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NEUROGENESIS AND APOPTOSIS IN THE DEVELOPMENTALLY REGULATED LOSS OF SPINAL CORD REGENERATION

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Abstract

Unlike the adult mammal, the chick can successfully regenerate its spinal cord until embryonic day (E) 13. Multiple factors may contribute to the subsequent loss of regenerative capacity, although most research has concentrated on axonal re-growth inhibition as a key issue. The number of viable cells remaining in the spinal cord could also be important and may be affected by cell survival and cell replacement. In this thesis the early response of the chick spinal cord to injury has been investigated, focusing on cell death and the potential to replace lost cells by neurogenesis.

Pharmacological reduction of haemorrhage after injury at E15 resulted in reduced apoptosis and cavitation, suggesting that blood-borne factors, such as the serine protease thrombin, may cause apoptosis. Endogenous thrombin expression and activity after injury was investigated. Thrombin was not up-regulated after injury at E15; however, evidence suggests that the activity of other serine proteases was increased. In parallel, in organotypic slice cultures, exogenous thrombin treatment did not increase apoptosis. These results provide new information about the contribution of serine proteases to apoptosis in the chick, suggesting that, although thrombin is not of primary importance, other serine proteases could play a greater role.

Next, the contribution of neurogenesis to regeneration at E11 was examined. Changes in the expression and phosphorylation of the early neuronal marker, doublecortin, in response to injury were observed. Although increased proliferation in the grey matter was observed, no increase in neurogenesis after injury was detected. Surprisingly, ongoing neurogenesis was discovered in the normal spinal cord at E11. These results challenge established views about the timing of neurogenesis in the chick spinal cord and suggest that ongoing proliferation and neurogenesis may contribute to the regenerative capacity at this stage.

This thesis presents insights into factors involved in the early response of the chick spinal cord to injury, providing new information about the contribution of neurogenesis and cell survival to regenerative capacity.

I, Katherine Ann Whalley, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abbreviations

AIF	Apoptosis-inducing factor
APC	Activated protein C
ATP	Adenosine tri-phosphate
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
BMP	Bone morphogenic protein
BrdU	Bromo-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CRMP	Collapsin response-mediated protein
CSPG	Chondroitin sulphate proteoglycan
DEPC	Diethylpyrocarbonate
DEVD-FMK	Biotin-X-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-CH_2F
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide-triphosphates
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglion
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediametetracetate
EGF	Epidermal growth factor
EtOH	Ethanol
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAP-43	Growth associated protein-43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPI	Glycosylphosphatidyl-inositol
GTP	Guanosine triposphate
H&E	Haemotoxylin and Eosin
HCl	Hydrochloric acid

HSPG	Heparin sulphate proteoglycan
IF	Intermediate filament
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MAG	Myelin-associated glycoprotein
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinase
MARK	MAP/ Microtubule affinity regulatory kinase
MOPS	4-Morpholinepropanesulfonic acid
NeuN	Neuronal nuclear antigen
NGF	Nerve growth factor
NgR	Nogo-66 receptor
NIMP	Nogo-interacting mitochondria protein
NMDA	N-methyl-D-aspartate
NT-4/5	Neurotrophin-4/5
OMgp	Oligodendrocyte myelin glycoprotein
PAR-1	Protease-activated receptor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKA	Protein kinase A
PN-1	Protease nexin-1
PNS	Peripheral nervous system
PPACK	D-Phe-Pro-Arg-chloromethylketone
PSA-NCAM	Poly-sialylated-neural cell adhesion molecule
RGC	Retinal ganglion cell
RNA	Ribonucleic acid
ROCK	RhoA-associated kinase
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TGF	Transforming growth factor
tPA	Tissue plasminogen activator
TM	Thrombomodulin
TUNEL	TdT-mediated dUTP nick-end labelling
VEGF	Vascular endothelial growth factor

Chapter 1. Introduction

1.1. Structure and function of the spinal cord

The central nervous system (CNS) is composed of two continuous structures, the brain and the spinal cord. The spinal cord consists of a small, roughly cylindrical column of nervous tissue, housed within the vertebral column and extending from the medulla oblongata at the base of the brain to the level of the first or second lumbar vertebrae (Haines 1997a). Although the structural organisation of the spinal cord is simple in comparison to the brain, its importance for the function of the CNS, and indeed the entire body, cannot be underestimated. The spinal cord provides the means by which the brain is able to interact with the rest of the body and the environment.

The principle cell types in the CNS are neurons and glia, with sub divisions of each providing the morphological and functional diversity which characterises the nervous system (Haines *et al.* 1997). In general, neurons make up the wiring of the nervous system, integrating and conveying electrical signals from one part of the CNS to another while glial cells have structural and homeostatic roles, maintaining the microenvironment of the spinal cord. In reality, however, the roles of different cell types are more complex and overlap significantly. Neurons are sub divided according to their morphological features, localisation, function, and neurotransmitter type. Spinal cord glia are divided into two main types; astrocytes, which have functions that include provision of structural support and maintenance of neuronal metabolism, and oligodendrocytes, which produce the myelin sheath that is essential for fast neuronal conductance (Jessen 2004).

The spinal cord is arranged around the central canal, which is continuous with the brain's ventricular system and through which cerebrospinal fluid passes. Closest to the central canal is the grey matter, butterfly shaped in cross section, in which all neuronal cell bodies are located. Surrounding the grey matter lies the white matter, which can be divided into a number of longitudinal columns, containing ascending and descending myelinated neuronal fibre tracts (Fig. 1.1.). The nervous tissue is surrounded by three fibrous layers of meninges which provide protection for the delicate structures beneath (Haines 1997b).

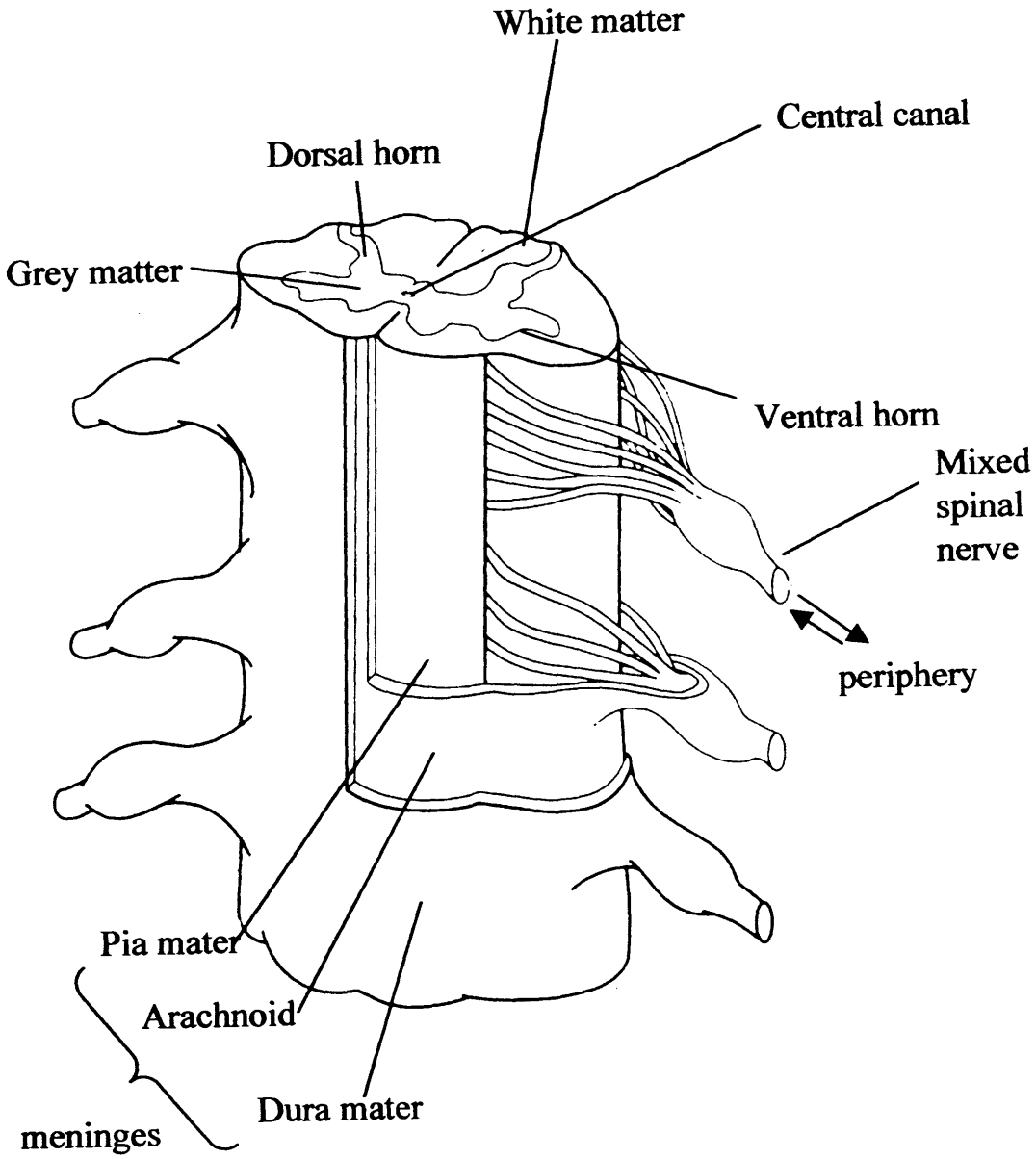


Fig.1.1 Structure of the adult human spinal cord. Schematic diagram illustrating the structural features of the adult human spinal cord. Adapted from Gupta, 1997.

A supply of oxygenated blood reaches the spinal cord via three main arteries - the anterior and posterior spinal arteries, and the segmental artery - which branch into the smaller blood vessels which penetrate the spinal cord (Mautes *et al.* 2000). Similarly, the anterior and posterior spinal veins are responsible for the drainage of blood from the spinal cord. The walls of blood vessels throughout the CNS consist of a specialised endothelial structure, the blood brain barrier, which strictly regulates substances entering and leaving the CNS (Huber *et al.* 2001; Ballabh *et al.* 2004)

In the human, thirty-one pairs of spinal nerves, containing a mixture of afferent (sensory) and efferent (motor) neurons, branch off from the spinal cord to innervate the periphery. Sensory information gathered by receptors in the periphery reaches the dorsal part of the grey matter via neurons that have their cell bodies in the dorsal root ganglia, situated just outside the spinal cord. In the grey matter information is integrated and conducted to higher control centres via white matter tracts which include the spinocerebellar, spinothalamic and spinoreticular tracts. Likewise, motor information is conducted from the motor cortex or brainstem nuclei to the spinal cord via four major tracts; the reticulospinal tract, the vestibulospinal tract, the rubrospinal tract and the corticospinal tract. The ventral part of the grey matter contains the cell bodies of primary effector neurons which exit the spinal cord to convey commands to muscles and glands in the periphery. Additional functions of the spinal cord include the maintenance of body homeostasis and the generation of rapid responses to harmful external conditions via short neuronal circuits, independent of higher control, termed reflex arcs. The spinal cord is therefore a pivotal component of the CNS, without which the brain would be isolated and unable to fulfil its functional roles.

1.2. Development of the spinal cord

At the three layer stage of embryogenesis, a midline portion of the dorsal ectoderm becomes specialised towards a neural fate by signals from a region of the underlying mesoderm, called the organiser (Stern 2005). The specialised region, termed the neural plate, goes on to give rise to the entire CNS. The mechanism by which the neural plate arises is still controversial. Studies in *Xenopus* embryos suggested that the inductive factors are principally inhibitors of bone morphogenetic protein 4 (Bmp4), giving rise to the default model of neural induction, whereby the ectoderm automatically adopts a

neural fate unless prevented from doing so by Bmp4 (Frisen *et al.* 1998; Stern 2005). However, evidence from studies of chick embryo development suggests that positive signalling from factors such as fibroblast growth factor (Fgf) and Wnt proteins may also be required for neural induction, which may begin at an earlier stage than previously thought (Bally-Cuif and Hammerschmidt 2003; Stern 2005).

The neural tube, the basic structure of the developing CNS, forms from the neural plate in a process termed neurulation as outlined in Fig 1.2 (Smith and Schoenwolf 1997; Copp *et al.* 2003). Neurulation also gives rise to the neural crest, a group of ectodermal cells which migrate peripherally from the dorsal part of the neural tube and form the peripheral and enteric nervous systems as well as a range of other cell types including melanocytes, and some skeletal cells (Burns 2005).

The walls of the neural tube initially consist of a pseudostratified layer of highly proliferative multipotent progenitor cells, the neuroepithelium, from which all CNS neurons and glia are ultimately derived. As these cells proliferate, the cell body moves backwards and forwards across the width of the neuroepithelium, making contact with the inner and outer edges of the tube via long processes (Hollyday 2001). The position of the cell body within the epithelium at any given moment corresponds to its current cell cycle stage. Between mitosis (M) and the DNA synthesis (S) phase, cells move away from the lumen of the neural tube before returning for the next round of division. Cell division that results in the birth of two daughter cells that are themselves both stem cells is termed symmetric division. As proliferation continues, asymmetric division, in which one daughter cell exits the cell cycle and migrates away from the lumen of the neural tube, also takes place. This generates a second layer of cells, the intermediate zone, in which differentiation into specific cell types is initiated. Eventually, a third layer, the marginal zone, forms, containing neuronal axons and glial cells. The intermediate and marginal zones later develop into the grey and white matter of the adult spinal cord. The control of symmetric versus asymmetric division may depend upon intrinsic factors such as the distribution of certain proteins in the daughter cells and extrinsic regulation by signalling factors such as Notch (Ikeshima-Kataoka *et* al, 1997, Shen *et al*, 1997, Zhong *et al,* 1996, Lewis, 1996, Frisen *et al*, 1998).

