reaction to CNS injury in the failure of regeneration, as opposed to any intrinsic failings.

Additionally, sensory neurons of the dorsal root ganglia have been noted to extend their central axon for considerable distances into peripheral grafts (Richardson et al., 1980; Richardson and Issa, 1984), or into the spinal cord itself (Neumann and Woolf, 1999), providing the peripheral axon was cut prior to the central axotomy. In other words, priming of DRG neurons increases their regenerative potential. This effect has been shown to correlate with elevated levels of cAMP within the neuronal cell bodies, and can be mimicked by exogenous application of the nucleotide (Cai et al., 2001).

Overall, adult neurons have been shown to retain many more characteristics of embryonic systems than previously believed. Neurogenesis has been detected in the adult human brain using Bromo-deoxyuridine (BrdU) labelling (Eriksson et al., 1998). Similarly in other animals, adult neurogenesis has been observed (Gould and Gross, 2002).

It is also noteworthy that the typical response to traumatic insult in the adult spinal cord is an abortive attempt at regeneration; axonal sprouting is a common observation (Bernstein and Bernstein, 1980). This suggests that the cells do possess some residual growth properties, but these are not sufficient to effect adequate repair.
These studies, in different ways, have demonstrated the capacity for axonal regeneration and suggest that a variety of factors, including local environment, reaction to traumatic insult, and intrinsic neuronal characteristics, may all contribute to the lack of regeneration seen in adult vertebrates.

1.3.3 Lessons from development

Given that regeneration of severed axons can take place, it is important to establish the reasons why it does not. Successful regeneration, when it occurs, is likely to encompass many of the processes seen during neural development, namely cell division, axonal extension and guidance, and the formation of appropriate connections. Thus, it seems logical that clues can be found by analysis of developmental processes for candidates of future therapeutic promise. It is apparent that young nerves have more regenerative potential that those found in the adult. If adult neurons could be encouraged to behave in a more embryonic fashion, then perhaps more effective regeneration could be achieved.

In recent years, a group of molecules have been identified which are believed to be crucial in supporting effective regeneration. Many of these regeneration-associated genes (RAGs) are also found in developing neurons. Many RAGs have been identified by comparing gene expression of injured, and uninjured peripheral nervous tissue, either by RNA differential display (Livesey and Hunt, 1998), or using cDNA microarrays (Bonilla et al., 2002).

One of the most intensively studied RAGs is growth-associated protein-43 (GAP-43). GAP-43 is upregulated during peripheral nerve regeneration and is present at high levels during development in axonal growth cones (Meiri et al., 1986) (Benowitz and Routtenberg, 1997). Transgenic mice overexpressing GAP-43 develop extensive aberrant axonal connections, suggesting a role in growth cone guidance and function (Aigner et al., 1995). Mice lacking GAP-43 die shortly after birth and display signs of
delayed axon projection within the brain (Strittmatter et al., 1995). GAP-43 is not upregulated following injury to CNS axons; upregulation may enhance the regenerative potential of normally non-responsive neurons (Yankner et al., 1990).

Other RAGs include cytoskeletal proteins, neurotrophins and their receptors, neuropeptides, and cytokines (Bonilla et al., 2002).

The sensory neurons of the DRG are a commonly used tool in regeneration research. Lesion of the peripheral process results in up-regulation of RAGs within the sensory neurons. This is sufficient to greatly enhance their regenerative potential. Normally, lesioned central axons of DRGs cannot re-grow and re-enter the spinal cord. Following RAG upregulation, such re-growth is possible. Conditioning lesions are hardly applicable to human therapy and so future research is likely to be focussed on modulating RAG expression by other means.

Differences in repair efficiency between embryonic and adult systems may also be explained by different environmental influences, for example in availability of growth factors or presence of axon-repelling stimuli.

There are several groups of molecules, present in development that function to guide the axons of the nervous system to their appropriate targets. Given that these factors can operate by actively repelling axons, might it be possible that the presence of such repulsive cues could negatively influence the process of axonal regeneration following nerve injury in adulthood?

One such group of axon-repulsing molecules are the semaphorins. These were originally identified from brain tissue using a growth-cone collapse assay (Luo et al., 1993). The semaphorin family of proteins has members also found in insects, implying a high degree of conservation through evolution. Although all semaphorin family members are thought to inhibit axonal outgrowth, most experimental research has
been performed on collapsin/Semaphorin 3A (Taniguchi et al., 1997b; Messersmith et al., 1995). Semaphorins can either be secreted, or act whilst membrane-bound, and all possess a distinctive 'sema' protein domain of around 500 amino acids. Their effects are mediated by a receptor complex of plexin, and neuropilin-1 (Takahashi et al., 1999). Downstream signalling is believed to be mediated by cytoplasmic collapsin-response mediator proteins (CRMPs) and activation of the small GTPase rac1, although the precise details of signal transduction are largely unknown (Goshima et al., 1995; Jin and Strittmatter, 1997; Goshima et al., 2002). The overall effect of semaphorins on responsive axons is induction of growth-cone collapse by altering cytoskeletal organisation. During development, semaphorins are crucial in the guidance of sensory neurones; peripheral extensions of the DRG avoid regions in which collapsin is expressed (Giger et al., 1996), and transgenic animals lacking Sema3A are found to have an excess of sensory fibres (Taniguchi et al., 1997a). In the brain, Sema3A is important in the initial outgrowth of pyramidal axons from the cortex (Polleux et al., 1998).

The ephrins represent a second large group of inhibitory guidance molecules of importance during development. Much research concerning ephrins focuses on their role in the retino-tectal system and the formation of retinotectal maps during the development of the visual system (Tessier-Lavigne, 1995). Ephrin gradients are crucial in this process. Animals lacking ephrin A5, for example, suffer from incorrect guidance and mapping of retinal axons in the midbrain (Frisen et al., 1998). Ephrins are implicated in many other developmental processes, including the establishment of motoneurone specificity along the rostrocaudal axis, and in the pathfinding of commisural and corticospinal axons (Imondi and Kaprielian, 2001; Coonan et al., 2001; Kullander et al., 2001)

Ephrins are classed as either 'A' or 'B' type depending on whether they are GPI anchored or cross the membrane, and function via tyrosine kinase Ephrin Receptors
(EphRs). Clustering of EphRs is believed to be important in receptor activation. Interestingly, the transmembranal ephrin ligands are known to transduce a signal across the membrane, clouding the distinction between receptor and ligand (Kullander and Klein, 2002). This reverse signalling is likely to be of importance in cell-cell communication and fine tuning of the developing nervous system.

Surprisingly, little research has been conducted concerning the potential role of semaphorins or ephrins in the failure of axonal regeneration during adulthood.

Semaphorin 3A was noted to be upregulated following CNS injury in the adult, but not neonatal rat (Pasterkamp et al., 1999). This study concluded that semaphorin induction is a key feature of glial scar formation, upregulation being restricted to fibroblast components of the scar. Sema 3A is also downregulated following peripheral nerve injury, implying that its removal may be a prerequisite for effective axonal repair (Pasterkamp et al., 1998).

Ephrin B3 has also been shown to be induced in the rat following spinal cord injury (Miranda et al., 1999). Similarly, the Ephrin A3 receptor is upregulated (Willson et al., 2002).

However, despite an abundance on in vitro evidence confirming the inhibitory effect of semaphorins and ephrins on axonal outgrowth, as yet no in vivo studies have confirmed that inhibition of their effects results in enhanced regeneration following nerve injury. It seems likely that these inhibitory molecules will be of some importance following nerve injury, either in directly impeding regeneration, or in the successful guidance of regenerating fibres to their appropriate targets.

Another class of repulsive molecules present in the CNS are those associated with myelin; these include nogo, MAG, and OgMP. The influence of these proteins has been demonstrated extensively in vitro, and in vivo blocking experiments have also produced
encouraging results. These factors will be discussed in more detail in section 1.7, and in Chapter 7.

1.3.4 Clues from peripheral nerve regeneration

It has been recognised for a considerable time that injury to the PNS can be repaired effectively, allowing regain of function in adult mammals (Ramon y Cajal, 1928). A full understanding of the processes involved in PNS regeneration may offer clues as to the mechanisms through which regeneration is abortive in the CNS. One major difference concerns the populations of myelinating cells present; the resident myelinating cells in the PNS are the Schwann cells, whilst in the CNS oligodendrocytes are present. Could underlying differences in the response of these cells to spinal injury account for the variations in regenerative potential? This will be discussed further in Section 1.6.

Another possibility lies in the differences seen between resident immune populations. The microglial cells present in the CNS are not found in the PNS. Furthermore the myelin-debris clearing actions of inflammatory cells in the periphery are known to be particularly robust. Monocytes are important in removing the assorted debris associated with Wallerian degeneration, and this process occurs more rapidly than in the CNS (Beuche and Friede, 1984; George and Griffin, 1994). It is likely that an effective macrophage-mediated clearance of the injury site following nerve injury in the periphery is crucial in promoting effective regeneration.

1.3.5 Animal models of spinal injury

Much of the current understanding of spinal cord injury has been generated using various animal models. These allow molecular analysis of the cascades following neural trauma to be made, and permit potentially beneficial therapeutic strategies to be tested.
Many current studies utilise the rat as a model: the rat spinal cord is well characterised in its response to trauma (Taoka and Okajima, 1998). The rat is chiefly utilised as a non-regenerating model; the pathology seen after injury is very similar to that seen following human injury (Metz et al., 2000). Commonly, experimental hypotheses are tested by local or systemic administration of agents to the rat, followed by behavioural analysis to evaluate effectiveness. Injuries can be standardised using controlled weight-drop apparatus, which allow a metal rod of known weight to drop onto the exposed surface of the spinal cord from a designated height (Metz et al., 2000). Other types of injury include hemisections (Bregman et al., 1997; Dyer et al., 1998), and complete transections (Coumans et al., 2001; Morin-Richaud et al., 1998) of the rat spinal cord.

Historically, the dog and the cat were popular models for spinal injury research (Windle, 1980). Much work concerned with investigating intrinsic spinal cord pattern generating circuits used cats following spinal transection (Rossignol et al., 2000). In recent years, use of such large mammals in spinal cord research has become much less common.

In addition to providing information about the processes that occur during unsuccessful regenerative attempts, animal models can also provide examples of systems that can effectively repair the injured spinal cord. These models can provide vital clues regarding the conditions required for successful repair. Often molecules found to be important in regenerating models will go on to be tested in non-regenerating models, such as the rat.

Urodele amphibians are capable of regenerating many body parts, including limbs, jaw, and spinal cord (Carlson, 1998; Thouveny and Tassava, 1998). Often tail amputation is used to investigate the mechanisms behind the successful repair of the spinal cord. Such studies in amphibians have helped develop the hypothesis that an ependymal
cell response is important in promoting spinal cord regeneration (Zhang et al., 2000; Nordlander and Singer, 1978).

The experimental model used in this study is the embryonic chick. This model combines the benefits of the non-regenerating and regenerating models described above. It has been known for some time that the immature nervous system is able to regenerate following injury to a much greater extent than adult systems. Using the embryonic chick, both regenerating and non-regenerating systems can be examined in the same species. Comparisons between these states can then be made, providing clues to the mechanisms responsible for abortive regeneration in the adult.

The accessible nature of avian embryos allows surgical manipulations to be made at early stages of development with relative ease. Following experimental spinal cord transection at various stages of development, it was demonstrated that the chick is capable of effective repair and functional recovery, providing the injury occurs prior to a pivotal point in embryogenesis (Shimizu et al., 1990). The periods of development where regeneration can occur were termed ‘permissive’, whereas later stages when repair is ineffective are known as ‘restrictive’. By injecting horseradish peroxidase caudal to sites of spinal cord injury during the permissive period, retrogradely labelled neuronal cell bodies were detected in the brainstem and in more cranial regions of the spinal cord, indicating that these neurons had extended processes beyond the injury site. This regeneration was observed in chicks at E5, E8, and E10, but not in chicks at E15 (Shimizu et al., 1990; Hasan et al., 1991). Subsequent research revealed that the final stage at which regeneration can occur in the chick is E13 (coinciding with onset of myelin basic protein expression) and crucially demonstrated that the regenerative effects seen were due to re-growth of existing nerve fibres, rather than extension of late-developing brainstem-spinal connections. This distinction was made by double labelling nerve fibres with fluorescent markers; firstly in a lumbar location to label all descending axonal fibres, then, following a thoracic spinal injury and 7-8 days of
regeneration, a second injection was made in the lumbar spine. This second injection was caudal to the injury site, thus only neuronal cell bodies whose axons had grown *through* the injury site would be retrogradely labelled with the second marker. The detection of double-labelled axons indicated that the same neurons had axonal projections to the lumbar region of the spinal cord before and after injury, i.e. regeneration had occurred (Hasan et al., 1993). Recent electrophysiological experiments have confirmed that functional neuronal circuitry is re-established following spinal transection during permissive stages of development (Sholomenko and Delaney, 1998).

The chick model of spinal cord regeneration remains underused. Initial studies identified myelin proteins as being important in the transition from permissive to restrictive states (discussed later in section 1.6). However, few other potentially crucial factors have been investigated in this model. Indeed the response of the chick to spinal injury has not been fully characterised. This thesis aims to address some of the unresolved issues surrounding the injured chick spinal cord and to investigate some of the most recent advances in the field using this versatile model.

### 1.4 GROWTH FACTORS AND REGENERATION

Growth factors play many crucial roles during development, and recently have been demonstrated to be of possible benefit following traumatic spinal cord injury. During development various growth factors promote cell survival, proliferation, and migration, attributes of benefit in modulating successful outcome following spinal trauma.

Applications of various growth factors have resulted in functional and histological improvements in outcome following CNS injury; I shall detail a few of the most significant factors in terms of potential future therapeutic interest.
Administration of **neurotrophin-3** (NT-3) results in enhanced axonal sprouting at the site of spinal cord injury in the rat (Schnell and Schwab, 1990). NT-3 is believed to influence corticospinal tract extension during development and local application following spinal cord injury can also attract CST axons and promote limited functional recovery (Houweling et al., 1998). An important aspect of neurotrophin-mediated reactions following spinal cord injury is the neuron specificity of each growth factor. Individual factors may promote re-growth of some neuronal pathways and not others. For example, NT-3 treatment can promote reticulospinal and rubrospinal axon growth, but does not affect raphe-spinal, or vestibulospinal tracts (Ye & Houle 1997). Grafting of NT-3 expressing cells at sites of rat spinal cord hemisection has also been observed to promote survival of neurons in Clarke's nucleus, which are normally reduced in number by 30% following spinal injury (Himes et al., 2001; Himes et al., 1994). NT-3 administration can also promote survival of red nucleus neurons, and can prevent spinal cord injury-induced cell loss completely when combined with foetal spinal cord transplantation (Bregman et al., 1998).

**Brain-derived neurotrophic factor (BDNF)** has also been demonstrated to have beneficial effects upon axonal regeneration. BDNF is detected at higher concentrations in the embryonic rat spinal cord than in adult, suggesting it may contribute to axonal growth seen after spinal injury in the neonate. Although BDNF was transiently elevated following injury to the adult spinal cord, the levels of expression seen during development were not achieved (Nakamura and Bregman, 2001). Other studies have reported similar transient upregulation of BDNF mRNA following spinal trauma (Ikeda et al., 2001). Application of BDNF-secreting fibroblasts following spinal injury in the rat was shown to promote regeneration of rubrospinal connections by Liu et al. (1999). This procedure also resulted in some recovery of forelimb function. The success of foetal transplants following spinal injury is improved by administering BDNF in conjunction with NT-3: the growth of severed descending tracts was enhanced and atrophy of rubrospinal cell bodies greatly reduced (Bregman et al., 1997; Bregman et
al., 1998). The mechanism for these effects is likely to be via upregulation of RAGs (Broude et al., 1999). Interestingly, the combination approach involving BDNF and embryonic spinal transplants has been noted to be effective at promoting functional recovery from spinal injury even when initiated 2-4 weeks from the time of initial damage (Coumans et al., 2001).

**Nerve growth factor (NGF)** can also promote axonal regeneration. When administered by adenovirus, NGF supported re-growth of severed central DRG axons into the spinal cord, re-establishing functional connections (Romero et al., 2001). Similarly, application of NGF by minipump results in extensive projection of sensory axons into the spinal cord following lesion (Oudega and Hagg, 1999). NGF also promotes some sprouting of CST axons at the site of spinal cord injury in the adult rat, and in the uninjured spinal cord of the developing rat pup (Schnell and Schwab, 1990). NGF is also neuroprotective for neurons in the hippocampus, preventing glutamate-induced excitotoxicity in vitro (Mattson et al., 1995; Semkova et al., 1996). Induction of NGF by the β2 adrenoceptor agonist clenbuterol may also partially explain the agent's beneficial effect in the rat following spinal injury (Zeman et al., 1999), or ischemia by carotid artery occlusion (Culmsee et al., 1999b; Culmsee et al., 1999a).

**Basic Fibroblast Growth Factor (FGF2)** can improve functional outcome following experimental spinal cord injury in the rat, either by direct injection or continuous administration by minipump (Rabchevsky et al., 2000; Rabchevsky et al., 1999; Teng et al., 1998; Teng et al., 1999). The normal response of the injured spinal cord is to elevate synthesis of FGF2; this has been demonstrated by analysis of mRNA and protein levels (Follesa et al., 1994; Mocchetti et al., 1996). The same elevations are observed following injury to the brain (Frautschy et al., 1991). FGF2 aids neuronal survival following spinal trauma (Lee et al., 1999; Teng et al., 1999) and following chemical injury to the brain (Nozaki et al., 1993; Kirschner et al., 1995). The mechanism of action possibly involves inhibition of apoptosis as FGF2 has been shown
to prevent caspase-3 activation in neuronal cells *in vitro* (Miho et al., 1999). FGF2 has many important roles during development that may help explain its beneficial effects following injury. Neuronal proliferation and differentiation are controlled, at least in part, by FGF2 signalling (Qian et al., 1997; Alvarez et al., 1998). FGF2 is not essential for embryogenesis however, transgenic mice lacking FGF2 are viable, although they possess neurological defects (Dono et al., 1998), and repair wounds to the skin less effectively (Ortega et al., 1998). Evidence from urodele amphibians also indicates that local increases in FGF2 levels are a vital component of effective spinal cord repair processes, possibly by initiating proliferation of neural progenitors from ependymal cells (Zhang et al., 2000).

The effects of FGF2 however, may not always be beneficial; FGF2 is a key mediator of glial responses following spinal trauma that can have negative influences on outcome. FGF2 upregulation is detectable in young rats following CNS injury at greater levels after the developmental onset of a scarring response (Smith et al., 2001). Furthermore, application of FGF2 to rat brain can directly trigger gliotic responses, potentially contributing to the inhibitory environment of the CNS for axonal re-growth (Eclancher et al., 1996).

### 1.4.1 Specificity of growth factor requirements

An important aspect of neurotrophin-mediated reactions following spinal cord injury is the neuron specificity of each growth factor. Individual factors may promote re-growth of some neuronal pathways and not others. For example, NT-3 treatment can promote reticulospinal and rubrospinal axon growth, but does not affect raphe-spinal, or vestibulospinal tracts (Ye & Houle 1997).

Considering this, it is likely that any future therapeutic intervention would require the application of a number of factors to ensure re-growth of as many spinal tracts as possible. The temporal requirements of treatment must also be considered. Certain
factors may be required for initial survival of neurons, but may not be best suited for the promotion of neurite extension.

1.5 THE GLIAL SCAR

Histological observations made by Windle in the 1950s following experimental spinal cord injuries indicated that astrocytes form a dense cellular meshwork that effectively seals the site of spinal injury from normal tissues (Windle and Chambers, 1950; Windle et al., 1952). This led to the hypothesis that such glial scars formed a physical barrier to axonal growth, preventing regeneration in the CNS. At the time these observations were made, astrocytes were thought to be largely inert support cells; indeed the term neuroglia is translated literally as ‘nerve-glue’. In recent years however there has been a rapid surge in understanding of the multiple properties and functions of glial cells in vivo. The inhibitory qualities of glial scars have been much more fully defined and have been shown to include chemical, as well as physical barriers to axonal growth.

1.5.1 Formation of the glial scar following injury

Following CNS damage there is a well-documented glial response. Astrocytes around the injured zone become ‘activated’, increasing their expression of glial fibrillary acidic protein (GFAP) and becoming hypertropic. Proliferation and migration of glial populations have also been reported following injury to the CNS (Kernie et al., 2001). The ‘mature’ glial scar is comprised of astrocytes, a web of associated extracellular matrix (ECM), and other cell types including fibroblasts, macrophages and monocytes. Collagen molecules are also deposited forming an ectopic basal lamina. This ECM structure is found normally in the spinal cord only in the vasculature and pial membranes. It is believed to act as a barrier to regeneration in the context of the glial scar (Feringa et al., 1980). Treatment with anti-collagen IV antibodies, or the iron chelator α-α-dipyridyl, prevented basement membrane formation and improved
regeneration (Stichel et al., 1999a; Stichel et al., 1999b). However, other researchers have reported negative results using similar protocols (Weidner et al., 1999).

The principal mechanisms behind glial scarring mediated axonal growth inhibition are the production of various inhibitory molecules rather than the structural composition of the scar itself. Several potent inhibitors of axonal growth have been identified as being synthesised by reactive astrocytes.

### 1.5.2 Production of inhibitory molecules by astrocytes

The most extensively studied, and perhaps most important of the inhibitory molecules synthesised by astrocytes are the chondroitin sulphate proteoglycans (CSPGs).

CSPGs are a vast family of molecules composed of a central core protein, with multiple glycosaminoglycan chains covalently attached. Crucial during embryonic development, these molecules were also found to have neurite inhibiting properties in vitro. Rapid upregulation of CSPGs has been observed following CNS injury (Lemons et al., 1999).

Much of the observed expression of CSPGs in the CNS is associated with the glial scar and specifically with astrocytes (Snow et al., 1990; McKeon et al., 1995; McKeon et al., 1991; Thon et al., 2000; McKeon et al., 1999), although additional cell types can contribute to overall CSPG levels, including inflammatory cells (Jones et al., 2002).

Recent evidence has suggested that CSPG expression following CNS injury may be one of the most crucial inhibitory influences affecting re-growing axons. Davies and colleagues (1997) carefully transplanted DRG neurons onto sections of rat brain. They found that axonal extension could occur across normally non-permissive tissues including white matter tracts. Furthermore, in instances when axonal regeneration was impeded, a synchronous elevation in local CSPG was detected. This is in keeping with
other studies in which nerve growth has been promoted over adult CNS tissue (Pettigrew et al., 2001; Pettigrew and Crutcher, 1999). Although these studies utilise transplanted DRG cells which are likely to have elevated expression of RAGs due to axotomy, and thus could be expected to regenerate to a greater extent than naïve neurons, the point that normal white matter is more permissive for regeneration than injured is clear (Davies et al., 1999). The fact that CSPGs are upregulated following spinal cord injury is also well established (Lemons et al., 1999; Niederost et al., 1999; Fitch and Silver, 1997a; Pindzola et al., 1993). Crucially, ablation of CSPGs by specific enzymatic degradation has been shown to enhance the regenerative potential of the spinal cord on sections (Zuo et al., 1998b), and in vivo (Bradbury et al., 2002).

In addition to CSPGs, other inhibitory signalling molecules have been demonstrated to be upregulated following spinal injury; these include members of the ephrin and semaphorin families (Pasterkamp et al., 1999); (Miranda et al., 1999).

The physiological reasons behind the production of inhibitory proteins around sites of injury are not clear. It has been speculated that they may function to prevent inappropriate new axonal connections, modulate growth factor responses, alter cellular migrations, or may simply aid in the sealing off of damaged regions from normal tissues (Fitch and Silver, 1999).

### 1.6 OLFACTORY ENSHEATHING GLIA

Despite the seemingly adverse influence of astrocyte populations following spinal cord trauma, it is not the case that all glial cells have an inhibitory effect on regenerating axons. Perhaps the most remarkable demonstration of functional recovery following spinal injury is that seen after transplantation of olfactory ensheathing glia (OEG). These cells are found in the olfactory bulb, one of the very limited regions of the adult CNS that retain the ability to regenerate throughout adulthood. Olfactory neurons are
constantly replaced throughout life, new projections are continually being made from the PNS to central neuronal targets. Transection of the olfactory nerve is also effectively repaired (Graziadei and Monti Graziadei, 1980). The reason why these neurons are able to regenerate and grow upon apparently inhibitory substrates is not due to any particular properties of the neurons themselves, rather it is the unique nature of the olfactory glial population that allows axonal extension. The OEG surround invading axons and act to shield them from the inhibitory environment of the CNS. OEG also express axon-growth promoting molecules on their cell surface.

OEG have shown to be effective when transplanted from the olfactory bulb to the site of nerve injury. Addition of OEG to transected dorsal root entry zones allows DRG axons to re-enter the spinal cord and project to appropriate laminae, a process normally prevented by the inhibitory nature of the CNS (Ramon-Cueto and Nieto-Sampedro, 1994).

Application of OEG has also been shown to help overcome one of the drawbacks of peripheral tissue ‘bridges’ being used to join opposed stumps of transected spinal cord. Although axons can enter and extend though these bridges, they rarely leave them to re-enter the spinal cord due to inhibition caused by glial scarring and the CNS environment. However, when OEG were injected into the opposed stumps of the spinal cord at the time of peripheral bridge insertion, axons were seen to leave the PNS tissue and extend for distances of up to 1.5 cm through the caudal spinal cord. When injected in this way, OEG spread throughout the immediate spinal tissue and promoted axonal growth through areas of glial scarring, as detected by GFAP immunostaining, in addition to white and grey matter (Ramon-Cueto et al., 1998).

The success of these experiments led to the effectiveness of OEG alone being evaluated following experimental spinal cord transection. Again, purified OEG were injected into the stumps of transected rat spinal cord, although no peripheral tissue bridge was inserted. Rats receiving OEG transplants displayed remarkable functional
recovery, regaining locomotor and sensory functions in the hindlimbs. Anatomically, treated spinal cord showed visible signs of repair, and histological analysis revealed extensive regeneration of cortico-spinal, raphe-spinal, and coerulo-spinal tracts into the caudal spinal cord stump (Ramon-Cueto et al., 2000)

Despite the relatively poorly developed olfactory system in man, an equivalent OEG cell has been identified from human patients with similar properties to the rat OEGs described above (Barnett et al., 2000). It seems likely that application of purified human populations of these cells could form part of future spinal cord injury treatment.

1.7 THE INHIBITORY INFLUENCE OF MYELIN IN THE ADULT CNS

1.7.1 Identification of myelin as an inhibitory factor

One immediate question raised following the successes of PNS grafting (David and Aguayo, 1981) was concerning the precise components of the CNS responsible for preventing axonal extension. A key discovery was made when it was noted that proteins associated with CNS myelin were highly inhibitory for neurite extension. Caroni and Schwab (1988) analysed components of myelin present in central, but not peripheral nerve fibres using an *in vitro* cell-spreading assay. By separating the myelin proteins by size, and assaying each fraction for inhibitory activity, Two membrane fractions of 250 and 35 kDa (Neurite Inhibitor (NI)-250 & NI-35) were identified which were responsible for the inhibitory properties of myelin in the adult rat. Furthermore, a monoclonal antibody, IN-1, raised against the NI-250 membrane fraction was shown to be effective at neutralising its inhibitory effects: pre-treatment of myelin, or of cultured oligodendrocytes with IN-1 improved permissiveness in each case (Caroni and Schwab, 1988). The same researchers went on to demonstrate that the non-permissive qualities associated with oligodendrocytes were limited to mature cells: immature
oligodendrocytes were seen to make cell contacts with neuronal cells and neurites in a culture system, whereas mature oligodendrocytes actively repelled the same cell types (Schwab and Caroni, 1988). This supported earlier work by other groups using the optic nerve as a substrate for axonal outgrowth; immature optic nerve supported retinal ganglion cell axonal extension to a greater extent than adult fibres (Shewan et al., 1995).

Further research revealed that the onset of IN-1 specific inhibitory activity in the developing rat spinal cord occurred after the period of axonal extension, but before the onset of myelin formation, a timeframe that the authors conclude is typical of an oligodendrocyte-specific localisation (Caroni and Schwab, 1989). Further experiments in which tissue sections of adult spinal cord and brain were used as substrate matter for growth of axons in culture provided more evidence for an oligodendrocyte-specific localisation of the CNS’s non-permissive nature. The axons failed to grow on tissue sections of white matter, whilst they adhered and extended neurites on areas of grey matter and sciatic nerve. The addition of IN-1 to the culture system improved the permissiveness of the white matter, supporting the notion that the 250 and 35 kDa myelin protein fractions were responsible for the observed effects (Savio and Schwab, 1989). Immunohistochemical analysis of IN-1 on frozen sections of adult rat CNS also provided evidence that NI-250 was restricted to a mature oligodendrocytic localisation (Rubin et al., 1994). Importantly, it was demonstrated that human CNS myelin also prevents axonal extension, and that a high molecular weight protein, analogous to NI-250, and sensitive to IN-1 was responsible (Spillmann et al., 1997).

Using time-lapse video recording to observe the interactions between growing axons and oligodendrocytes in vitro, Bandtlow et al. (1990) confirmed that cellular contact was required for the prevention of neurite extension. The morphology of oligodendrocyte-mediated inhibition was also observed; the growth cones of the axons were seen to rapidly stop growing, and to collapse in a third of cases. Again, IN-1
administration was sufficient to prevent the inhibitory effects of the mature oligodendrocytes.