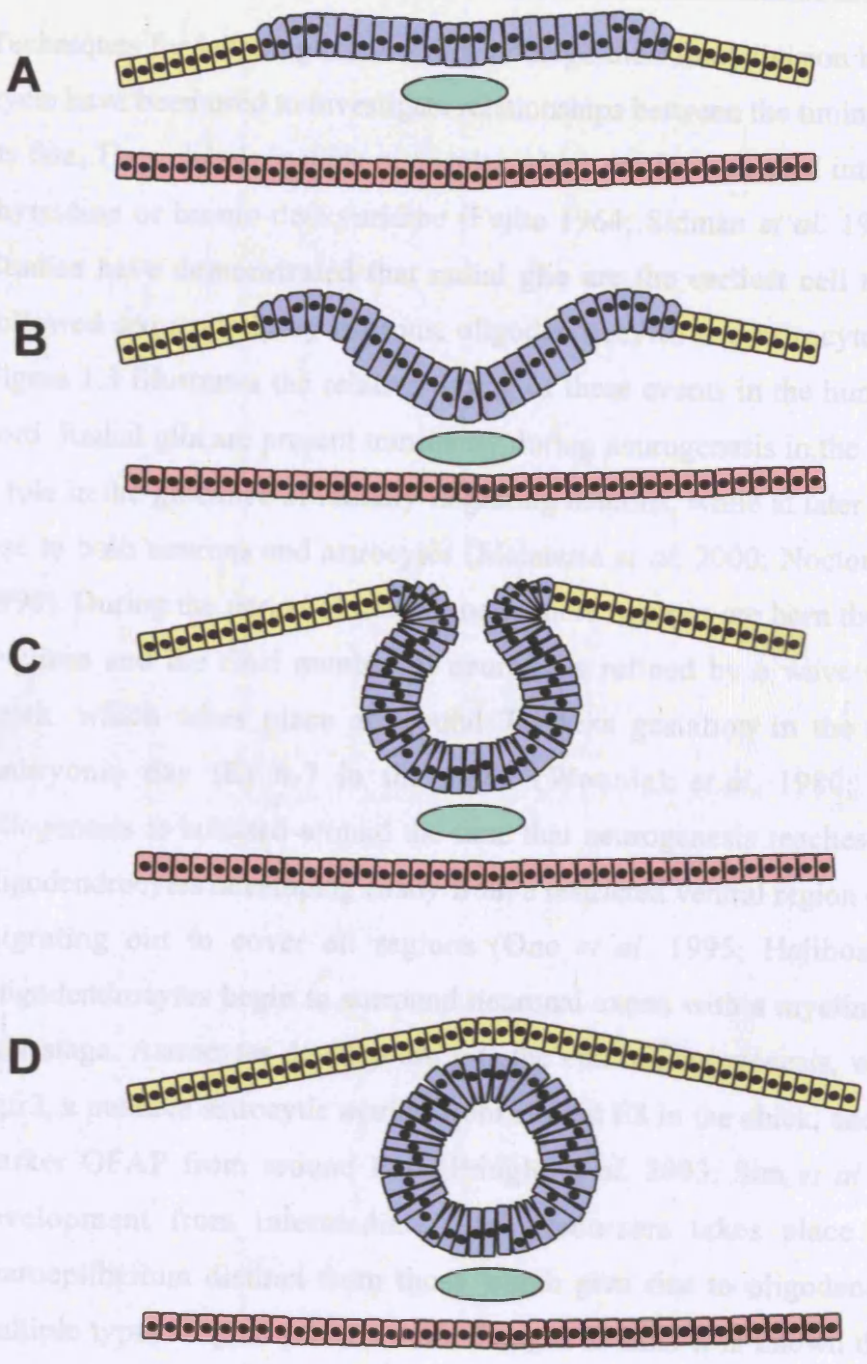
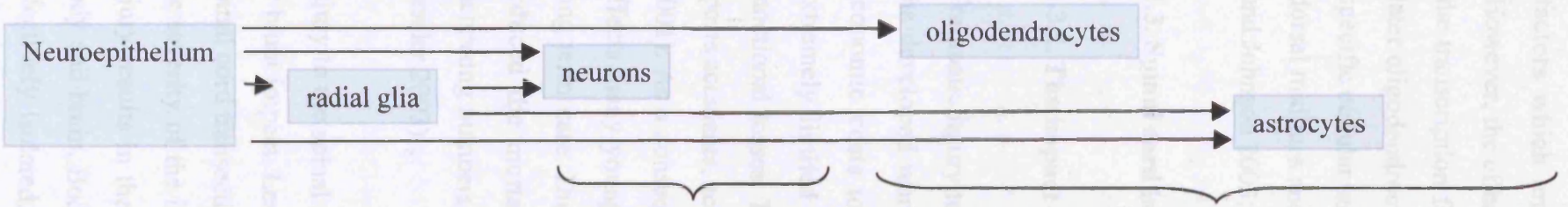


Fig. 1.2. Principles of neurulation. A) During neural tube closure, a region of the dorsal ectoderm (yellow) lying above the notochord (green) thickens to form the neural plate (blue) and elevates to form the neural folds. **B)** The neural plate begins to fold, forming the neural groove. **C)** Continued folding causes the neural folds to be brought together at the midline. **D)** Finally, the neural folds fuse to form a continuous neural tube. Adapted from Frisen *et al*, 1998.

Techniques for labelling cells as they undergo their final division before exiting the cell cycle have been used to investigate relationships between the timing of a cell's birth and its fate. These labels include molecules which are incorporated into DNA such as ^{3}[H]-thymidine or bromo-deoxyuridine (Fujita 1964; Sidman *et al.* 1959; Gratzner 1982). Studies have demonstrated that radial glia are the earliest cell type to differentiate, followed sequentially by neurons, oligodendrocytes and astrocytes (Lee *et al.* 2000). Figure 1.3 illustrates the relative timing of these events in the human and chick spinal cord. Radial glia are present transiently during neurogenesis in the spinal cord and have a role in the guidance of radially-migrating neurons, while at later stages they can give rise to both neurons and astrocytes (Malatesta *et al.* 2000; Noctor *et al.* 2002; Hatten 1990). During the period of neurogenesis more neurons are born than will ultimately be required and the final number of neurons is refined by a wave of programmed cell death. which takes place at around 7 weeks gestation in the human and around embryonic day (E) 6-7 in the chick (Wozniak *et al.* 1980; Oppenheim 1991). Gliogenesis is initiated around the time that neurogenesis reaches its conclusion with oligodendrocytes developing firstly from a restricted ventral region of the CNS and then migrating out to cover all regions (Ono *et al.* 1995; Hajihosseini *et al.* 1996). Oligodendrocytes begin to surround neuronal axons with a myelin sheath at a slightly later stage. Astrocytes develop towards the end of neurogenesis, with cells expressing Fgfr3, a putative astrocytic marker from around E8 in the chick, and the later astrocytic marker GFAP from around E10 (Pringle *et al.* 2003; Sim *et al.* 2002). Astrocytic development from intermediate glial precursors takes place in regions of the neuroepithelium distinct from those which give rise to oligodendrocytes. Although multiple types of glial precursor are thought to exist it is known that some astrocytes develop from radial glial cells via transdifferentiation (Pixley and de Vellis 1984; Gray and Sanes 1992; Chanas-Sacre *et al.* 2000; Doetsch *et al.* 1999; Liu and Rao 2004).

The differentiation of neural progenitor cells into neurons and glia involves a series of decision-making steps in which cells first become post-mitotic and then commit to a specific lineage (Hollyday 2001). The mechanisms underlying regulation of these decisions are complex and not yet fully determined. However, it is known that neurogenesis and gliogenesis are initiated in progenitor domains in a specific temporal and spatial pattern and that this pattern is determined early. Within a progenitor cell, neurogenesis is controlled in part by the expression of pro-neural genes, such as the



Species	Radial glia born	Neurogenesis	Programmed cell death	Gliogenesis		
				Oligodendrocytes	Myelination	Astrocytes
human	By 12 weeks gestation (Weidenheim, 1994)	4 – 8 weeks gestation (Wozniak *et al*, 1980)	7 weeks gestation (Wozniak *et al*,1980)	7 weeks gestation (Hajihosseini *et al*, 1996)	20 weeks gestation (Grever *et al*, 1997; Tanaka *et al*, 1995)	Unknown
chick	Unknown	E3 – E9 (Fujita, 1964)	E6 – E7 (Oppenheim, 1991)	E5 – E6 (Ono *et al*,, 1995)	E13 (Ono *et al*,, 1995)	E8 -E19 (Pringle *et al* 2003)

Fig 1.3. Temporal birth of neurons and glia in the developing spinal cord. Figure at top shows the approximate sequential order of birth of glia and neurons in the developing spinal cord. Table shows studies which have recorded the approximate dates at which each cell type/ event is observed in the human and chick spinal cord.

basic helix-loop-helix (bHLH) transcription factors, while gliogenesis is promoted by factors which antagonise bHLH proteins (Bally-Cuif and Hammerschmidt 2003). However, the effect of different genes varies with development. Certain factors, such as the transcription factor Olig2, are involved in specification firstly of neurons and then later oligodendrocytes (Lu *et al.* 2002). The neural tube is also patterned spatially into specific cellular subtypes by diffusible signals arising from structures in the ventral and dorsal midlines and from tissues adjacent to the developing cord (Hollyday 2001; Helms and Johnson 2003; Stemple 2005).

1.3. Spinal cord injury

1.3.1. The impact of spinal cord injury

Traumatic injury to the spinal cord affects between 11.5 and 53.4 people per million in the developed world, with life-shattering consequences for individuals and enormous economic costs to society (Sekhon and Fehlings 2001). The human CNS has an extremely limited capacity for self-repair and damage usually results in permanent functional losses. The principle causes of spinal cord injury are road traffic accidents, sports accidents, accidents at work, falls in the home and violence (Sekhon and Fehlings 2001). As a consequence, unlike many neurodegenerative diseases, spinal cord injury affects many young, otherwise healthy individuals with a consequent requirement for long term care. During the 20th century, improvements in medical care substantially reduced the mortality rates associated with spinal cord injury and consequently increasing numbers of individuals are living with chronic spinal cord injury (Houle and Tessler 2003).

Injury to the spinal cord can take several forms, most frequently involving compression or blunt impact. Less commonly, a penetrating injury may result in partial or complete spinal cord transection. Regardless of injury type, the functional outcome depends upon the severity of the injury and the spinal cord level at which it takes place. Spinal cord injury results in the disruption of the axonal tracts carrying information between the body and brain. Body areas innervated by nerves arising at or below the injury site are effectively isolated, losing sensory, motor and autonomic function. The outcome of spinal cord injury may therefore range from paraplegia, in which only the lower half of

the body is paralysed, to tetraplegia in which assistance for functions such as breathing may be necessary (Profyris *et al.* 2004). Secondary complications associated with the loss of these functions may also arise, including urinary tract infections, pressure sores and neurogenic pain.

1.3.2. Pathophysiology of spinal cord injury

Injury to the central nervous system in the adult mammal invokes a series of pathophysiological events which culminate in the functional impairments experienced by the patient. Characteristic features of spinal cord injury include neuronal and glial death, axonal degeneration and demyelination, the formation of large fluid filled cavities within the spinal cord and the generation of a glial scar. Many of these processes also occur, to an equal or lesser extent, in lower vertebrate and avian species.

Primary and secondary injury

Cellular and tissue damage following injury to the mammalian CNS is divided into two broad stages, termed the primary and secondary injury (Dumont *et al.* 2001). The primary injury occurs immediately upon the traumatic disruption of spinal cord tissue, resulting in the immediate destruction of tissue and haemorrhage due to rupture of cell membranes and blood vessel walls. Damage tends to be focused in the delicate, highly vascularised grey matter at the cord centre due to the concentration of shearing forces in this region, and is localised to the injury site (Profyris *et al.* 2004). Although the primary injury results in significant disruption, overall damage is vastly expanded by a series of delayed injury processes, triggered by the initial insult, which collectively make up the secondary injury (Fig. 1.4). These processes include vascular alterations, biochemical activity and cellular responses and trigger cell death by both necrotic and apoptotic mechanisms (Hausmann 2003). The extent of the secondary injury response after injury in species that demonstrate a greater regenerative response, such as the embryonic chick (see section 1.4), is unknown, and a reduction in these mechanisms might make a contribution to spinal cord regeneration in these species.