Further evidence implicating oligodendrocytes in contributing to the establishment of a non-permissive CNS environment comes from experiments conducted using the embryonic chick. As described above, the embryonic chick can effectively repair damage to the spinal cord, resulting in functional recovery, providing the injury occurs prior to E13 (Shimizu et al., 1990; Hasan et al., 1991; Hasan et al., 1993). This transitional period coincides with the onset of myelination in the chick spinal cord. Furthermore, by injecting antibodies raised against galactocerebroside (GalC) and complement from E9 - E12, the process of myelination is delayed in the chick spinal cord, and the transition from permissive to restrictive states does not occur until E15 (Keirstead et al., 1992). Similar results were obtained in post-hatching chicks following spinal injury and subsequent disruption of myelin structure within the spinal cord. Injection of antibodies raised against GalC, or O4, along with injection of serum complement proteins resulted in unravelling of myelin sheaths, a process that was reversible following cessation of treatment. After 2-4 weeks of recovery, 9-14% of descending brainstem-spinal connections were seen to have regenerated across the lesion site, as detected by double retrograde labelling techniques. No voluntary locomotion was recovered in any treated chick, although electrophysiological analysis indicated that some connections had been re-established (Keirstead et al., 1995). A similar strategy achieved comparable results in later experiments performed on the adult rat, promoting the idea that general myelin disruption may promote enhanced axonal regeneration following spinal injury in all vertebrate systems (Dyer et al., 1998).

This experiment supports the notion that oligodendrocytes, or myelin itself, play a critical role in preventing axonal regeneration following spinal injury.

Experiments in other models of CNS regeneration also provide evidence for the fundamental role of inhibitory myelin proteins in preventing axonal growth. Goldfish are
capable of CNS regeneration. However, when retinal cells from goldfish were cultured in the presence of mammalian CNS myelin, axonal extension was inhibited and growth cone collapse occurred in an IN-1 sensitive manner. The same retinal cells can grow freely on fish myelin, suggesting that an absence of NI-250/35 proteins in the fish CNS may account for its regenerative capability (Bastmeyer et al., 1991)

Perhaps the most crucial development in the field of IN-1 related prevention of axonal inhibition was the demonstration that it was also effective in vivo. This was first shown by implanting IN-1 producing tumour cells into the CNS of young rats, then performing spinal cord transection. In treated animals, massive sprouting was seen at the lesion site, and elongation of axons was observed up to 11 mm caudal to the site of injury. Control animals managed only around 1 mm regeneration (Schnell and Schwab, 1990; Schwab and Brosamle, 1997). IN-1 treatment following experimental lesion of the pyramidal tracts in adult rats resulted in recovery of forearm grasping functions, as compared to controls (Z'Graggen et al., 1998).

The timing of IN-1 treatment following injury is an important factor in determining outcome. It had been shown in adult rats that delay of IN-1 administration for 8 weeks greatly reduces its effectiveness in promoting regeneration. However, the same study concluded that shorter delays, of up to a few weeks, may not prevent beneficial effects following IN-1 treatment (von Meyenburg et al., 1998).

1.7.2 The Cloning of Nogo

The identification and purification of the bovine equivalent of the rat NI-250/35 inhibitory myelin proteins provided a breakthrough in the field. The protein, of 220 kDa, was shown to be similarly effective at inhibiting neurite outgrowth and cell spreading in vitro, and was also sensitive to the effects of IN-1. The relatively large quantities of protein obtained allowed peptide microsequencing analysis to be performed, and six short peptide sequences were published (Spillmann et al., 1998). This provided a
starting point for the cloning of the gene encoding N1-220/250, and two years later the
gene sequence was simultaneously published by three separate laboratories (Chen et
al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). The new gene, named Nogo,
encodes 3 protein products (Nogo-A, B, & C) via alternative splicing (Figure 1.8). The
largest splice form, Nogo-A, encodes the N1-220/250 protein that is recognised by the
IN-1 antibody. All isoforms contain two large hydrophobic domains, believed to be
membrane-spanning regions, although the precise topology of the molecule within the
membrane remains a contentious issue (Figure 1.9): the transmembrane domains are
sufficiently large (~50 amino acids) to allow multiple passes through the lipid bilayer. It
has yet to be conclusively established whether the large N-terminal domain, present
only in Nogo-A, is located intra-, or extra-cellularly. One striking feature of the Nogo-A
molecule is that it possesses more than one biologically active neurite-inhibiting
domain. The Nogo-66 domain, situated between the two transmembrane regions, is
common to all Nogo isoforms and has been shown to be located extracellularly, as
demonstrated by immunostaining on live, non-permeabilised cells. In the same study,
COS-7 cells expressing myc-tagged Nogo, either at the N, or C terminus, displayed no
cell surface myc-immunoreactivity, suggesting that both ends of the protein are found
in the cytosol (GrandPre et al., 2000), although this has been contested by other
laboratories (Martin Schwab, personal communication). The C-terminal domain
containing the transmembrane regions, and the Nogo-66 segment, displays 70%
homology with a group of proteins known as the reticulons (GrandPre et al., 2000).
This family has related sequences in the worm genome, suggesting it is well conserved
through evolution (Iwahashi et al., 2002). Analysis of other members of the reticulon
family does not shed much light on potential functions for the Nogo proteins. So far,
reticulon proteins have been proposed as markers for certain lung cancers (Senden et
al., 1997), and Reticulon-1 expression is known to be regulated by ethanol (Schafer et
al., 2001; Schafer et al., 1998). Each member of the family, including all Nogo isoforms,
possesses an endoplasmic reticulum retention motif, and indeed immunostaining with
anti-Nogo antibodies on permeabilised Nogo-transfected Cos cells suggested an ER-
like localisation. The function of the reticulon proteins remains elusive, although only the Nogo proteins have neurite-inhibiting properties in vitro (GrandPre et al., 2000).
Figure 1.8 Schematic representation of the 3 Nogo isoforms. Location of the common Nogo-66 domain is indicated, as is the region responsible for promoting apoptosis in tumours (Li et al. 2001). The approximate location of regions recognised by antibodies used in this study are indicated.

Figure 1.9 Proposed topologies of the Nogo-A molecule. The precise orientation of Nogo-A is a contentious and important issue. A. The model proposed by Fournier et al. (2001). Only the Nogo-66 domain is extracellularly located. B. Alternative Nogo-A topology: the amino-domain is also extracellularly located, permitting physiological interaction with other cells in a non-injured environment. C. Intracellular topology for Nogo-A: the presence of an ER-retention motif may mean than neither of the active domains is present on the cell surface.
The N-terminal domain, present in Nogo-A and partially in Nogo-B is unique and displays no similarities to other protein motifs. The region can act as a soluble molecule and can inhibit neurite outgrowth to a greater extent than Nogo-66 (Prinjha et al., 2000). Antibodies raised against Nogo-A specific peptides (AS-472) inhibit the effects of Nogo on neurite outgrowth and label mature oligodendrocytes in a manner identical to IN-1. In addition, injection of AS-472 into dissected rat optic nerve allows DRG neurons to enter and extend processes for long distances; pre-immune serum had no effect (Chen et al., 2000).

1.7.3 A Nogo Receptor

The mechanisms through which the Nogo-A specific domain of Nogo exerts its effects remain largely unknown. The structure of Nogo-A is not similar to any other known inhibitory factor, such as the ephrins or semaphorins, nor does it contain any obvious domain characteristic of a guidance molecule, such as Ig, or EGF domains.

There are some clues however regarding the probable mode of function of the Nogo-66 domain. This region is known to act via Nogo Receptor (NgR). This receptor molecule is specific to Nogo-66 and does not interact with the Nogo-A specific domain. NgR is brain-specific and its expression is sufficient to render cells sensitive to the inhibitory effects of Nogo (Fournier et al., 2001). NgR acts to inhibit neurite outgrowth: it does not affect non-neuronal cell morphology, which seems to be a Nogo-A specific effect.

NgR is brain specific in the adult mouse. The protein is 473 amino acids in length and contains eight leucine-rich repeat domains and a glycosylphosphatidylinositol (GPI) anchorage site (Figure 1.10). Much of the structure of NgR is unique and is shows no similarity to other mediators of axonal repulsion or their receptors (Fournier et al., 2001). Very recent data has confirmed that blocking NgR using a short peptide
sequence promoted enhanced axonal regeneration following spinal injury *in vivo* (GrandPre et al., 2000).

It should be remembered that NgR, in acting via Nogo-66, is reactive with all three Nogo isoforms. This implies that the function of the receptor goes further than the process of neurite inhibition. Nogo-B and Nogo-C affect neurites to a lesser extent than Nogo-A, if at all *in vivo*. The expression of Nogo proteins has also been detected outside the nervous system, although a comprehensive expression analysis has not been performed. Could Nogo-NgR interactions be performing different functions in different tissues? This question remains to be answered, as expression analysis of NgR has yet to be investigated, as does an examination of possible downstream targets of the receptor.

The amino-terminal Nogo-A specific domain however operates by an unknown mechanism. It is highly probable that a second Nogo-receptor remains to be discovered. There are problems however with the concept of Nogo receptors: for them to be of physiological use, they would require Nogo proteins to be expressed extracellularly. This remains a contentious issue. It seems apparent, that due to ER-retention domains, the majority of Nogo within a given cell is located at the ER (GrandPre et al., 2000). Very little Nogo is present at the plasma membrane, and evidence exists to suggest that even when located at the cell surface, most of the molecule is in the cytosol, with the exception of the Nogo-66 domain. It is difficult to imagine how a receptor could interact with the Nogo-A specific region in this location. One theory is that Nogo-A only acts to inhibit neurite outgrowth following damage to oligodendrocytes, and its subsequent release to the extracellular environment. Other groups favour a topology in which the N-terminal region is present extracellularly, where it acts to prevent inappropriate arborisation of nerve fibres in adulthood.
Figure 1.10 The Nogo-66 receptor
LRR- leucine rich repeat domains; LRRCT- leucine rich repeat C-terminal domain; TM/GPI –transmembrane/glycosylphosphatidylinositol linkage (from Fournier et al. 2001)
1.7.4 Nogo function?

The physiological function of the Nogo proteins remains unknown. Application of anti-nogo antibodies to uninjured CNS results in axonal sprouting, suggesting that normal function may be to prevent this after completion of neuronal circuits during development (Buffo et al., 2000). Inhibition of these restrictions may explain the elevated sprouting observed after anti-Nogo antibody treatment following experimental CNS lesion (Raineteau et al., 1999; Wenk et al., 1999; Raineteau et al., 2001). Little research has been performed on Nogo-B, or Nogo-C, although Nogo-B has been identified as a pro-apoptotic gene in human tumours (Li et al., 2001).

1.7.5 Myelin Associated Glycoprotein (MAG)

The Nogo proteins are not the only inhibitory factors known to be present in CNS myelin. MAG has also been shown in vitro to be a potent inhibitor of neurite outgrowth. Unlike Nogo however, the expression of MAG is not restricted to the myelin of the CNS; it is also to be found in the peripheral nervous system. The presence of MAG in the periphery and its role as an inhibitor of axonal regeneration are not entirely contradictory. It is well known that myelin clearance is a crucial step in the effective repair pathway present in the PNS.

MAG is thought to play a vital role in the process of myelination itself and is one of the first myelin-specific proteins to be expressed during development. It was also the first identified molecule present in myelin to inhibit neurite outgrowth: it was demonstrated to prevent neurite extension from cultured DRG neurons, an effect reversible by administration of monoclonal anti-MAG antibody (Mukhopadhyay et al., 1994). Interestingly, MAG has also been demonstrated to act as a soluble molecule in a dose-dependent fashion to prevent axonal extension, a mechanism that may be of significance following myelin damage upon spinal injury (Tang et al., 1997)
Some groups have cast doubt upon the significance of MAG in abortive regeneration. Using a MAG-deficient mouse, it was shown that MAG-/- myelin extracts were equally poor at allowing axonal extension as wild type myelin. Similarly, experimental lesions in optic nerve or corticospinal tract also failed to regenerate to any greater extent than controls (Bartsch et al., 1995). These data are in apparent contradiction with later studies in which MAG-/- myelin was shown to extensively enhance axonal extension in cultured adult DRG neurons in comparison to wild type myelin controls. Moreover, a differential effect was observed, dependent on the developmental stage of neuron examined. MAG was seen to inhibit mature axonal growth, but promote neonatal neurite extension. Myelin from knockout animals had the opposite effect; it enhanced growth from adult, and inhibited growth from neonatal cells (Shen et al., 1998).

Such developmentally regulated responses to neurite inhibitors are not unique to MAG. Cellular responses to Nogo are also dependent on the maturity of the neurons involved (Bandtlow and Loschinger, 1997) This study demonstrated that neuronal response to contact with Nogo differed between immature and mature neurons: immature cells experienced a transient elevation in intracellular calcium, whilst mature neurons had a more sustained response, resulting in growth cone collapse.

Such observations of differential responses to environmental cues offer a potential new approach to the problem of overcoming such obstacles following spinal injury. Rather than alter the extracellular environment, might it be possible to alter the nature of the neurons, rendering them capable of ‘ignoring’ negative environmental cues? Fundamental to such an approach is the identification of the molecular reasons underlying neuronal susceptibility to the inhibitory influences of myelin. Components of the molecular pathway conducting the inhibitory signal from environmental myelin cues are beginning to be unravelled. The phosphatase enzyme NERPP-2C has been shown to act downstream of such inhibitory stimuli. The enzyme is only detectable in mature
neurons, and blockade of activity by antisense oligonucleotides can allow axons to overcome normally inhibitory myelin growth substrates (Labes et al., 1998).

As described earlier, expression of RAGs can mediate neuronal responses to environmental cues, such as myelin proteins. It was recently shown that cellular levels of cyclic AMP play a major role in establishing whether a cell retracts upon encountering an inhibitory stimulus. Immature neurons were noted to contain higher levels of cAMP than mature cells: also, by experimentally raising the levels of cAMP in mature neurons, they became less sensitive to the effects of myelin and could grow over previously inhibitory substrates, such as myelin (Cai et al., 2001).

1.8 PROSPECTS FOR THERAPY

The surge in our understanding of the processes through which regeneration occurs, and does not occur in the context of the spinal cord has greatly elevated levels of optimism in the field. Progress has been made, and it seems likely that therapeutic tools can be developed in time. One thing seems clear however, that no single factor is likely to provide a complete solution. Rather, it is probable that multiple strategies will be simultaneously adopted. One can envisage that in the near future, spinal cord trauma will be treated rapidly with new agents designed to minimise secondary injury processes. These may modulate inflammation within the CNS, help reduce levels of free radicals, or block the effects of excitotoxic neurotransmitters. Such strategies would reduce the number of patients developing serious debilitating paralysis and loss of sensory function. In addition to prevention of secondary damage, it is likely that regeneration of severed axonal tracts will also be a therapeutic aim. Administration of growth factors, or drugs designed to alter endogenous growth factor expression may improve neural survival at the site of injury, and could also prevent neuronal cell death or atrophy at distant locations in the brain. Additional pharmacological methods of enhancing RAGs within neurons may be developed, increasing the potential of cells to
grow new processes. The inhibitory effects of myelin proteins could additionally be overcome by administration of antibodies, or specific blocking peptides. Degradation of CSPGs would also improve the overall permissiveness of the CNS for axonal regeneration. These strategies could potentially benefit those with chronic injuries, perhaps in combination with transplantation of foetal or stem cell grafts, or of administration of olfactory ensheathing glia.

The problems presented by spinal cord injury can be addressed from a number of directions. It is probable that research in the near future will result in at least some of these strategies reaching clinical use. Given that significant gains in mobility, sensation, and in quality of life can be gained from modest survival or regeneration of a few axonal tracts, it seems certain that those with spinal injuries can expect a brighter future.

1.9 EXPERIMENTAL STRATEGY

The overall aim of this study was to investigate the developmental transition from permissive to restrictive regenerative states in the embryonic chick spinal cord. This useful model permits analysis of regenerating and non-regenerating systems within the same species. The mechanisms responsible for the switch from regeneration competent to incompetent states that occurs around E13 has not been fully characterised however, especially with respect to recent advances in the understanding of processes and molecules involved in spinal injury.

Firstly, the morphological response of the embryonic chick spinal cord to experimental injury was examined in greater detail than previous studies (Chapter 4). Would fundamental differences exist in the immediate response of the permissive and restrictive state spinal cord to trauma? This was investigated histologically and by using TUNEL analysis.
Next, molecular differences between the permissive and restrictive states were examined in more detail. Could changes in neural proliferation or differentiation account for loss of regenerative potential? Other prospective factors were also investigated, addressing the role of growth factors, inhibitors of axonal extension, and mediators of secondary tissue damage mechanisms, to the change in spinal cord repair efficiency seen at E13. This analysis involved use of immunohistochemical techniques, in addition to RT-PCR, and computer-based image analysis.

A potentially novel method of manipulating growth factor expression in the spinal cord was examined in Chapter 6. A pharmacological means of enhancing FGF2 levels in the spinal cord was attempted for the first time in the chick. Would this approach enable non-regenerating stages of chick development to improve prognosis following spinal injury? And what does this tell us about the role of growth factors in the short-term response to neural trauma? The effect of drug treatment on FGF2 levels was explored using semi-quantitative RT-PCR, subsequent assessment of spinal injuries was performed by histological and TUNEL analysis.

Finally, the chick model of regeneration was used to investigate the myelin-associated inhibitor of axonal extension Nogo-A, and its receptor NgR. These molecules are currently attracting much attention in the field of spinal cord injury research, but have yet to be examined in the chick system. Could onset of Nogo-A, or NgR, expression during chick development define the beginning of the restrictive period for regeneration? Or would Nogo-A be down regulated following spinal injury during permissive periods? It was also hoped that analysis of Nogo expression in an embryonic system might provide clues regarding the unknown physiological function of the molecule. Given the exciting nature of the Nogo proteins and the current explosion of international interest following recent publications, experiments examining Nogo and NgR were extended to also include embryonic human samples. This analysis involved
use of immunohistochemistry, Western Blotting, RT-PCR, gene sequencing, and cell culture techniques.
CHAPTER 2. MATERIALS
2.1 General reagents

Unless otherwise specified the reagents used were obtained from Sigma-Aldrich (Poole, UK)

2.2 Preparation of solutions

2.2.1 Phosphate Buffered Saline (PBS)

PBS was prepared using 1 PBS tablet from Oxoid (Basingstoke, UK) per 100ml distilled water, resulting in a final concentration of 0.16 M NaCl, 0.003 M KCl, 0.008 M Na$_2$HPO$_4$, 0.001 M KH$_2$PO$_4$. PBS-TWEEN was prepared using 5 ml TWEEN-20 per litre PBS.

2.2.2 Paraformaldehyde (PFA)

PFA was prepared as a 4% solution by dissolving 16 g PFA in 400 ml PBS. The mixture was heated in a 65°C water bath until clear, before being transferred to ice and stored in 20 ml aliquots at -20°C.

2.2.3 Lysis buffer for Immunoblotting (RIPA)

100 ml RIPA (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate (DOC), 0.1% Sodium dodecyl sulphate (SDS), 50 mM Tris (ph 8.0) was prepared by mixing:

- 5 ml 3M NaCl
- 1 ml NP-40
- 0.5 g DOC
- 1 ml 10% SDS
2 ml 2.5M Tris (pH 8.0)

91 ml Distilled water

Protease Inhibitor Cocktail (P8340-Sigma) was added freshly before use at a dilution of 1 in 100.

2.2.4 Electrophoresis Sample Buffer (for Western Blot)

5 ml of buffer was prepared as follows:

- Glycerol 1 ml
- β-mercaptoethanol 0.5 ml
- 10% SDS 3.0 ml
- 1M Tris-HCl (pH 6.8) 1.25 ml
- Bromophenol Blue 2 mg

Storage: -20°C in 50μl aliquots.

2.2.5 5x Gel Running Buffer

600 ml buffer was made by adding:

- 9.0 g Tris Base
- 43.2 g Glycine
- 3.0 g SDS

to an empty measuring cylinder and making the volume up to 600 ml using distilled water. The solution was diluted 5-fold prior to use.
2.2.6 Protein Transfer Buffer

One litre of transfer buffer contained the following components:

- 5.8 g Tris Base
- 2.9 g Glycine
- 2.75 ml 10% SDS
- 200 ml Methanol
- to 800 ml Distilled water

2.2.7 TBE buffer

10X TBE Buffer was prepared as follows:

- 108 g Tris base
- 55 g Boric Acid
- 9.3 g EDTA

add Distilled water to 1000ml

2.2.8 Acid Alcohol

100 ml of Acid Alcohol fixative contained:

- 95 ml Ethanol
- 5 ml Acetic Acid

The solution was stored at -20°C until required.
2.2.9 Ponceau Red Solution

20% Ponceau S was dissolved in 30% trichloroacetic acid, 30% sulfosalicylic acid.

2.2.10 Citric Acid Buffer

500 ml 0.01M Citric Acid Buffer was prepared as follows:

2.1 g Citric Acid

500 ml dH₂O

pH to 6.0 with NaOH

2.2.11 Diaminobenzidine (DAB)

Sigma FAST™ 3,3' Diaminobenzidine tablets were dissolved in 5 ml distilled water with shaking. The solution was then filtered using a 0.4 µm sterile filter (Millipore) to remove insoluble particles prior to use.

2.2.12 Methyl Green Solution

500 ml of Methyl Green staining solution was prepared as follows:

4.1 g Sodium Acetate (0.1M)

Add 500 ml distilled water

pH to 4 using Acetic Acid

Add 2.5 g Methyl Green (0.5 %)

The resulting solution was allowed to dissolve with magnetic stirring for 20 minutes, then filtered prior to use.
2.2.13 Hoechst Dye

Staining of cell nuclei was performed using Hoechst 33258 at 1.2 mg/ml dilution in water. This stock was further diluted 1 in 500 prior to use.

2.3 Source of chicken tissues

Fertilised white leghorn chicken eggs were provided by J.K. Needle and Co, Polyndon Farm, Herts, UK. Eggs were incubated in a humidified forced draft incubator at 37.5°C (Curfew Ltd., Essex, UK).

2.4 Source of human tissues

Human embryonic tissue was obtained via the MRC and Wellcome Trust funded Human Developmental Biology Resource. Materials were acquired with consent and ethical approval from recent terminations. Pre-embedded paraformaldehyde fixed tissue sections of human material were also obtained from the same source.

2.5 Anaesthetic & drug treatments

Tricaine (Ethyl 3-aminobenzoate methanesulfonate salt) and diazepam (7-Chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one) were obtained from Sigma, UK.

2.6 Primary antibodies

Primary antibodies raised against the proteins listed below were used in this study. The dilution of antibodies used in immunohistochemistry or Western blotting is indicated.
2.6.1 CS-56

CS-56 is a monoclonal IgM antibody raised against ventral membranes of chicken gizzard fibroblasts: It reacts specifically with Chondroitin Sulphate Proteoglycans, and was obtained as an ascites fluid from Sigma and used at a 1 in 40 dilution for immunohistochemistry.

2.6.2 GFAP

This antibody, supplied by DAKO, was raised against GFAP isolated from bovine spinal cord using a rabbit host. The purified IgG fraction reacts with GFAP from a number of species, including human, mouse, rat, sheep, chicken, and kangaroo. It was used at a dilution of 1 in 100 for immunocytochemistry.

2.6.3 H3

Anti-phospho-Histone H3 (Upstate Biotechnology, Buckingham, UK) specifically recognises the phosphorylated 17Kd histone H3 which is found only in mitotic cells. The antibody was raised in the rabbit, using amino-acids 7-20 of the human H3 as immunogen. H3 was used for immunohistochemistry at a dilution of 1 in 100.

2.6.4 Heparan Sulphate (10E4)

This antibody is a purified monoclonal IgM raised against the 10E4 epitope of heparan sulphate. It was isolated from the ascites of BALB/c mice inoculated with 10E4. The reactivity is specific to heparan sulphate and no binding to chondroitin sulphate, dermatan sulphate, or hyaluronan occurs. It was obtained from Seikagaka America and used at a dilution of 1 in 100 for immunohistochemistry.
2.6.5 Neu-N

Neu-N is a monoclonal antibody raised against purified cell nuclei from mouse brain. The affinity purified immunoglobulin was purchased from Chemicon International Inc, Temecula, CA. The antibody reacts specifically with (Neu)ronal (N)uclei of most neuronal cell types, with the exception of Purkinje, mitral, and photoreceptor cells. It labels only post-mitotic cell nuclei. Neu-N was used at 1 in 100 dilution for immunohistochemistry.

2.6.6 RT-97

RT-97 is a monoclonal antibody obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Raised in the mouse using rat high molecular weight neurofilaments as antigen, it was used for immunohistochemistry, and immunocytochemistry at a dilution of 1 in 100. It is known to react with axon-specific neurofilaments of human, chick, and rat origin.

2.6.7 AS-472

Antiserum 472 is a rabbit polyclonal antibody raised against a short peptide sequence specific to the protein Nogo-A. It was a generous gift from the laboratory of Martin Schwab (Institute for Brain Research, Zurich). AS-472 was used at 1 in 2000 dilution for immunohistochemistry and 1 in 3000 for Western blotting.

2.6.8 Nogo-A (D-19)

D-19 is an affinity-purified goat polyclonal antibody raised against a peptide from the internal region of human Nogo-A. It is claimed to be specific to Nogo-A, with no reactivity with other Nogo family members. Obtained from Santa Cruz Biotechnology, it was used for immunohistochemistry and Western Blotting at a dilution of 1 in 100, and 1 in 200, respectively.
**2.6.9 Nogo (N-18)**

N-18 was acquired from Santa Cruz Biotechnology. It is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the amino terminus of human Nogo-A. N-18 reacts with Nogo-A and Nogo-B. It was used for immunohistochemistry at a dilution of 1 in 100 and Western Blot at 1 in 200.

**2.6.10 Nogo Receptor**

Nogo Receptor antibody was a generous gift from the laboratory of Dr. Stephen Strittmatter (Department of Neurology and Section of Neurobiology, Yale University School of Medicine, U.S.). It was prepared by immunising rabbits with a Nogo Receptor- GST fusion protein as described in Fournier et al. (2001). The antibody was used at 1 in 100 dilution for immunohistochemistry.

**2.6.11 Basic Fibroblast Growth Factor (FGF2)**

Anti-FGF2 neutralising polyclonal antibody raised in rabbit using bovine FGF-2 as an immunogen (AB-33-NA; R&D Systems) [50 μg/ml for immunohistochemistry; 2 μg/ml for Western Blotting]. This antibody is highly specific and has been shown not to cross-react with a wide range of other growth factors (Bandtlow et al., 1993)

**2.6.12 PCNA**

PCNA (PC10) is a mouse monoclonal IgG<sub>2a</sub> antibody derived by fusion of spleen cells from a BALB/c mouse immunised with recombinant PCNA with Sp2/0-Ag14 myeloma cells (Santa Cruz Biotechnology). It reacts against the PCNA p36 protein expressed at high levels in proliferating cells of human, murine, insect and yeast origin and was used at 1 in 100 dilution for immunohistochemistry.
2.7 Secondary antibodies

The following secondary antibodies were obtained from Jackson Labs (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA):

- Goat anti-rabbit (FITC) – used 1 in 100
- Goat anti-rabbit (TRITC) - used in 50
- Donkey anti-goat (FITC) – used 1 in 100
- Donkey anti-mouse (TRITC) – used 1 in 50
- Donkey anti-mouse IgM (TRITC) - used 1 in 50

The following secondary antibodies were obtained from DAKO:

- Goat anti-rabbit (biotin) – used 1 in 600
- Goat anti-mouse (biotin) – used 1 in 600
- Goat anti-rabbit (HRP) - used 1 in 500
- Goat anti-mouse (HRP) - used 1 in 500
- Rabbit anti-goat (HRP)- used 1 in 600

All the above sera are anti-IgG unless otherwise stated.

2.8 Histological reagents and equipment

Glass staining troughs and racks, storage containers and all histological stains were obtained from BDH. Sections were cut on a Microm HM330 rotary microtome using disposable blades (both from Raymond Lamb, UK) or using a Bright 5040 Cryostat, Model OTF (Bright Instrument Company Ltd, Huntington, UK). Heraeus Instruments
(Essex, UK) supplied the T6030 wax oven. Glass microscope slides, coverslips and paraffin wax, melting point 57-58°C (Lambwax), were obtained from Raymond Lamb. HistoClear was from National Diagnostics (Atlanta, GA, USA). Slides were mounted in dextropropoxyphene (DPX; BDH), or using Citifluor (Citifluor Ltd, UK) for fluorescent mounting. Hydrophobic marker (PAP pen) was from DAKO, UK. A Hinari 900W Microwave oven was used for antigen retrieval when necessary.

2.9 Microscopy

Microdissection and digital imaging of large specimens was carried out under a Zeiss (Carl Zeiss, 7082 Oberkochen, Germany), SV11 stereomicroscope. Brightfield microscopy was performed on a Zeiss Axioplan 2 microscope, using objective lenses of x 2.5, x 5, x 10, x 20, x 40, and x 63 (oil immersion) magnification. Digital images were captured electronically using a Zeiss Kontron ProgRes 3012 digital camera (Imaging Associates Ltd, Thame, Oxon, UK), version 2.0 of the associated software and stored and labelled in Adobe Photoshop v6.0 (Adobe Systems Europe, Edinburgh, UK). Fluorescent images were viewed under Zeiss Axioplan microscope using fluorescent illumination and were digitally scanned using a Hamamatsu digital camera (C4742-95, Hamamatsu Photonics KK, Japan) directly into Openlab software (version 3.03, Improvision Ltd, improvement.com).
2.10 Terminal transferase-mediated dUTP nick end labelling (TUNEL)

Apoptotic cells were detected using an ‘In Situ Cell Death Detection Kit - Fluorescein’ from Roche Molecular Biochemicals, Mannheim, Germany. Catalogue number 1684795.