Haemorrhage

In a typical spinal cord injury, mechanical trauma causes damage to the blood vessel network resulting in localised haemorrhage within the injured part of the cord. Within

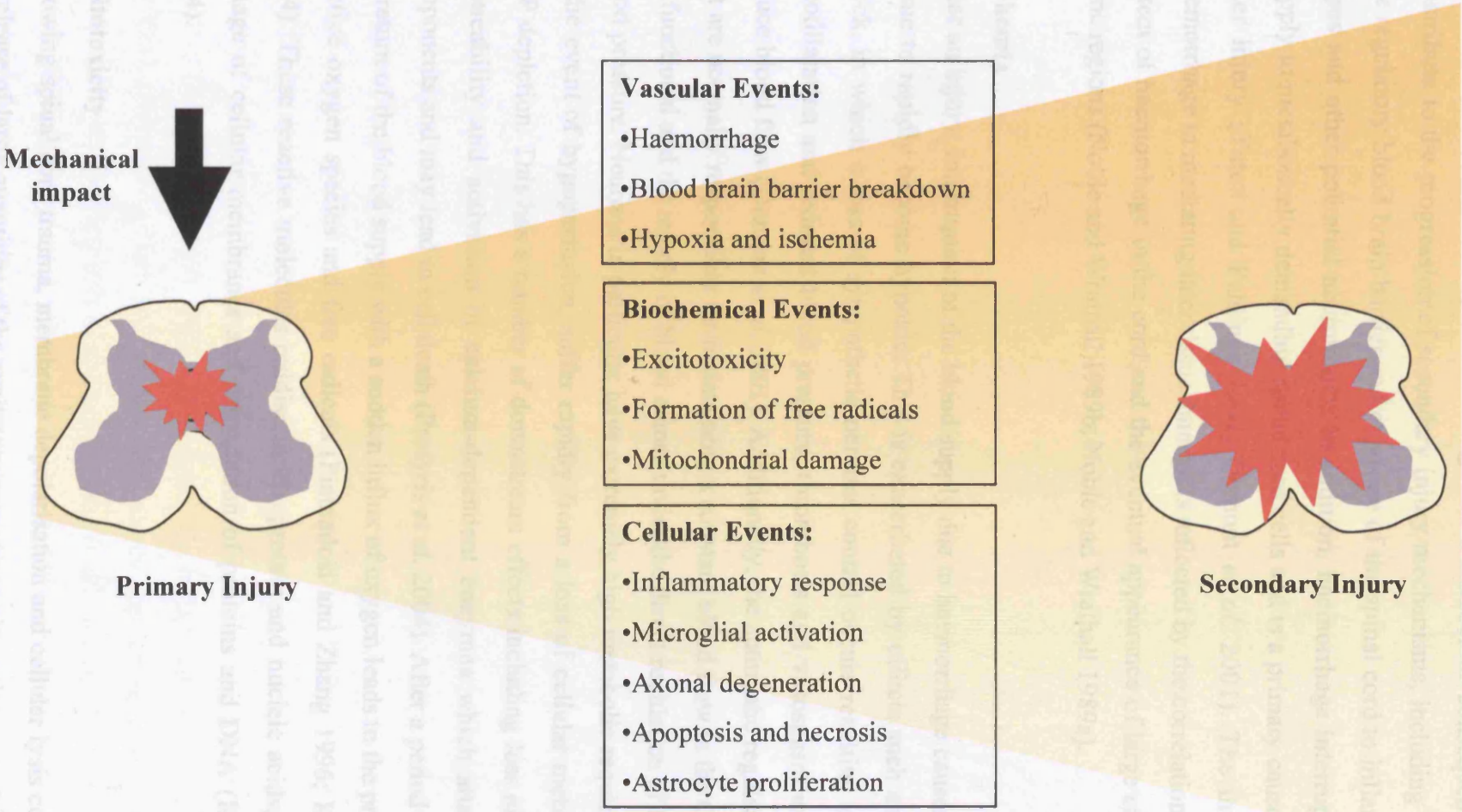


Fig. 1.4. Summary of secondary injury mechanisms after spinal cord injury in the mammal. Adapted from Hausmann, 2003

the first 24 hours after injury, this haemorrhage spreads extensively within the cord (Noble and Wrathall 1989b). Haemorrhage has a number of consequences that may contribute to the progression of secondary injury mechanisms, including disruption of the regulatory blood brain barrier and exposure of the spinal cord to inflammatory cell types and other potential neurotoxins. In addition, haemorrhage interrupts the blood supply to metabolically demanding spinal cord cells and is a primary cause of ischemia after injury (Tator and Fehlings 1991; Dumont *et al.* 2001). The importance of haemorrhage in mediating later tissue damage is reflected by the correlation between the extent of haemorrhage in the cord and the eventual appearance of large cavities in the same regions (Noble and Wrathall 1989b; Noble and Wrathall 1989a).

Ischemia

After an injury, interruption of the blood supply due to haemorrhage causes spinal cord tissue to rapidly become hypoxic. This is exacerbated by effects such as neurogenic shock, in which a loss of sympathetic nervous control occurs resulting in peripheral vasodilatation and reduced blood pressure, thrombosis and vasospasm which further reduce blood flow (Dumont *et al.* 2001). Additionally, the automatic regulatory systems that are normally responsible for maintaining a constant blood flow in the CNS become dysfunctional and the supply of blood comes under the direct regulation of the systemic blood pressure. Neurons in particular have extremely high metabolic requirements and, in the event of hypoperfusion, suffer rapidly from a loss of cellular metabolism and ATP depletion. This has a number of downstream effects including loss of membrane permeability and activation of calcium-dependent enzymes which attack cellular components and may lead to cell death (Profyris *et al.* 2004). After a period of hypoxia, the return of the blood supply with a sudden influx of oxygen leads to the production of reactive oxygen species and free radicals (Piantadosi and Zhang 1996; Kwon *et al.* 2004). These reactive molecules oxidise lipids, protein and nucleic acids, leading to damage of cellular membranes and degradation of proteins and DNA (Kwon *et al*, 2004).

Excitotoxicity

Following spinal cord trauma, membrane depolarisation and cellular lysis contribute to the release of large quantities of the excitatory neurotransmitter glutamate from neurons and glia. Extracellular glutamate levels are further increased by compromise of the

active mechanisms by which it is recovered by cells, due to the loss of cellular metabolism. Excessive stimulation of ionotropic glutamate receptors causes further membrane depolarisation and an increase in the intracellular levels of calcium and sodium (Choi 1987). Increased intracellular sodium causes water to enter the cell down an osmotic gradient, resulting in oedema and cell lysis. Increased intracellular calcium levels are associated with a number of additional detrimental effects including disruption of mitochondrial activity and activation of destructive proteolytic enzymes. Additionally, continued membrane depolarisation results in further neurotransmitter release causing an escalation of the excitotoxic effect (Faden and Simon 1988; Choi 1988; Choi 1992; Liu *et al.* 1991; Liu *et al.* 1999a).

Inflammation

Although the CNS is generally regarded as an immune-privileged area, injury and disruption of the blood brain barrier initiates an inflammatory response (Hausmann 2003). An influx of neutrophils within the first hours after injury is followed by the recruitment of macrophages and lymphocytes from the periphery and activation of resident microglial cells (Popovich *et al.* 1997; Popovich and Jones 2003). Cytokine release leads to further inflammatory cell recruitment and the inflammatory response escalates over several days after the injury. The issue of whether this inflammatory response is of harm or benefit to the injured spinal cord has been the subject of much controversy; however, a consensus now suggests that it represents a 'dual-edged sword' (Bethea and Dietrich 2002; Kwon *et al.* 2004). Inflammation makes a significant contribution to the expanding secondary injury. Macrophages and microglia cause tissue destruction as well as releasing cytokines which can contribute to oxidative stress by stimulating the generation of reactive oxygen species and nitric oxide (Leskovar *et al.* 2000; Satake *et al.* 2000). It has been shown that microinjection of an activator of macrophages and microglia into the spinal cord without causing physical damage results in a persistent inflammatory response that leads to significant secondary damage and cavitation (Fitch *et al.* 1999). On the other hand, the inflammatory response can have beneficial effects. For example, macrophages secrete certain molecules and growth factors which could contribute to wound healing (DeKosky *et al.* 1994; Bethea and Dietrich 2002; Nguyen *et al.* 2002). Macrophages also contribute to the removal of harmful debris and molecules that are inhibitory to future axonal re-growth. It has even been suggested that the diminished inflammatory response observed in the CNS in

comparison to other tissues underlies the poor repair of this region and functional improvements have been demonstrated after re-introduction of inflammatory cell types (Rapalino *et al.* 1998; Schwartz *et al.* 1999; Moalem *et al.* 1999).

Apoptotic cell death

Cell death can occur as a result of two alternative mechanisms, necrosis or apoptosis, characterised by their biochemical pathways and accompanying morphological changes. Necrosis is a passive form of cell death in which cell swelling and lysis usually occur and an inflammatory response may be initiated (Kwon *et al.* 2004). In contrast, apoptosis is a genetically-programmed form of cell death involving protein synthesis and a regulated series of biochemical events, which does not stimulate an inflammatory response.

Apoptosis has a variety of non-pathological functions during development and in tissue homeostasis, and alterations in its regulation are associated with several disease areas (Thompson 1995; Henderson 1996; Savitz and Rosenbaum 1998). Apoptosis may be triggered by external stimuli or in response to internal signalling. The activation of members of the caspase family of cysteine proteases represents a critical convergent step for classical apoptotic pathways and results in the cleavage of key survival related proteins and, ultimately, cell death (Cohen 1997; Thornberry and Lazebnik 1998; Kuan *et al.* 2000). Although the pathways leading to caspase-dependent cell death have been intensively investigated, apoptotic cell death has also been observed in the absence of caspase activation, leading to the concept of caspase-independent apoptotic pathways (Stefanis *et al.* 1999; Yaginuma *et al.* 2001; Oppenheim *et al.* 2001; Abraham and Shaham 2004). This is a growing field of research, in which release of proapoptotic proteins, such as apoptosis-inducing factor (AIF), from mitochondria, may be of key importance (Lorenzo and Susin 2004). By either mechanism, apoptotic cell death culminates in DNA fragmentation, cell shrinkage and phagocytosis by surrounding cells.

Ischemia and excitotoxicity can trigger both necrotic and apoptotic mechanisms. Additionally, exposure of spinal cord cells to neurotoxic molecules and inflammatory cells during haemorrhage may contribute to cell death (Noble and Wrathall 1989b; Bethea and Dietrich 2002). There is evidence for caspase-dependent apoptosis taking

place in the spinal cord after injury in both experimental models and human patients (Crowe *et al.* 1997; Liu *et al.* 1997; Lou *et al.* 1998; Emery *et al.* 1998; Yong *et al.* 1998; Springer *et al.* 1999; Citron *et al.* 2000a; Li *et al.* 2000). Apoptosis has been shown to take place in two waves after spinal cord injury and lasts 3 weeks after the injury in rats and 8 weeks in humans (Liu *et al.* 1997; Emery *et al.* 1998). An early phase of apoptosis of neurons and glia close to the injury site is followed by the delayed apoptosis of oligodendrocytes at a distance from the injury (Liu *et al.* 1997; Shuman *et al.* 1997; Li *et al.* 1999). Delayed apoptosis of microglia in the white matter has also been observed (Casha *et al* 2001, Shuman *et al, 1997)*

Axonal degeneration

As a result of a traumatic mechanical injury to the spinal cord the axons of surviving neurons may be severed or damaged. Additionally, neurons close to the lesion may die, leaving behind axonal fragments. Damaged axons degenerate over the course of days to years after the injury in a process termed Wallerian degeneration, contributing to the ongoing secondary injury process (Profyris *et al.* 2004). This degeneration involves de-myelination, axonal swelling and axonal lysis. Ultimately the debris generated by axonal degeneration is cleared by the arrival of macrophages and microglia. Oligodendrocytes that rely on their association with axons for neurotrophic support may subsequently die due to the removal of this supply.

Glial scar formation

One of the later events to occur after spinal cord trauma is the formation of the so-called glial scar, which forms over several weeks after the injury (Fawcett and Asher 1999). The formation of the glial scar is directly related to the inflammatory response and may be associated with increased TGFβ2 expression after injury (Fitch *et al.* 1999; Lagord *et al.* 2002). Similarly, other inflammation-related molecules, including interleukin (IL)-1, interferon-γ (IFNγ) and FGF2 have been associated with glial scar formation (Silver and Miller 2004). Microglia and macrophages initially invade the injury site, acting to clear up the debris from degenerating axons and oligodendrocytes. As the lesion site is cleared, oligodendrocyte precursors enter the site and begin to differentiate. Following this, astrocytes migrate to the injury site where they proliferate and become hypertrophic, generating large quantities of intermediate filaments (Profyris *et al.* 2004).

Eventually these cells fill the cavities left by the injury. During this process a barrier is effectively created between the region of injury site and the rest of the spinal cord. In cases where the meningeal membranes surrounding the spinal cord have been disrupted by the injury this is compounded by the entry of meningeal cell types which, together with the astrocytes, form a dense scar matrix. The action of glial cells and the formation of the scar after spinal cord injury has both beneficial and harmful consequences. The entry of glial cells into the injury site helps to stabilise the spinal cord, repair the blood brain barrier and re-establish the microenvironment (Bush *et al.* 1999). The segregation of the injured tissue at the lesion site may also help to protect the rest of the spinal cord from further damage. In support of this, studies have shown that removal of reactive astrocytes using a transgenic approach to delete GFAP expressing cells exacerbates tissue damage after CNS injury (Faulkner *et al.* 2004; Bush *et al.* 1999). However, components of the glial scar including oligodendrocyte precursors and reactive astrocytes have been demonstrated to be extremely inhibitory to future axonal re-growth (Silver and Miller 2004; Properzi and Fawcett 2004).

1.3.3. Experimental models of spinal cord injury

Much of what is known about the secondary injury mechanisms that take place in the injured mammalian spinal cord has been derived from animal models. A range of different models has been developed, each designed to mimic aspects of the situation in the traumatised human spinal cord. The extent to which they are able to do this dictates the usefulness of the model. Two common rodent models are spinal cord contusion, usually involving a controlled weight drop into the dorsal surface of the spinal cord, and spinal cord compression in which a weight is placed onto the cord for a set duration (Noble and Wrathall 1989b; Noble and Wrathall 1989a; Mautes *et al.* 2000; Young 2002). These models mimic the most common forms of human spinal cord injury and pathophysiological changes observed in these models correspond well to human spinal cord injury pathology. However, the complex nature of these types of injury can complicate the interpretation of results designed to examine distinct aspects of spinal cord injury, such as axonal regeneration. A third commonly used model involves transection, or more usually hemisection, of the spinal cord (Rosenzweig and McDonald 2004). In human spinal cord injury, transection alone is relatively rare; however these models are useful for measuring axonal regeneration since they allow researchers to

ensure that all descending and ascending tracts are completely transected. In addition to these models, systems that demonstrate the capacity to regenerate after injury, such as the embryonic chick spinal cord, can be useful in understanding the response of the spinal cord to injury and these will be discussed in greater detail in section 1.4.