2.11 Western blotting equipment

Polyacrylamide gels were prepared according to tables 2.1 and 2.2. Autorad apparatus was used to run polyacrylamide gels. Blotting of protein from the gel to membrane was carried out using a Trans-blot SD semi-dry transfer cell (both from Bio-Rad). The enhanced chemiluminescence reagent (ECL) for detecting proteins on membranes was provided by Amersham International, Little Chalfont, Bucks, UK. X-ray film used was Kodak X-OMAT AR from Genetic Research Instrumentation (GRI, Braintree, Essex, UK). Autoradiographic cassettes and intensifying screens were supplied by GRI, Hybond-C Extra nitrocellulose membrane from Amersham and 3MM chromatography paper by Whatman International Ltd (Maidstone, Kent, UK). Blotto was from Santa Cruz.
### Table 2.1 Components of 5% Stacking gel for protein separation. Total volume 8 ml

<table>
<thead>
<tr>
<th>Solution Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide Mix (ml)</td>
<td>1.3</td>
</tr>
<tr>
<td>H$_2$O (ml)</td>
<td>5.5</td>
</tr>
<tr>
<td>1.5M Tris (pH8.8) (ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>10% SDS (μl)</td>
<td>80</td>
</tr>
<tr>
<td>10% Ammonium Persulphate (μl)</td>
<td>80</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>8</td>
</tr>
</tbody>
</table>

### Table 2.2 Components for preparation of resolving gels for protein separation (15ml)

<table>
<thead>
<tr>
<th>Solution Components</th>
<th>6%</th>
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<th>10%</th>
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<td>30% Acrylamide Mix (ml)</td>
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<tr>
<td>H$_2$O (ml)</td>
<td>7.9</td>
<td>6.9</td>
<td>5.9</td>
<td>4.9</td>
</tr>
<tr>
<td>1.5M Tris (pH8.8) (ml)</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>10% SDS (μl)</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>10% Ammonium Persulphate (μl)</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>
2.12 Reverse transcriptase polymerase chain reaction (RT-PCR)

TRI REAGENT was used for RNA isolation. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), random hexamer primers, Recombinant RNasin Ribonucleotide Inhibitor, Taq polymerase and Deoxynucleotide Triphosphates (dNTPs) were obtained from Promega. Oligonucleotides were designed manually and custom synthesised by Genosys (Cambridge, UK). Primer sequences are given in table 2.3. A Programmable Thermal Cycler (MJ Research Inc, PTC-100 Boston, MA, USA) was used throughout. Thermostable PCR tubes were purchased from Elkay Laboratory Supplies Ltd. (Hampshire, UK)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>Expected Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHICK FGF2</td>
<td>AGCGGCTCCTACTGCAAGAAC</td>
<td>CTTCCGTGACCGGTAAGTG</td>
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</tr>
<tr>
<td>CHICK GAPDH</td>
<td>CAGTGAGAAAGTGGAGGTCA</td>
<td>GACACCATCACAACATGG</td>
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<tr>
<td>CHICK NGF</td>
<td>GGACCAAGAGGGACTGCACAT</td>
<td>GATTTCCTGCTGAGCACACA</td>
<td>355</td>
</tr>
<tr>
<td>CHICK Nogo</td>
<td>CGTTCTTTGTCATAGTCCCCA</td>
<td>TTGGAGTCTGATGCTGTG</td>
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</tr>
<tr>
<td>CHICK Nogo-A</td>
<td>AGCTGAAGAAATGCAGCTAGA</td>
<td>GTGCACAGGTTTCAGTCT</td>
<td>839</td>
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<tr>
<td>CHICK QUEK-1</td>
<td>GCCAGCAAGTGGGAGTTTC</td>
<td>CTCCTGAGATATGCCAGAGATTTC</td>
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</tr>
<tr>
<td>HUMAN BETA-ACTIN</td>
<td>TGCTACTCCAGGCTGTCTAT</td>
<td>GATGGAGTTGAGGTAGTTT</td>
<td>543</td>
</tr>
<tr>
<td>HUMAN Nogo RECEPTOR</td>
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<td>TTCTGCACGCCAGGCCCCAGA</td>
<td>717</td>
</tr>
<tr>
<td>HUMAN Nogo-A</td>
<td>GCTCTTTCTGCTGCATCTGAG</td>
<td>TGCTCTGATTTTACCTCCAGC</td>
<td>672</td>
</tr>
</tbody>
</table>

Table 2.3 Primer sequences and expected size of products

2.13 Nucleic acid separation and sequencing

Agarose was supplied by Gibco BRL. Agarose gels were run in the Horizon horizontal gel electrophoresis system (Gibco BRL). Sequencing reactions were performed using
the BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Warrington, UK) and automated sequencer (AB1377 Perkin Elmer).

2.14 Molecular size markers

Rainbow™ markers (RPN 756; Amersham International, Little Chalfont, Bucks, UK) were used to size proteins. 1Kb and 123 base pair ladders were used for sizing DNA and RNA bands (Gibco BRL).

2.15 Centrifuges

Samples were centrifuged in a Heraeus Biofuge 13R (Heraeus instruments, Brentwood, Essex, UK) or a Sigma 113 microfuge (Sigma, Osterode, Germany).

2.16 Tissue culture media and equipment

Tissue culture grade Petri dishes and other plastics were supplied by Philip Harris (Stone, Staffs, UK) and Nunc Inc (Naperville, IL, USA). Millipore filters were obtained from Millipore Corp (Bedford, MA, USA). DMEM and L15 media were provided by Gibco BRL (Paisley, Scotland). Thirteen mm diameter round glass coverslips were obtained from Raymond Lamb.

2.16.1 Spinal cord culture medium

500 ml of medium for maintaining human spinal cord explants in culture was prepared as follows:

\[
\begin{align*}
360 \text{ ml} & \quad \text{DMEM-Glutamax} \\
40 \text{ ml} & \quad \text{Foetal Calf Serum} \\
4 \text{ ml} & \quad \text{Penicillin/Streptomycin solution}
\end{align*}
\]
Medium was stored at 4°C until needed.

2.17 Sera

Normal goat serum was from DAKO. Fetal calf serum (FCS) was supplied by Sigma.
CHAPTER 3. METHODS
3.1 Egg Maintenance

Fertilised White Leghorn chicken eggs were maintained in a humidified forced flow incubator at 37°C until the required developmental stage. The first day of incubation was considered to be embryonic day (E) 0. Prior to incubation, eggs were stored at 8°C. Eggs were incubated on their sides in cardboard egg racks. This ensured that windowing could be performed on the eggshell directly above the developing embryo.

3.2 Egg Surgery

Chick embryos on which surgery was to be performed were all windowed at E7. It was found that windowing eggs of an older age resulted in poorer survival due to the more extensive network of blood capillaries present in the chorioallantoic membrane at later stages. At E7, a window could be opened without damage to blood vessels. The egg could then be re-opened at a later stage for surgical intervention.

3.2.1 Windowing

Eggs were removed from the incubator and placed on a specially constructed egg-holder. This consisted of a plasticine ring of around 5cm diameter wrapped in silver foil. This helped hold the egg securely in position during manipulations. A small puncture was then made in the eggshell above the air-space, i.e. at the blunt side, using a metal seeker. This had the effect of lowering the level of the albumin and chorioallantoic membrane inside the egg, although the puncture did not itself break any internal membranes. The top surface of the egg was then windowed. This was done by carefully boring a small hole in the shell using blunt serrated forceps without damaging the underlying membranes. This small opening was then used to prise small sections of shell up from the shell membrane. The hole was gradually widened until an area of around 2cm² of shell membrane was exposed. This was then carefully peeled away.
using forceps. When successful, the chorioallantoic membrane remained undamaged by this procedure and could be seen approximately 1 cm below the eggshell, sitting on the lowered albumin. Occasionally, difficulties arose when the chorioallantoic membrane remained stuck to the shell membrane, making atraumatic removal of the shell membrane impossible. It was found in some cases however that gentle tapping of the membrane, or simply waiting for a short time, resulted in the chorioallantoic membrane 'dropping' from the shell membrane, allowing windowing to proceed. Following windowing, eggs were sealed using Sellotape. The seal was made as airtight as possible; this is important to prevent loss of moisture during incubation, and to prevent bacterial infection. Occasionally, more than one piece of tape was necessary to ensure adequate sealing. Eggs were then labelled using a marker pen and returned to the incubator until required.

3.2.2 Surgical Procedures

Spinal cord transection was performed at developmental stages E7, E11, or E15. Eggs were windowed prior to surgery (except in cases of E7 transection), and only chicks that appeared to be developing normally were used for transection. In the case of E15 chicks, anaesthesia was administered prior to surgery by way of tricaine application to the chorioallantoic membrane as used in previous studies (Zemanova et al., 1993). 0.5 ml tricaine was applied diluted in PBS to achieve a final concentration of 0.75 g/kg.

The tape seal was removed from the egg using forceps and a specially constructed glass hook was used to lift the embryo into the window in the shell. The hook was made from a glass pasteur pipette; the opening at the end was sealed using a Bunsen burner, and the terminal end of the pipette was also curved into a hook shape by heating. Hooks of suitable size were selected for each developmental stage of embryo used. In order to hook the neck of the chick embryo, it was necessary to break through the chorioallantoic membrane which contains an intricate network of blood vessels. Early attempts to tear a precise hole in the membrane between vessels using forceps
were not always successful and were time consuming. It was found to be more effective to simply push the blunt, curved part of the glass hook through an avascular point in the membrane. This technique resulted in minimal, or no bleeding and minimised the time required for each operation. The chick was then gently lifted using the glass hook and manipulated in the window of the eggshell, allowing access to the thoracic region of the spine. A pair of sharp forceps were then used to puncture the skin at either side of the dorsal column and perform a crush of the spinal cord. Pressure was maintained with the forceps fully closed for a few seconds, then the forceps were extracted and the chick carefully lowered back into the egg. The egg was then re-sealed as described above and returned to the incubator.

Operated animals remained in the incubator for a maximum of 4 days before sacrificing by decapitation and processing for tissue sectioning as described below.

3.2.3 Animal Numbers

Overall survival from surgical manipulation was low. The number of animals 'n' indicated below represent the number of chicks surviving surgery until the required postoperative timepoint:

**Histological analyses.**

E7 spinal cord injuries: 24 hour time point (n = 5)
4 day time point (n = 3)
sham-operated animal (n = 2)

E11 spinal cord injuries: 24 hour time point (n = 6)
4 day time point (n = 4)
sham-operated (n = 2)

E15 spinal cord injuries: 1 hour time point (n = 2)
24 hour time point (n = 8)
2 days time point (n = 2)
3 days time point (n = 4)
4 days time point (n = 4)

Diazepam experiments:
E10 diazepam treated animals (n = 6)
E10 DMSO treated animals (n = 4)
E10 untreated (n = 5)
E14 diazepam treated animals (n = 4)
E14 DMSO treated animals (n = 3)
E14 untreated controls (n = 4)

Diazepam treatment, followed by spinal cord injury:
E10 animals: 24 hours time point (n = 6)
E10 animals: 4 days time point (n = 2)
E14 animals 24 hours time point (n = 5)

For transverse section analysis, immunostaining was performed on cross sections of thoracic spinal cord from at least 3 different animals. At least 6 sections from each animal were analysed. Representative images are shown.

3.3 Tissue Processing

Much of the data generated in this project has come from the analysis of wax, or cryo-embedded tissue sections on glass slides. Here I will detail the techniques used in the preparation, staining, and analysis of tissue sections.

3.3.1 Dissection and Fixation

Tissues to be prepared for sectioning were dissected rapidly and transferred immediately into cold paraformaldehyde (PFA) or Carnoys fixative. Typically when
spinal cords were to be examined, the entire dorsal column was removed around the thoracic region. This allowed much more rapid removal of the cord and also ensured its integrity. In some cases, particularly at late stages of development, it was deemed necessary to remove the spinal cord from the dorsal column; this was done by carefully slicing along the dorsal midline using a clean scalpel. It was then possible to 'open' the vertebral column using forceps to pull apart the overlying vertebrae. Once exposed the spinal cord was carefully lifted whilst snipping the connections leading from the dorsal root ganglia using iris scissors.

Generally, the spinal cords were embedded inside the dorsal column, and it was found to be extremely difficult to remove intact spinal cords from the dorsal column at all in very young embryos (younger than 10 days).

Tissues were fixed overnight at 4°C in PFA or for 30 minutes at room temperature in Carnoy's fixative.

3.3.2 Wax Embedding

Embedding is the process by which the aqueous content of tissues is replaced with paraffin wax, allowing thin tissue sections to be prepared. All of the incubations described below were performed in 30ml Falcon tubes, or in 7ml glass bijoux, depending on the size of sample. The length of time samples were left in solutions was usually 30 minutes per incubation, however in some large samples this was increased to 1 hour. Residual PFA was removed from fixed tissues by washing twice in PBS at 4°C. The gradual replacement of water by alcohol in the samples was initiated by transfer of the tissues into a solution of 50% PBS: 50% Ethanol, before replacement with a 70% ethanol solution in water. Once tissues were in 70% ethanol, they could be stored for future use. The embedding procedure continued with solutions of 85%, 95%, and 100% Ethanol. This final ethanol step was repeated to ensure that all water had left the tissue sample. The ethanol, which now saturated the specimens, was gradually
replaced with the synthetic solvent Histoclear™. Following 3 changes of Histoclear, the
samples were incubated in a solution of 50%Histoclear: 50% Paraffin Wax at 70°C in
an oven. Three further incubations in 100% Paraffin Wax followed, ensuring complete
penetration of the tissue. The samples were then transferred to a plastic mould and
orientated appropriately. The wax was then allowed to set, and after cooling for
approximately 1 hour, the tissue was ready to be sectioned.

3.3.3 Cryo Embedding

Tissues that were to be sectioned using a cryostat were positioned in the correct
orientation within plastic embedding moulds in OCT compound. When the tissue was
suitably aligned the plastic mould was placed into a plastic trough containing
isopentane. The isopentane was chilled prior to this using an icebox full of dry ice. OCT
rapidly solidifies when exposed to low temperatures. This provides rigidity to the tissue
sample, allowing thin cryosections to be cut. Embedded specimens were stored at –
70°C until required.

3.3.4 Preparation of Coated Glass Slides

Prior to sectioning, glass slides were prepared by coating with Poly-L-Lysine. This
ensured good adhesion of tissue sections to the glass and prevented loss of tissues
during slide processing. A 10% solution of Poly-L-Lysine was prepared in distilled
water (~ 250ml volume). This was poured into a black plastic slide box, into which
racks of clean glass slides were immersed. After 5 minutes of bathing, the racks were
removed, blotted on paper towels and dried in a 37°C incubator for 3 hours. The slides
were then placed back in their box for storage until needed.

3.3.5 Paraffin Tissue Sectioning

Wax tissue blocks for sectioning were firstly trimmed to an appropriate size using a
razor blade. Sections were then cut at a thickness of 7µm using a microtome. Short
strips of adjoined sections were transferred onto pools of distilled water upon coated glass slides. The slides were heated using a Slide Dryer and when all creases were observed to flatten, the excess water was removed using a tissue and the slides transferred to metal slide racks. These racks were placed in a warm incubator (25°C) overnight prior to further processing.

3.3.6 Cryo Tissue Sectioning

Blocks of cryo-embedded tissue were mounted onto pre-chilled metal chucks using OCT. These were then allowed to solidify by placing the chucks onto the quick freeze plate within the cryostat. Sections were cut at a thickness of 10 μm and were immediately transferred onto coated slides by carefully placing the slide onto the section. Several tissue sections were mounted on each slide. After cutting, slides were stored in metal racks and stored at -70°C until required.

3.3.7 Dewaxing

The majority of tissue sections used in this study were paraffin wax embedded. Wax however, is not miscible with aqueous solutions, meaning that any histological stain or antibody applied would not gain access to tissues on the slide. It is thus necessary to re-hydrate paraffin sections, removing the wax from the tissue, and replacing it with water (i.e. a reversal of embedding). This was done by immersing the slides in a Coplin jar containing Histoclear™ at room temperature for 5 minutes. Typically, to ensure complete removal of all wax residues from tissue sections, a second immersion in Histoclear™ was performed for a further 5 minutes. Histoclear™ is a synthetic solvent which has effectively replaced Xylene in laboratories performing immunohistochemistry. It is highly effective at dissolving wax, but is non-toxic.

After visual confirmation that all traces of wax were removed from the slides, they were then transferred to a Coplin jar containing 100% ethanol. Histoclear™, like wax is not
water-soluble, however it will mix with ethanol. Ethanol therefore is used as a ‘middle ground’ between solvent and water-filled tissues. Sections were immersed in 100% ethanol for 5 minutes at room temperature, then transferred to fresh 100% ethanol for a further 5 minutes to ensure complete removal of all Histoclear™. Now the water content of the tissue was gradually increased by submerging the slides in Coplin jars containing 95%, 85%, and 70% ethanol for 5 minutes, before finally transferring the slides to jars of distilled water. After 5 minutes immersion in water, the tissues were ready for further processing.

3.4 Haematoxylin and Eosin staining

Haematoxylin and Eosin (H&E) staining was used to assess tissue morphology and pathology in fixed sections.

The protocol adopted was as follows: dewaxed sections were immersed in Coplin jars containing haematoxylin for 10 minutes. Slides were then transferred to a fresh Coplin jar containing distilled water for 1 minute, before being placed in a slide rack inside a glass trough. Tap water continually flowed through the glass trough via a piece of rubber tubing, providing an efficient rinsing system. After 5 minutes in running tap water, the slides were briefly dipped in acid alcohol solution (5 % acetic acid, 95% ethanol). After dipping in acid alcohol, slides were returned to the running tap water bath. The initial haematoxylin staining always resulted in a very strong blue colour. The immersion in acid alcohol served to lower the intensity of this staining to a suitable level. This usually required 2 or 3 seconds, although further dips in acid alcohol were sometimes required to achieve a desirable level of blue colouring. The slides were next immersed in eosin solution for 10 minutes before returning them to the flowing tap water for around 1 minute. The slides were then placed in 95% ethanol for 1 minute, then 100% ethanol for 3 minutes before finally being transferred to Histoclear. It was noted that extensive washes following eosin treatment resulted in the washing away of
too much red colouring. For this reason the final washes were brief and slides appeared to be over-stained when they were placed in 95% ethanol. However more red colour is lost during the ethanol stages, and by the end of the experiment a satisfying red colour was achieved. After 5 minutes in Histoclear solution, the slides were mounted using DPX solution and a clean glass coverslip.

3.5 Immunohistochemistry

Immunohistochemistry was performed to allow detection of specific proteins in situ on tissue sections. The protocol utilised varied between antibodies; the optimal conditions for one antibody were often found to be not ideal for another. I will describe in detail all of the steps involved, then list whatever adaptations and omissions from the complete protocol that were used for individual antibodies.

3.5.1 Removing OCT

In cases where cryo-embedded tissue sections were used for immunohistochemistry, it was necessary to remove the embedding compound OCT from the slides prior to further processing. This was achieved by immersing the slides in Coplin jars containing distilled water for 10 minutes at room temperature. This was repeated twice to ensure complete removal of OCT. Following this frozen tissue sections were treated in the same manner as wax embedded specimens. It was noted however that cryosections typically adhered less well to the glass slides, and were thus more prone to falling off during immunostaining. Care was taken then to avoid excessive shaking and jolting of frozen sections during experiments.

3.5.2 Antigen Retrieval

In some cases, it was found that immunohistochemistry was unsuccessful due to restricted access of antibodies to the tissue antigens. This can be caused by protein
cross-linking during fixation. Several techniques have been developed to 'undo' some of the cross-links caused by fixatives, allowing antibody access and thus antigen localisation. In this study I used a citric acid buffer treatment to 'retrieve' some antigens (see table 3.1 for details). Dewaxed slides were placed in a plastic rack and immersed in a plastic trough containing ~300ml citric acid buffer (0.5M citric acid, pH 6.0). This container was then sealed with SaranWrap and placed in a microwave oven. The SaranWrap was punctured in several places to prevent its rupture by steam. The container was then microwaved for 8 minutes at 60% power, causing the citric acid buffer to boil. The slides were allowed to cool, then transferred to a Coplin Jar containing distilled water. Complete removal of citrate was ensured by immersing the slides in PBS for 5 minutes at room temperature.
Antibodies requiring Antigen-Retrieval
(Citric acid buffer treatment)

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Table 3.1 Primary antibodies requiring antigen retrieval

3.5.3 Endogenous Peroxidase Block

In experiments where Horseradish Peroxidase (HRP) was used to visualise immunostaining, it was important to block the activity of any endogenous peroxidase enzyme that may be present in the tissue section. If this were not done, it would be impossible to distinguish between real, antibody-mediated signal, and non-specific background labelling. The function of endogenous peroxidases was inhibited by immersing the slides in a Coplin jar containing 2% hydrogen peroxide for 15 minutes at room temperature. This had the effect of over-stimulating the enzyme, effectively preventing its function using its natural substrate. After the incubation, the slides were rinsed of all hydrogen peroxide by immersion in 2 changes of PBS for 5 minutes.
3.5.4 Serum Block

One mechanism by which non-specific staining can occur during immunohistochemistry is via the interaction of immunoglobulins with tissues that contain charged or hydrophobic sites. That is, antibodies can bind to tissues due to their protein structure or charge, independent of the specificity of its antigen recognition site. This type of non-specific binding can be minimised by pre-blocking of the tissue section with non-immune serum. By doing this, all charged or hydrophobic sites that may be present should become occupied, or blocked, by proteins or immunoglobins. After this has been done, it is safe to apply the specific antibody of interest, knowing that the only tissue sites with which it will be free to interact with, are those antigens to which it has been specifically raised. To block non-specific protein interactions in this study, 10% normal goat serum diluted in PBS was used. In some experiments the PBS used to dilute the serum also contained 0.5% TRITON. Slides were removed from their Coplin jar using forceps and were dried of any excess solution. Tissue sections on each slide were carefully surrounded by a hydrophobic ring using a PAP pen. The purpose of this was to prevent any antibody solution from running from the tissue section during incubation. It was also found that smaller volumes of antibody solution could be used when the sections were circled in this way. Typically, about 3 tissue sections were encompassed by a single ring although this varied with the size of each tissue section. A second area of the slide was usually isolated for negative control experiments. That is, a further group of sections was circled on the same slide and treated identically to the first, although no primary antibody was applied to these sections. Once the tissues had been circled in this way, the slides were placed face-up in a humid chamber, and 100μl of blocking solution applied to the experimental and control rings. The humid chamber was constructed from a plastic slide trough and contained moistened tissue paper. This prevented excessive evaporation of antibody solutions during prolonged incubation periods. Slides were raised from the base of the chamber on a rack formed by two 5ml pipettes. This permitted easy removal from the
chamber and minimised the risk of spillage. Slides were blocked in this way for 1 hour at room temperature.

3.5.5 Application of Primary Antibody

Antibodies were diluted optimally prior to application. The dilution required was determined by titration. A range of dilutions for each antibody was tested initially, based roughly on information provided in data sheets, or from previous usage by other groups. The dilution providing optimal signal with minimal background was selected for all subsequent experiments. The dilutions used for each antibody are detailed in Chapter 2. Typically, 100μl of primary antibody was prepared for each section.

Antiserum was diluted in the same solution utilised for non-specific protein blocking (10% goat serum in PBS / PBS-TWEEN). Primary antibody solutions were applied immediately following blocking without washing. The blocking solution was carefully poured from the slide onto a folded tissue, the block was then replaced with antibody solution. In the case of negative control tissue sections, the blocking solution was not replaced. Primary antibodies were applied overnight at 4°C before any excess antibody was rinsed from the tissue by immersion in a Coplin jar contain PBS for 10 minutes with gentle shaking. This was repeated 3 times to ensure all unbound antibody had been washed from the tissue. In some experiments, PBS containing 0.5% TWEEN was used to minimise background staining (see Chapter 2).

3.5.6 Application of Secondary Antibody

The selection of secondary antibody varied with the species from which the primary was obtained, and with the desired choice of detection method. The secondary antibodies used were usually raised in goat, and directed against either rabbit or mouse immunoglobulins. For HRP detection systems, the secondary antibody was either conjugated directly to HRP, or to biotin. In the case of fluorescent immunodetection, the secondary chosen was conjugated to FITC, or Rhodamine. In
addition, when secondary immunodetection was used, Hoechst nuclear dye was also added to the secondary antibody mixture at a dilution of 1 in 1000. This resulted in all nuclei staining blue in colour under fluorescent illumination. Details of the individual secondaries used can be found in table Chapter 2. Optimal dilutions of secondary antibodies were obtained empirically, and dilutions were made in the same solution as was the primary antibody (usually 10% goat serum). Secondary antibodies were applied for 1 hour at room temperature in a humid chamber. In the case of fluorescent secondary antibodies, the humid chamber was surrounded with aluminium foil to prevent the fluorescence from becoming ‘bleached’ by exposure to light. Unbound secondary antibody was then rinsed from the sections with three 10-minute washes in PBS or PBS-TWEEN.

3.5.7 Double Immunostaining protocols

When the location of 2 proteins was to be examined simultaneously in the same tissue section, both primary antibodies were applied optimally diluted at the same time. It was important that the antibodies selected were raised in different species, allowing specific detection of each. Secondary antibodies were again applied together- optimally diluted in the same tube. In these experiments, the secondary antibodies used for double labelling were all fluorescently conjugated. Different fluorochromes were selected to detect each primary antibody.

3.5.8 Avidin-Biotin Complex Application

In experiments where a biotin-conjugated secondary was used, a complex of Streptavidin and Biotinylated horseradish peroxidase was used to detect the secondary antibody localisation. This complex has high affinity and specificity for biotin. Since each secondary antibody is conjugated to many molecules of biotin, many Streptavidin-Biotin-HRP complexes can bind to each secondary antibody. This results in an amplification in the strength of signal detected.
10 µl of solution A (Streptavidin in 0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, 15mM NaN₃, pH 7.2) and 10 µl solution B (Biotinylated horseradish peroxidase in 0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, 15mM NaN₃, pH 7.2) from the StreptABComplex/HRP kit (DAKO) were added to 1ml of PBS and incubated at room temperature for 30 minutes to allow complexes to form. The volume of ABC solution prepared was dependent on the number of slides (~100µl per section). After this pre-incubation step, 100µl of ABC solution was applied to each section in the same manner as the application of primary or secondary antibody. Slides were then incubated for 40 minutes at room temperature in a humid chamber before washing of unbound ABC from the tissue with 3 changes of PBS with shaking for 10 minutes.

3.5.9 Visualisation of HRP

HRP or biotin conjugated secondary antibodies required the same method of visualisation. The processes described thus far result in the localisation of several enzyme molecules to the antigen of interest in situ. In order to visualise the location of these molecules, the enzyme is provided with a substrate that forms an insoluble coloured product.

100µl of filtered DAB solution was applied to each section after careful drying of excess PBS with a tissue. The development of the colour reaction was then monitored under a dissecting microscope. The reaction was stopped, usually between 30 seconds and 2 minutes following the development of a suitably contrasting brown colour, by immersion of the slides in a Coplin jar containing distilled water.

3.5.10 Methyl green counterstaining

Peroxidase-labelled sections were counterstained with methyl green to allow visualisation of non-immunostained tissues. The stain results in a mild green/blue colour, which labelled all cell nuclei. Methyl green also stained developing cartilage.
particularly strongly. To counterstain, slides were transferred to a Coplin jar containing Methyl Green for 10 minutes at room temperature. Slides were then lifted using forceps and washed in 3 changes of distilled water, each wash lasting around 5-6 seconds in total. Following the 3rd wash, most of the excess green colour had been removed, and the slides were immersed in butanol for 5 minutes in a fume hood. This resulted in effective dehydration of the tissue sections prior to their transfer to a Coplin jar containing Histoclear. After 10 minutes in Histoclear, slides were mounted using DPX mountant and a clean glass coverslip. Mounted slides were allowed to dry for 1 hour in a fume hood before being examined using a light microscope.

3.5.11 Digital Capture of Images

Following experimental procedures, images were captured electronically using a digital camera and scanned directly into Photoshop or OpenLab. Alterations to brightness and contrast were made as necessary.

3.5.12 Surface area measurements

Measurements of blood vessel surface area were made using OpenLab software. Sections of spinal cord immunostained with anti-laminin antibody were imaged and stored as JPEG files. These were then opened in the OpenLab program. Using the freehand selection tool, the outline of each spinal cord was traced. The measurements window was then used to obtain the total surface area of the spinal cord. Next, the ‘shrink around image’ option was used to select the blood vessels within the outlined area. This tool defines regions within the selected area as ‘positive’ by analysing differences in colour density: all regions of dark colour on a white background would be included. It was important to have maximum contrast between the positively labelled vasculature and the negative background, so no counterstaining was used in the immunohistochemical procedure. In each case, the program’s estimation of blood vessels was checked visually. Occasionally it was necessary to adjust the sensitivity of

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the ‘shrink around image’ function. This was done by changing the tolerance of the
‘lasso’ function. In performing this analysis, the area of each spinal cord was usually
measured in steps. The OpenLab system cannot process very large images using
‘shrink around image’, and it was also important not to include the central canal in the
region to be analysed as this would be interpreted as positive staining by the software.
Typically, spinal cords in each section were divided into 4 quadrants and the surface
areas tallied to obtain the total surface area of the spinal cord. The surface area of the
blood vessels within this region was then expressed as a percentage of total spinal
cord area.

3.6 Preparation of human tissues

Embryonic human tissue sections were obtained from the Human Developmental
Biology Resource and were provided already paraformaldehyde fixed and embedded in
paraffin wax. Fresh tissue samples for RNA or protein extraction, or for explant culture,
were acquired from recent terminations. These tissues were collected on site by MRC
staff and transported to the laboratory for dissection in ice-cold L15 buffer.

3.7 Explant Culture

Embryonic human spinal cord samples were obtained from the Human Developmental
Biology Resource. Spinal cords were carefully diced using sharpened forceps and
iridectomy scissors into pieces of around 1mm diameter. These were then transferred
to a 24-well tissue culture dish containing gelatin-coated glass coverslips. These were
prepared by flaming glass coverslips using IMS to render them sterile. The sterile
coverslips were then transferred to the wells of the dish and 200 μl of 4% sterile gelatin
solution added. The gelatin solution formed a solid jelly when stored at 4°C and so
needed heating in a 37°C waterbath until it became liquid. The gelatin solution was
applied for 2 minutes then removed using a Gilson pipette; this left a thin coating of
gelatin on each coverslip. Approximately three spinal cord explants were positioned on each coverslip before addition of 500μl of organ culture medium. The tissue culture dish was then placed in a tissue culture incubator at 37°C and 5% CO₂. Explants were examined using an inverted microscope and explant medium was replaced every 7 days.