1.3.4. Current and prospective treatments

At present, treatment for spinal cord injury cannot provide any anatomical repair and most therapies are directed towards rehabilitation, coping with disability and prevention of various associated consequences of the injury. These areas have shown great progress, resulting in greatly reduced mortality, yet there is clearly great demand for therapeutics which can improve long term functional recovery (Blight 2002).

Whilst complete repair of the injured spinal cord remains a distant goal, small improvements in neuronal circuitry could have enormously beneficial consequences for patients. This might extend to regaining the control of bladder function, breathing without a respirator or small gains in limb movement rather than a complete restoration of locomotion. However, a stumbling block in the development of therapeutic agents arises in the translation of treatments that show promise in rodent models to the clinic. This may be related to the reliance on locomotor function as the usual outcome measure in preclinical studies (Blight 2002). Consequently, only one pharmacological agent, the steroid methylprednisolone, has been licensed for spinal cord injury (Bracken *et al.* 1998). As will be described below, this treatment is the subject of much controversy and is not prescribed in the UK, although it is licensed for use in the US. Although the past fifteen years have seen a number of other clinical trials launched, most of these have demonstrated little positive effects (Blight 2002). Meanwhile, basic research continues to identify potential approaches for improving outcome and trials of several of promising agents are imminent. As our understanding of the pathophysiological processes taking place after spinal cord injury continues to improve it is hoped that trials of prospective therapies will produce better outcomes. This may be aided by a consideration of the responses to trauma that take place in systems which can more successfully repair after injury.

1.4. Spinal cord regeneration

A number of organs in the human body are able to self-repair to varying extents throughout adult life. These include the liver, skin and the peripheral nervous system (PNS). In contrast, the CNS does not spontaneously regenerate once it has been damaged, leading to significant functional losses as outlined above. The lack of regeneration observed in the adult mammalian CNS is in striking contrast to the robust regeneration that is observed after injury in a number of other systems (Fig 1.5). These include adult mammalian systems such as the peripheral nervous system, lower vertebrates, such as fish and certain amphibian species, and embryonic systems, such as the chick spinal cord (Ferretti *et al.* 2003).

1.4.1. Regeneration in the peripheral nervous system

Not all adult mammalian neurons are incapable of regeneration. In contrast to the CNS, PNS neurons demonstrate a remarkable capacity for repair after injury (Fawcett and Keynes 1990). Injury to the PNS results in the activation and proliferation of Schwann cells, the myelinating glial cells that are unique to the PNS. These cells are thought to represent a key element of the regenerative response of the PNS neurons, aiding axonal re-growth by clearing cellular debris and expressing and secreting a number of molecules which support axon growth, contributing to a process termed Wallerian degeneration (Fawcett and Keynes 1990; Son *et al.* 1996). Primary afferent (sensory) neurons also have the unique feature of interacting with both PNS and CNS tissue, crossing the junction between the two at the dorsal root entry zone (DREZ). Sensory neurons can robustly regenerate their peripheral processes after an injury, yet show limited capacity to re-enter the CNS by crossing the DREZ after an injury (Chong *et al.* 1999). Interestingly, pre-conditioning of sensory neurons by transection of the peripheral process has been demonstrated to increase the regenerative response following a later injury to the central process (Richardson and Issa 1984; Neumann and Woolf 1999). PNS neurons therefore represent a useful model for comparison with CNS neurons within the same species and additionally allow comparison of the response of peripheral and central parts of the neuron.

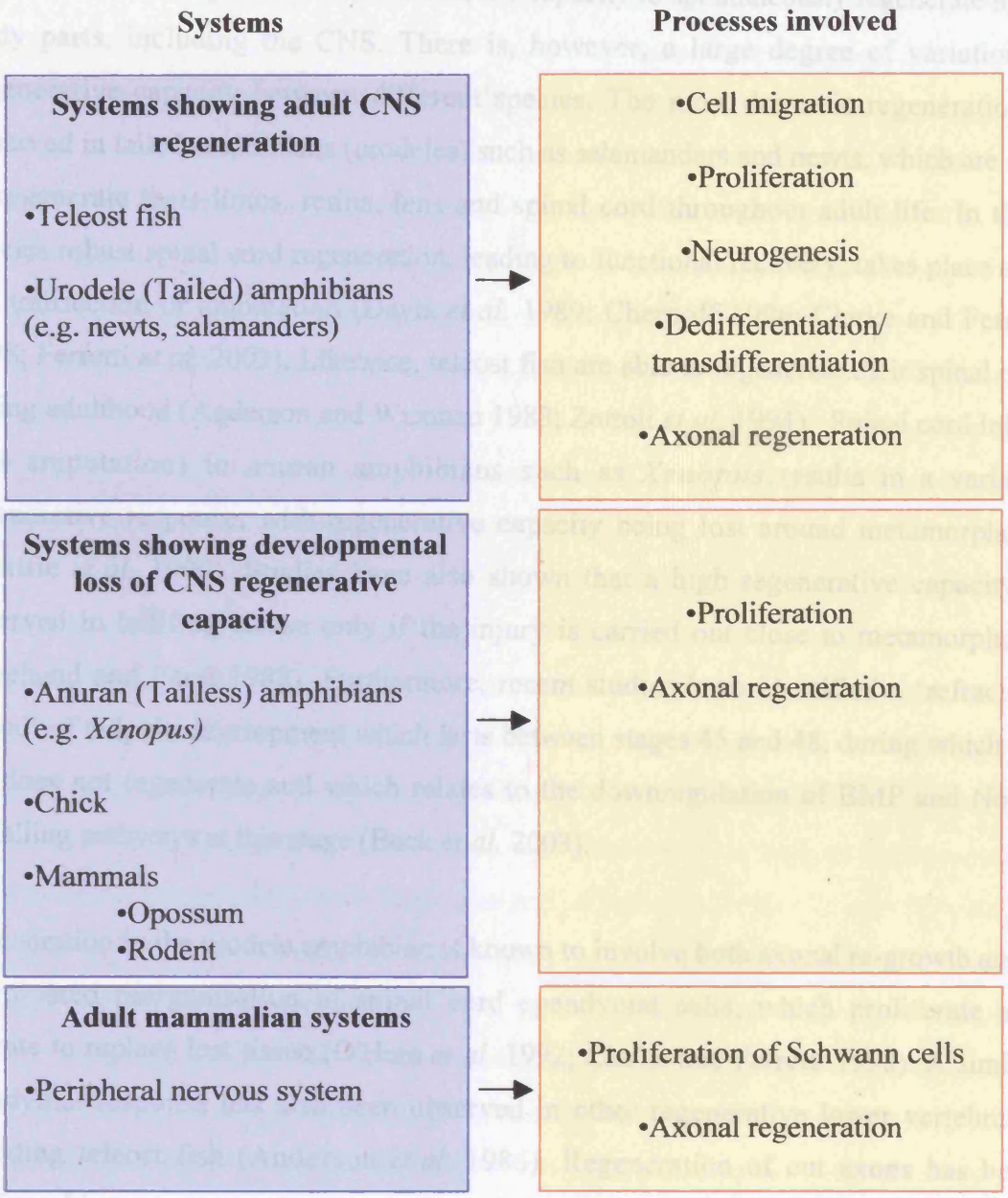


Figure 1.5. Common spinal cord regeneration model systems. Systems showing different regenerative capacities have been used to study responses to spinal cord injury. Figure shows the three main classes of regenerative models and some processes that have been demonstrated to contribute to the regenerative response in each model

1.4.2. Regeneration in lower vertebrate species

Certain fish and amphibians demonstrate the capacity to spontaneously regenerate many body parts, including the CNS. There is, however, a large degree of variation in regenerative capacity between different species. The most dramatic regeneration is observed in tailed amphibians (urodeles) such as salamanders and newts, which are able to regenerate their limbs, retina, lens and spinal cord throughout adult life. In these species robust spinal cord regeneration, leading to functional recovery, takes place after tail transection or amputation (Davis *et al.* 1989; Chernoff 1996; Clarke and Ferretti 1998; Ferretti *et al.* 2003). Likewise, teleost fish are able to regenerate their spinal cord during adulthood (Anderson and Waxman 1983; Zottoli *et al.* 1994). Spinal cord injury (tail amputation) in anuran amphibians such as *Xenopus* results in a variable regenerative response, with regenerative capacity being lost around metamorphosis (Beattie *et al.* 1990). Studies have also shown that a high regenerative capacity is observed in bullfrog larvae only if the injury is carried out close to metamorphosis (Forehand and Farel 1982). Furthermore, recent studies have identified a 'refractory period' of tadpole development which lasts between stages 45 and 48, during which the tail does not regenerate and which relates to the downregulation of BMP and Notch signalling pathways at this stage (Beck *et al,* 2003).

Regeneration in the urodele amphibian is known to involve both axonal re-growth and a coordinated reorganisation of spinal cord ependymal cells, which proliferate and migrate to replace lost tissue (O'Hara *et al.* 1992; Clarke and Ferretti 1998). A similar ependymal response has also been observed in other regenerative lower vertebrates including teleost fish (Anderson *et al.* 1986). Regeneration of cut axons has been confirmed in regenerating bullfrog larvae. Brainstem-spinal neurons were labelled with two different retrograde tracers applied to the lumbar cord before and after the injury and double-labelled cells were subsequently observed in the brainstem (Forehand and Farel 1982). The high regenerative capacity of these species has been attributed by some to the retention of developmental properties in the spinal cord but may also be the result of other factors including a high intrinsic capacity of neurons to regenerate, reduced glial scarring or an ability to recruit progenitor cells in response to injury (Holder and Clarke 1988; Chernoff 1996).

1.4.3. Regeneration in embryonic higher vertebrates

Higher vertebrates, including avian and mammalian species, demonstrate a high degree of regenerative capacity in their spinal cord during embryonic development which is lost as development progresses (Nicholls and Saunders 1996). These systems can therefore provide information about factors affecting loss of regenerative capacity in a model that has greater relevance to the adult mammal than lower vertebrates. The most useful systems for experimental manipulation are those that do not have to be accessed *in utero*, allowing complex manipulations to be carried out which would not be tolerated by most mammalian embryos. These models include the embryonic chick, which can be accessed while in the egg, and marsupials such as the opossum, which are born early during development and undergo a significant portion of their spinal cord development postnatally, while attached externally to the mother.

The embryonic chick spinal cord has been demonstrated to undergo complete anatomical and functional repair after transection injury up until embryonic day 13 (E13), after which this faculty is lost (Shimizu *et al.* 1990; Hasan *et al.* 1991). Recovery has been assessed by examining the re-growth of axonal tracts by retrograde labelling, electrophysiological recordings and behavioural measurements such as the capacity to hatch unaided (Hasan *et al.* 1993; Sholomenko and Delaney 1998). In order to confirm that neurites that grow across the injury site are derived from the regeneration of damaged axons, rather than continuing nervous system development, a double labelling strategy similar to that used in the anuran amphibian has been employed (Hasan *et al.* 1993). The developing opossum, either *Didelphis Virginia* or *Monodelphi domestica*, has been shown to have a similar developmentally-regulated capacity for regeneration. In organotypic cultures or *in vivo* the opossum spinal cord has been demonstrated to undergo complete anatomical and functional recovery up to 12 days after birth, beyond which this capacity is lost (Treherne *et al.* 1992; Saunders *et al.* 1998; Martin *et al.* 2000). These studies have also demonstrated that axonal re-growth takes place in the regenerating cord, and that synaptic connections are made although it has not been fully established whether the re-established connections are normal (Lepre *et al.* 1998; Saunders *et al.* 1998). The embryonic chick and opossum spinal cord are extremely useful models for investigating spinal cord regeneration. The developmental loss of regenerative capacity allows for direct comparisons of a regenerating and non-

regenerating system within the same species. In this thesis, the embryonic chick spinal cord was used to investigate mechanisms that contribute both to spinal cord regeneration and to its loss with progressing development.

1.4.4. What can we learn from regenerating systems?

The ultimate goal of spinal cord injury research is to identify strategies to gain successful regeneration in the mammalian spinal cord, with a wide variety of approaches under consideration. To narrow the field of potential therapeutic strategies it would be beneficial to determine the factors responsible for the marked lack of regeneration in the mammalian CNS. To this end, knowledge gained about the regenerative process in other species and systems, such as the embryonic chick spinal cord, will be useful. Although these systems differ in many ways from the adult mammal, they provide a supplementary experimental approach to models that more closely mimic the human injury. In particular it is hoped that this approach may lead to the identification of factors that are of key importance for successful regeneration

1.4.5. Factors affecting regenerative capacity

As described above, enormous variation in the ability of the CNS to self-repair after an injury exists among species and between developmental stages. Determining the factors that govern these differences in spinal cord regenerative capacity will increase our understanding of why the human spinal cord is unable to regenerate.Unfortunately, no single factor is likely to be responsible for determining regenerative ability and the multitude of different elements that may be involved make this a complex issue. In the following sections, a simplified model for considering the factors involved in spinal cord regeneration will be outlined. Next, a number of potential contributory elements will be discussed in relation to their role in determining the regenerative capacity in different systems and how they might be targeted therapeutically in a non-regenerating system.

1.4.6. Conditions for successful regeneration

The term 'regeneration' has been interpreted in a number of different ways; however, for the purposes of this thesis, regeneration is defined as the restoration of functional connectivity within the spinal cord following an injury. This concept is comparable to limb, or whole organ regeneration, and is likely to involve processes such as cellular proliferation and migration in addition to the re-growth of axons. It is useful to begin by considering what conditions must be met for successful spinal cord regeneration to take place. Fig. 1.6 summarises a simplified view of these conditions, which can be divided into two broad complementary requirements for regeneration.