3.8 Immunocytochemistry of cultured cells

Cells grown on coverslips were also stained using antibodies. All solutions were applied in 100μl volumes which was sufficient to entirely coat the coverslip. Antibodies were diluted in L15 medium. Coverslips were removed from the 24-well culture dish using sharpened forceps and transferred to small pedestals (lids from screw-capped ependorf tubes). This allowed the coverslips to be manipulated easily. Cells were fixed by application of ice-cold acid alcohol or 4% PFA solution for 8 minutes. In some cases, fixation of the cells was not performed until after application of antibodies. This was done when cell-surface proteins were under investigation. After fixation, coverslips were lifted by forceps and excess solution removed by touching the side of the coverslip on some folded tissue paper. The coverslip was then washed by dipping in three 30ml tubes containing L15. Each dip lasted around 3 or 4 seconds. The primary antibody was then applied and left for 1 hour at room temperature. Following this, the primary antibody was blotted off, and the coverslip washed as before. The secondary antibody was then applied and incubated for 30 minutes at room temperature. The coverslip was then washed a further 3 times in L15, followed by a brief rinse in PBS, then distilled water. Finally, Citifluor was used to mount the coverslips on clean glass slides. The cells were then examined using a fluorescent microscope.
3.9 TdT-mediated dUTP nick end labelling (TUNEL)

Cells undergoing apoptotic cell death were detected using a TdT-mediated dUTP nick end labelling kit. This kit preferentially labels apoptotic nuclei by using the enzyme terminal transferase to add nucleotides to breaks, or 'nicks', which characteristically appear in DNA at early stages of apoptosis.

Wax tissue sections were used for TUNEL and were de-waxed as described previously for immunohistochemistry. It was then necessary to permeabilise the tissue sections by incubation with Proteinase K (DAKO, UK) at a concentration of 10 μg/ml. The Proteinase K solution was made up to the correct dilution in Tris-HCl then pre-incubated at 37°C for 30 minutes. Following this, 100μl was applied to each tissue section in a humidified chamber for 15 minutes at room temperature. Over-digestion with PK was noted to result in false positive staining in all cell nuclei, and so the pre-incubation and timing of PK application was critical to the success of the TUNEL experiments performed.

Following the PK digestion, slides were rinsed in 2, five-minute changes of PBS. The tissue sections were then carefully dried of excess PBS using a tissue. TUNEL reaction mixture was prepared on ice immediately prior to application. The kit comprises of 2 solutions; enzyme solution, containing TdT in storage buffer, and labelling solution containing fluorescein conjugated dUTPs in reaction buffer. For each slide analysed, 5μl of enzyme was mixed with 45μl of labelling solution. After vortexing, 50μl of the TUNEL solution was added to each section. For negative control slides, 50μl of labelling solution was added to sections without addition of enzyme.

All TUNEL treated slides were placed in a humid chamber wrapped in aluminium foil to prevent bleaching of fluorescence by light, and transferred to a 37°C incubator for 60 minutes. Slides were then washed in three changes of PBS (5 minutes per solution), again foil was used to minimise exposure to light during these washes. Finally sections
were briefly dried and mounted using Citifluor and a glass coverslip. TUNEL positive cells were observed using a Fluorescent microscope and photographed. Typically, morphological changes were also examined to confirm the fidelity of the labelling.

### 3.10 Immunoblotting

To detect specific proteins from tissue samples, and to evaluate the fidelity of antisera, immunoblotting, alternatively known as Western Blotting was performed.

#### 3.10.1 Protein sample preparation

Tissue samples for immunoblotting were rapidly dissected, weighed, and were typically stored at -70°C until required. Tissues were then homogenized in ice-cold lysis buffer, containing 1% Protease Inhibitor Cocktail. This prevented protein degradation during tissue processing. 1 ml of lysis buffer was used per 100 μg tissue. A syringe was used to break down the tissues, the sample being drawn repeatedly through progressively narrower gauge needles until no fragments of tissue were visible. The samples were then placed on ice for 30 minutes, before being centrifuged at 13,000g for 30 minutes at 4°C. The supernatant was then removed and divided into 100 μl aliquots in 0.5 ml eppendorf tubes for storage at -70°C.

#### 3.10.2 Determination of protein concentration

In order to ensure adequate and equal amounts of protein were loaded in each lane, it was necessary to determine the protein concentration of each sample used for immunoblotting. This was done using a BCA protein assay kit (Pierce, USA).

This procedure involves the generation of a coloured product following the following enzymic reaction.
1. Protein (peptide bonds) + Cu$$^{2+}$$ → tetradentate-Cu$$^{1+}$$ complex

2. Cu$$^{1+}$$ + Bicinchoninic acid (BCA) → BCA-Cu$$^{1+}$$ complex (purple coloured)

A series of known concentration standards were prepared using bovine serum albumin (BSA), which was supplied at a concentration of 2mg/ml. Standard concentrations ranging from 25 to 1500μg/ml were prepared and were analysed at the same time as the unknown samples.

A working reagent (WR) was prepared by mixing the supplied Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2M sodium hydroxide) and Reagent B (4% cupric sulphate solution) in a 50:1 ratio. 1ml of WR was then applied to 50μl volumes of known, or unknown protein concentration.

Following a brief vortexing, the mixtures were incubated at 37°C for 30 minutes, then cooled to room temperature. The cooling of the reaction mix effectively slows down the colourimetric reaction to the extent that further colour development occurring during the measurement process is insignificant. All tubes were measured on a spectrophotometer at a wavelength of 562nm and compared to a blank standard containing water. Each sample was measured twice and an average reading used for calculations.

The readings from the BSA standards were plotted in a standard curve and this was used to extrapolate the protein concentration of each unknown.

3.10.3 Preparation of polyacrylamide gels

Polyacrylamide gels were prepared using the components detailed in Table 2.1 and 2.2. The amount of polyacrylamide contained in the gel was determined by the nature of the protein under investigation; large molecular weight proteins required thicker gels.
with more acrylamide for adequate resolution, and vice versa. An Autorad gel tank was used which requires 2 gels of approximately 7ml volume to be run simultaneously.

10x10 cm glass plates were cleaned carefully using detergent, water, and ethanol. These were then positioned in the casting mould separated by two 1.5 mm gel spacers. The casting mould presses the glass plates tightly against a rubber seal; the watertightness of this seal was checked by filling the space between the plates with approximately 5 ml of water. Following this, the water was removed, and the resolving gel mixture was carefully poured into the space between the glass plates using a 10ml plastic pipette. The gel was poured to a height of around 5cm, allowing space above for the gel comb and for 1cm depth of stacking gel. Care was taken to prevent the generation of bubbles when pouring the gel. Once poured, the gel surface was overlain with water-saturated butan-1-ol to ensure a flat surface was obtained. After allowing around 30 minutes for the resolving gel to set, the layer of butanol was poured off, and the gel washed briefly with 2 changes of distilled water. The stacking gel was then poured to the very top of the glass plates before insertion of the gel combs. This prevented accidental insertion of bubbles into the gel surrounding the wells. After the combs had been inserted, the area around the surface of the gel was covered with Sellotape to minimize gel retraction during setting. After 30 minutes to allow for setting of the gel, the 2 gel moulds were removed from the casting frame and attached to the electrode frame, then placed in the gel tank. The tank and the internal buffer reservoir were then filled with running buffer (25mM Tris, 192mM glycine, 0.1% SDS) and the gel combs removed; the unit was now ready for the loading of proteins.

3.10.4 Protein sample preparation

The volume of each protein sample required was calculated using the protein concentrations obtained previously using the BCA Protein Assay Kit (as described above). Equal amounts of protein were loaded in each well (10-90 µg). Proteins were
mixed with an equal volume of loading buffer (20% glycerol, 10% β-mercaptoethanol, 5% SDS, 0.25M Tris-HCl pH 6.8, bromphenol blue) and denatured by heating in a heating block for 5 minutes at 100°C. Samples were next placed on ice to prevent reformation of secondary and tertiary structures.

3.10.5 Sample loading and running of gel

The proteins were loaded into the wells using a 20μl Gilson pipette. It was found that the first and last well of each gel was most likely to produce distorted protein bands and so these were avoided if possible. Molecular Weight standards were loaded in one lane to establish the size of any protein band revealed.

After loading the gel was run at 60V until the protein front had passed through the stacking gel and entered the resolving gel, after this the voltage was increased to 100V. Whilst operating, the gel tank was checked frequently, and if necessary, the internal buffer reservoir was topped up with running buffer. Immediately after the dye front had passed completely from the gel, the current was switched off and the gels removed from the apparatus.

3.10.6 Semi-dry Blotting of Proteins

Following electrophoresis, the glass plates were removed from their mounts and carefully separated using the gel spacers as levers. The stacking gel was then cut from the resolving gel using a clean razor blade. The resolving gel was then immersed in transfer buffer (48mM Tris, 39mM glycine, 0.037% (v/v) SDS, 20% (v/v) methanol) for 15 minutes. A ‘sandwich’ was then constructed on the anode of a semidry blotter. This comprised of 3 sheets of absorbent blotting paper, followed by a sheet of nitrocellulose membrane; the polyacrylamide gel, and 3 further sheets of blotting paper. All the components were cut to the same size as the gel, and soaked in transfer buffer prior to sandwich construction. The cathode was then positioned on top of the sandwich and
semidry blotting was performed at 12V for 40 minutes. After this time, the membrane was removed and rinsed in PBS. To check for protein transfer, the membrane was stained using Ponceau S. Following visualization of proteins on the membrane, the location of each protein ‘smear’ was marked by numbering the lanes at the top of the membrane using a pencil. If different lanes required analysis by different antibodies, then the membrane was cut into appropriate sections at this point using scissors. The red staining was then removed by immersing the membrane in two changes of PBS.

### 3.10.7 Application of antibodies

Blotted membranes were blocked overnight at 4°C using 5% Blotto prepared freshly using PBS and containing 0.5% Tween-20. Primary antibodies were diluted in Blotto and made up to a volume of 3ml. Membranes were rolled inside 30 ml Sterilin tubes with the protein side facing inwards. Care was taken to avoid any overlap of membranes inside the tube. Antibodies were applied to the tube and the tubes placed on a mechanical roller at 4°C. Larger 50 ml tubes were occasionally required for multiple membranes and in this case 6 ml of antibody was prepared. Primary antibodies were applied for 2 hours, then excess was removed by washing with 2 changes of Blotto for 15 minutes each at room temperature.

Appropriate HRP-conjugated secondary antibodies were applied in a similar way to the primaries and were incubated at room temperature for a period of 30 minutes. The membranes were then washed thrice with Blotto for 15 minutes, before a final 5 minutes rinse in PBS.

### 3.10.8 ECL detection of proteins

Enhanced Chemiluminescence (ECL) is a highly sensitive method of detecting HRP-conjugated secondary antibodies. This system relies on the HRP/hydrogen peroxidase catalysed oxidation of luminol under alkaline conditions. This reaction releases light as
a by-product. The presence of chemical enhancers, such as phenols greatly enhances the quantities of light released (this being the enhanced aspect of ECL). This emitted light can be detected by exposure to blue-light sensitive autoradiography film.

The 2 provided reagents from an ECL kit (Amersham Pharmacia Biotech, UK) were mixed in a 1:1 ratio to a volume sufficient to cover the membrane (usually 1ml of each reagent was used). The mixture was applied to the membrane for 1 minute exactly, and then blotted off using absorbent paper. The blot was then wrapped in Saran Wrap, avoiding excessive wrinkling, and taped into a film cassette. The membrane was then exposed to X-ray film under safe lighting conditions. A number of exposure times were attempted in each case, ranging from 30 seconds to 15 minutes. Following exposure, the film was developed mechanically; labelled protein bands were visible as regions of darkened film. The size of each marker lane was then added to the exposed film using a marker pen by overlaying the transparent exposure on top of the original membrane.

3.11 RNA Extraction

RNA was extracted from chick or human tissues using TRI reagent (Sigma, UK). Tissues were fragmented in 1ml TRI reagent, either fresh or following storage at –70°C. A narrow gauge needle was used to break up the tissue by repeatedly drawing the solution through the syringe. Occasionally a broader needle was required to initiate fragmentation, particularly in older tissue samples. RNA was then isolated according to the manufacturer’s instructions and was re-suspended in 20μl of distilled water.

3.12 Reverse Transcription

cDNA was constructed from extracted RNA using M-MLV Reverse Transcriptase according to the supplied guidelines. 1μg of RNA, as determined by spectrophotometry, was used per reaction and random hexamers (Promega) were
used as primers. cDNA was stored at -20°C until required. Negative controls were performed in which no enzyme was added to the mixture.

3.13 Polymerase Chain Reaction (PCR)

PCR was used to amplify portions of specific gene sequence, confirming their presence, or offering quantitative information. Primers of 20-22bp lengths (see Table 2.3) were designed from gene sequences obtained from GenBank. Oligonucleotides were custom synthesised by Genosys (Cambridge, UK).

PCR was performed in 50μl reactions using Taq DNA Polymerase (Promega) using thin-walled PCR-tubes. For each reaction, the following was required:

- dH₂O 31.8 μl
- x10 Buffer 5 μl
- dNTPs (2mM) 5 μl
- Sense Primer 20pmol/μl 0.5 μl
- Antisense Primer 20pmol/μl 0.5 μl
- Taq Polymerase 0.2 μl
- cDNA 3 μl

Following addition of all reagents, the PCR tubes were briefly vortexed to ensure complete mixing before being transferred to a PCR machine.

The following general PCR program was utilised:

Step 1. 94°C for 3 minutes

Step 2. 94°C for 30 seconds
Step 3. $T_A$ for 1 minute

Step 4. 72°C for 1 minute

Step 5. GOTO Step 2. 'X' times.

Step 6. 72°C for 10 minutes.

Where $T_A$ is the Annealing temperature of the reaction, and $X$ is the number of cycles. These variables were individually determined for each PCR reaction based on primer length, characteristics, and levels of RNA expressed (genes with few copies of transcript required more cycles of amplification). The annealing temperature $T_A$ for each primer pair, was calculated by the formula:

$$T_A = 69.3 + (0.41 \times \text{GC}) - (650 \div n).$$

where $n =$ length of oligo primer, and GC% is the percentage of G and C content in the oligo, typically 55% (11/20).

Following PCR amplification products were stored at 4°C until being run on an agarose gel.

3.13.1 **Agarose gel analysis of PCR products**

Agarose gels (1-2 %) were prepared by adding the appropriate weight of agarose to 100ml TBE in a glass flask. The mixture was then boiled in a microwave oven for 2 minutes to allow the agarose to dissolve. 4 µl of ethidium bromide was then added and mixed thoroughly by gentle swirling of the flask. The gel was then poured into a previously prepared gel mould containing a suitable comb. The gel was allowed to cool for 30 minutes at room temperature before removal from the mould and transfer to a
gel tank. The tank was then filled with TBE until the entire gel was submerged. After filling the tank, the gel comb was removed, allowing the loading of PCR products.

Samples were prepared for loading by mixing 16μl of PCR product with 4μl of Orange G loading buffer. Each tube was then vortexed to mix thoroughly and then briefly spun in a centrifuge to collect the sample at the bottom of the tube. 16μl of each sample was then loaded into an appropriate well using a Gilson pipette. An appropriately sized DNA ladder was loaded into the first well of each gel allowing the size of each band to be determined. Typically a 1Kb (Promega) ladder was used.

Following loading, gel tanks were closed and attached to a power source. Electrophoresis was performed at 70-100V and was stopped when the dye front had crossed around ¾ of the length of gel.

3.14 Imaging of gels

Gels were visualised by transillumination under ultra violet light using a digital camera. Alphalmager (Alpha Innotech Corporation) 1200 computer software was used to analyse gel images and perform densitometry analysis. Gels were either printed immediately or stored as JPEG image files for future reference.

3.15 DNA sequencing

3.15.1 Purification of PCR products

PCR products were sequenced directly following gel imaging. The appropriate band was carefully excised using a clean scalpel blade under UV illumination. DNA was then purified using a QIAGEN gel extraction kit according to the manufacturer’s instructions. This kit utilises the DNA-binding properties of a silica-gel membrane. Impure DNA samples are applied at high salt concentrations and pH>7.5, conditions favouring
membrane binding. The sample is then washed with ethanol-containing buffer before the DNA is eluted with low salt, low pH buffer as a purified solution.

3.15.2 Cycle Sequencing

Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Warrington, UK). 4 μl of Ready reaction mix was added to a 0.5 μl Eppendorf tube containing 1 μl of primer (from original PCR reaction, diluted to 3 pmol/μl), and 20 ng DNA. The final volume was made up to 20 μl with distilled water.

This reaction mixture was made for both sense and antisense PCR primers, resulting in simultaneous sequencing of the product in both directions. The mixture was placed in a PCR machine and the following program initiated:

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration/Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>10 minutes 96°C</td>
</tr>
<tr>
<td>Step 2</td>
<td>1 minute 96°C</td>
</tr>
<tr>
<td>Step 3</td>
<td>30 seconds 50°C</td>
</tr>
<tr>
<td>Step 4</td>
<td>4 minutes 60°C</td>
</tr>
<tr>
<td>Step 5</td>
<td>Goto Step 2 25 times</td>
</tr>
<tr>
<td>Step 6</td>
<td>END</td>
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</tbody>
</table>

Following this reaction, the products were removed from the PCR machine and 50μl ethanol and 2μl NaOAc was added to promote precipitation of the DNA.

The mixture was then left at room temperature for 45 minutes, and then spun at 13,000rpm in a centrifuge for 20 minutes. The supernatant was removed, and the pellet washed in 250μl of 70% ethanol by vortexing. The mixture was spun again for 5 minutes, then the washing procedure was repeated. Following the second wash all
excess ethanol was removed and the pellet air-dried for 5 minutes. The sample was now ready for sequencing, which was performed using an automated sequencer (AB1377 Perkin Elmer) by trained colleagues.

Analysis of output sequences was performed using GeneJockey and internet-based software.

3.16 Statistical Analysis

Unpaired Student’s t-tests were utilised to compare populations of diazepam treated versus untreated animals. This allowed any potential differences in means of each group to be established. This statistical method was appropriate as the populations to be examined were independent of each other and of normal distribution. The probability (p-value) obtained represents the likelihood of the null hypothesis being true (i.e. no difference between groups). A p value of < 0.05 was deemed to be significant. Unpaired t-tests were performed using SigmaPlot software, and graphs represent mean values ± standard error of the mean error bars.
CHAPTER 4. THE RESPONSE OF THE CHICK SPINAL CORD TO INJURY
4.1 INTRODUCTION

The embryonic chick has been previously demonstrated to effectively repair spinal cord damage, providing the injury occurs prior to a pivotal point in development, occurring around E13 (Shimizu et al., 1990; Hasan et al., 1991). It is apparent however that although previous studies have provided adequate evidence for functional recovery following injury at permissive stages for regeneration – by means of locomotor activity (Shimizu et al., 1990), retrograde labelling of regenerated axons (Hasan et al., 1993), or electrophysiological measurements (Sholomenko and Delaney, 1998), a detailed morphological analysis of injury responses, particularly at non-permissive stages has not been published.

Most previous studies have implicated the onset of myelination as a crucial, if not defining, point in spinal cord development, responsible for the onset of non-permissiveness (Keirstead et al., 1992; Keirstead et al., 1995). Interestingly, few other possibilities have been considered in this model. Given the plethora of molecular cascades triggered by traumatic neural injury, it seems unlikely that a single molecular factor will be solely, or even primarily responsible.

Cell survival is a fundamental issue following spinal cord damage. Mechanical trauma will inevitably result in some degree of necrotic cell loss. This ‘primary injury’ however is frequently not the major contributor to cell death in the spinal cord. Secondary mechanisms are initiated immediately following initial insult, and these processes can go on to encompass a region more extensive than the primary injury site (Mautes et al., 2000). A crucial point remains that, these progressive secondary injury mechanisms may provide an avenue of therapeutic intervention; they may be reduced in severity, or prevented from occurring.

The nature of secondary injury mechanisms are diverse. Factors such as haemorrhage within the spinal cord will result in the release of many cytotoxic, or specifically
neurotoxic substances into the CNS. The blood-brain barrier acts to prevent most water-soluble molecules from accessing the brain and spinal cord. Spinal damage will frequently compromise this barrier, allowing neurons and glia to contact potentially damaging substances. Although all cell death occurring in the spinal cord upon initial trauma will be due to necrosis, it has recently been reported that secondary tissue damage can result in the apoptotic death of neurons, glia, and oligodendrocytes (Liu et al., 1997; Li et al., 1999; Newcomb et al., 1999). Because apoptosis is an active process, the prospect of intervention exists, perhaps by supplying extrinsic survival factors, or specific anti-apoptotic agents.

Although secondary tissue damage is a well-documented phenomenon, and it undoubtedly results in a more severe pathology, the precise extent to which it interferes with regeneration is yet to be established in the embryonic chick. I aimed to address the issues of whether embryonic spinal cord is affected less by secondary factors, or is better equipped to deal with the traumas they present during permissive periods for regeneration.

To achieve this, I have examined the early responses of the chick spinal cord to injury at both regeneration competent, and incompetent stages of development to gain insight into the processes through which secondary injury factors propagate and extend the site of initial injury. Previous studies in which chick spinal cords have been injured have concentrated primarily on late stage events in the repair process, such as behavioural recovery, or re-growth of axons as measured by retrograde labelling (Hasan et al., 1993; Hasan et al., 1991; Shimizu et al., 1990). Spinal cords were analysed morphologically and by TUNEL.
4.2 RESULTS

4.2.1 The response of the E7 chick spinal cord to injury

Thoracic spinal cord injuries were performed on chick embryos on the 7th day of incubation as described in Chapter 3. Overall surgical survival was low, with as few as 5% of chicks reaching E11, although around 35% survived at least 24 hours. Mortality was presumably due to the extensive nature of the injury sustained, and the degree of embryonic manipulation required. Previous studies have reported poor survival following spinal transection in the chick (Shimizu et al., 1990). Sham operated embryos also experienced lower survival rates than unopened eggs, but showed no signs of spinal damage (n=2, not shown). Surviving embryos (n=8) were sacrificed and the vertebral column rapidly dissected before fixation. The spinal cords were then embedded and sectioned in the longitudinal plane.

At E7, the chick spinal cord is capable of functional regeneration, and is still in some respects, relatively immature. The neuronal populations of the spine are post-mitotic, and most descending axonal tracts from the brain have passed the thoracic region, where the experimental injury was performed (Okado and Oppenheim, 1985).

24 hours following spinal transection, the site of injury was clearly visible using H&E staining, although no separation of cranial and caudal stumps was observed (Fig 4.1A). The damage seen was consistent with that caused by the initial trauma, i.e. primary tissue damage. There was little evidence of haemorrhage or oedema - signs of secondary injury processes in action. By 4 days post-injury, the spinal cord was displaying evidence of repair (Fig 4.1B). The opposed stumps had fully re-joined and the site of injury was difficult, or impossible to detect, this being consistent with observations made by Shimizu and colleagues following spinal transection at E5 (Shimizu et al., 1990). Neurofilament staining confirmed the integrity of the axonal tracts at this time (Fig 4.1B). These findings are in keeping with previous studies,
supporting the notion that immature spinal cord tissue is capable of seamless repair
and full morphological recovery. It could be interpreted that the E7 spinal cord
environment is highly permissive for regeneration, and that no major inhibitory
influences exist following neural damage. No disruption to normal tissue architecture
was observed, indicating that re-growing axons simply re-established their original
routes, unhindered by local environmental cues. Alternately, the intrinsic growth
properties of these nerves may enable them to effectively ‘ignore’ otherwise growth
retarding chemical influences.
Figure 4.1 Longitudinal sections of E7 spinal cord injuries. A. 24 hours after surgery, the site of injury (arrows) is detectable by H&E staining. Overall spinal cord structure is intact. B. 4 days after surgery: section is stained using RT97 to detect neurofilaments (brown), counterstain is methyl green- the site of injury cannot be distinguished from normal tissues. w = white matter, g = grey matter, v = vertebrae. Scalebar = 400 μm in A, 1 mm in B.
4.2.2 The response of the E11 chick spinal cord to injury

Spinal cord injuries were performed at E11 using the same surgical methods employed at E7 (see methods). Survival of embryos following surgery was better than that observed at E7; around half of all operated animals survived for 1 day, although far fewer chicks remained alive after 4 days. Again, embryos were fixed and sectioned in the longitudinal plane. The E11 chick spinal cord is able to effectively repair spinal cord damage (Steeves et al., 1994). By this stage the cord is effectively mature; all ascending and descending axonal tracts are known to be complete (Okado and Oppenheim, 1985).

Histologically, after 24 hours, the injured E11 spinal cord (n=6) looked similar to the equivalent E7 injury. Visible damage was largely restricted to the immediate vicinity of the primary injury site (Fig 4.2, Fig 4.3). Spinal cord tissue appeared normal in morphology at distances greater than 0.5 mm from the point of transection.

There was little evidence of secondary damage, although a few red blood cells were usually visible at the injury site, occasionally these were found within the ependymal canal adjacent to the injury (Fig 4.2b). Analysis of longitudinal serial sections indicated that the injury was complete, and that the degree of damage did not display much variation along the dorso-ventral axis (Fig 4.2c).

By 4 days post-injury (n=4), effective repair was underway: spinal cords no longer had a gap between the transected stumps and any infiltrating blood cells had been effectively cleared. In one case, visible re-routing of descending fibres was apparent (Fig 4.4a). In this embryo, axons of the ventral funiculus were seen to diverge laterally, presumably avoiding some inhibitory influences within the immediate environment. Neurofilament staining was employed to confirm the axonal nature of these fibres (Fig 4.4b).
These results support previous observations that E11 chick spinal cord is capable of functional regeneration. The overall structure of the spinal cord is rapidly re-established following transection. In addition, there is morphological evidence for re-tracking of axonal fibres, indicative of regeneration. Interestingly, as at E7, there appears to be little contribution of secondary tissue damage to E11 spinal injury. The injuries sustained are concise, and would be expected to have occurred mainly from the primary insult.

It is interesting that visible re-routing of descending pathways was evident. The implications of this may be that local inhibitory factors and influences are present in the spinal cord following E11 injury. The descending axons however seem capable of forming new pathways down the spinal cord. This implies that although at E11, some axon-repulsive factors are released after damage, the spinal cord axons are still intrinsically capable of overcoming the obstacle, although some may be diverted via less inhibitory locations.

These observations were then compared with analogous injuries performed during the restrictive period for spinal cord regeneration.
Figure 4.2 Longitudinal H & E stained section of E12 chick spinal cord injured at E11. A. Following spinal cord transection at E11, the site of injury is clearly visible 24 hours later (arrow). Some blood is present at the injury site, and has travelled along the ependymal canal for a short distance. B. High magnification view of ependymal canal from A. Scalebar = 1mm in A, 50 μm in B.
Figure 4.2C. Serial sections through an E11 spinal cord injury, after 24 hours. Image A is most dorsal, D is ventral. Images are approximately 50 µm apart. Scalebar = 1mm.
Figure 4.3 Chick spinal cord injured at E11 and sectioned longitudinally 24 hours later. As in the previous figure, the spinal injury has clearly separated the spinal cord, and limited haemorrhage is visible. Scalebar = 1mm
Figure 4.4 Longitudinal section of E11 spinal cord injury after 4 days. A. H & E staining: the spinal cord has undergone significant recovery. Point of injury is indicated (arrows). Ventral funiculus is visible (vf) and can be seen re-routing axons laterally. B. RT-97 immunostaining of adjacent section confirming that the fibre tracts present in (A) are axons. Scalebar = 500μm in A, 1 mm in B.
4.2.3 The response of the E15 chick spinal cord to injury

A total of 14 successful spinal cord injuries were performed at E15, any morphological changes between this restrictive stage and earlier injuries being particularly of interest. Survival of E15 embryos was better than that of younger chicks; typically 50-60% of injured animals survived 24 hours, and around 20% reached E19 (the latest time-point examined). As before, spinal cords were dissected within the vertebral column and sectioned in the longitudinal plane. De-calcification was employed in some late stage embryos to permit sectioning of vertebrae, which begin to become mineralised around E15.

By E15, the embryonic chick spinal cord has entered the restrictive period for repair and no functional recovery is possible (Steeves et al., 1994). Spinal cord injury performed at this stage resulted in considerable pathological differences compared to E7, or E11 injuries discussed above.

As early as 1 hour post-injury, considerable differences were evident: The extent of haemorrhage within the injured cord was noted to be much more extensive (Fig 4.5A). Furthermore, this immediate bleeding encompassed areas of the spinal cord distal from the point of injury. Commonly the junction between white and grey matter allowed flow of blood to regions cranial and caudal to the initial injury site (Fig 4.6A). White matter in close proximity to areas of haemorrhage displayed unusual morphology after 1 hour, appearing swollen, presumably due to local oedema (compare Figs 4.6B, with 4.6C). The accumulation of blood around the injury site was not a transient phenomenon. Analysis of E15 injuries after 8 hours indicated that haemorrhage had spread for up to 6 mm total distance along the spinal cord. In addition, fluid-filled cavities were beginning to form (Fig 4.5B).

This pathology was also evident in spinal cord injuries after 24 hours. In these cases, cavitation was more pronounced, extending for several millimetres. Pooling of blood cells was also evident, as were regions of tissue debris. The disruptive effects of spinal
cord injury at E15 seemed to encompass all cell types, affecting both white and grey matter (Fig 4.7A). Analysis of serial sections confirmed that the injury pathology was present throughout the spinal cord. The degree of cavitation was observed to vary along the dorso-ventral axis, although normal spinal cord architecture was severely disrupted at all levels (Fig 4.7B).