First, in order for functional regeneration to occur, there must be enough viable cells present within the spinal cord. This includes both neurons that send projections long distances up and down the cord, and interneurons, which make the local connections that are responsible for the fine control of neuronal activity and glial cells. There is evidence that only a fraction of the original neuronal cell count needs to survive an injury in order for significant functional recovery to take place (Fehlings and Tator 1995). The number of viable cells can be influenced both by the degree of cell loss after the injury and by the ability of the spinal cord to generate replacement cells to take over from those lost.

Second, the neurons must be able to re-grow disrupted axons or grow new axons to restore neuronal pathways. This can be controlled both by the intrinsic capacity of the cell for axonal re-growth and by the permissiveness of the spinal cord environment to axonal re-growth, which is influenced by the presence or absence of growth-promoting or inhibitory molecules. Regenerating axons must also be re-myelinated, requiring the presence of surviving oligodendrocytes. Furthermore, limited benefit is likely to be gained by axon re-growth unless the connections established are functional and do not lead to abnormal neuronal activity. Axonal path finding and synapse formation must therefore be adequately controlled.

In a robust regenerative response to injury, such as that taking place in the pre-E13 chick, all of these steps must take place. Failure to meet any or all of these conditions might result in the loss of regenerative capacity, such as that observed in the post-E13

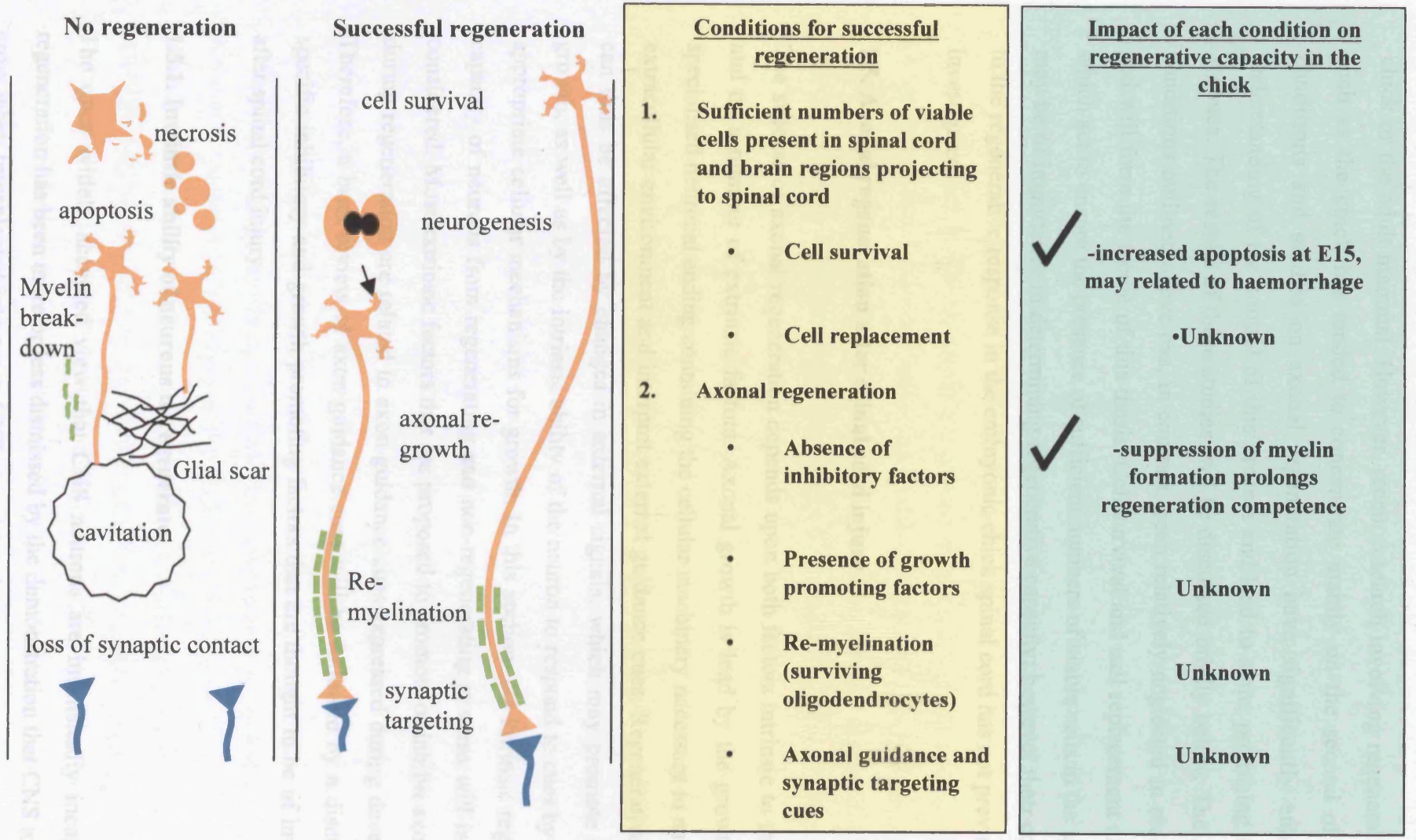


Fig. 1.6. Conditions necessary for successful spinal cord regeneration. Figures illustrate the typical challenges to neuronal regeneration occurring in the non-regenerative spinal cord and the processes contributing to successful regeneration. The hypothesised conditions for successful regeneration are listed (yellow box), while the green box on the right indicates whether these have been investigated previously for a role in the loss of regenerative capacity occurring in the embryonic chick spinal cord

chick or the adult mammal. However, recent research involving regenerative species such as the chick has tended to concentrate mainly on the second of these two conditions and studies on axonal regeneration have significantly enhanced our understanding of the control of this process and lead to some promising therapeutic avenues. The results of these studies will be discussed briefly below. The first of the conditions outlined above has, in contrast, been relatively neglected in recent studies and will form the theme of this thesis. Cell survival and cell replacement after injury, which act to ensure the presence of sufficient numbers of viable cells in the spinal cord, may be of importance in determining regenerative capacity; however their contribution to the regenerative response in the embryonic chick spinal cord has not previously been investigated.

1.5. Axonal regeneration after spinal cord injury

The success of axonal regeneration depends upon both factors intrinsic to the neurons and on a number of extrinsic factors. Axonal growth is lead by the growth cone, a specialised neuronal ending containing the cellular machinery necessary to navigate the extracellular environment and interpret external guidance cues. Regenerative capacity can thus be affected by changes in external signals, which may promote or inhibit growth, as well as by the intrinsic ability of the neuron to respond to cues by initiating appropriate cellular mechanisms for growth. In this section, the intrinsic regenerative capacity of neurons from regenerating and non-regenerating systems will initially be considered. Most extrinsic factors that are proposed to promote or inhibit axon growth during regeneration are related to axon guidance cues expressed during development. Therefore, a brief review of axon guidance cues will be followed by a discussion of specific inhibitory and growth promoting factors that are thought to be of importance after spinal cord injury.

1.5.1. Intrinsic ability of neurons to regenerate

The once widely accepted view that CNS neurons are intrinsically incapable of regeneration has been more or less dismissed by the demonstration that CNS axons can grow when transplanted into a non-CNS or embryonic environment (Richardson *et al.* 1980). However, studies on the growth of different types of CNS neurons *in vitro* have

demonstrated that there can be marked differences in the intrinsic ability of neurons to regenerate on the same substrate (Fawcett 1992). For example, neurons that are capable of extensive axonal outgrowth include cerebellar deep nuclei neurons, while cerebellar Purkinje cells have a limited growth capacity (Properzi and Fawcett 2004). The intrinsic capacity of sensory neurons to grow into the CNS following dorsal column lesion can be increased by preconditioning lesions (Neumann and Woolf 1999). Additionally, embryonic neurons have in a number of cases been shown to be able to grow in the adult CNS where adult CNS neurons apparently cannot (Wictorin and Bjorklund 1992; Li and Raisman 1993; Shewan *et al.* 1995). Indeed, a number of studies have indicated that the intrinsic regenerative capacity of neurons diminishes with age. In some studies the environment in which neurons are situated does not affect this loss of regenerative capacity with age. However in other studies this developmental transition occurred only in an inhibitory environment suggesting the onset, at later developmental stages, of responsiveness to inhibitory signalling factors (Condic 2001; Li *et al.* 1995; Shewan *et al.* 1995).

Despite this evidence, the molecular changes underlying differences in intrinsic regenerative capacity are poorly understood. A greater understanding of these changes should provide a basis for enhancing regeneration in neurons that show a weaker intrinsic axonal re-growth capacity.

Several growth-associated molecules have been shown to be upregulated after injury in regenerative neurons, such as growth-associated protein (GAP) 43 and leukocyte protein 1 (L1) (Chaisuksunt *et al.* 2000). GAP43 is upregulated after a conditioning lesion in DRG neurons and is thought to be involved in the subsequently improved regenerative response (Chong *et al.* 1994). Axotomy of rubrospinal neurons resulted in a shorter-lasting up-regulation of growth-associated genes to PNS neurons with no concurrent regenerative improvement (Tetzlaff *et al.* 1991). Addition of these factors to non-regenerative neurons has produced only a moderately enhanced regenerative response, suggesting that other key factors remain to be identified (Buffo *et al.* 1997; Bomze *et al.* 2001). Expression of growth related signalling molecules such as integrins has also been demonstrated to be reduced in more mature neurons, providing another strategy for improving the intrinsic ability of adult CNS neurons to regenerate by

artificially upregulating integrin expression to levels observed in less mature neurons (Condic 2001).

Intracellular factors that alter the response of neurons to a potentially inhibitory environment might also be of importance. One such factor might be cyclic AMP (cAMP), which has been shown to promote axonal growth even in inhibitory environments, although in these studies pre-treatment with cAMP before injury was necessary (Cai *et al.* 1999; Neumann *et al.* 2002). Reduced cAMP levels have been suggested to underlie reduced regenerative capacity in more mature neurons (Cai *et al.* 2001). cAMP levels may also govern the response of neurons to axonal guidance cues (Ming *et al.* 1997; Song *et al.* 1997). Administration of a phophodiesterase inhibitor, Rolipram, which prevents cAMP degradation, has been demonstrated to improve regeneration in experimental models of spinal cord injury (Nikulina *et al.* 2004).

1.5.2. Axonal path finding and synaptic targeting

Although strategies for enhancing the intrinsic regenerative capacity of neurons or for removing environmental inhibition may result in greater axonal growth, this does not mean that functionally relevant connections will be formed. Axonal growth during development involves the strict direction of growth cones down the correct pathways by various secreted or membrane bound guidance cues (Mueller 1999). The growth cone contains actin-rich finger-like protrusions called filopodia which probe and interact with the environment of the advancing neurite. The filopodia express a wealth of receptors which, via intracellular signalling pathways that involve the Rho family of small GTPases, determine the direction of axon growth (Mueller 1999). Rearrangement of the actin cytoskeleton, which is regulated by Rho family members including RhoA, Rac and Cdc42, may result in retraction of filopodia and growth cone collapse or neurite advancement and filopodia formation (Mueller 1999; Nobes and Hall 1995). The response of the growth cone to a given guidance cue has also been shown in some cases to be affected by intracellular levels of cAMP (Ming *et al.* 1997; Song *et al.* 1997). It is likely that a similarly strict regulation of axonal re-growth will be required during regeneration to ensure that appropriate connectivity is restored. Importantly, axonal guidance involves inhibitory as well as growth-promoting cues, which has relevance for strategies that aim to universally reduce growth inhibition or augment growth promotion

in the CNS environment. Table 1.1 summarises some of the principle guidance cues that are involved in axonal growth during development. Growth cone receptors and environmental guidance cues may need to be restored to a developmental state for regeneration to take place. However, little is known about the re-expression of these molecules in the injured spinal cord and most of the work investigating the potential role of these molecules in CNS regeneration has been carried out on the projection from retinal ganglion cells (RGC) in the eye to the superior colliculus in the brain.

Overall, it is apparent that axon guidance during development is subject to a complex array of cues and signalling pathways. To recapitulate such signalling from scratch in the regenerating nervous system is therefore a daunting task. Nevertheless, in systems that do regenerate, sufficient guidance cues could be presumed to be either already present or up-regulated in response to injury. Encouragingly, studies on RGC regeneration have indicated that some of these molecular cues continue to be expressed in the adult mammalian CNS long after development is complete. On the other hand, the continuing presence or even up-regulation of some chemorepellant molecules might contribute to the inhibition of axonal growth.

1.5.3. Growth-promoting factors

As described above, axonal regeneration involves the interaction of receptors present on the growth cone surface with factors present in the environment that may support and encourage growth. It can therefore be presumed that the presence or absence of such growth promoting factors might contribute significantly to the overall success of regeneration. Potentially growth-promoting factors include molecules located in the extracellular matrix (ECM), such as laminin, fibronectin and some of the chemoattractive molecules described above. In addition neurotrophic and growth factors, secreted by neighbouring cells, can influence the intrinsic activity of the growth cone. There is some evidence for differences in the expression of growth promoting factors in regenerative and non-regenerative systems. For example, heparan sulphate proteoglycan (HSPG) and laminin, both of which are known to enhance neurite growth, have been demonstrated to be down-regulated with development of the optic nerve, which corresponds to a reduction in regenerative capacity (Cohen *et al.* 1987; Hantaz-Ambroise *et al.* 1987). The expression of molecules such as laminin and HSPG in

Table 1.1. Axon guidance molecules in the developing and adult CNS

Guidance cue	Growth cone receptor	Details	Chemoattractive or chemorepulsive?	Role in axon guidance after injury?
Semaphorins •Secreted •Membrane bound	•Neuropilin receptors (He & Tessier-Lavigne, 1997) •Plexins (Winberg *et al*, 1998)	•More than 30 members identified so far (Chen 1997). •May be secreted or membrane bound •All possess 500aa extracellular SEMA domain	Chemorepellants but may act under some circumstances as chemoattractants (Mueller, 1999, Wong *et al*, 1997)	•Changes in semaphorin expression (including up-regulation of type-3 semaphorins) have been reported after injury (DeWinter *et al*, 2002, Pasterkamp *et al* 1998) •May guide regenerating neurons or inhibit regeneration as part of glial scar
Ephrins	•Eph receptors •Two classes Eph-A and Eph-B, according to their preferred ligand	•Ephrin-A class are GPI-linked to cell membrane •Ephrin-B class are transmembrane proteins	Mediate growth cone collapse but chemoattractive properties have also been described (Holmberg & Frisen, 2002)	•Ephrins and Eph receptors (Ephrin B2 and Eph B2) up-regulated by reactive astrocytes after injury (Bundesen *et al*, 2003)
Netrins	•Deleted in colorectal cancer (DCC) receptors •UNC5H receptor	•4 family members	Either, depending on receptor: •DCC receptors promote attraction •UNC5H receptor mediates inhibition (Hong *et al*, 1999, Keino-Masu *et al*, 1996, Mueller, 1999)	Netrins and receptors down-regulated in some injury models but other studies have questioned their role. (Ellezam *et al*, 2001, Deiner *et al*, 1997, Petrausch *et al*, 2000),
Slits	Roundabout (Robo) receptors	•3 family members •Secreted ligands	Both chemorepellant and chemoattractive	
Others: **•Neurotrophins** **•Myelin proteins** **•CSPGs/Tenascin**	•See later sections			

regenerative species such as lower vertebrates or the embryonic chick has not been documented. However, further support for the importance of such factors comes from studies, mainly involving neurotrophic factors, in which exogenous application of growth-promoting factors has been shown to enhance regeneration.

The neurotrophins are a group of polypeptides, including BDNF, NGF, GDNF and NT-4/5, which play a number of roles during development of the nervous system including promoting neuronal survival and regulating axon guidance. These factors could therefore have the dual benefit of reducing cell death as well as encouraging axon growth after injury (Cheng *et al.* 2002). Neurotrophins have been applied singly or in combination at the injury site, via injection or by transplantation of cells expressing the protein, with some limited success (Schnell *et al.* 1994; Menei *et al.* 1998; Grill *et al.* 1997; Liu *et al.* 1999b; Namiki *et al.* 2000; Coumans *et al.* 2001; Shumsky *et al.* 2003). In an alternative approach, neurotrophins have been applied directly to the neuronal cell body in the brain. Application of BDNF or NT-4/5 in the brainstem has been demonstrated to result in an up-regulation of growth-associated genes and improved regeneration of axotomised neurons (Kobayashi *et al.* 1997). Using a similar approach it was shown that CNS axons could be stimulated to re-grow a full year after injury (Kwon *et al.* 2002). However, neurotrophin treatment alone has only rarely been demonstrated to induce regeneration in the CNS and it is likely that such treatment would need to be combined with other treatments that might improve the permissiveness of the environment in which the axons must grow (Bradbury *et al.* 1999)

1.5.4. The inhibitory environment

The concept of permissive and non-permissive environments for axonal extension after injury was prompted by the discovery that damaged mammalian CNS axons are able to grow into peripheral nervous system grafts but do not behave in the same way in the damaged CNS environment (David and Aguayo 1981). Since this discovery, intensive research into potential inhibitory molecules has resulted in the identification of two main sources of inhibitory signalling molecules in the injured spinal cord: myelin and the glial scar.

Myelin-derived inhibitory molecules

Degenerating myelin deposited at the injury site has been suggested to form the main inhibition to axonal re-growth in the immediate aftermath of the injury, a proposition which is supported by several lines of enquiry. *In vitro* studies have demonstrated that axonal outgrowth is inhibited on substrates containing myelin (Schwab and Caroni 1988). Immunisation against myelin or suppression of myelin formation by x-irradiation during development has been shown to promote regeneration in the injured spinal cord (Savio and Schwab 1990; Huang *et al.* 1999; Bregman *et al.* 1995; Caroni and Schwab 1988; Schnell and Schwab 1990). Chemically-induced lesions of non-myelinated axons in the CNS that avoid damage to neighbouring myelinated tracts result in regeneration which does not occur when myelinated axons are also damaged (Berry 1982). On the other hand, some researchers have cast doubt on the concept of myelin as an inhibitory factor for regeneration, questioning the evolutionary basis for such inhibition (Raisman 2004). Furthermore, studies have demonstrated that PNS axons are able to regenerate within both intact and degenerating spinal cord white matter (Davies *et al.* 1997; Davies *et al.* 1999). Additionally, it has been suggested that in some cases CNS myelin may actually benefit axon regeneration by inhibiting erroneous branching and promoting directed growth down myelin-lined channels (Raisman 2004). Despite this controversy, several different molecular components of myelin have been identified as potential contributors to the inhibition of axonal re-growth. These include the Nogo proteins, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp), as summarised in Figure 1.7.

The most prominent of the inhibitory myelin proteins over recent years has been Nogo-A. The Nogo gene was cloned in 2000, followed by the identification of three isoforms, Nogo-A, Nogo-B and Nogo–C, of which Nogo-A appears to have the greatest role in the nervous system. In addition to inhibiting axon re-growth via the Nogo-66 receptor (NgR, Fig 1.7) Nogo proteins also interact with mitochondrial proteins such as Nogo-interacting mitochondria protein (NIMP), anti-apoptotic proteins such as Bcl-2 and axonal proteins such as Caspr, suggesting a variety of endogenous roles for these proteins (Teng *et al.* 2004). Nogo-A is expressed on CNS oligodendrocytes, while NgR is expressed on axons, consistent with a role in axonal outgrowth inhibition by Nogo-A (Huber *et al.* 2002; Wang *et al.* 2002b). Furthermore, NgR is not expressed in the

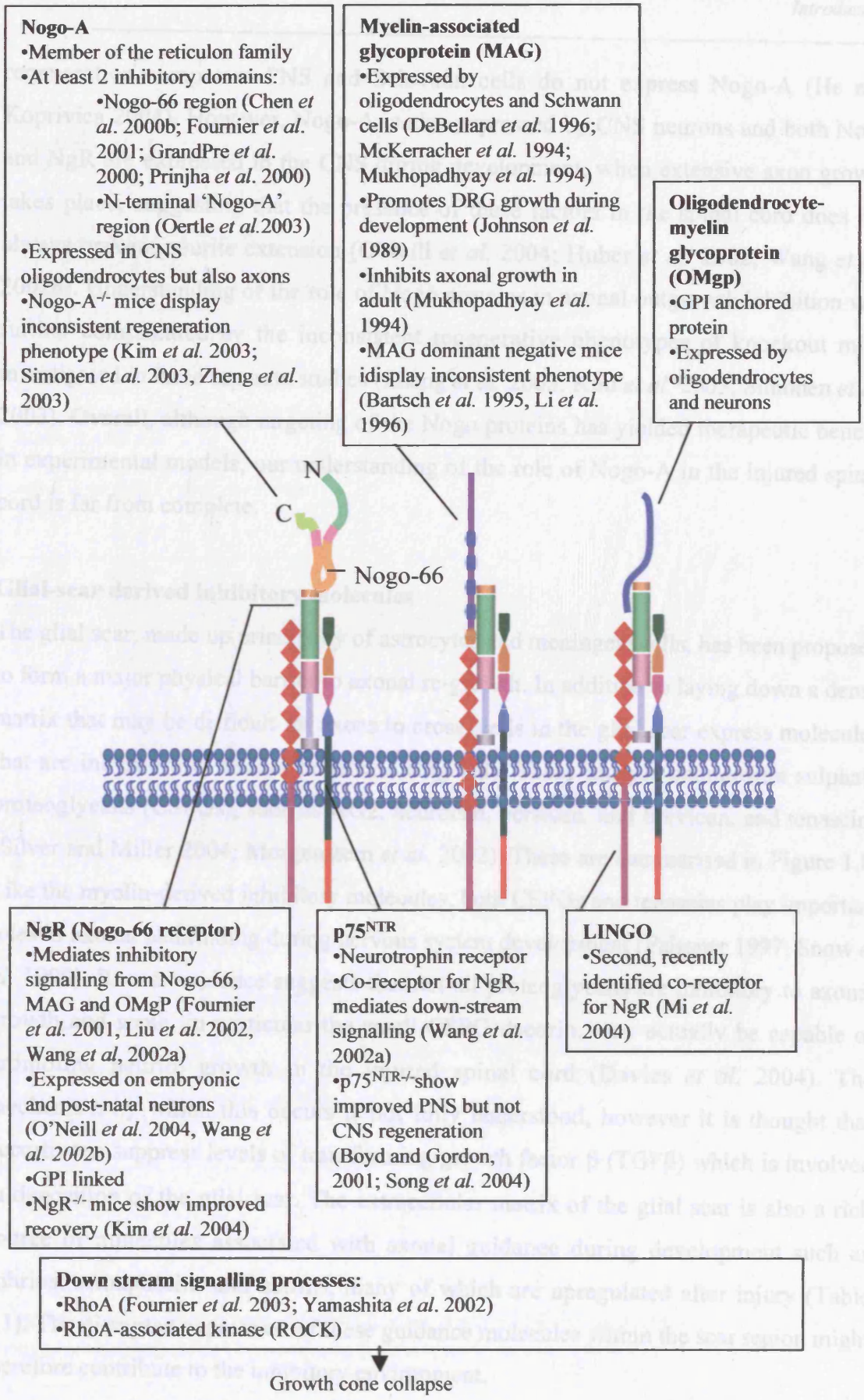


Fig 1.7. Properties of the principal myelin-derived inhibitory molecules. Illustration adapted from Sandvig *et al*, 2004.

regeneration-competent PNS and Schwann cells do not express Nogo-A (He and Koprivica 2004). However, Nogo-A is also expressed by CNS neurons and both Nogo and NgR are expressed in the CNS during development, when extensive axon growth takes place, suggesting that the presence of these factors in the spinal cord does not always prevent neurite extension (O'Neill *et al.* 2004; Huber *et al.* 2002; Wang *et al.* 2002b). Understanding of the role of Nogo proteins in axonal outgrowth inhibition was further complicated by the inconsistent regenerative phenotypes of knockout mice investigated in three separate studies (Zheng *et al.* 2003; Kim *et al.* 2003; Simonen *et al.* 2003). Overall, although targeting of the Nogo proteins has yielded therapeutic benefit in experimental models, our understanding of the role of Nogo-A in the injured spinal cord is far from complete.

Glial-scar derived inhibitory molecules

The glial scar, made up principally of astrocytes and meningeal cells, has been proposed to form a major physical barrier to axonal re-growth. In addition to laying down a dense matrix that may be difficult for axons to cross, cells in the glial scar express molecules that are inhibitory to axonal re-growth (Fig 1.8). These include chondroitin sulphate proteoglycans (CSPGs), such as NG2, neurocan, versican, and brevican, and tenascins (Silver and Miller 2004; Morgenstern *et al.* 2002). These are summarised in Figure 1.8. Like the myelin-derived inhibitory molecules, both CSPGs and tenascins play important roles in axonal pathfinding during nervous system development (Faissner 1997; Snow *et al.* 1990). Recent evidence suggests that not all proteoglycans are inhibitory to axonal growth and some, in particular the small CSPG decorin, may actually be capable of promoting neurite growth in the injured spinal cord (Davies *et al.* 2004). The mechanism by which this occurs is not fully understood, however it is thought that decorin can suppress levels of transforming-growth factor β (TGFβ) which is involved in deposition of the glial scar. The extracellular matrix of the glial scar is also a rich source of molecules associated with axonal guidance during development such as ephrins, semaphorins and netrins, many of which are upregulated after injury (Table 1.1). The disrupted expression of these guidance molecules within the scar region might therefore contribute to the inhibitory environment.

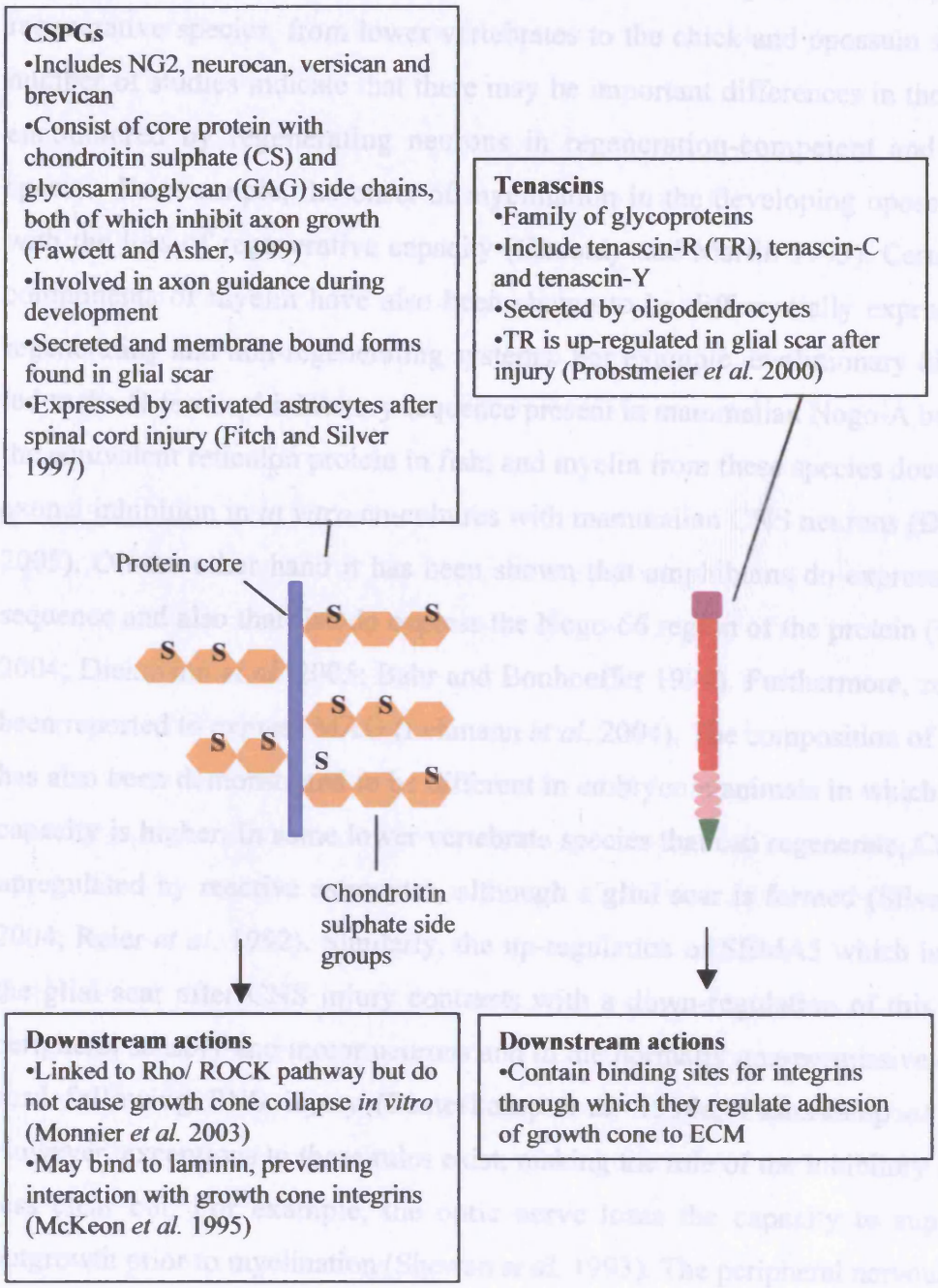


Fig 1.8. Properties of the principal glial-scar derived inhibitory molecules. Illustrations adapted from Properzi and Fawcett and Faissner, 1997.