By 3 days post-injury, the damaged cords were seen to contain large cavities encompassing regions of tissue debris and blood cells (Fig 4.8A). By this stage, signs of injury maturation were evident. Surviving regions of spinal cord became partitioned from the fluid filled cavities by the formation of a smooth, endothellum-like border (Fig 4.8B-D). This process may be the initial step toward the synthesis of a glial scar. The separation of surviving tissues from the noxious products of secondary tissue cascades is likely to be crucial in preventing further extension of the damaged area. However, the process of partitioning is not fully complete at this stage; some areas have only a partial cellular layer between cavity and cord, whilst it is missing entirely in other regions (Fig 4.8D).

The E15 spinal cord injury appears to have become effectively 'mature' by E19, 4 days after surgery. Large cavities persist in the cord (Fig 4.9, Fig 4.10), although these have been effectively 'shut off' from unaffected areas by the completion of a smooth cellular border, encompassing the cavity. Large islands of tissue debris also remain within these cavities. The surviving white matter of the injured spinal cord is characterised by an abundance of axonal blebs, typical of retracting axons following axotomy (Fig 4.10 C, D).

By E15, the spinal cord can be thought of as effectively mature, the process of myelination is well underway. Clearly some aspects of spinal maturation, perhaps associated with myelin deposition render the spinal cord more likely to trigger secondary injury cascades, or, more susceptible to their effects.
Figure 4.5 Early responses of the E15 chick spinal cord to traumatic injury. A. E15 spinal cord – fixed 1 hour after spinal cord injury: Extensive haemorrhage is present at the site of injury, and at locations extending for about 2mm cranial to the injury site. White matter in close proximity to blood appears swollen and oedematous (arrow). B. E15 spinal cord 8 hours after spinal cord injury: Pooling of blood is apparent, and blood is observed several millimetres from the site of injury. The border between white and grey matter seems to provide a main route of blood transport from the injury site to more distal locations (arrows). Signs of cavitation are already present (asterisk). Scalebar = 1 mm.
Figure 4.6 Early responses of the chick spinal cord to traumatic injury (2). A. E15 spinal cord, 1 hour after spinal cord injury: image is of region cranial to lesion site. Blood originating from the site of initial trauma is seen extending to more cranial regions via the junction between white and grey matter (arrows). B. Area of normal white matter in the E15 chick spinal cord. C. White matter from injured spinal cord - 1 hour post-injury. Nearby bleeding has resulted in swelling of the white matter, severely disrupting its linear structure and possibly affecting function. Scalebar = 400 μm in A, 5 μm in B and C.
Figure 4.7A The response of the E15 chick spinal cord to injury after 24 hours. A. E16 spinal cord, injured at E15: Extensive cavitation and haemorrhage is present, extending for distances of over 5 mm. Substantial damage has occurred to spinal cord structure with both white and grey matter affected: grey matter morphology is particularly disrupted (asterisk). Here, vertebral displacement indicates the site of spinal injury, illustrating the cranial and caudal spread of secondary tissue damage. Scalebar = 1mm
Figure 4.7b H & E stained sections through an E15 spinal cord injury: 24 hours after transection. Image A is the most dorsal, D most ventral. Images are approximately 35, 92, 115, and 450μm apart. Scalebar = 1mm
Figure 4.8 Embryonic chick spinal cord – 3 days following injury at E15.  

A. Cavitation is extensive and extends for several millimetres 3 days post injury. Cellular debris (asterisk) appears to be becoming more separate and distinct from unaffected tissue.  

B. C. A smooth epithelial-like border is present around most of the cavity edge, separating the cavity (asterisk) from unaffected tissues (arrow).  

D. In some areas, the border between cavity (asterisk) and unaffected tissue (arrow) is not complete. Scalebar = 1mm in A, 100 μm in B, C, & D.
Figure 4.9 E15 spinal cord injury – 4 days post-lesion. A. Spinal cord cavity is well developed after 4 days. It has become increasingly fluid-filled (asterisk). B, C. The border between cavity (asterisk) and spinal cord (arrow) has become well defined and is complete. Scalebar = 1mm in A, 100 μm in B & C.
Figure 4.10 Second example of E15 spinal cord injury after 4 days. A large fluid filled cavity has formed (asterisk), with a solitary island of cellular debris (arrow). The border between cavity and spinal cord is well defined. B, C. Numerous axonal blebs (arrows) are present in the surviving white matter, typical of retracting axons following axotomy. Scalebar = 1 mm in A, 5 μm in B, & C.
4.2.4 The contribution of apoptosis following spinal cord injury

The results described above clearly indicate a difference in the extent of secondary tissue damage occurring between permissive and restrictive periods of development in the chick embryo. Secondary cascades typically result in necrotic cell death, due to a variety of cytotoxic mechanisms. However, recent evidence has suggested that programmed cell death may play a contributing role following injury to the spinal cord (Lou et al., 1998; Crowe et al., 1997; Liu et al., 1997).

To investigate whether apoptotic cell death may be involved in the injury responses of the chick spinal cord, I performed TUNEL analysis on longitudinal sections of injured spinal cord.

Following spinal cord injury at E11, TUNEL analysis confirmed that apoptotic cell death was occurring at the immediate injury site 24 hours after transection (Fig. 4.11A). In keeping with the effective repair of the spinal cord, and lack of secondary tissue death, no TUNEL positive nuclei were detected 4 days post-injury (Fig 4.11B). Uninjured control animals (n=2) had very few TUNEL positive cells within the spinal cord at E12 (not shown).

In the case of E15 spinal cord injury, TUNEL analysis indicated that an abundance of apoptotic cell death was occurring in the cord 24 hours after injury, affecting apparently all cell types and spreading cranially/caudally to the same extent as haemorrhage (Fig. 4.11C). Interestingly, apoptotic cell death was also present in the E19 spinal cord (the latest time point examined), following injury at E15. These cells were especially abundant in the tissue islands found within cavities, although TUNEL positive cells were also found in the surrounding regions of white matter. This observation is indicative of a constant and prolonged production of cell-death inducing stimuli via secondary mechanisms and seems likely to contribute to the failure of functional regeneration at non-permissive stages of development.
The results detailed above indicate a difference in the extent of apoptosis in response to spinal insult between restrictive and permissive regenerative states. In order to clarify exactly what this difference was, a detailed examination of the onset, and cessation of TUNEL following injury was performed. A number of time-points following spinal cord injury were examined for the presence of TUNEL positive cells above background levels. Results of these experiments are summarised in Figure 4.13.

Following either E11 or E15 spinal cord injuries, no TUNEL positive cells were detected earlier than 8 hours post-injury. This suggests that the positive cells were undergoing programmed cell death, rather than necrosis. The specificity of TUNEL for apoptotic over necrotic cells has been questioned by some researchers, however the delay in detection seen in this study is indicative of an active process. TUNEL is specific for blunt ended ‘nicks’ in DNA, a relatively late-stage event in the apoptotic pathway. It is probable that the enzymic cascade, beginning with extrinsic factors triggering cellular receptors and cumulating in cleavage and activation of caspases, would take several hours to occur. Morphological analysis of cells positive for TUNEL also supports the notion that apoptosis was occurring. The labelled cells were observed to be small and rounded, with evidence of nuclear condensation as confirmed by labelling nuclei with Hoechst dye which allowed clear visualisation of apoptotic bodies. Examples of typical nuclei undergoing programmed cell death from the vicinity of an E15 spinal cord injury are shown in Figure 4.12.

In addition to morphological confirmation, treatment with diethyl pyrocarbonate was attempted, as this has been shown to reduce the number of false positive cells seen when using the TUNEL assay (Stahelin et al., 1998). No obvious difference in number of TUNEL positive cells was observed between treated or untreated groups (not shown). For these reasons, it can be assumed that the cells detected by the TUNEL assay were indeed genuine apoptotic cells.
In the case of E11 spinal injuries, apoptotic cell death could not be detected 48 hours after the initial injury. This contrasts with the prolonged apoptotic response seen following E15 injuries, which still contained many apoptotic bodies 72 hours post-injury.

Clearly an elevated and prolonged apoptotic response occurs during the restrictive period of regeneration that seems likely to contribute to the failure of regeneration at this time.
Figure 4.11 The contribution of apoptosis to spinal cord injury at regeneration competent, and incompetent stages of development. A. E11 spinal cord injury, 24 hours after transection: TUNEL positive cells (green) are located at the immediate injury site, but do not extend more than 500 μm cranial or caudal to the lesion. B. E11 spinal cord injury, analysed using TUNEL 3 days post-injury: no positively labeled cells are present. C. E15 spinal cord injury, sectioned 24 hours after surgery, many TUNEL positive cells are present, extending for distances of several millimetres along the spinal cord. D. E15 spinal cord injury – 4 days post lesion: TUNEL positive cells are still abundant within cavities of the injured cord, and to a lesser extent in the surrounding regions. Scalebar = 500 μm in A-C, 200 μm in D.
Figure 4.12  High magnification view of apoptotic cell bodies from E16 spinal cord, injured at E15. Many pyknotic nuclei (arrowheads) are present (A-F), strongly indicative of apoptotic cell death. Scalebar = 1 μm in all panels.
**Fig 4.13** Onset of apoptosis following spinal cord injury at permissive or restrictive periods for regeneration.

Spinal cord transections were performed and embryos fixed after various time-points, then were sectioned and analysed by TUNEL. Sections were scored as being TUNEL positive (+), highly positive (++), or negative (-), compared to unoperated controls.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>2</th>
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<th>6</th>
<th>8</th>
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4.3 DISCUSSION

The results described in this chapter highlight important differences between the period when the chick spinal cord can and cannot effectively recover from spinal injury. Morphological analysis indicates that E15 spinal cord is affected to a much greater extent by secondary tissue damage than at E11. It seems likely that this fact will influence greatly the effectiveness of spinal repair. Most studies concerning spinal injury concentrate on specific factors that inhibit or promote axonal regeneration. Indeed in terms of offering hope to chronically affected patients with spinal injury this research is both useful and necessary. However the results described in this chapter highlight that early intervention is preferable. It seems likely that one of the major reasons the E15 spine cannot regenerate axons is that injuries sustained at E15 are intrinsically more severe than those at E11. E15 spinal cord may also contain more inhibitory factors preventing axonal extension, or less growth promoting neurotrophins, but it may also be the case that the extent of cell death may provide a far more imposing obstacle.

4.3.1 Secondary tissue damage in the chick spinal cord: implications for regeneration.

The results of this study indicate that secondary tissue damage, triggered by traumatic injury to the chick spinal cord is likely to be a major contributor to the failure of regeneration seen at late stages of development and adulthood. Furthermore, it is clear that vascular compromise plays a major role in the spread of neural injury beyond the initial injury site. Haemorrhage was widespread in the injured E15 cord, but was present at much lower levels at earlier developmental stages.

It is worth pointing out however that the observations made in this chapter were of a qualitative nature, and thus were open to subjective bias. Although the observed differences in morphology between E11 and E15 injuries were large, a more
quantitative method of analysis would clearly be preferable and allow more definite conclusions to be drawn. Other investigators have adopted various methodologies to generate such quantitative measurements. In mice, longitudinal midsaggital sections of spinal cord have been used to provide an estimate of total cavitation. Sections were selected that included profiles of the central canal rostral and caudal to the injury site. Of these, only the section with the largest lesion area was used for image analysis. Measurements were made by 2 blinded assessors. (Inman et al., 2002). This provides a standardised technique and allows rapid analysis of many samples, although presumptions are made that the cavities analysed are of a uniform shape and are of greatest volume at the centre of the spinal cord. Another method of analysis is to stain many sections and add the areas of cavitation together to estimate total volume. Takami et al. employed such an approach, staining every 5th slide using cresyl violet and using computer image analysis software to measure cavity volume (Takami et al., 2002). This approach can also be adopted using transverse spinal cord sections, although the total number of section analysed may be greater (Gorio et al., 2002).

Perhaps one of the most comprehensive analyses of cavity formation was adopted by Duerstock and Borgens. Serial sections from injured guinea pig spinal cord were used to construct a three-dimensional computer model. This allows accurate comparisons to be made between treatments and controls in respect to changes in spinal cavitation/tissue sparing (Duerstock et al., 2000; Duerstock and Borgens, 2002).

Adopting either of these approached to the data presented is one way in which these results could be improved. It should be noted however that for any method of measurement, a clear distinction must be made between ‘cavity’ and ‘normal’ tissue. The studies mentioned above were performed on animals with ‘mature’ spinal cord injuries; the timepoints examined ranging from 1 week to 8 weeks. In mature cavities, the boundary between normal and damaged tissue is clear: a new basal lamina is usually formed. Demarcation of the boundary of the cavity should be possible by immunostaining with laminin, a marker of these laminae. In this study however, the
distinction between damaged and spared tissue was often unclear, reflecting the point that cavitation was actively progressing. Only at timepoints of 4 days post injury did clear partitioning of the cavity seem complete. Prior to initiating any measuring protocol, clear definitions must be made, and these should be enforced by an observed blind to the injury type.

Thus the results described in this chapter must be taken at face value. Numerical measurements are required to permit statistical analyses, only then can assumptions be made concerning the significance of differences between injuries during different stages of development. Nevertheless, in all of the specimens examined in this study, the extent of damage seen along the rostro-caudal axis was elevated by at least 10 fold in E15, as compared with E11 injuries.

Given this, the observation that hemorrhage appears to be more widespread at restrictive periods could lead to a hypothesis implicating post-injury bleeding as the crucial factor influencing clinical outcome following traumatic spinal cord injury. What mechanisms are responsible for the elevated extent of hemorrhage observed at non-regenerating stages of development? It is possible that development of an extensive capillary network within the spinal cord around the transitional period of E13 could contribute to failure of regeneration. I have examined this further in Chapter 5.

That these vascular events are greatly enhanced during non-permissive periods for regeneration is potentially of great interest. Periods in chick spinal cord development were originally defined as ‘permissive’ and ‘restrictive’ to reflect differences in local tissue environment, affecting the capacity of severed axons to re-grow. Alterations in expression of various inhibitors of regeneration have been proposed as explanations for the generation of a non-permissive environment, although the contribution of secondary pathogenesis has not previously been considered.

The development of mature cavities within the injured chick spinal cord has not been previously described. Earlier studies by Shimizu described the formation of microcysts
following injury at E15, although morphological analysis is limited (Shimizu et al., 1990). The large cavities described in this study are typical of those found following spinal cord injury in the rat (Taoka and Okajima, 1998), and human (Kakulas, 1999b).

The results described in this chapter imply that propagation of initial injury, possibly initiated primarily by components of the blood, could contribute to, or define, the transition during development to a non-regenerating cord. This hypothesis is not incompatible with the concept of developmentally regulated inhibitors of regeneration becoming more abundant with age. It is entirely possible that massive extension in the extent of spinal injury would result in far greater availability of axon-repulsing molecules to the local environment. Indeed, following his experiments that indicated that one of the axon-inhibiting domains of the Nogo-A molecule was located intracellularly, Strittmatter proposed that the domain in question may only be active when released following damage to oligodendrocytes (GrandPre et al., 2000). Given this, inhibitory molecules need not be developmentally regulated to contribute to the transition from permissive to non-permissive environments; an increase in their bioavailability following injury would suffice. Indeed, results in chapter 7 indicate that Nogo-A and Nogo receptor, potent inhibitors of regeneration, are present during early embryogenesis.

Supporting this idea is the observation that some re-direction of axonal fibres was observed following E11 spinal injury (Fig 4.3), but not following E7 injury (Fig 4.1). It is possible that axon repulsing substance are present, and possibly the limited secondary injury response at E11 restricted their release into the local environment, allowing effective re-growth of fibres.

Secondary tissue damage mediated by vascular compromise following spinal injury has been previously described (Mautes et al., 2000), however significant secondary effects have not been identified in the chick. Overall, the pathology described in this chapter is remarkably similar to that in other experimental models, such as the rat, and is also
analogous to that seen in cases of human spinal cord injury (Kakulas, 1999b). For these reasons, the chick spinal cord may be considered of potential interest in future regenerative studies.

4.3.2 Apoptotic cell death following spinal injury

This study has demonstrated for the first time that apoptotic cell death is involved in the response to spinal trauma in the chick. It is also the first time that apoptosis has been examined in any model of embryonic spinal injury. These data support the hypothesis that apoptosis is of crucial importance in cases of CNS injury, and may represent a means of therapeutically improving prognosis following injury to the spine.

One potentially important point is the type of cell affected by the secondary injury induced apoptosis described above.

In these experiments apoptosis was largely limited to the injury site and to sites affected by secondary reactions. Following initial trauma, the spread of apoptotic cell death appears to encompass all cell types within the spinal cord. At more distal locations, TUNEL positive cells were concentrated around regions where blood had also reached, strongly suggestive of a causal relationship. The spread of blood fluids along the injured cord however was not uniform. Commonly streams were found along the boundary of white and grey matter. It is possible that physical cord characteristics, determining which cells are exposed to toxic infiltrates may influence the distribution of cell death following injury to a greater extent than individual cellular susceptibility.

Recent publications have examined the cell type and timeframe of apoptotic cell death in other models of spinal cord injury.

It has been shown that following weight-drop injury in the rat, there are waves of apoptotic cell death, reflecting differing cellular populations. Apoptotic neurons, detectable after 8 hours, were absent 24 hours after injury. Glial cells however were observed to undergo apoptosis at the immediate injury site for several days, and were
found at cranial and caudal locations in the white matter as late as 14 days post-injury (Liu et al., 1997). Lou and colleagues also identified apoptotic nuclei in rat following spinal cord weight drop injury, as positive cells were seen after 4 hours, but were absent after 24 hours (Lou et al., 1998). Lou's study however, did not identify the cells types involved, nor are the figures of sufficient resolution to distinguish between neuronal or glial cell death. Studies in rat brain again found apoptotic cell death following cortical impact (Newcomb et al., 1999). Levels of programmed cell death were maximal one day after injury, were reduced by 3 days, and absent 14 days post-injury. Other investigators have established that caspase-3 mediated enzymic cascades are involved in post-injury responses in the rat spinal cord (Springer et al., 1999). The likelihood that programmed cell death processes may affect pathological outcome was supported by experiments performed by Liu and colleagues. Administration of cyclohexamide, which blocks de novo protein synthesis, and thus the process of apoptosis, resulted in less severe injury characteristics following spinal cord weight drop injury in the rat (Liu et al., 1997).

Apoptosis of oligodendrocytes has been observed following acute spinal injury in the monkey and rat (Crowe et al., 1997). Furthermore apoptosis of oligodendrocytes has been observed at long distances from the injury site, and for long periods of time after injury (Li et al., 1999). The death of oligodendrocytes has been shown to correlate with axonal degeneration, as demonstrated by co-localisation with Beta-Amyloid Precursor Protein – an indicator of axonal injury (Casha et al., 2001).

Although precise identification of the apoptotic cell types was not established in my own study, it seems likely that many of the moribund cells were neuronal, especially at early time-points after injury. It is more contentious whether the late stage TUNEL positive cells observed after 4 days in the chick spinal cord injury model reflect a late dying population of oligodendrocytes triggered to die by degeneration of their associated axons. Firstly, the timescale differs from previous studies: Liu et al (1997), observed a late wave of glial cell death in the white matter beginning 7 days after
spinal cord injury. Such a late time-point was not examined in this study. However, degenerating axons are present in the 4-day post injury chick white matter, as illustrated by the presence of abundant axonal blebs and associated oligodendrocytes that could contribute to the apoptotic cell death seen 4 days after injury.

Another important point of note is that because much of the cell death in the injured spinal cord is apoptotic in nature, and as the apoptotic process takes around 8 hours to reach completion, the prospect of therapeutic intervention may exist. It may be possible to rapidly administer appropriate agents to either prevent apoptosis directly, or neutralise the toxic effects of specific noxious components of the secondary injury cascade. The observation that cell death continued up to 72 hours post injury in this model suggests that timely intervention could ‘rescue’ a great number of cells within the spinal cord. Even though research aimed at regenerating severed axons is making rapid progress, it may be advantageous to save them from damage in the first place by limiting the devastating effects of secondary injury.
CHAPTER 5. DEVELOPMENTAL ANALYSIS OF FACTORS INFLUENCING SPINAL CORD REPAIR AND REGENERATION
5.1 INTRODUCTION

The chick embryo is a classical model in the field of developmental biology and is generally well characterised in terms of morphological and molecular changes occurring during embryogenesis. It is apparent however that certain gaps are present in the literature; in particular, data concerning the later (E7-E18) stages of thoracic spinal cord development in the chick are scarce. To address this, I have examined the expression of a number of factors likely to be of key importance in the developmental transition from regeneration permissive to restrictive states in the chick embryo seen at this time.

Firstly, the extent of cell division within the spinal cord was examined between E7 and E18. An understanding of the proliferation dynamics of the cord during this crucial period may help explain why regeneration does not occur after E13. Does the ependymal canal lose the ability to produce new cells of neural lineage around the transitional period? A robust ependymal response is one of the mechanisms believed to permit effective repair following spinal injury in amphibian species (Benraiss et al., 1999; Zhang et al., 2000). Analysis of proliferation in situ also allows the identification of distinct cellular populations that may be expanding around the transitional period. Expansion of cell populations which may contribute to the inhibitory nature of the adult CNS are likely to play a role in the permissive/restrictive transition.

To investigate this further, the onset of maturation of the cellular populations of the spinal cord was also examined. Although the origin and maturation of oligodendrocytes within the chick spinal cord is well documented (Ono et al., 1995), specific information regarding terminal differentiation of neuronal cells, astrocytes, and blood vessels within the thoracic chick spinal cord is remarkably scarce.
Finally, the expression of factors known to be important during the processes of development and regeneration were examined around the same transitional period. The growth factors FGF2 and NGF are both heavily implicated in regenerative responses (see Chapter 1), although their contribution to regenerative failure in the chick remains undetermined. Similarly, chondroitin sulphate proteoglycans are known to be potent inhibitors of axonal regeneration, yet their distribution during the late stages of chick spinal cord development has not been fully investigated.

Overall, the chick spinal cord is poorly characterised with respect to many of the influences likely to affect the regenerative process around the transitional stage E13.1 aimed to address this by performing immunohistochemistry to detect proliferating cells (H3, PCNA), mature neurons (Neu-N), mature astrocytes (GFAP), blood vessels (HSPG, laminin), FGF2, and CSPG. RT-PCR analysis was also used to detect NGF mRNA.

5.2 RESULTS

5.2.1 Analysis of proliferation in the developing chick spinal cord

The population of dividing cells in the chick spinal cord was investigated in 2 ways. Initially, the presence of phosphorylated histone H3 was examined by immunostaining. H3 labels only cells that are currently in the M-phase of the cell cycle. The antibody detected a small population of cells and showed strong nuclear staining (Fig 5.1). The low numbers of labelled cells were insufficient to provide an indication of overall cellular proliferation. Proliferation was marked in the cells of the ependymal tube at the earliest stages examined (Fig 5.1A, B). At later stages, positively labelled cells were found chiefly in the developing white matter, although occasional labelled ependymal cells were detected (Fig 5.1C-F)
The Proliferating Cellular Nuclear Antigen is expressed by proliferating cells and has a longer half-life than H3. Expression is maintained through M and S phases of the cell cycle. This has the effect of labelling dividing cells, and those that have recently divided. PCNA analysis allowed a more general picture of proliferation within the spinal cord to be established.

In E8 spinal cords, strong labelling was observed in the ependymal layer, although other cells in the mantle and marginal zones were also stained (Fig 5.2A). By E10, many more cells of the marginal zone were positively labelled, particularly in ventral regions. The ependymal cells were also positive (Fig 5.2B). At E12, proliferation was markedly increased in the developing white and grey matter; many presumptive glial cells were present, and some blood vessels were also labelled (Fig 5.2C). This pattern of expression was similar in all later time points examined, with a gradual increase in levels of white matter and blood vessel staining (Fig 5.2D-F).
Figure 5.1 Immunohistochemical analysis of H-3 in the developing chick spinal cord. 

A. Transverse section of E8 spinal cord: positive staining (brown) is present in cells of the ependymal zone (arrow). Few cells are labelled in the marginal or mantle zones. 

B. High magnification view of (A), showing positive ependymal cells. 

C. E9 spinal cord showing positive staining in the ependymal layer, and in occasional marginal zone cells. 

D. E12 spinal cord: H3 staining is present in the developing white matter and in some grey matter cells. 

E. E13 spinal cord: staining is present in white matter. 

F. E17 spinal cord. Occasional positively stained cells are present in the grey and white matter. Ependymal cells are still positive at E17 as highlighted by insert. Scalebar =200 μm, except B =50 μm.
Figure 5.2 Immunohistochemical analysis of PCNA in the embryonic chick spinal cord. A. Transverse section of E8 spinal cord. Positively labelled cells (brown) are mainly present in the ependymal zone (arrow). Occasional cells are also stained in the mantle and marginal regions. B. E10 spinal cord: ependymal zone is positively labelled, as are numerous individual cells of the marginal zone, particularly in the most ventral aspect. C. E12 spinal cord: most cells of the developing white matter are labelled in addition to those of the ependyma. Blood vessels are also seen to express PCNA (arrow). D. E13 spinal cord: PCNA is chiefly detected in the developing white matter and ependymal canal. E. E15 spinal cord: white matter is robustly stained with many blood vessels labelled in addition to the ependymal cells. F. E17 spinal cord: PCNA expression is still strong in the white matter and ependymal cells. Scalebars = 200μm
5.2.2 Analysis of Neu-N in the developing chick spinal cord

The onset of maturity within neuronal populations of the spinal cord was investigated using anti-Neu-N antibody. Neu-N is a well-characterised antigen, which is present in a wide range of neuronal subtypes (Mullen et al., 1992). Of particular interest is the fact that it is only detectible in post-mitotic cells and as such can be considered a marker of neuronal maturation (Sarnat et al., 1998).

At the earliest point examined (E7), Neu-N positive cells were present in the ventral aspects of the spinal cord and in the DRG (Fig 5.3A). These positive cells most likely represent motoneuron and sensory neuron populations and indicate that they are effectively mature. There remain however, a considerable population of cells within the mantle layer that do not express Neu-N. These negative cells may reflect a population of immature neuronal cells or glial precursors. No positively labelled cells were detected in the ependymal, or marginal zone of any section examined, confirming the specificity of the staining. Examination of later developmental stages revealed that the number of Neu-N positive cells in the grey matter progressively increased with time (Fig 5.3A-C). By E12 (Fig 5.3D), all neurons expressed the marker protein, a pattern that was maintained in all other stages examined (Fig 5.3D-F).
Figure 5.3 Analysis of Neu-N in the developing chick spinal cord. A. E7 spinal cord: positive staining (brown) is present in ventral regions (arrows) and in sensory neurons of the DRGs (arrowheads). B. E8 spinal cord: labelled cells are found in more dorsal locations (arrow), in addition to prominent ventral staining and most cells of the DRG. C. E9 spinal cord: most cells of the grey matter and DGRs are positively labelled. D, E, F. E12, E15, E17 spinal cord: all neurons of the grey matter are stained. Scalebars=200 μm
5.2.3 Analysis of GFAP in the developing chick spinal cord

The onset of glial cell maturation in the developing spinal cord is a potentially crucial regulatory component of the response to spinal injury.Astrocytes respond in a well-documented fashion to trauma. Typically, they become ‘activated’, as detected by upregulation of the marker protein GFAP. Activated glial cells are known to be a key contributor to the cocktail of inhibitory molecules that characterises the non-permissive environment of the adult CNS following injury. Surprisingly the developmental point at which mature astrocytes appear in the chick spinal cord has not been established. Studies in the chick are restricted to post-hatching animals (Kalman et al., 1998), and thus the possible role of glial maturation affecting regenerative potential has yet to be addressed.

This shortfall was investigated by analysing GFAP expression in the developing chick embryo spinal cord by immunohistochemistry. Initial efforts using PFA, or Carnoy fixed tissue samples were unsuccessful. Unfixed cryosections of spinal cord were finally used to detect GFAP protein within the spinal cord from E11 to E17 using immunofluorescence. This procedure resulted in poorer morphology of spinal cord sections than obtained using paraffin embedding.

GFAP was present primarily in regions of white matter at E11 (Fig 5.4A). The protein was also detected in the Schwann cells associated with the DRGs. Subsequent analysis of later developmental stages revealed a similar pattern of expression; GFAP was found chiefly in white matter in E13-E17 spinal cords (Fig 5.4B-D). The extent of positive labelling appeared to increase with time, although this was difficult to quantify using fluorescent labelling.
Figure 5.4 Immunohistochemical detection of GFAP in transverse cryosections of embryonic chick spinal cord. A. E11 spinal cord: GFAP staining (green) is found in peripheral regions of white matter, and in Schwann cells located in the DGR. Occasional staining was evident in cells of the grey matter. Counterstain is Hoechst nuclear dye (blue). B. E13 spinal cord. Positive labelling is found in the white matter to a greater extent than seen at E11. C. E15 spinal cord: The entire white matter region is strongly labelled. D. E17 spinal cord: GFAP labelling is still largely restricted to white matter, although several positive cells are present in the ventral grey matter. Scalebars = 200µm
5.2.4 Analysis of FGF2 in the developing chick spinal cord

As detailed in the introductory chapter, evidence exists implicating a lack of neurotrophic support in the failure of spinal cord regeneration. One of the molecules most strongly believed to play a role is basic fibroblast growth factor (FGF2). FGF2 is known to be up-regulated following spinal cord injury at both the protein (Mocchetti et al., 1996), and the mRNA level (Follesa et al., 1994). In addition, increases in FGF2 expression following tail amputation in the newt are known to be associated with a proliferative response by neural progenitors (Zhang et al., 2000). FGF2 knockout mice show defects in wound healing (Ortega et al., 1998), and injections of FGF2 following spinal cord injury help boost survival of motoneurons (Teng et al., 1999; Teng et al., 1998).

Given this, I decided to investigate whether the distribution of FGF2 during development was consistent with a potential role in the transition from regeneration competent to incompetent states.

FGF2 protein was initially detected within the neuronal populations of the E8 spinal cord (Fig 5.5A). No positive labelling was observed in the marginal zones (Fig 5.5B). The population labelled at E8 is likely to represent a largely immature neuronal cell type: comparisons with Figure 5.3 indicate that few neurons express Neu-N at this stage. Indeed, one of the known functions of FGF2 during development is to promote neuronal progenitor cell differentiation (Murphy et al., 1990).