The importance of the inhibitory environment for regenerative capacity

Axonal regeneration is an essential component of spinal cord repair in many regenerative species, from lower vertebrates to the chick and opossum spinal cord. A number of studies indicate that there may be important differences in the environment encountered by regenerating neurons in regeneration-competent and incompetent species. For example, the onset of myelination in the developing opossum coincides with the loss of regenerative capacity (Ghooray and Martin 1993). Certain inhibitory components of myelin have also been shown to be differentially expressed between regenerating and non-regenerating systems. For example, evolutionary divergence has led to the N-terminal inhibitory sequence present in mammalian Nogo-A being absent in the equivalent reticulon protein in fish, and myelin from these species does not result in axonal inhibition in *in vitro* co-cultures with mammalian CNS neurons (Diekman *et al*, 2005). On the other hand it has been shown that amphibians do express the Nogo-A sequence and also that fish do express the Nogo-66 region of the protein (Klinger *et al.* 2004; Diekmann *et al.* 2005; Bahr and Bonhoeffer 1994). Furthermore, zebrafish have been reported to express MAG (Lehmann *et al.* 2004). The composition of the glial scar has also been demonstrated to be different in embryonic animals in which regenerative capacity is higher. In some lower vertebrate species that can regenerate, CSPGs are not upregulated by reactive astrocytes, although a glial scar is formed (Silver and Miller 2004; Reier *et al.* 1992). Similarly, the up-regulation of SEMA3 which is observed in the glial scar after CNS injury contrasts with a down-regulation of this molecule in peripheral sensory and motor neurons and in the normally non-permissive dorsal spinal cord following PNS injury (Pasterkamp *et al.* 1998a; Pasterkamp *et al.* 1998b). However, exceptions to these rules exist, making the role of the inhibitory environment less clear cut. For example, the optic nerve loses the capacity to support neurite outgrowth prior to myelination (Shewan *et al.* 1993). The peripheral nervous system has also been reported to up-regulate many postulated inhibitory factors such as CSPGs after injury, although it is thought that the rapid clearance of these molecules might explain the apparent lack of axonal re-growth inhibition (Pasterkamp *et al.* 1998a).

The contribution of some of these factors to regeneration has been investigated in the embryonic chick. For example, it has been shown that the chick spinal cord is capable of regeneration only at stages prior to myelination and experimental delay in myelination has been demonstrated to extend the permissive period for axonal

regeneration (Keirstead *et al.* 1992; Keirstead *et al.* 1995). Additionally, the regeneration-competent period is associated with a low ratio of CSPG expression to expression of the growth promoting HSPG; this ratio was demonstrated to increase after E13 (Dow *et al.* 1994).

Strategies for overcoming the inhibitory environment

The multitude of inhibitory factors that has been identified in the injured spinal cord suggests that removal or inhibition of any single factor is unlikely to result in significant improvements in regeneration. However, a number of different strategies have been pursued in experimental models, and these are summarised in Fig 1.9. Interestingly, it has also been shown that neurons can grow in an inhibitory environment if provided with certain growth factors prior to exposure to the inhibitory signalling, via a mechanism involving cAMP (Cai *et al.* 1999). Downstream signalling involves protein kinase A (PKA) and genes including Arginase I, which catalyses the synthesis of polyamines, possibly leading to rearrangement of the cytoskeleton (Cai *et al.* 2002; Spencer and Filbin 2004). Similarly, enhancing retinoid signalling by transduction of sensory neurons with the retinoic acid receptor RARβ can promote regeneration across inhibitory substrates via mechanisms which probably involve cAMP signalling (Wong *et al.* 2006) These mechanisms might also explain the higher innate regenerative capacity of immature neurons because prenatal DRG neurons have been shown to have higher cAMP and Arginase 1 levels than more postnatal neurons, and PKA inhibitors blocked spontaneous regeneration in the embryonic rat spinal cord (Cai *et al.* 2001; Cai *et al.* 2002). Thus an alternative strategy to aid axonal re-growth would be to treat neurons to render them unresponsive to inhibitory signals. A drawback is the requirement for cAMP elevation prior to contact between the axon and the inhibitory substrate. The inhibitory environment might also be overcome by transplantation of synthetic or biological bridges permissive to axon growth. Transplantation of peripheral or embryonic nerves enhances regeneration in animal models but has a number of ethical and practical drawbacks for clinical use (David and Aguayo 1981; Iwashita *et al.* 1994). A range of different synthetic and biodegradable materials have been developed including collagen based gels, fibronectin mats or tubes made of polycarbonates (Geller and Fawcett 2002; Montgomery *et al.* 1996; Plant *et al.* 1997; Steuer *et al.* 1999). These may be implanted in conjunction with cell transplantation strategies, which will be outlined in greater detail below.

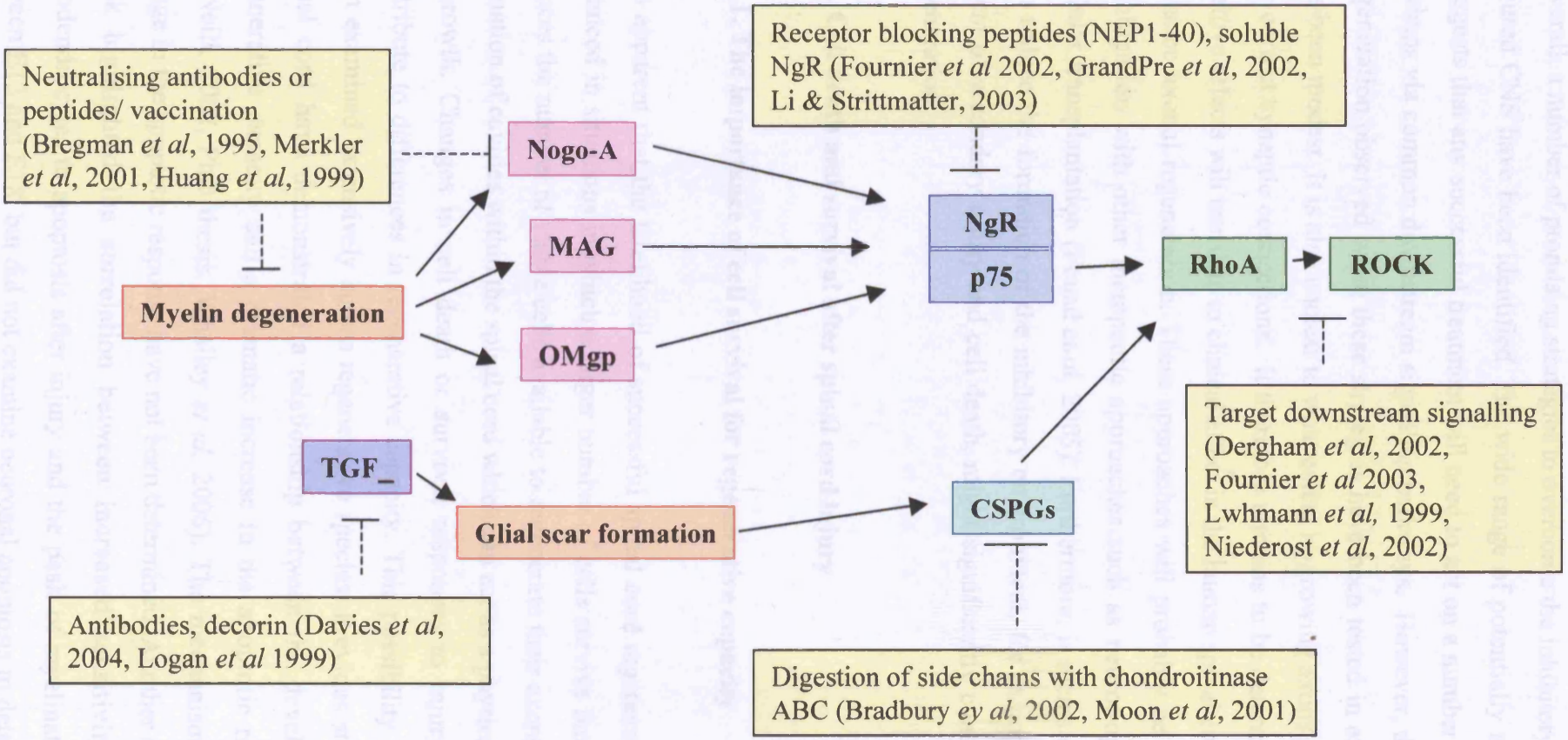


Fig 1.9. Strategies to overcome the inhibitory environment. Schematic diagram, illustrating the components of the signalling pathways which inhibit axonal growth which have been targeted experimentally to improve spinal cord regeneration

Overall, a number of promising strategies to overcome the inhibitory environment of the injured CNS have been identified. The wide range of potentially inhibitory molecules suggests that any successful treatment will need to act on a number of different targets, perhaps via common downstream signalling pathways. However, the magnitude of the regeneration observed when these strategies have been tested in experimental models has been modest. It is also unclear to what extent re-growing axons are able re-establish the correct synaptic connections. It therefore remains to be determined whether these positive effects will translate to clinical effect in the human spinal cord and lead to long-distance axonal regeneration. These approaches will probably be most successful in combination with other therapeutic approaches such as neurotrophin treatment and cellular transplantation (Fouad *et al.* 2005). Furthermore, it seems clear that strategies that reduce the formation of the inhibitory environment, for example by reducing the extent of secondary injury and cell death, might significantly contribute to improved regeneration.

1.6. Cell death and survival after spinal cord injury

1.6.1. The importance of cell survival for regenerative capacity

It is apparent that the likelihood of successful spinal cord regeneration will be vastly enhanced in situations in which a larger number of cells survive the injury. Cell death reduces the number of viable cells available to regenerate their axons and results in the formation of cavities within the spinal cord which can act as a physical barrier to axonal re-growth. Changes in cell death or survival responses to injury might therefore contribute to differences in regenerative capacity. This possibility has, however, not been examined extensively across regenerative species. Previous studies in the chick spinal cord have demonstrated a relationship between the developmental loss of regenerative capacity and a dramatic increase in the apoptotic response to injury (O'Neill, 2002, PhD thesis, Whalley *et al,* 2006). The mechanisms underlying this change in the apoptotic response have not been determined. Another recent study in the chick highlighted the correlation between increased sensitivity of developing oligodendrocytes to apoptosis after injury and the peak of myelination which occurs between E13 and E18, but did not examine neuronal apoptosis in detail (McBride *et al.* 2003).

Table 1.2. Neuroprotective strategies targeting secondary injury mechanisms. Table shows major strategies which have been tested in pre-clinical animal models and clinical trials.