The E9 spinal cord has a similar pattern of FGF2 labelling to E8 (Fig 5.5C). Large neuronal cell bodies in the ventrolateral regions of the grey matter were strongly stained (Fig 5.5D). These large cells were similarly labelled by Neu-N at this stage (Fig 5.3B). These cells most likely are motoneuron populations of the cord, which are one of the first neuronal populations to differentiate (Hollyday and Hamburger, 1977). This
image also illustrates that the sensory neurons of the DRG are also FGF2-positive. These neurons are derived from neural crest, and are not of ependymal origin. The regulation of cell division of neural crest cells and their derivatives by FGF2 has been previously described (Murphy et al., 1994).

Change occurs in the expression pattern of FGF2 by E12. At this stage, positive labelling is present in the marginal regions of the spinal cord (Fig 5.5E). The ventral areas of the white matter are especially strongly stained (arrow). This staining is likely to be due to the birthing and maturation of glial populations occurring at this time. The production of oligodendrocytes occurs much earlier in the avian spinal cord (Ono et al., 1995), and thus the FGF2 populations observed here are likely to be astrocytic, or to represent changes in oligodendrocyte gene expression associated with the onset of myelination. The ependymal canal is also still strongly positive at this stage: combined with the continued expression of PCNA in these cells shown earlier (Fig 5.2C), this suggests that the proliferation of neural progenitors is maintained.

By E15, the levels of FGF2 in the grey matter declined so that white matter FGF2 was more strongly labelled (Fig 5.5G) Individual large neuronal cells within the grey matter, probably motoneurons (based on cell shape and ventral location), maintained strong FGF2 expression, as did the ependymal canal (arrows). The distribution of FGF2 positive cells at E17 was similar to that seen at E15. Again, grey matter levels of FGF2 were lower than at earlier stages of development although individual cells maintained higher levels (Fig 5.5H). The ependymal canal continued to express FGF2.

Overall, the levels of FGF2 in neuronal populations decreased after the transition from regeneration permissive to restrictive states, while the levels of white matter expression were elevated at stages later than E12.
Figure 5.5 Immunodetection of FGF2 in transverse sections of embryonic chick spinal cord. A. E8 spinal cord: FGF2 labelling is restricted largely to the developing grey matter. The white matter is unstained (arrow). B. High magnification of E8 spinal cord showing differential staining in white matter (w) and grey matter (g). C. E9 spinal cord: pattern of staining is similar to that recorded at E8. Large ventrally located cells are particularly well stained (arrow), as are the DRGs. D. High power view of ventrally located grey matter. E. E12 spinal cord: in addition to grey matter labelling, FGF2 is detectable in the white matter, especially in ventral regions (arrow). The cells of the ependymal canal are strongly positive. F. High power view of boundary between grey and white matter. G. E15 spinal cord: intensity of grey matter labelling has declined, although staining is still present in cells of the ependymal canal (arrow) and in ventral neuronal populations. H. E17 spinal cord: intensity of white matter staining is stronger than grey matter labelling. Scalebars = 200μm, except B, D, F which are 50 μm.
5.2.5 Analysis of heparan sulphate proteoglycans in the developing chick spinal cord.

Heparan sulphate proteoglycans (HSPGs) have been identified as crucial components in FGF-signalling pathways. Referred to as low-affinity receptors, expression of HSPGs are crucial for FGF activity. Given this, differential HSPG expression during embryogenesis could contribute to differential regenerative responses by modulating FGF-signalling.

For this reason, HSPG expression was examined in the developing chick spinal cord. The HSPGs however reflect a vast family of related molecules, differing in extent and complexity of side chains and core proteins. The antibody used in this study detected only a distinct sub-population of HSPGs. Unfortunately; this population was largely absent from the spinal cord at the stages of development examined in this study (E7-E17- Fig 5.6). Positive staining was found on the pial surface throughout.

Interestingly, blood vessels were found to be strongly labelled only at a distinct point of development, from E14 to E15 (Fig 5.6D-E). FGF2 signalling is crucial in angiogenesis, and this transient wave of HSPG expression in blood vessels could reflect an important stage in blood vessel maturation.

Overall, the specificity of the antibody used in this analysis to particular HSPG populations did not permit any general conclusions to be drawn concerning the prospective role of HSPGs in the regenerative transition seen in the chick spinal cord during development.
Figure 5.6 Immunohistochemical detection of heparan sulphate proteoglycan in transverse sections of embryonic chick spinal cord. A. E7 spinal cord: only the ventral aspects of the pial membrane are positive (arrow). B. E10 spinal cord: staining is similar to E7. Insert shows high magnification view of ventral pial surface (arrow). C. E11 spinal cord: expression in the pial membrane has extended to more ventral regions. D. E14 spinal cord: positive labelling in the pial membrane is more intense than previous stages of development. The pial membrane staining now encompasses the entire circumference of the spinal cord. A few blood vessels are also faintly positive. E. E15 spinal cord: the pial membrane is strongly labelled, as are many blood vessels within the grey and white matter (arrows). F. E17 spinal cord: no blood vessels are positively stained at this stage of development, although the pial membrane is still labelled. Scalebars = 200 μm.
5.2.6 Analysis of NGF in the developing chick spinal cord.

Application of NGF following spinal cord trauma results in improved prognosis (Bloch et al., 2001; Romero et al., 2001). NGF also plays important roles during neural development, being of particular importance in mediating the survival of sensory neurons, specifically those with axons that are unmyelinated, or possess only a thin layer of myelin (Bibel and Barde, 2000). Unlike FGF2, NGF is not up-regulated following spinal injury in the adult rat (Follesa et al., 1994). I therefore hypothesised that higher NGF levels during early development, as compared to later developmental stages, may contribute to the favourable environment for axonal re-growth at this time. This was investigated by RT-PCR analysis of NGF expression at various points during development. cDNA from chick spinal cords of E11, E13, and E15 was synthesised. Each sample was prepared from a pool of 3 spinal cords from different animals. It was observed that NGF levels increased over the transitional period defining the regenerating / non-regenerating chick spinal cord (Fig. 5. 7). This suggests that absence of NGF in the spinal cord itself after E13 is not a contributing factor in explaining regenerative failure.

5.2.7 Analysis of chondroitin sulphate in the developing chick spinal cord.

Chondroitin sulphate proteoglycans are known to be potent inhibitors of axonal growth and regeneration. They have been demonstrated to be upregulated following injury to the spinal cord (Lemons et al., 1999; Zuo et al., 1998a). Indeed adult DRG neurons have been shown to extend axons for long distances in the normally inhibitory environment of the adult spinal cord providing that transplantation is atraumatic, and no CSPG is produced by astrocytes around the site of transplantation (Davies et al., 1999). This research implicates CSPGs as being the major inhibitory influence following CNS injury. In addition, enzymatic degradation of CSPGs on sections of adult
spinal cord increases the extent of axonal growth of DRG neurons cultured on it (Zuo et al., 1998b).

Previous studies have examined the absolute quantities of chondroitin and heparan sulphate in the chick spinal cord and reported an elevation in the ratio of CSPG:HSPG of 50% over the transitional period around E13 (Dow et al., 1994). The cellular location of CSPGs over this developmental period however has not been determined. Expression of CSPGs in different cell types, in addition to quantitative differences, could result in marked alterations in the response of the spinal cord to injury.

To address this issue, immunohistochemical analysis was performed on transverse sections of chick spinal cord from developmental stage E9 to E17 using the antibody CS-56. At E9, positive immunostaining was noted in the marginal zone of the spinal cord, although labelling was weak (Fig 5.8A, B). The levels of staining in the marginal areas gradually increased with development (Fig 5.8C, D), and by E12, all axonal fibres in the developing white matter were positive (Fig 5.8E, F). At later developmental stages, CSPG immunoreactivity was observed to spread to areas of the grey matter (Fig 5.8G, H).
Figure 5.7 RT PCR analysis of nerve growth factor in the embryonic chick spinal cord. cDNA from spines of E11, E13, and E15 embryos were amplified using NGF-specific primers. GAPDH primers were used as controls to standardise cDNA levels. Distilled water was used as a negative control. Ladder is 1Kb.
Figure 5.8 Immunodetection of chondroitin sulphate proteoglycan in the embryonic chick spinal cord using the antibody CS-56. A. Transverse section of E9 chick spinal cord: CS-56 positive cells are present in the DRG, and in the marginal regions of the spinal cord (arrows). B. High magnification of E9 white matter. C. E10 spinal cord: pattern of labelling is similar to that seen at E9, with more ventral staining evident. D. High power view of ventral aspect of E10 spinal cord. E. E12 spinal cord: CSPGs are detected in all areas of the developing white matter. F. High magnification of E12 white matter, staining is also evident in the pial membrane (p). G. E15 spinal cord: All regions of the spinal cord are equally well labelled by CS-56. H. E17: The widespread expression pattern is maintained at later stages of development. Scalebars = 200 µm, 50 in B, D, F
5.2.8 Blood vessel development in the chick spinal cord

From the results described in Chapter 4, it is apparent that the response of the chick spinal cord to injury differs significantly between permissive and restrictive states. One of the most striking observations made was that the volume of blood in the immediate vicinity of the injury site was much greater during the restrictive period. Secondary tissue damage can extend and worsen the extent of CNS injuries through a plethora of molecular cascades, many of which are associated with haemorrhage and loss of vascular integrity. Numerous potentially toxic substances could be delivered to sites of neuronal injury via leakage from ruptured vessels. One question, which remains unanswered, is the issue of whether a simple escalation in number, size, or arborisation in spinal capillaries could contribute to the loss of regenerative potential seen during development.

To address this, the contribution of angiogenesis to the transition from permissive to restrictive states was investigated. Firstly, RT-PCR was performed to analyse the degree of vascularisation of the spinal cord at different developmental stages. The endothelial cell marker QUEK-1 was chosen as a suitable gene for investigation as it is highly specific to endothelial cells (Wilting et al., 1997; Eichmann et al., 1993). The levels of QUEK-1 transcript were seen to increase with development, indicating a rise in number of blood vessels corresponding with the transition from permissive to restrictive states (Fig 5.9).

To investigate this further, I performed immunohistochemistry on Carnoy fixed, paraffin embedded transverse sections of chick spinal cord using an antibody specific to laminin. Laminin is a crucial component of basal laminae and has been used by other investigators to label blood vessels in situ (Casella et al., 2002). The anti-laminin IgG strongly labelled all blood vessels in the chick, in addition to the pial membrane, and muscle. Analysis of spinal cords from various stages of development revealed a
significant elevation in numbers of blood vessels with time (Fig 5.10A-F). This was confirmed by measurements of blood vessel surface area using OpenLab software as described in the Methods chapter. Total percentage surface area of blood vessels in the spinal cord increased exponentially during the period of development corresponding with the transition from regeneration competent to incompetent stages (Fig 5.11).
Figure 5.9 Expression of QUEK-1, the avian homologue of the VEGF (vascular endothelial growth factor) tyrosine kinase receptor Fik-1 was determined by RT-PCR analysis of normal chick spinal cord at different developmental stages. Note the increase in QUEK-1 mRNA with development.
Figure 5.10 Immunohistochemical detection of laminin in transverse sections of embryonic chick spinal cord. A. E7 spinal cord and surrounding tissues: spinal cord staining is largely restricted to radial processes originating from the ependymal zone. B. E9 spinal cord: the pial surface is clearly labelled (p), as are the DRGs. A few blood vessels are evident in the spinal cord C. E11 spinal cord: pial membranes and ependymal canal are labelled, as are a few small blood vessels (arrow). D. E13 spinal cord: Several large blood vessels are present within the white and grey matter (arrows). E. E15 spinal cord. Many positively stained blood vessels are present which are elaborately branched. F. E17 spinal cord: The extent of blood vessel branching is much greater than earlier stages of development. Scalebar = 0.5 mm
Fig 5.11  Analysis of blood vessel surface area with development in the embryonic chick spinal cord.

Transverse sections of chick spinal cord were stained using anti-laminin antiserum; total percentage blood vessel surface area was then calculated as compared with total spinal cord area using OpenLab software as described in Chapter 3. Results are expressed graphically ± standard error.
5.3 DISCUSSION

5.3.1 Neural maturation and regeneration

An understanding of the maturity of the chick spinal cord is crucial in drawing conclusions regarding regenerative responses. The process of neurogenesis is complete at early stages of development: 95% of lateral column motoneurons are born by E4, with the remainder appearing by E6 (Hollyday and Hamburger, 1977). The data presented here however demonstrates that the chick spinal cord is still highly proliferative at later stages of development.

Of particular interest are the ependymal cells of the spinal cord, which continue to divide at E17, although to a lesser extent as clearly demonstrated by H3 reactivity. This reflects the production of glial cells, which are believed to arise from immature neuro-ectodermal cells. These are born in the ependymal zone, and migrate laterally prior to further division and terminal differentiation (Goldman, 2001). Ependymal cells are thought to be key regulators of the effective repair processes initiated following spinal cord injury in amphibian species (Egar and Singer, 1972; Benraiss et al., 1999; Zhang et al., 2000). It remains to be determined whether the ependymal canal of the chick embryo can contribute to regeneration at early stages of development, perhaps by producing new neurons in response to local damage.

Surprisingly, this study is the first to examine GFAP expression in late stage chick embryos. My results indicate that GFAP is present at E11, mainly in the white matter of the spinal cord, and that this pattern is maintained until E17. These results suggest that mature astrocytes are present in the spinal cord at times when regeneration can occur. Such information is vital in the creation of hypotheses governing the generation of a non-regenerating spinal cord around E13.
GFAP is also present much earlier in development, as it is expressed by radial glia (Holder et al., 1990). These cells line the ependymal canal and guide proliferating neurons to more lateral locations along radial fibres. Recent evidence has suggested that radial glia may also be involved in neurogenesis (Malatesta et al., 2000; Noctor et al., 2001). Radial glia are generally not maintained throughout development; the only GFAP positive cells in the adult CNS are mature astrocytes (Edwards et al., 1990).

Astrocyte reactions have been implicated in the generation of a non-permissive environment following spinal injury. It is therefore of interest that astrocytes are present in the chick spinal cord at periods when effective regeneration can occur.

Astrocytes participate in the generation of physical and chemical barriers to axonal growth (Fitch and Silver, 1997b). They are known to become activated following spinal injury, and to produce inhibitory substances, such as chondroitin sulphate proteoglycans. Indeed, when astrocytic activation is avoided, neurons can extend processes on normally non-permissive substrates (Davies et al., 1997; Pettigrew et al., 2001; Pettigrew and Crutcher, 1999).

The observation that mature astrocytes are present at times when the spinal cord can effectively self-repair indicates either that: the contribution of astrocytic reactions to the failure of spinal cord regeneration is not absolute, or that the astrocytes do not yet react in the same manner as that which occurs following injury to the adult CNS. Alternately, intrinsic properties of the embryonic spinal cord may allow re-growing axons to overcome any inhibitory barriers created by astrocytes.

The morphological observations made in Chapter 4 indicate that no obvious glial response occurs following E11 spinal injury. Thus, it may be likely that although the astrocytes express GFAP at this time, they do not yet react to injury to the same extent as during adulthood, or non-permissive periods of development.
It was also noted that PCNA was present in the white matter at higher levels at developmental stages later than E13; it is likely that many of these dividing cells were astrocytic (Goldman, 2001). It is possible that this late surge in astrocyte numbers could contribute to the inability of the spinal cord to regenerate at these stages.

5.3.2 FGF2 in development and regeneration

The results described in this chapter indicate a change in localisation of FGF2 in the chick spinal cord occurring over the transitional period for spinal cord regeneration. At stages prior to E13, FGF2 is chiefly found in neuronal populations, whereas during the non-permissive period, FGF2 is more abundant in white matter. Such a change may have implications for the regenerative response of the chick spinal cord.

The appearance of FGF2 in white matter coincides with PCNA labelling in this region. Thus, the positive cells are likely to represent a proliferative population at this stage of development. The most likely group of cells responsible are astrocytes as discussed above.

The level of FGF2 present in neuronal cells also appears to decline around the transitional period. This may have implications upon neuronal survival following injury. Perhaps local concentrations of growth factors, including FGF2, are sufficient at permissive stages to permit survival of most neurons. Indeed, application of FGF2 following spinal injury has been shown to enhance neuronal survival and promote some functional repair (Teng et al., 1998; Teng et al., 1999; Rabchevsky et al., 1999; Rabchevsky et al., 2000). Also, in the amphibian spinal cord, upregulation of FGF2 is associated with the proliferation of neural progenitor cells within the ependymal canal (Zhang et al., 2000).

It is of interest that in the adult rat, the normal response following spinal cord injury is an up-regulation of FGF2 in the spinal cord: mRNA levels are increased by 6 hours,
and protein is found at greater concentrations 1 day post-injury (Mocchetti et al., 1996; Follesa et al., 1994). Similar results have been obtained following cortical brain injury in the rat (Frautschy et al., 1991). This demonstrates that simple elevation of FGF2 is not sufficient to promote successful regeneration. It is likely that timing is important. It may be necessary for FGF2 to be present immediately following injury for beneficial effect. Such a role would implicate the growth factor in neuronal survival, rather than active promotion of nerve outgrowth. Indeed, FGF2 does promote survival of certain neuronal subtypes in vitro, and can also inhibit neuronal apoptosis (Pataky et al., 2000; Miho et al., 1999).

Finally it should be remembered that the influence of FGF2 following CNS injury may not always be beneficial. It is feasible that elevated levels of FGF2 following injury may contribute to the formation of glial scars via astrocyte activation (Eclancher et al., 1996; Smith et al., 2001).

5.3.3 Heparan Sulphate Proteoglycans and spinal repair

The nature of the HSPG antibody used in this study did not allow definite conclusions to be drawn concerning the possible role of these proteoglycans in the transition from regeneration competent to incompetent states. The particular molecules identified were largely specific to the pial membranes, and were also visible on blood vessels during a short window of development. Other studies using the same antibody have also described staining mainly on basal lamina (Oshiro et al., 2001). The 10E4 epitope is associated with the sulphate chains of the HSPG molecule, and treatment with heparitinase abolishes reactivity (David et al., 1992). Changes in ratios of heparan to chondroitin sulphate proteoglycans have been demonstrated to correlate with transition from permissive to restrictive states in the embryonic chick (Dow et al., 1994). The results presented in this chapter however would suggest that this effect is due to elevated levels of CSPG, rather than diminished HSPG activity (see 5.3.5).
5.3.4 A role for nerve growth factor?

Absolute levels of NGF also increased in the spinal cord from E11 to E15. This result suggests that high concentrations of NGF at permissive stages are not a significant factor in determining whether regeneration will occur. The E15 spinal cord, which cannot regenerate, has more NGF than the E11 cord. It is possible that the cellular location of NGF in different cell types could contribute to differential effects at different stages, although this was not investigated in this project. The absence of a functioning NGF-specific antibody did not permit detailed in situ analysis.

5.3.5 Chondroitin Sulphate: implications for repair

CSPGs are known to play crucial roles in development, particularly in processes such as axonal guidance. The results described in this chapter reveal that in the permissive period for regeneration, CSPGs are largely restricted to the marginal zones of the developing spinal cord. At times where regeneration does not occur, CSPGs can be additionally detected in the neuronal populations of the spinal cord. Are these results consistent with a role for CSPGs in the transitional switch from permissive to restrictive states? There is no doubt that CSPGs are present within the spinal cord at times when it can regenerate, albeit at lower levels. It is possible therefore, that the difference between the two regenerative states may be a quantitative one, and that there is simply more CSPG around later in development, preventing further axonal growth. Indeed at stages of development where axonal growth is complete, it would be beneficial to prevent further aberrant connections being made. This is suggestive of a model where neuronal connections are 'cemented' in place once complete.

Another possibility is that the location of CSPGs within the spinal cord could be more important than overall levels of expression. The specific cell types producing CSPGs may be different in permissive and restrictive states. The results presented in this chapter indicate that this is indeed the case. In the permissive stages, CSPGs can be
found only in the marginal regions, restricting its expression to oligodendrocytes or astrocytes. This is supported by the data presented earlier demonstrating onset of GFAP expression in the white matter around the same point of development. Taken together, these data could represent the onset of astrocyte maturation at this time, with an associated increase in CSPG expression. Such a maturation of glial cells during development may play a major role in the prevention of effective regeneration. CSPGs are clearly of significance in preventing regeneration in the adult spinal cord, as demonstrated by a recent study in which degradation of CSPG at the site of spinal injury resulted in enhanced functional recovery (Bradbury et al., 2002). The appearance of CSPGs and GFAP at this time also coincides with the upregulation of FGF2 within glial populations of the white matter. This also could have significant implications for the outcome of spinal injury, given that FGF2 in astrocytic populations may have non-beneficial effects following CNS trauma; glial scarring may be triggered presenting additional barriers to regeneration.

In the restrictive stages however, in addition to intensification of white matter staining, the grey matter is positive, suggestive of neuronal staining. Of course, further validation is necessary in the form of double immunostaining to confirm exactly which cell types are expressing CSPG in each case.

Although the cell types expressing CSPGs have not been specifically identified, the results detailed here indicate a definite difference in quantity and location of staining between permissive and restrictive states. Given the inhibitory nature of CSPGs in terms of neurite outgrowth, and the published differences in relative levels of CSPGs:HSPGs observed at these stages (Dow et al., 1994), it seems likely that CSPG expression does contribute to the establishment of a non-permissive environment during embryogenesis.
5.3.6 Blood vessel development and secondary tissue damage

The results detailed in this chapter indicate that the spinal cord's blood supply in the chick embryo is established early in development, but becomes much more elaborate and branched particularly after E13.

The findings of my experiments are in keeping with previous research. The last investigation examining blood vessel development in the chick CNS was performed in 1946: animals were perfused with dye allowing visualisation of the vasculature (Feeney and Watterson, 1946). Initial protrusion of blood vessels into the spinal cord was noticed at E6 in this study, with subsequent arborisation and branching through development, although no quantification was performed. The application of modern staining procedures has complemented this vintage data, providing a more complete picture of the dynamics of vascular development in the embryo.

The results described above and in the previous chapter provide strong circumstantial evidence implicating a role for blood vessel development in regenerative failure at late stages of development, and in adulthood. The surface area of blood vessels in the embryonic cord increases exponentially around the transitional period of development, coinciding with an elevation in injury-induced haemorrhage and cell death (Chapter 4). It could be hypothesised that increased bleeding after trauma at restrictive periods of development directly results in cell death, and thus prevents effective regeneration.

What components of blood could possibly be involved in these cytotoxic processes?

Simple osmotic imbalance may account for some cell lysis and necrosis, although many specific cytotoxic components of blood plasma have been identified. Members of the serine protease family of proteins, such as thrombin, are known to be neurotoxic. These molecules are key regulators of the clotting cascades necessary for blood coagulation. Experiments using E5 chick motoneuron cultures demonstrated that
thrombin induced caspase-3 dependent apoptotic cell death via interactions with the thrombin receptor PAR-1 (Turgeon et al., 1998). Similarly, thrombin can initiate programmed cell death in rat neurons and astrocytes (Donovan et al., 1997), and in various human tumour cell lines (Ahmad et al., 2000). Further experimentation is required to test whether changes in blood composition with development, or simply the presence of blood itself, are responsible for propagation of secondary injury reactions. If the blood of an E15 chick is injected into the site of an E11 spinal injury, will there be an increase in secondary tissue damage? If cytotoxic components of blood are developmentally regulated. E15 blood would be expected to promote much more cell death than that taken from E11 embryos.

Overall, it seems that the connection between blood vessel development and enhanced secondary reactions, leading to cell death- some of which is apoptotic, is clear. That this leads directly to failure of successful regeneration has not been examined in this study, although this seems likely.
CHAPTER 6. MANIPULATION OF GROWTH FACTORS IN THE SPINAL CORD.
6.1 INTRODUCTION

One of the more attractive prospects for future therapeutic intervention following spinal cord injury is the development of pharmacological treatments aimed at minimising initial trauma and secondary damage, and enhancing the regenerative capabilities of the spinal cord. Administration of the synthetic corticosteroid methylprednisolone is the only current therapy for acute spinal injury and is believed to improve outcome by reducing secondary injury processes, although the precise mechanism of action is largely unknown, and the agent's efficacy has recently been questioned (Bracken et al., 1990; Taoka and Okajima, 1998; Rabchevsky et al., 2002).

Methylprednisolone treatment is by no means an actual cure, and much scope exists for improving the context of the damaged spinal cord in terms of cell survival and axonal regeneration.

As described previously, growth factors are believed to play crucial roles in the injured spinal cord (Follesa et al., 1994; Mocchetti and Wrathall, 1995). Generally, the effect of factors is beneficial; they can both enhance neuronal survival, and stimulate axonal regeneration (Miho et al., 1999; Schnell et al., 1994).

Given the effects of FGF2 on regeneration, both in vitro, and in vivo (Murphy et al., 1990; Pataky et al., 2000; Teng et al., 1998; Teng et al., 1999), I attempted to pharmacologically manipulate its expression in the chick spinal cord to improve prognosis following spinal cord injury at non-permissive periods of development.

Recent work has demonstrated that the benzodiazepine diazepam can elevate levels of FGF2 in the rat spinal cord, at the mRNA and protein level (Gould et al., 1999). Similar results have also been obtained in the rat brain (Gomez-Pinilla et al., 2000). Given these data, I attempted to duplicate this effect in the chick embryo.
6.2 RESULTS

6.2.1 Diazepam elevates levels of FGF-2 mRNA in the embryonic spinal cord

E14 chick embryos were administered diazepam by topical administration to the chorioallantoic membranes. The agent was given at a final concentration of 10 mg / kg; control animals received vehicle alone, or no treatment. Chicks were sacrificed 24 hours after treatment in keeping with previous studies and a 1 cm region of thoracic spinal cord was removed for RNA extraction. RT-PCR was performed on each mRNA sample using primers designed to amplify FGF2 and GAPDH. Densitometry analysis was performed after gel separation of PCR products; the amount of FGF2 mRNA in each sample was then expressed as a ratio of GAPDH mRNA to allow for small variations in total cell number. Results are shown in Figure 6.1. Chick embryos treated with diazepam had significantly more FGF2 mRNA than untreated, or vehicle treated controls (p=0.03). It was noted that DMSO (vehicle only) treated animals seemed to have lower expression of FGF2, although this was not significant.

In order to establish whether the spinal cord's response to diazepam was developmentally regulated, the experiment was repeated using younger, E10 chick embryos (Fig 6.2). In this case, although the overall pattern was similar to the effect seen at E14, diazepam had no significant effect on FGF mRNA expression.
Figure 6.1 The effect of diazepam administration on FGF-2 mRNA levels in E14 chick spinal cord. One day after administration spinal cords were collected and RT-PCR performed. Bands on gel were measured using a densitometer. Plot of ratios between FGF-2 and GAPDH mRNA indicates that diazepam induces expression of FGF-2. Ratio of FGF2:GAPDH following diazepam administration (n=4) was significantly elevated (p=0.03) compared to untreated (n=4), or DMSO treated (n=3) animals. Bars indicate standard errors of mean.
Figure 6.2. The effect of diazepam administration on FGF-2 mRNA levels in E10 chick spinal cord. Experiment was repeated as for E14. mRNA was collected from diazepam treated (n=6), untreated (n=5), or DMSO only (n=4) chick spinal cords. RT-PCR analysis was performed using specific primers to amplify FGF2 and GAPDH. No significant difference was detected between the 3 groups.
6.2.2 Diazepam Induced Elevation In FGF2 Does Not Improve Prognosis Of Spinal Cord Injury

Given the observed effect on growth factors levels seen at E14 by administration of diazepam, I investigated whether increasing FGF2 levels in this way may provide some form of beneficial effect following spinal cord injury at E15 (i.e. during the non-permissive period for regeneration). Given the previously described severity of spinal cord injuries performed at E15 (see chapter 4), and the known roles of FGF2 cell signalling on cell survival, I examined the effect of diazepam treatment on survival of neuronal cells by analysing spread of secondary injury damage and extent of apoptotic cell death.

Chicks were administered diazepam at E14 as described above, prior to spinal cord injury at E15. Animals were then sacrificed 24 hours later and vertebral columns removed, fixed, and processed for histology.

H & E analysis of chicks treated in this way showed no noticeable reduction in extent of spinal injury (n=5) as compared to normal, untreated spinal cord injuries (Fig. 6.3). Indeed, the overall extent of damage, and degree of cavitation appeared greater in diazepam treated chicks, although this was difficult to quantify.

To assess whether diazepam treatment actually worsened spinal cord injuries, E10 diazepam treated animals were operated upon at E11, a stage where effective regeneration can occur. Again the spinal cords were dissected and fixed after 24 hours, before processing for histological analysis.

In diazepam treated E11 injuries (permissive period for regeneration), spinal cord damage was observed to be much greater than in untreated embryos (n=8) (Fig. 6.4A, B). Disruption to normal spinal architecture was evident for long distances caudal, and cranial to the initial injury site. TUNEL analysis revealed that apoptotic cell death was
also greatly elevated in drug treated animals (Fig 6.4 C, D): positive cells were located at sites distant from the initial injury. It was however noted that two out of six animals treated with vehicle only also had extension of the local injury site, as compared to untreated controls, suggesting that DMSO may contribute to the effect (Fig 6.4E, F).

Given however that the E11 spinal cord is capable of functional recovery following spinal injury, it was investigated whether the enhanced injury caused by diazepam/DMSO administration could be successfully repaired. Would the haemorrhage and cell death seen 1 day after injury be transient and absent at later stages of recovery?

To answer this, E11 injuries were examined after 4 days of recovery. These spinal cords still showed extensive disruption, characteristic of an injury performed during the non-permissive state. Cavities were seen to form, and many retracting axonal blebs were present (Fig 6.5E). These morphological observations were indicative of late stage injuries seen following E15 transection, and appeared similar to adult injuries performed in the rat or human. Although later stages of development were not investigated, it seems unlikely that this pathology would be reversed by adulthood.
Figure 6.3 The effect of prior diazepam administration on pathology of E15 spinal cord injury. A. E16 chick spinal cord, injured at E15. A large cavity (asterisk) is present in the spinal cord containing cellular debris. B. Second example of E16 spinal cord injured at E15. The resultant cavitation (asterisk) extends for several millimetres. Scalebar = 1mm
Figure 6.4 Pathology of spinal cord injury following prior administration of diazepam/DMSO at E10. A. E12 spinal cord, injured at E11: Signs of secondary tissue damage are present. There is evidence of bleeding (asterisk), and the spinal cord caudal to the site of injury appears swollen and oedematous (arrowhead). Point of injury is indicated with arrow. B. Second example of E12 spinal cord, injured at E11: Again, haemorrhage is present, and caudal spinal cord is displaying signs of degeneration (arrowhead). C. TUNEL of E12 spinal cord, injured at E11. Positively labelled cells extend cranially and caudally from the point of injury. D. Second example of TUNEL from E12 spinal cord, injured at E11: This view demonstrates the extent of caudal cell death. Arrow indicates point of injury. E. Control animal treated with DMSO alone labelled by TUNEL. Cell death is apparent at sites cranial and caudal to the site of injury. F. TUNEL labelling of untreated spinal cord injury: positive cells are found only in regions close to the injury. Scalebar = 200 μm in A + F, 400 μm in B, C, D & E.
Figure 6.5 Effect of diazepam administration on pathology of E11 spinal cord injury – sectioned at E15.
Diazepam administration 24 hours prior to injury has resulted in cavitation and haemorrhage within the spinal cord. Overall pathology is similar to that of an injury performed during the restrictive period for regeneration. Scalebar = 0.5 mm
6.3 DISCUSSION

The results detailed in this chapter have demonstrated that the chick spinal cord behaves in the same manner as the rat in response to diazepam administration. This further strengthens the notion that the post-E15 chick embryos is a valid and relevant model for further spinal cord injury research. However, although growth factor expression was successfully upregulated by pharmacological means, diazepam’s influence has proven ineffective at protecting the chick spinal cord from the effects of traumatic injury. Indeed the treatment appears to worsen, not improve the extent of secondary tissue damage seen following initial trauma. The vehicle used in this study however may have adverse effects per se on the spread of secondary injury and further work is required to clarify this issue.

6.3.1 Implications for regeneration

Although specific molecular causes of the effects described remain elusive, the observed potentiation of secondary tissue damage mechanisms seen in treated E11 spinal cord injuries demonstrates a few key points. Firstly, the E11 spinal cord is intrinsically capable of responding to spinal trauma is a way rendering regeneration unlikely. That is, the factors responsible for the spread of necrotic, and apoptotic cell death, and the mechanisms through which cavitation occurs are in place. Laminin immunostaining revealed that E11 spinal cord contains far fewer capillaries than at E15 (Fig 5.10). It could be argued that the extended and extensive response to spinal injury at E15 was directly related to the presence and density of blood vessels at the site of injury. Perhaps insufficient blood is flowing at E11 to allow flooding of neural compartments? This data however demonstrates that blood can flood into the E11 spinal cord after injury, i.e. the network of capillaries already in place at E11 is sufficient to flood the immediate and surrounding regions with serum and blood proteins.
These data provide further supportive evidence that cavitation, as seen following E15 spinal injury, may be caused directly by infiltration of blood into the CNS.

If this hypothesis is true, the question then remains -why does it not usually do so? The fact that the normal E11 cord does not immediately fill with blood following injury without pharmacological intervention suggests that some protective mechanism(s) is in place that prevents this from happening. Of course, spinal injury will cause some initial local haemorrhage due to primary tissue damage. This implies some further mechanism must come into play, causing much more comprehensive leakage into the cord, perhaps by altering blood vessel permeability.

The molecular means by which spinal cord pathology was altered following diazepam/DMSO administration are not understood in this case. The application of 2 bioactive agents, both of which have multiple effects, makes interpretation of these results difficult. Potentially, some alteration in blood vessel permeability could have affected the E11 cord in this way, as could potentiation of immune responses.

Given the altered pathology following injury, is the diazepam- treated E11 spinal cord capable of functional recovery? It remains undetermined whether the effects of diazepam on E11 injuries would adversely affect regeneration. On the basis of morphological observation made 4 days following injury, it appears likely that this would be the case. The severity of injuries is far greater than normally seen, resembling the type of injury response observed at non-permissive stages of development, or in adulthood. Such injury pathology is typically associated with severe impairments in motor function and sensation (Kakulas, 1984; Kakulas, 1999a). However, no functional measurements of recovery were made in this study. Behavioural analysis of post-hatchling chicks would be necessary to determine the effects of the injury on motor function.
Although these experiments have proved unsuccessful in enhancing regenerative responses to trauma, the strategy of growth factor manipulation remains valid and promising. Previous attempts to increase FGF2 concentrations following CNS injury have produced encouraging results. Injection of FGF2 directly following weight drop injury resulted in protection of 2 subsets of neurons in the spinal cord; ventral horn, and intermediolateral column motor neurons. Moreover, the surviving neurons also expressed more choline acetyl transferase, suggesting they were functional (Teng et al., 1998). Administration of FGF2 by minipumps has also proved a success. Rabchevsky implanted osmotic pumps into the lateral ventricle, and the lumbar sac, perfusing the injured spinal cord with FGF2 for 1 week. Behavioural analysis indicated that some functional recovery had occurred, although histological investigation indicated little difference between treated animals and controls in terms of tissue sparing (Rabchevsky et al., 2000).

The main difference between these studies and this investigation is the mode of growth factor delivery. In all cases so far, FGF2 has been administered directly. This method however is invasive: any injection of growth factor into the spinal cord will, in itself result in some degree of spinal injury. The attraction of pharmacological means is clear, such invasive procedures can be avoided, and the prospect of prolonged administration of factors is more economical.

Pharmacological growth factor manipulation has been attempted with some success in animal models of ischaemic cell death. Neuronal cell death in the hippocampus in response to cerebral artery occlusion can be prevented by administration of clenbuterol, a β2-adrenoceptor agonist (Culmsee et al., 1999b) This effect is believed to be mediated by induction of NGF expression (Culmsee et al., 1999a). Indeed clenbuterol has also proven to be effective in improving prognosis following spinal injury in rats, although the mechanisms responsible were not established (Zeman et al., 1999).
Manipulation of FGF2 by pharmacological agents is still of possible benefit in cases of spinal injury. One area in which the experimental design of this study could be improved is in the choice of vehicle. Although DMSO did not significantly alter levels of FGF2, it did seem to adversely affect the outcome of spinal transection. Indeed DMSO has been shown to exert a variety of biological effects, many of which could have affected the outcome of this study (Nakamuta et al., 2001; Chang et al., 1999). Further analysis of diazepam’s effects may be worthy of investigation, although the drug was found to be insoluble in water, and only poorly soluble in ethanol. The larger volumes of ethanol required to achieve effective diazepam concentrations proved to be toxic to the chick embryos, adversely influencing survival.

6.3.2 Mechanism of action?

Although beneficial effects on injury responses were not seen, diazepam treatment was noted to enhance levels of FGF2 in the chick spinal cord at E15. Diazepam acts via an allosteric site on GABA<sub>A</sub> receptors, potentiating the effect of GABAnergic signals (Bormann, 2000). The observation that younger (E11) embryos did not significantly upregulate FGF2 in response to diazepam may reflect developmental differences in GABA receptor expression at these times: GABA receptors could be found at lower levels, or be expressed in different cell types.

The mechanism through which GABA-A activation promotes FGF2 transcription is unclear. GABA is an inhibitory neurotransmitter and its receptor is a multi-subunit ion channel. Administration of diazepam has previously been shown to be neuroprotective in cases of experimental hypoxia; survival of hippocampal neurons was promoted (Schwartz et al., 1995; Schwartz et al., 1994). Activation of the GABA receptor results in hyperpolarisation of cells via an influx of chloride ions. It is possible that GABA activation could help counter some of the excitatory neurotransmitters released following CNS damage, preventing excitotoxicity. These same hippocampal neurons also show elevated levels of FGF2 following diazepam treatment (Gomez-Pinilla et al., 197...
2000). It is unclear whether the upregulation of FGF2 was directly related to the survival of these neurons in the hypoxia model.

Also of importance is the cell type in the spinal cord affected by the pharmacological treatment. In this study, I have not identified the cells with enhanced levels of FGF-2 after diazepam treatment. Previous studies in the adult rat spinal cord reported enhanced FGF-2 expression in 'astrocyte-like' cells, although a comprehensive double-labelling protocol was not adopted (Gomez-Pinilla and Dao, 1999). Astrocytes are known to express various GABA-A subunit mRNAs and respond to GABA stimulation in a similar manner to neurons (Bovolin et al., 1992; Kettenmann et al., 1987). Moreover, cultured astrocytes also respond to benzodiazepines, including diazepam (Backus et al., 1988); (Verkhratsky and Steinhauser, 2000).

In the adult, FGF-2 is expressed by astrocytes, but chiefly in response to local trauma. Typically, FGF-2 levels are increased after spinal injury, and also following experimental brain lesion (Follesa et al., 1994; Mocchetti et al., 1996; Frautschy et al., 1991). Such enhanced production of FGF2 may result in some degree of neuroprotection via paracrine signalling mechanisms.

Interestingly, corticosteroid treatment has also been observed to elevate FGF2 levels in cultured astrocytes (Magnaghi et al., 2000). This effect was thought to be mediated, at least in part, by activation of GABA-A receptors. Episodes of stress are also known to similarly elevate hippocampal FGF2, suggesting that this effect may be a protective mechanism in the rodent (Molteni et al., 2001). The increased astrocytes numbers seen later in development may explain the observation that diazepam can elevate FGF levels at E14, but not at E10.

Alternatively, diazepam may promote FGF2 upregulation in neurons themselves, as suggested by enhanced detection of FGF2 mRNA in the hippocampus following diazepam administration in the rat (Gomez-Pinilla et al., 2000).
CHAPTER 7. NOGO IN DEVELOPMENT AND REGENERATION
7.1 INTRODUCTION

As described in Chapter 1, myelin-associated proteins within the spinal cord are known to inhibit axonal extension and contribute to the establishment of a non-permissive environment within the adult CNS. Of the inhibitory myelin proteins, only Nogo has been shown to directly deter axonal regeneration \textit{in vivo} (Schnell and Schwab, 1990; Merkler et al., 2001; Raineteau et al., 1999; Bregman et al., 1995). However, little is known about the onset of Nogo expression during embryogenesis, and crucially the normal physiological role of the molecule has yet to be established.

To address this I have investigated the expression of Nogo during development in the embryonic chick and human. My original aim was to determine if onset of Nogo expression contributed to the transition from regeneration-competent to incompetent states which occurs in the chick embryo at around E13. Nogo-A is known to be the most potent isoform in preventing axonal growth, so my investigation concentrated chiefly on this molecule.

Cell culture experiments were also adopted to analyse the specific cell types expressing Nogo-A in the human spinal cord, and also to gain insight into the topology of Nogo-A within the membranes of human neural cells.

In addition, recent publications have identified Nogo Receptor (NgR) as the mediator of at least some of Nogo-A's inhibitory effects (Fournier et al., 2001; Ng and Tang, 2002). Again, no studies have previously examined the normal distribution of NgR through development. For this reason I have also investigated the distribution of NgR in chick and human embryos.

Overall this data has revealed some unexpected expression patterns for Nogo-A and NgR, and has provided some information regarding membrane topology. Together
these results allow some predictions to be made about possible physiological functions of Nogo-A and NgR. The molecules role in abortive regeneration is also discussed.

7.2 RESULTS

7.2.1 Partial Sequencing of Nogo-related mRNA in chick

Using expressed sequence tag (EST) data, sections of the chick Nogo-A gene were obtained. Chick Nogo-A specific primers were designed, and RT-PCR performed to confirm the presence of Nogo-A in E11 chick spinal cord cDNA (Fig 7.1A). The amplified gene fragment of approximately 940 bp was sequenced, allowing a portion of chick Nogo-A protein sequence to be established (Fig 7.1B). This sequence, of 381 amino acids was shown to be 45% identical and 62% similar to human Nogo-A at the protein level.

Chick cDNA samples were also probed with human primers specific to the C-terminal region of Nogo-A, this permitted the amplification of a 298 base pair fragment of chick Nogo-A C-terminal region. This sequence was found to show significant homology to human Nogo (Fig 7.1C), and BLAST analysis confirmed that the sequence was closely related to all 3 human Nogo genes and to several other members of the reticulon family.

7.2.2 RT-PCR analysis of Nogo in the chick spinal cord.

Chick specific Nogo primers were used to analyse mRNA from chick spinal cords taken from various stages of development using RT-PCR. There was no obvious regulation in mRNA levels with development from E11 to E17 (Fig 7.2).
Figure 7.1. Nogo-A in the embryonic chick. A. Amplification of a 900 bp Nogo-A specific region from E11 chick spinal cord cDNA. B. Alignment of partial Nogo-A protein sequence from E11 chick spinal cord with corresponding human data. Vertical lines and colons indicates identical and similar amino acids respectively. Section is 45% identical, 62% similar. C. Partial sequence of chick Nogo from C-terminal region 95% similar, 90% identical to human Nogo-A.

Figure 7.2 Expression of Nogo in the developing chick spinal cord as detected by RT-PCR. mRNA from E11, E13, and E15 spinal cords was tested for the presence of Nogo by RT-PCR using primers specific to chick Nogo; mRNA integrity was established by simultaneously checking for the presence of the ‘housekeeping’ gene GAPDH. Similar levels of Nogo were detected in all samples, regardless of developmental stage. Control samples were negative.
7.2.3 Immunohistochemical analysis of Nogo-A in the embryonic chick

In order to determine the precise spatio-temporal distribution of Nogo-A in the developing chick spinal cord, immunohistochemistry was performed on transverse, paraffin embedded tissue sections of embryos ranging from E3 to E18. Nogo-A was labelled using the polyclonal antibody AS-472 (Chen et al., 2000), and was visualised using peroxidase staining or fluorescein-conjugated secondary antibodies. Positive labelling was detected in the most ventro-lateral points of the E3 spinal cord (Fig 7.3A). In addition, the notochord and myotome were also labelled. Nogo expression extended dorsally within the spinal cord at E4 (Fig 7.3B) and E5 (Fig 7.3C), and the entire marginal layer was positive by E7 (Fig 7.3D). Strong staining was maintained in the developing white matter through stages E10 (Fig 7.3E), and E13 (Fig 7.3F). Nogo expression was also noted in peripheral nerve fibres; for example at E10, peripheral nerve bundles and accompanying artery (Fig. 7.3G), and the nerve fibres surrounding the oesophagus are Nogo-positive (Fig 7.3H).

To establish whether expression of Nogo-A might be altered following spinal cord injury, immunohistochemical analysis was performed on sections of injured E11 spinal cord. At this permissive stage, Nogo-A was seen to be present in close proximity to the injury site (Fig 7.4).

Immuno-localisation of Nogo largely overlaps with that of the neurofilament marker RT-97 at early stages of development, suggesting an axonal localisation (Fig 7.5). However, at much later developmental stages, no co-localisation with neurofilaments was observed; instead Nogo-A seemed to be present in small individual cell bodies within the white matter, a distribution consistent with an oligodendrocyte localisation (Fig 7.6).
Fig 7.3 Immunolocalisation of Nogo-A (brown) in transverse (A-H) and longitudinal (I) sections of chick embryos. Sections are counter-stained with methyl green to detect nuclei (green). A. In E3 spinal cord Nogo is present at the most ventrolateral point (arrows); in addition it is expressed in the notochord (arrowhead) and myotome (my). B. In E4 spinal cord Nogo is also present in more dorsal areas. C. The E5 spinal cord expresses Nogo in yet more dorsal regions, staining is also evident in the axonal tracts (at) projecting from ventral roots and in axons within the DRGs. D. By E7, Nogo is also expressed in the most dorsal aspects of the developing white matter. E. E10 spinal cord showing complete staining of all white matter; Nogo is also present in peripheral nerve fibres (p). F. E13 spinal cord; the entire white matter is Nogo-positive. G. Peripheral nerve (n) and artery (a) from E10 embryo express Nogo. H. Oesophagus from E10 embryo: Nogo is present in the enteric plexus.
Figure 7.4 Nogo-A is expressed at sites of spinal cord injury during the permissive period of regeneration. Longitudinal sections of spinal injuries performed at E11 were stained using AS-472. Nogo-A was found to be located between the opposed stumps of severed spinal cord in every case. 2 examples are shown above. Scalebar = 400μm
Figure 7.5 Co-localisation of Nogo and neurofilaments in the chick spinal cord as detected by the antibodies AS-472 and RT-97 respectively. A. Immunolocalisation of Nogo (green) in the E3 spinal cord (arrows) B. same section of E3 chick spinal cord showing expression of high molecular weight neurofilaments (red). C. E11 chick spinal cord stained for Nogo-A. D. E11 spinal cord displaying positive labeling of RT-97 in the white matter. Counterstain is Hoechst nuclear dye (blue). E. Merged image of (C) and (D). Regions of co-expression are orange in colour. F. High magnification view of region of co-expression. Scalebar = 50 µm
Figure 7.6 Double staining for Nogo-A and neurofilaments in the E17 chick spinal cord. Nogo-A labeling (green) was not closely associated with high molecular weight neurofilaments (red, as detected by RT-97) at this late developmental stage. Image is high magnification view of the ventral funiculus. Cartoon illustrates the region of spinal cord shown. Scalebar = 50 μm

Figure 7.7 Immunoblot of total protein extracted from embryonic chick spinal cord at different developmental stages (E11, E13, E15, E17) using the anti-Nogo-A antiserum AS472. Size of molecular weight standards are indicated. Published molecular weights of human (220 kDa) and rat (250 kDa) Nogo-A are shown.
7.2.4 Western Blot analysis of Nogo-A in the embryonic chick.

Immunoblotting was performed to determine the molecular weight of the presumptive chick Nogo-A protein. Samples of E11, E13, E15, and E17 chick spinal cords were probed with AS-472 and indicated the presence of a band of approximately 250 kDa, correlating well with the known size of rat Nogo-A (Chen et al., 2000). As seen by other groups, additional bands were detected (Fig 7.7).

7.2.5 Immunohistochemical analysis of Nogo-A in the embryonic human

The results obtained in the chick embryo suggested that the role of Nogo in spinal cord regeneration may be more complex than previously anticipated, and indicated a previously un-described contribution of the molecule to chick development. In order to determine whether the role of Nogo-A in development was common to other vertebrate species, it was decided to investigate its expression in the embryonic human. To ascertain the precise localisation of Nogo-A protein in the embryonic human spinal cord and surrounding tissues, immunohistochemical analysis was performed using antisera raised against Nogo-A (AS-472). Transverse paraffin sections of embryos ranging from 28 to 70 days gestation were analysed (Fig 7.8). No staining in the spinal cord was observed in 28 day embryos, although Nogo-A was present in the notochord, the myotome, and epidermis. (Fig 7.8A). By 49 days gestation, cells of the ependymal canal and floorplate stained positively for Nogo-A (Fig 7.8B). The developing white and grey matter was first noted to express Nogo-A in 55-day tissues and immunostaining appeared to be strongest in the ventral half of the spinal cord (Fig 7.8C). At the later stages examined, levels of Nogo-A expression appeared to be greater, and encompassed more dorsal regions of the cord (Figs 7.8D, 7.8E). Strong immunostaining was also observed in the striated musculature of the heart and trunk, and around the oesophagus (Fig 7.8F-H).
**Fig 7.8  Detection of Nogo-A in the embryonic human by immunohistochemistry.**

**A.** At 28 days gestation (dg), expression is limited to the notochord (n + insert), epidermis (e) and myotome (my). **B.** Nogo-A is present in the spinal cord by 45dg in the marginal zone. **C.** 55dg human embryo; Nogo-A staining is more pronounced in the developing white matter and appears to be strongest in ventral regions. **D.** By 61dg, strong ventral Nogo-A staining is pronounced in the spinal cord. Positive labeling is also evident in the DRG and skeletal muscle (sk). **E.** The latest stage examined, 70dg, robust Nogo-A labelling persists in white and grey matter. **F.** Skeletal muscle from 61d human embryo showing strong labeling for Nogo-A. **G.** Nogo-A is also present in the foetal heart (70 dg). **H.** Epidermal labeling as seen in 28 dg human embryo. Scalebar = 200 μm A-E, 500 μm in G, 50 μm in F & H.
7.2.6 Western Blot analysis of Nogo-A in the embryonic human

Immunoblotting confirmed that AS-472 was binding to a protein fraction of expected size for human Nogo-A (220 kDa) in spinal cord and muscle (Fig 7.9).

7.2.7 RT-PCR analysis of Nogo-A in the human spinal cord

In order to assess whether early Nogo-A expression is a general feature of vertebrate development, RT-PCR analysis was performed on embryonic human tissue samples using primers designed to specifically amplify Nogo-A and Nogo receptor (Fig 7.10). In the earliest sample analysed (45 days gestation) Nogo-A cDNA was detected, but NgR was not present. The presence of both transcripts was confirmed in 10 and 13-week human spinal cord samples. Myelin Basic Protein (MBP) expression was also examined to determine the relative maturity of oligodendrocytes within the spinal cord at the stages investigated. Only trace levels of MBP were found in the 45dg spinal cord sample, with robust expression detected at 10 and 13 weeks gestation. The integrity of each cDNA sample was established by performing PCR with primers specific for the housekeeping gene β-actin.
Figure 7.9 Immunoblot of human spinal cord proteins probed with AS-472. A single band of approximately 220 kDa was observed in samples from 10 weeks gestation spinal cord (lane A) and heart (lane B).

Figure 7.10 RT-PCR analysis of human embryonic spinal cord. A. 45 dg RNA sample B. 70 dg RNA sample. C. 75 dg RNA sample D. distilled water control
7.2.8 Analysis of the cellular localisation of Nogo/Nogo-A

Of crucial importance in establishing the physiological role of Nogo-A in the normal and injured spinal cord is the protein’s topology within the membranes of Nogo-expressing cells. The molecular structure of the protein indicates the presence of 2 putative transmembrane domains, in addition to an ER-retention motif. The position of Nogo within the plasma membrane remains a contentious issue with conflicting results being obtained by different laboratories. In order to address this problem, human embryonic spinal cord explant cultures were prepared; cells were allowed to migrate from the explant for 14 days and then stained using anti-Nogo antibodies. This procedure was performed on unfixed, and on fixed, permeabilised cells to examine the cell surface expression of the Nogo proteins.

Using the Nogo-A specific antibody D-19, unfixed cultures of 41-98 dg were found to contain very few Nogo-A positive cells. Although an accurate cell count was not performed due to the limited lifespan of fluorescent immunostaining, the number of positive cells on unfixed samples was estimated to be less than 1%. Positive cells were typically sparsely distributed, and were difficult to identify from morphology alone (Fig 7.11). Experiments using an antibody that also recognised Nogo-B (N-18) produced similar results, in terms of frequency of positive labelling and cell morphology (Fig 7.12). It was usual to find Nogo positive cells at the edges of cell clusters, or in regions of low cell density (e.g. Fig 7.11A, Fig 7.12C), although some exceptions were apparent where positive cells were found within dense cellular aggregates (Fig 7.11E).

Fixation of tissue prior to Nogo staining, either with acid alcohol, or 4% PFA, followed by permeabilisation of plasma membranes with detergent, resulted in quite different patterns of immunostaining. Many more cells were labelled, the majority of which exhibited a broad, flat morphology (Fig 7.13A, B). In addition, long thin processes were also positive for Nogo-A (Fig 7.13C, D). Double staining experiments using
neurofilament-specific antibodies confirmed that these processes were neuronal axons (Fig 7.15C). Again, repeating the experiment with the N-18 antibody to identify Nogo-A and Nogo-B yielded similar results (Fig 7.14). In experiments using late stage (98 days gestation) human embryonic material, Nogo staining in unfixed cells commonly resulted in non-uniform, or polarised expression within cells. Often this seemed to represent the leading edge of cells (Fig 7.14F), although similar punctate staining was observed on presumptive axons (Fig 7.15C).

Double labelling experiments designed to identify the Nogo positive cell population in unfixed cultures (i.e. those with cell-surface expression of the N-terminal domain) yielded no positive results. Co-localisation with RT-97, GFAP, and O4 could not be confirmed (not shown). The low numbers of Nogo-positive cells under unfixed conditions made this analysis difficult, and it is possible that some double labelling could be found if a larger scale study was performed although the limited availability of human tissues made this difficult to implement.

However, in fixed, permeabilised tissues, double labelling experiments indicated that Nogo was found in axons (Fig 7.15D), and in oligodendrocytes (Fig 7.15F). Interestingly, in 65 days gestation spinal cord cultures, no Nogo expression in oligodendrocytes was detected (Fig 7.15E). This suggests that a transitional switch occurs during development, turning Nogo expression on after a specific stage of maturation. Results from the chick embryo described earlier in this chapter also support this idea (Fig 7.6).

Double labelling with GFAP revealed that although Nogo and GFAP cells commonly overlap in culture, the molecules do not appear to be expressed by the same cell (Fig 7.15A,B)
Figure 7.11 Detection of Nogo-A protein by immunocytochemistry in unfixed human spinal cord explant cultures. A. Nogo-A (green) positive cell from 41 dg human spinal cord explant culture. Nuclei are stained with Hoescht dye (blue). B. Phase contrast image of A. Arrow indicates Nogo-A positive cell. C. Area of higher cell density from 41 dg human spinal cord culture: two Nogo-A positive cells are labeled (arrows). D. Phase contrast image of C. E. Nogo-A positive cells from 61 dg human spinal cord explant culture. F. Phase image of E. Scalebar = 50 μm
Figure 7.12 Immunocytochemical detection of Nogo-A in fixed human embryonic spinal cord cultures. A. 41 dg human explant culture: many broad, flat cells were positively labeled for Nogo-A (green). Nuclei are stained with Hoechst dye (blue). B. Phase contrast image of A. C. 61 dg human spinal cord culture. Nogo-A labeling was found in many large flat cells, and also in narrow processes, resembling axons (arrows). D. Phase image of C: presumptive axons are indicated (arrows). Scale bar = 50 μm.
Figure 7.13 Detection of Nogo-A/Nogo-B in unfixed human spinal cord explant cultures using the antibody N-18. A. 41 dg human spinal cord culture: two Nogo positive (green) cells are indicated. Nuclei are stained with Hoescht dye (blue). B. Phase contrast view of A. Nogo positive cells are indicated (arrows). C. Second example of Nogo positive cells from 41 dg culture. D. Phase image of C revealing morphology of labeled cells (arrows). E. Nogo positive cell from 61 dg unfixed human explant culture: cell appears to be attached to a long process. F. Phase view of E, positive cell is labeled (arrow). Scalebar = 50 μm.
Figure 7.14 Immunodetection of Nogo-A/Nogo-B in fixed and permeabilised human spinal cord explant cultures. A. 41 dg human culture: many large, broad cells were Nogo-positive (green). Nuclei were stained with Hoescht dye (blue). B. Phase contrast image of A. C. 61 dg human spinal cord culture: again many large flat cells were positively labeled. D. Phase image of C. E. Low magnification view of 61 dg human spinal cord culture: this area of high cell density is almost completely Nogo-positive. F. Nogo-positive cells from 98 dg spinal cord culture: restriction of Nogo expression to 'leading edge' processes was observed. Scalebar = 50 μm
Figure 7.15 Double Labeling of Nogo with neural markers. A. 65 dg human spinal cord explant culture (fixed and permeabilised): many cells and extending processes are Nogo-positive (green). GFAP positive cells are present (red), but do not appear to co-localise precisely with Nogo-expressing cells. B. Explant culture from 98 dg human spinal cord: again, Nogo positive, and GFAP positive cells appear distinct. C. 61 dg spinal cord explant. Nogo (green) and neurofilament (red) are co-localised in long processes (arrows), confirming axonal identity. Nuclei are stained with Hoescht dye (blue). D. 98 dg spinal cord culture. Nogo (green) is present within long axon-like processes: expression appears to be punctate. E. 65 dg human spinal cord culture stained for Nogo (green) and the oligodendrocyte marker O4. No co-localisation exists. F. High magnification of 98 dg spinal cord culture, labeled with Nogo and O4. Areas of double labeling appear yellow in colour (arrow). Scalebar = 50μm (A-D), 25 μm (E, F).
7.2.9 Nogo Receptor in the developing chick spinal cord

From the results described earlier in this chapter, it seems apparent that Nogo signalling is likely to be of importance in early development in the embryonic chick and human. Of crucial importance in understanding the role played by Nogo, and how it may be regulated, is the distribution of the receptors responsible for the transduction of the Nogo signal. Two distinct domains exist within the structure of Nogo-A, exerting similar inhibitory effects on neurite extension \textit{in vitro}, but mediated by different receptors. The 66-amino acid region located between the two transmembrane domains has been shown to exert its effects via Nogo Receptor (NgR). The second, more potent domain, found within the Nogo-A specific portion of the protein, functions independently of NgR, and presumably acts via a yet undiscovered receptor. The NgR molecule however may be thought of as the effector of general Nogo function: transfection of cells with NgR is sufficient to confer sensitivity to the effects of Nogo (Fournier et al., 2001; Fournier et al., 2001).

Expression of NgR during development remains to be characterised, and thus a longitudinal analysis was initiated in transverse sections of chick spinal cord. I hypothesised that since Nogo-A is present during permissive periods for regeneration, perhaps the transition from regeneration-permissive to restrictive states occurs via modulation of NgR expression during development. That is, NgR would not be present in the spinal cord at developmental stages when regeneration can occur.

7.2.10 Immunohistochemical analysis of Nogo Receptor in the embryonic chick

Western blot analysis indicated that the anti-NgR antibody detected a single band of around 60Kda in all human samples analysed (Fig 7.16). Chick samples also revealed a band of 60 Kda, although other weaker bands were present. The expected molecular size of NgR according to previous studies is 85 Kda (Fournier et al., 2001), however
the immunoblot presented by Fournier et al. also contains a band of around 60 Kda. The tissues examined here are also from different developmental stages. Changing levels of post-translational modification of nogo receptor protein could account for changes in estimated molecular weight with development.

Immunohistochemical analysis revealed little or no positive staining within the spinal cord at E3, though the notochord was clearly positive (Fig 7.17A). Between E4 and E9, staining was observed in the differentiating grey matter, and neurons within the dorsal root ganglia (DRG) were strongly labelled (Fig 7.17B-D). Most spinal neurons were positive by E10 (Fig 7.17E). NgR expression persisted in the neurons of the grey matter and DRGs until E13 (Fig 7.17F). By E14 (Fig 7.17G) however, and time points thereafter (not shown), NgR labelling was limited to a few individual neurons of the spinal cord. A distinctive temporal pattern of expression was also noted in the heart and developing musculature. The heart was positively labelled by E3 (Fig 7.18A), and the myotome by E5 (Fig 7.17B). Skeletal muscle was first noted to express NgR at E9 (Fig 7.17D, 7.18B), and by E10 all muscle fibres examined were positively labelled (Fig 7.17E). The pattern of muscle staining mirrored that of neuronal labelling in the spinal cord, and by E14 no muscle fibres were expressing NgR (data not shown). Expression was also observed in the retina of E5 chick embryos and seemed to be restricted to the peripheral retina (Fig 7.18C-E). In all cases, analysis of NgR staining under high magnification revealed a dotted staining pattern typical of membrane receptors (Fig 7.19J). With the exception of early developmental stages where NgR expression was seen in the notochord (Fig 7.17A) and myotome (Fig 7.17B), the localisation of NgR appeared to be mutually exclusive with that of Nogo-A (compare Figs 7.3E and 7.17E).
Fig 7.16 Immunoblot of Human and chick protein samples with the anti-Nogo Receptor antibody. Anti-NgR was used to probe protein samples from 45, 70, and 75 days gestation human embryos, and from E11, E13, E15, and E17 chick embryos. A band of around 60KDa was detected in all samples.
Fig 7.17  Detection of Nogo Receptor in the embryonic chick by immunohistochemistry

A. E3 chick embryo showing positive immunostaining in the notochord (n).  B. E4 chick embryo: NgR is present in the myotome (my), DRG, and some neuronal populations of the spinal cord.  C. E7 spinal cord: very faint NgR staining is present in some neurons.  D. E9 spinal cord: very faint labeling is evident in the neurons of the spinal cord; a few individual muscle fibres are strongly stained (sk).  E. E10 spinal cord: all neurons of the spinal cord and DRG are robustly labeled. There is also widespread staining within the musculature (sk).  F. E13 spinal cord: strong NgR staining is maintained in all neuronal populations of the grey matter and DRG.  G. E14 spinal cord: a few individual neurons of the grey matter are positively labeled. Most of the spinal cord and musculature has low/negative levels of staining.  H. NgR staining of E12 DRG neurons showing 'dotted' expression pattern. Scalebar = 200 µm A-G, 50 µm in H.
Figure 7.19 Detection of Nogo Receptor in the embryonic chick musculature and eye using immunohistochemistry.  

A. NgR was present in the heart of E3 chick embryos.  

B. High magnification view of individual muscle fibre labelling as seen at E9.  

C. E5 chick eye: NgR was expressed in the ganglion cells of the peripheral (p), but not central (c) retina.  

D. High magnification view of central retina: no staining is present.  

E. High magnification view of peripheral retina showing robust NgR expression. Scalebar = 300 μm in A, 200 μm in C, and 50 μm in B, D, & E.
7.2.11 Immunohistochemical analysis of Nogo-Receptor in the embryonic human

Given the considerable similarities between chick and human expression of Nogo-A, it was decided to examine whether NgR was also found in early human development, as seen in the chick.

In order to assess whether onset of NgR expression was developmentally regulated in human embryos, immunohistochemistry using antisera raised against NgR was performed on transverse sections of human spinal cord and surrounding tissues. The receptor was detected at 28 days of gestation in the myotome and notochord (Fig 7.19A), and was particularly strong in the heart (Fig 7.19F). By 45 days of gestation, expression was evident in the developing DRGs, and in a few individual cells of the grey matter (Fig 7.19B). In the spinal cord, NgR levels were highest at 55 days of gestation (Fig 7.19C) where the entire white matter seemed to be positively labelled, as were many ventrally located neurons within the grey matter. By 61 days of gestation though the muscle was strongly positive (not shown), spinal cord labelling was restricted largely to ventral neuronal cell bodies. Little staining of the white matter persisted, with the exception of the dorsal root entry zone, where projections from the DRG were clearly positive (Fig 7.19D). At the latest time point examined, 84 days of gestation, NgR staining was largely absent from the spinal cord, but was present in the DRGs (Fig 7.19E). A distinctive pattern of expression was noted in the 45 day retina, with the ganglion cells being positively labelled in peripheral, but not central areas (Fig 7.20A-C). The optic nerve was also NgR-positive (Fig 7.20).

7.2.12 RT-PCR analysis of Nogo Receptor In the human spinal cord

The expression data described above was supported by RT-PCR analysis of human spinal cord samples using primers designed to amplify Nogo Receptor. No mRNA was
detected in 45 day samples, although robust expression was evident at 70, and 75 dg (Fig 7.10).
Figure 7.20 Immunohistochemical detection of NgR in the embryonic human. A. 28dg human embryo: positive staining is present in the myotome (my) and notochord (n). B. 45dg spinal cord: NgR expressing cells are evident in the DRGs and in some regions of the grey matter. C. 55dg spinal cord: NgR appears to be present in the white matter, and in ventral regions of the grey matter. The DRGs are also positive. D. 61dg spinal cord: neurons of the DRGs and ventral grey matter are positively labeled. The white matter is largely negative, with the exception of the dorsal root entry point (asterisk). E. 84dg spinal cord. White and grey matter is mostly negative. There is however some staining in the DRG. F. 28dg human heart expresses NgR at high levels. Scalebar = 200 μm
Figure 7.21 Immunohistochemical analysis of Nogo Receptor in the human eye. 

A. 50dg human eye: NgR is present in peripheral (p), but not central (c) retina. Optic nerve (on) is also positive. Black pigmentation of retinal pigmented epithelium (rpe) is distinct from positive brown immunostaining.

B. High magnification view of peripheral retina from 50dg embryo. Staining is evident in ganglion cells.

C. High magnification of central retina from (A). NgR is not expressed. Scalebar = 200 μm in A, 50 μm in B, and C.
7.3 DISCUSSION

A note on antibody specificities.

Although the data presented in this chapter provides some evidence concerning the distribution of Nogo-A and NgR during embryogenesis, it is important to recognise the limitations of the experimental approach. The results obtained are heavily reliant on immunohistochemistry data; for conclusions to be drawn with confidence, it is necessary to be convinced that the antibody is indeed specific for the protein against which it was raised. The antibodies used in this chapter have been demonstrated to be specific in other publications (Chen et al., 2000; Fournier et al., 2001). However, it is noteworthy that their effectiveness has not been previously demonstrated for immunohistochemistry on chick tissues. The immunohistochemical data presented here is supported by the amplification of Nogo-A mRNA from E11 chick spinal cord (Figure 7.2), which confirms that Nogo-A is transcribed at this stage of development. Also in support of the staining observed is its specific nature: very little background staining is evident, suggesting that a single protein is being targeted. Moreover, the N-terminal region against which the AS472 antibody is targeted is somewhat unique, having very little similarity to other proteins. The 20 amino-acid sequence is more similar to chick Nogo-A than any other protein in the SwissProt database.

Western Blotting is a useful method of confirming antibody specificity. In this case, although AS472 was specific for a solitary band of 220 Kda in human tissues, several additional bands were evident in chick. The full-length chick version of Nogo-A has not yet been cloned, thus the precise molecular weight remains unknown. Differences in molecular weights of Nogo-A isoforms are known to exist between species, thus it is feasible that the strong band of around 280Kd may reflect the chick Nogo-A isoform. It cannot be discounted however that non-Nogo proteins are being detected. Attempts to replicate the experiments with commercially available Nogo-A antibodies proved unsuccessful; these sera reacted poorly with human and chick tissues and produced no staining. Ideally, blocking peptides should have been utilised. These would have
specifically inhibited Nogo-A binding, and thus would abolish all staining if the correct protein was being targeted. Unfortunately, these peptides were not made available, and stocks of the donated AS472 and anti-NgR are depleted. In-situ hybridisation experiments would also have been beneficial. These would confirm the presence of Nogo-A mRNA, and could provide valuable support for the immunohistochemical data. Time limitation has prevented the initiation of such analysis. As such, the data presented here must be considered as a first indication of possible localisation of Nogo-A and NgR in the chick.

7.3.1 Developmental Regulation of Nogo-A

The experiments described in this chapter, concerning the role of Nogo-A in the developing chick, produced some interesting, if unexpected, results. Firstly, the temporal and spatial distribution of Nogo-A was surprising. Given that the Nogo proteins were initially identified from a fraction of myelin, the presence of Nogo-A in the chick very early in development, prior to myelination, was unexpected. Human embryos also express Nogo-A prior to the onset of myelination, and the pattern of expression at this stage of development is remarkably similar between human and chick.

Published data would be predictive of Nogo-A becoming expressed around the time of myelination. The onset of IN-1 mediated inhibitory effects was investigated using a functional assay based upon fibroblast cell spreading (Caroni and Schwab, 1989). This study concluded that IN-1 sensitive effects were apparent in the spinal cord after nerve fibre growth, but prior to myelination. Importantly, this study examined only the functional effects of the Nogo molecule; the actual presence of Nogo at earlier stages of development was not investigated. The results described above support the hypothesis that Nogo is expressed in many tissues during early development, but becomes restricted to oligodendrocytes at later stages of embryogenesis. Furthermore, Nogo's inhibitory influence on axonal extension would seem not to be present, until at later stages of development when its expression in oligodendrocytes is upregulated.
The expression of Nogo-A in non-oligodendrocytes was of particular interest, this being suggestive of a novel function for the protein during development. Nogo-A expression was found in chick axons as early as E3, but was not axonally located by E17. Levels of Nogo-A found in chick axons was found to decline at time points later than E13, when myelination has begun in the spinal cord. Also, in human spinal cord explant cultures, Nogo labelling was absent from O4-positive oligodendrocytes in 65 days gestation samples, but double labeled cells were found in 98 days gestation cultures. In these same 98 dg cultures, Nogo expression was observed to become more restricted within other cell types, commonly Nogo was found only in leading edges of cells. This may reflect a gradual down-regulation of Nogo expression in non-oligodendrocytes with development and could indicate a change in Nogo function. The point of transition from a mainly neuronal to a chiefly oligodendrocyte location of Nogo is likely to represent an important stage in the development of a non-permissive environment for axonal regeneration. However, given that Nogo is present at permissive periods for regeneration, albeit in a different cell population, a change in Nogo function would be expected to occur if the molecule is indeed a prominent player in the creation of a non-permissive environment during development. That is, early Nogo expression may not inhibit axonal extension.

Alterations in Nogo expression with development may simply reflect changes in splicing or promoter usage. All three Nogo isoforms are derived from the same gene in this manner (Chen et al., 2000; GrandPre et al., 2000). Absolute levels of Nogo may not change, but different isoforms may assume precedence with ongoing development. This is supported by the observed expression of Nogo-A in embryonic muscle in human and rat (Fig 7.8F, (Josephson et al., 2001), but not in adult muscle (Chen et al., 2000; Li et al., 2001). However, these studies indicate that adult muscle strongly expresses Nogo-C. Thus, developmental changes in Nogo splice forms could reflect an important stage of tissue maturation.
The detection of Nogo proteins in neuronal cells is of particular interest. Nogo-A positive neurons were observed in human sections after 55 days of gestation, and were particularly abundant in ventral regions and in the DGRs, indicative of motor and sensory neuronal cell bodies (Fig 7.8). Nogo-A was also found in axons, in the chick embryo (Fig 7.3), and in human explant cultures (Fig 7.15). These observations are key in developing hypotheses concerning prospective roles for Nogo during early embryogenesis. Previous models of Nogo-A's inhibitory effects are based on axonal responses to environmental Nogo – located on oligodendrocytes. The discovery of Nogo-A expression in neuronal cells implies another function of the molecule. This is supported by the fact that neuronal Nogo-A topology seems to differ from that necessary for contact mediated neurite inhibition, i.e. the N-terminal domain is located primarily intracellularly. Presumably such novel functions of the Nogo molecule would be associated with an ER location.

Other Nogo-interacting proteins have been identified and deposited in the protein sequence databases SwissProt. Some of these may offer clues to the nature of such additional functions of the Nogo proteins. The recently identified Nogo-interacting mitochondrial protein implies a role for Nogo in mitochondrial function or metabolism for example (Hu et al., 2002).

The positive labelling of peripheral axons in the chick was also not expected. Nogo-A has been demonstrated in adult systems to be specific to the central nervous system. However, as with centrally located axons, positive staining of peripheral nerves was not detected at late stages of embryonic development. Previous studies have demonstrated that adult rat sciatic nerve expresses Nogo-B (Chen et al., 2000), perhaps indicating another change in isoform expression with development.

Detection of Nogo in myotome and notochord in both chick and human was certainly unexpected and is previously unreported. Later in development, differentiated skeletal muscle was also strongly labelled in the human, a finding supported by other studies.
Adult muscle however does not express Nogo-A (Josephson et al., 2001; Chen et al., 2000). These findings support the notion that Nogo-A is important in the early maturation and development of skeletal muscle fibres. Regions of striated muscle in the human heart were also strongly Nogo-A positive. The exact time-point when expression of Nogo-A is lost in the musculature was not determined in this study; the latest stage examined was 70 days of gestation. It would be intriguing if down-regulation of Nogo-A in muscle and neurons occurred at the same point of development and would be suggestive of a common function in both. Nogo-A was not found to be present in the musculature of the chick embryo; this could be due to differences in the structure of avian, and mammalian muscle fibres.

7.3.2 Developmental Regulation of Nogo Receptor

There is currently no published data regarding the expression of Nogo Receptor during development. What is known is that NgR is present on adult neurons and is sufficient to confer sensitivity to Nogo mediated axonal repulsion upon unresponsive cells. Information regarding embryonic expression is based mainly on one piece of functional in vitro data: E7 chick DRG neurons are less responsive to Nogo than E13 neurons (Fournier et al., 2001).

The results of this study indicate that NgR is widely expressed during early development, perhaps implicating a function beyond that of axonal repulsion. NgR was first detected in the notochord of both human and chick embryos. Interestingly, Nogo-A was also detected in this organ at this stage of development in both species. The notochord is important in establishing ventral identity during spinal cord development (Gilbert, 2000): the function played by Nogo/NgR in this tissue remains a mystery.

Neuronal expression of NgR was also detected in both species in both spinal cord and DRGs. Surprisingly NgR protein was present in E7 DRGs, a population which according to Strittmatter is non-responsive to Nogo (Fournier et al., 2001). This result
could be explained by the fact that NgR is likely to function via interactions with another, membrane-spanning protein (Ng and Tang, 2002). It is possible that developmental regulation of this unidentified subunit may confer sensitivity to Nogo function in neurons. However, transfection with NgR alone is sufficient to confer Nogo sensitivity upon E7 DRG neurons (Fournier et al., 2001), suggesting that additional cellular apparatus necessary for signal transduction must be in place.

Of particular interest in chick development was the striking mutually exclusive nature of Nogo-A and NgR expression observed. NgR is largely restricted to neuronal cell bodies within the spinal cord, and to skeletal muscle fibres in the periphery. Conversely, Nogo-A was found on axonal fibres at this stage of chick development. Overall, this relationship was interpreted as being suggestive of a role in axonal guidance or synapse formation: NgR was found consistently on the targets of axonal innervation, whilst Nogo-A was located on the nerve fibres themselves. There are problems with such a hypothesis however: the period of synaptic formation with muscle fibres occurs at earlier stages than the peak of NgR labelling seen in this study. Indeed the expression could be described as occurring immediately after this process. So, could the role of NgR be to finalise the process of synapse formation? Or to somehow prevent further innervation by new nerve fibres? Either of these hypotheses is consistent with the spatiotemporal pattern of expression. It should be remembered that the relationship between Nogo-A expressing cells, and NgR expressing neurons is an inhibitory one, thus axonal repulsion from a previously innervated muscle fibre remains a possibility.

It should also be remembered that Nogo-A and NgR may not interact at all, depending on molecular topology. NgR mediates the effects of the Nogo-66 domain, which is common to all Nogo isoforms. Thus, Nogo-B or Nogo-C could also interact with NgR during early development. The contribution of the B, and C isoforms has not been
addressed in this study. Also, interaction with NgR requires cell surface expression of Nogo-66.

The retinal expression of NgR was quite distinctive. Labelling of peripheral, but not central retinal ganglion cells was evident, and was identical in chick and human tissue sections. The functional implications of such differential staining are unclear. It is possible that intrinsic differences in neuronal maturation within the retina are reflected in the NgR staining. Neural differentiation is initiated in the chick eye at E2 in central regions, then progresses peripherally (Fischer and Reh, 2000). Thus the central retina is the most mature, perhaps explaining differences in NgR expression. Also of interest is the distinctive pattern of NgR expression seen on individual cells under high magnification. In all cases, a ‘speckled’ appearance was observed, on muscle fibres and neuronal cells. This staining pattern resembles typical plasma membrane labelling, although it is difficult to confirm whether it is localised to synapses without the use of electron microscopy.

In addition to staining within neuronal cell bodies, some positive labelling was also detected in the white matter of some human embryos. Of particular interest was the specific white matter labelling of the dorsal root entry zone, seen in 61dg embryos. The significance of this distinct pattern remains elusive.

7.3.3 Topology and function of the Nogo proteins

The data presented here on the cellular localisation of Nogo in human spinal cord cultures supports the hypothesis that Nogo-A is present on the extracellular surface of oligodendrocytes, albeit at low levels. This view is in keeping with the concept of oligodendrocytes acting via Nogo-A expression to inhibit axonal growth, or arborisation in vivo. My data is the first to examine this topology in human cells, and supports the model where both inhibitory domains of the molecule are located extracellularly, but only in a minority of cells (Fig 1.9A). However, my data also suggests that far greater
levels of Nogo N-terminal domain are located intracellularly, in a wider range of cell types, including neurons and their axons. This pool of Nogo probably resides in the ER of the cells, based on the presence of an ER-retention motif within the reticulon domain of the protein.

The topology of the Nogo proteins within individual cells is crucial to our development of ideas regarding their function. Published functional data indicates that the 2 active domains of Nogo-A are expressed extracellularly. IN-1 dependent, contact-mediated axonal and fibroblast inhibition by oligodendrocytes has been demonstrated. This requires extracellular expression of Nogo-A's N-terminal domain (Chen et al., 2000; Bandtlow et al., 1990). In addition, separate experiments have shown the effects of the Nogo-66 domain by conferring cell sensitivity via transfection with NgR; again this effect requires cell surface expression (Fournier et al., 2001) However, it is not apparent if single cells express both domains extracellularly at the same time. Strittmatter could detect the Nogo-66 domain on unfixed cells, but could not detect myc-labeled C, or N-terminuses. This implies that both ends of the protein reside within the plasma membrane, including the N-terminal located inhibitory domain. Other groups however claim to have detected extracellular N-terminal domains on unfixed cells using specific antibodies (Martin Schwab, personal communication).

So, given that as early as 41 dg, the N-terminal domain of Nogo-A is located on the cell surface of neural cells, what can be deduced about the topology and function of the Nogo-A protein at this time?

Most Nogo-A seems to be located intracellularly. This however does not provide information regarding the cellular location. The Nogo-A molecule could be associated with the plasma membrane, but with only the Nogo-66 domain located extracellularly. Or, the molecule could be completely distinct from the plasma membrane, and be ER-associated.
Immunohistochemical analysis indicates that early in human development, neuronal cells appear most likely to express Nogo-A (my work, (Huber et al., 2002), (Josephson et al., 2001)). Thus Nogo-A is certainly present in human spinal cord prior to myelination, and is likely to be playing some role in embryogenesis.

Overall, the differences seen using a Nogo-A specific, and a Nogo-A/Nogo-B antibody were slight: similar cells types were stained with each in both unfixed and fixed cultures. Thus I was unable to ascertain whether Nogo-B, or Nogo-C were expressed in the culture system, as the Nogo antibody may have been only detecting Nogo-A. No Nogo-B, or Nogo-C specific antibodies are available commercially as yet. The similar results obtained using 2 different antibodies also suggests that both antibodies are detecting the correct protein in vitro. Non-specific binding of unrelated antigens would be unlikely to be identical using different antisera.

It is feasible that functional roles for Nogo proteins during early development are similar to those of other reticulon family members. These share homology with the Nogo proteins over the transmembrane C-terminal region. However, little research has been published on these proteins, although the growing significance of the Nogo family may stimulate new studies. Reticulons are known to be expressed in certain tumours (Senden et al., 1997), and in adipocytes (Morris et al., 1999). The localisation of reticulons to the endoplasmic reticulum is the reason they are so named.

My own data shows that Nogo Receptor is expressed during development in the spinal cord at later stages than the onset of Nogo-A expression (Fig 7.10, Fig 7.19). This is not inconsistent with a possible role of Nogo-A during early development: Nogo-A has 2 distinct domains, and it is likely that the N-terminal domain is acting via a second receptor at this time.
7.3.4 Nogo's influence on spinal cord regeneration

Inhibition of Nogo has been demonstrated to enhance axonal outgrowth and promote a degree of repair following spinal cord injury (Schnell and Schwab, 1990; Merkler et al., 2001; Raineteau et al., 1999; Bregman et al., 1995). The data presented in this chapter however disproves my hypothesis that onset of Nogo-A expression is crucial in the developmental transition from regeneration-competent to incompetent states in the chick embryo. Given the similarities between chick and human expression patterns, it is also likely that onset of Nogo-A expression in human embryos does not define a point from which spinal regeneration cannot occur. It is possible that other factors help characterise this transitional period. The elevated severity of secondary injury cascades described in Chapter 4 are likely to contribute, perhaps by increasing the availability of inhibitory influences such as Nogo to regenerating axons, or by causing a degree of cell death too great to be overcome. It is possible that extracellular Nogo release following spinal damage could contribute to failure of regeneration; given the fact it can operate as a soluble ligand (Prinjha et al., 2000).

That regeneration can occur in the presence of Nogo is clear. In this study, severed E11 chick spinal cord expresses Nogo-A between opposed stumps that will go on to regenerate. Neurons can clearly overcome Nogo's inhibitory influence at this stage of development, if indeed Nogo is inhibitory at this point. Of potential crucial importance is the cell type, and the cellular location of Nogo at this time. As discussed earlier, it is possible that extracellular localisation of Nogo-A's N-terminal domain on oligodendrocytes is necessary for prevention of regeneration following nerve injury. Nogo's presence earlier in development is likely to be on other cell types, and its function at that time may not necessarily be associated with axonal repulsion.

Another point worthy of consideration is the orientation of Nogo within the spinal cord. Time-lapse video experiments have indicated that cell-cell contact is necessary for the axon growth repelling nature of Nogo (Bandtlow et al., 1990). Furthermore, it has been
demonstrated that the inhibition of axonal growth mediated by Nogo is affected by geometry: axons appear to be able to grow along myelin tracts, but are restricted in growing across them (Pettigrew and Crutcher, 1999). For these reasons, the presence of Nogo early in development would not be expected to inhibit normal axonal growth, providing that extension was proceeding in the correct direction. However, damage to spinal architecture would inevitably result in disruption to white matter geometry, and this would presumably inhibit axonal re-growth. This model is supported by experiments by Pettigrew where rat spinal cords were injured, then immediately frozen to prevent glial reactions. Cryosections of such spinal injuries supported parallel axonal growth along uninjured white matter tracts. Damaged white matter repelled axons, or resulted in short non-parallel sprouting (Pettigrew et al., 2001).

Experiments in which the structure of myelin has been disrupted immunologically have resulted in enhances axonal regeneration following injury, both in the embryonic, and posthatchling chick (Keirstead et al., 1992; Keirstead et al., 1995) and in adult rat (Dyer et al., 1998). These experiments involve administration of GalC antibodies and complement, resulting in an unravelling of myelin sheaths (Keirstead et al., 1995). Presumably such an effect would allow greater flexibility to re-growing axons, permitting more effective repair following injury. Perhaps significantly, these experiments did not target Nogo proteins directly, but disrupted myelin as a whole. Thus, only Nogo directly associated with myelin would be affected. This could be crucial, as widespread immunological disruption of all Nogo-expressing cells is likely to have serious consequences, affecting many cell types, including developing neurons and muscle according to the data presented in this thesis.

Several possibilities exist to explain why the presence of Nogo-A early in development does not prevent effective spinal repair. Firstly, Nogo-A is expressed in different cell types at these stages of development. This in itself could be enough, although Nogo-A may also have a different function at early stages of development, perhaps related to
the endoplasmic reticulum. Although NgR is present in early embryogenesis, the receptor mediating Nogo-A’s N-terminal domain effects may not be expressed at this time. Thus axons would be non-responsive to the effects of the N-terminal domain. Additionally, the release of Nogo-A into post-injury spinal cord may be less, due to less severe secondary tissue injury responses. This seems likely to be true, as the results from chapter 4 indicate. It is possible that any, or all, of these reasons may be applicable.
CHAPTER 8. FINAL DISCUSSION
This thesis has gone some way in characterising the embryonic chick model of spinal cord regeneration. This underused system has previously been utilised for demonstrating the influence of myelin on the effectiveness of regeneration. Here however, I have analysed thoroughly the histopathology of spinal cord injuries at both permissive and restrictive periods for regeneration, and have characterised the model with respect to recent advances in the field of axonal regeneration.

Crucially, I have shown that secondary injury processes affect the spinal cord to a greater extent at later points of development (Chapter 4). This observation is of significance in interpreting why the adult spinal cord cannot effect successful repair, in contrast with the embryonic nervous system. Indeed the pathology of spinal injury in the chick resembles that seen in the rat or human to a greater extent than previously described. The process of cavitation is similar in each case, validating the chick as an accurate model of human spinal injury.

Previous studies have implicated the onset of myelination as defining the point at which effective regeneration can no longer occur (Keirstead et al., 1992; Keirstead et al., 1995). It is likely that substantial expansion of spinal cord injuries via secondary mechanisms will contribute to failure of regeneration, probably to a greater extent than the expression of specific inhibitors of axonal growth, or the profile of regeneration-promoting neurotrophins. Also of importance is the role of cell death following spinal trauma. The number of cells dying was observed to be greater in non-regenerating stages of development; furthermore, much of the cell death was apoptotic in nature. This is in keeping with other recent studies that have identified programmed cell death as a significant contributor to cell loss following spinal injury (Lou et al., 1998; Newcomb et al., 1999; Liu et al., 1997). This has exciting therapeutic implications: the severity of spinal cord injuries could be minimised by rapid treatment with anti-apoptotic agents. My results indicate that apoptotic death persists for at least 4 days in
the injured chick spinal cord, suggesting that a large number of cells could be rescued by such intervention.

Besides the pathological observations described here, many other factors were investigated for potential contributions to the transition from regeneration competent to incompetent states (Chapter 5). Surprisingly no previous studies had performed such analyses. I found that the axon-repulsing chondroitin sulphate proteoglycans was expressed at higher levels in the chick spinal cord at restrictive periods. Also it was found in neuronal populations, in addition to expression in marginal regions as seen earlier in development. It is likely that this reflects the generation of a non-permissive environment within the spinal cord.

Given the differences in degree of secondary injury processes, the vascularisation of the spinal cord was also examined. This revealed an exponential elevation in percentage surface area of blood vessels in the spinal cord during the developmental period E7-E17. This may be a key reason behind the elevated extent of haemorrhage observed after E15 spinal injury, compared to E11.

In addition, the expression of FGF2 was investigated, revealing a transition from chiefly neuronal to glial localisation over the same developmental period. FGF2 is beneficial when applied following spinal cord injury, and the loss of expression from neuronal population may be of significance in influencing cell survival after trauma.

This was investigated further by attempting to manipulate FGF2 levels in the embryonic chick spinal cord by pharmacological means. The benzodiazepine drug diazepam has been shown to elevate brain and spinal cord levels of FGF2 (Gomez-Pinilla et al., 2000; Gomez-Pinilla and Dao, 1999). These results were successfully replicated in the E15 chick. However, the diazepam treatment did not improve outcome following spinal cord injury, as analysed by histology. Indeed the treatment worsened outcome following E11 spinal injury, although at this developmental stage FGF2 expression was
not altered by diazepam. Despite the fact that injury prognosis was not improved, the worsening of E11 injuries provided some insight into the reaction of the embryonic chick to spinal damage. The diazepam treatment resulted in initiation of secondary injury mechanisms not usually seen at this developmental stage. This demonstrates that the toxic cascades necessary for prolonged cell death and cavitation are present, but are not normally triggered following spinal injury. Understanding why this is so may provide valuable clues in developing strategies to reduce injury escalation in human patients.

Finally I have used the chick model to investigate the contribution of Nogo-A to the prevention of axonal regeneration. My initial hypothesis that Nogo-A would only be expressed after E13 in the chick spinal cord was immediately proved incorrect. Nogo-A was found at very early stages of development, in cells other than oligodendrocytes. This study was extended to include human embryos and yielded similar results. Furthermore analysis of spinal cord injuries during permissive developmental stages indicated that Nogo-A was present in close proximity to regenerating axons. My subsequent hypothesis, that Nogo-A's effect was mediated then by onset of Nogo Receptor at E13 was also found to be incorrect. Nogo Receptor is also expressed at early stages of development in both chick and human. These data imply a role for Nogo/Nogo Receptor in developmental processes that may include axonal guidance or synapse formation. Experiments performed on human embryonic cultures also provide evidence for a developmental switch from neuronal to oligodendrocyte expression, such a transition may help explain the onset of a non-permissive environment within the adult CNS.
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