Strategy-	Process(es) targeted	Pre-clinical studies	Clinical success?
Glutamate antagonists	excitotoxicity	Showed significant effects in SCI models (Gaviria *et al.* 2000)	No significant improvement (gacyclidine) in phase 2 trials (unpublished)
Calcium channel blockers	Ischaemia	Mixed results with nimodipine (Fehlings *et al.* 1989; Haghighi *et al.* 1993)	No benefit shown in prospective trial (Pointillart *et al.* 2000)
Interleukin-10	Inflammation	Protective in experimental models (Brewer *et al.* 1999, Plunkett *et al.* 2001)	Not yet tested
Steroids (e.g. methylprednisolone)	•Free radical formation •Lipid peroxidation •Cytokine production •iNOS activity (Carlson and Gordon, 2002)	Mixed results in animal experiments (Young *et al.* 1988; Koyanagi and Tator, 1997)	•Clinical trials results showed effect at 8h after injury, but are controversial (Bracken *et al.* 1992, Bracken *et al*, 1997, Hurlbert 2001) •Approved for SCI treatment in the US, but rarely elsewhere.
GM-1 gangliosides	•excitotoxicity	Provided neuroprotection in experimental models (Ferrari and Greene, 1998)	•Clinical trials did not show evidence of improved outcome (Geisler *et al.* 2001)
Minocycline	•Caspase activity •Microglial activation •iNOS activity	Improves outcome in experimental models (Lee *et al.* 2003b, Stirling *et al.* 2004, Wells *et al.* 2003)	Not yet tested
Neurotrophins	•Trophic support	Reduce apoptosis in experimental models (Koda *et al.* 2002)	Not yet tested

Evidence for the pathological effects of haemorrhage in the spinal cord also comes from two studies in which treatment with recombinant thrombomodulin reduced haemorrhage at 24 hours after injury and also resulted in neuroprotection and reduced cavitation after a compression or contusion injury (Taoka *et al.* 1998; Festoff *et al.* 2004). However, treatment with thrombomodulin did slightly increase haemorrhage within the first three hours after injury and the authors have attributed the effects of the drug to the effects of thrombomodulin on thrombin signalling, which will be discussed further below (Festoff *et al.* 2004). Furthermore, changes in the haemorrhagic response may contribute to the regenerative capacity of the spinal cord. In the developing chick spinal cord, the age-related increase in apoptosis in the spinal cord after injury corresponds to a dramatic increase in the extent of haemorrhage within the cord (Whalley *et al.* 2006; O'Neill 2002). This developmental increase in haemorrhage reflects the rapid development of the spinal cord vascular system which occurs at around the time that the chick loses its ability to regenerate its spinal cord (O'Neill 2002; Whalley *et al.* 2006). Moreover, pharmacologically increasing the amount of haemorrhage in the chick spinal cord at normally regenerative stages was shown to result in an increase in cavitation and apoptosis to levels equivalent to those normally seen only at non-regenerative stages (O'Neill 2002; Whalley *et al.* 2006). It is therefore of interest to consider potential factors which might be responsible for the link between apoptosis and haemorrhage after injury.

1.6.4. Serine Proteases

The correlation between haemorrhage and apoptosis after spinal cord injury points to the potential involvement of blood-borne factors in triggering apoptosis in the injured CNS. Blood contains a multitude of different factors that could be considered contenders for this role, not least the inflammatory molecules and cells that are known to contribute to secondary injury. However, evidence suggests that the serine proteases, more usually associated with their roles in haemostasis, also make excellent candidates for linking haemorrhage with apoptosis in the nervous system.

Introduction to serine proteases

The serine proteases are a large family of proteolytic enzymes with serine residue at their catalytic site (Stone and Maraganore, 1992). Members of the family include

thrombin, activated protein C (APC), plasminogen, plasmin and the plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase (Turgeon and Houenou 1997). These molecules are all key players in a series of complex interacting pathways which regulate the formation and breakdown of fibrin-based blood clots (Fig.1.10). Most serine proteases are expressed as inactive precursors which are subsequently activated by the proteolytic action of an enzyme higher in the cascade. Serine protease activity is further regulated by a protein superfamily called 'serpins', including protease nexin-1 (PN-1), a potent thrombin inhibitor and neuroserpin, a tPA inhibitor (Osterwalder *et al.* 1996; Sommer *et al.* 1987). Serpins act primarily by forming complexes with serine proteases that are subsequently internalised by nearby cells (Pike *et al.* 2005). Additionally, the activity of serine proteases may be regulated by other inhibitors which include the thrombin inhibitor thrombomodulin. Thrombomodulin is a chondroitin sulphate proteoglycan that combines with thrombin to activate protein C, which has opposing actions to thrombin (Pindon *et al.* 1997).

In addition to their well-characterised roles in the circulatory system the serine proteases may have important roles in many other parts of the body. They have been associated with inflammation, tissue remodelling, cell migration and tumour formation (Turgeon and Houenou 1997). One area in which they are believed to have an important function is the nervous system. In particular, the action of thrombin in the nervous system has been extensively investigated, due to the early identification of its receptor and elements of its signalling pathway (Vu *et al.* 1991). Additionally, the plasminogen activator, tPA, has been known to have a role in CNS development for many years (Krystosek and Seeds 1981; Sumi *et al.* 1992) Although much of the original work concentrated on the role of these molecules in the peripheral nervous system, recent focus has moved to the central nervous system, with emerging evidence for crucial roles in the brain and spinal cord (Gingrich and Traynelis 2000).

The discovery of a specific thrombin receptor and the identification of its novel 'tethered ligand' mechanism of action, whereby cleavage of the N-terminal domain by thrombin or another serine protease, exposes a new amino terminus that can bind and activate the receptor, was of key importance in uncovering the function of serine proteases in the CNS (Rasmussen *et al.* 1991; Vu *et al.* 1991; Coughlin 2000). Protease

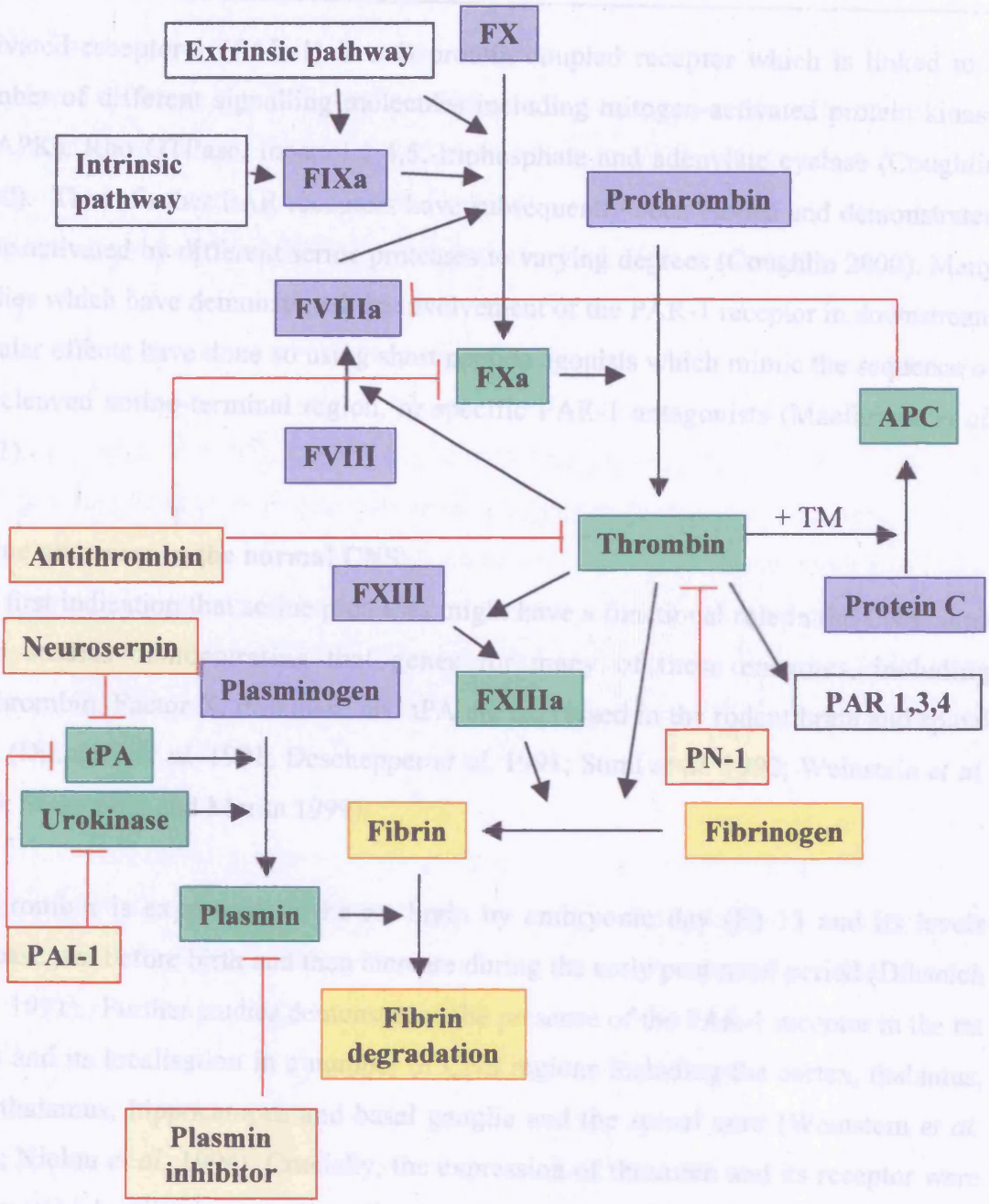


Fig. 1.10. Components of the coagulation cascade. Schematic diagram illustrating the principle interactions (simplified) underlying coagulation/ fibrinolysis. Inactive serine protease precursors are shown in blue boxes, the activated proteases are shown in green boxes. Serine protease inhibitors (serpins) are shown in red boxes. Abbreviations: a= activated APC = activated protein C, F = coagulation factor, , PAI-1 = plasminogen activator inhibitor -1, PAR = proteinase activated receptor, PN-1 = protease nexin -1, tPA = tissue plasminogen activator,, TM = thrombomodulin. Adapted from Gingrich and Traynelis, 2000 and the Chromogenix website (www.chromogenix.com).

activated receptor-1 (PAR-1) is a G protein coupled receptor which is linked to a number of different signalling molecules including mitogen-activated protein kinase (MAPK), Rho GTPase, inositol 1,4,5,-triphosphate and adenylate cyclase (Coughlin 2000). Three further PAR receptors have subsequently been cloned and demonstrated to be activated by different serine proteases to varying degrees (Coughlin 2000). Many studies which have demonstrated the involvement of the PAR-1 receptor in downstream cellular effects have done so using short peptide agonists which mimic the sequence of the cleaved amino-terminal region, or specific PAR-1 antagonists (Macfarlane *et al.* 2001).

Serine proteases in the normal CNS

The first indication that serine proteases might have a functional role in the CNS came from studies demonstrating that genes for many of these enzymes, including prothrombin, Factor X, urokinase and tPA are expressed in the rodent brain and spinal cord (Dihanich *et al.* 1991; Deschepper *et al.* 1991; Sumi *et al.* 1992; Weinstein *et al.* 1995; Shikamoto and Morita 1999).

Prothrombin is expressed in the rat brain by embryonic day (E) 13 and its levels decrease just before birth and then increase during the early post-natal period (Dihanich *et al.* 1991). Further studies demonstrated the presence of the PAR-1 receptor in the rat brain and its localisation in a number of CNS regions including the cortex, thalamus, hypothalamus, hippocampus and basal ganglia and the spinal cord (Weinstein *et al.* 1995; Niclou *et al.* 1994). Crucially, the expression of thrombin and its receptor were shown to co-localise in a number of brain regions, although, in contrast to prothrombin expression patterns, PAR-1 becomes more restricted in the adult brain (Weinstein *et al.* 1995). In the rat spinal cord, PAR-1 is expressed exclusively in the grey matter and in ependymal cells, with no expression observed in the white matter (Niclou *et al.* 1994). Although thrombin and PAR-1 are known to be expressed in the developing chick spinal cord, a detailed analysis of their pattern of expression has not been carried out.

The expression of urokinase and tPA has also been examined in the developing rat CNS (Sumi *et al.* 1992). Urokinase was shown to be expressed early during development (from E12.5) in developing neurons in the spinal cord and continued to be expressed into adulthood, while tPA was found to be concentrated in floor plate cells (Sumi *et al.*

1992). The expression of plasminogen activators has not been examined in the chick spinal cord in detail; however studies in the cerebellum, cerebral hemispheres and optic lobe have demonstrated that only urokinase is expressed in the chick CNS and that expression levels are elevated during periods of neuronal migration and synapse formation (Scicolone *et al.* 1997; Pereyra-Alfonso *et al.* 1997; Scicolone *et al.* 1998).

Accompanying the serine proteases in the brain, a range of different serpins have also been demonstrated to be expressed by neural cells (Wagner *et al.* 1991; Niclou *et al.* 1994; Krueger *et al.* 1997). PN-1 is thought to be the predominant thrombin inhibitor in the brain, produced by both astrocytes and neurons and localised around blood vessels in the brain (Choi *et al.* 1990). In the rat spinal cord PN-1 is expressed from E18 and is predominantly located in the small glial cells in the grey and white matter as well as in motorneurons (Niclou *et al.* 1994). Thrombomodulin is expressed by endothelial cells and astrocytes (Pindon *et al.* 1997; Pindon *et al.* 2000). Again, little is known about the expression patterns of these serpins in the developing chick spinal cord.

The function of serine proteases in the normal CNS is not fully understood. However these molecules are believed to have important roles in nervous system development, plasticity and homeostasis (Turgeon *et al.* 2000). A number of *in vitro* studies point to an involvement for serine proteases in morphogenesis and differentiation of neurons and glia. Activation of PAR-1 causes cell rounding and neurite retraction in cultured neuroblastoma cell lines and motorneurons via activation of Rho GTPase (Gurwitz and Cunningham 1988; Suidan *et al.* 1992; Jalink and Moolenaar 1992; Jalink *et al.* 1994; Brewer 1995; Turgeon *et al.* 1998). Likewise, inhibitors of thrombin such as PN-1 and hirudin promote neurite outgrowth (Monard *et al.* 1983; Gurwitz and Cunningham 1990). In contrast plasminogen and plasmin have been reported to promote neurite outgrowth in explant cultures (Nagata *et al.* 1993; Pittman and DiBenedetto 1995). In astrocyte cultures, thrombin has a mitogenic effect and can affect astrocyte morphology and stellation (Perraud *et al.* 1987; Cavanaugh *et al.* 1990; Nelson and Siman 1990). Thrombin also plays a role in programmed cell death during nervous system development. It has been demonstrated that inhibition of thrombin by PN-1 results in a reduction in programmed cell death in motorneurons *in vivo* in the chick embryo whereas activation of PAR-1 with peptide ligands can stimulate apoptosis *in vivo* in the chick embryo and in purified motorneuron cultures (Turgeon *et al.* 1999; Houenou *et al.*

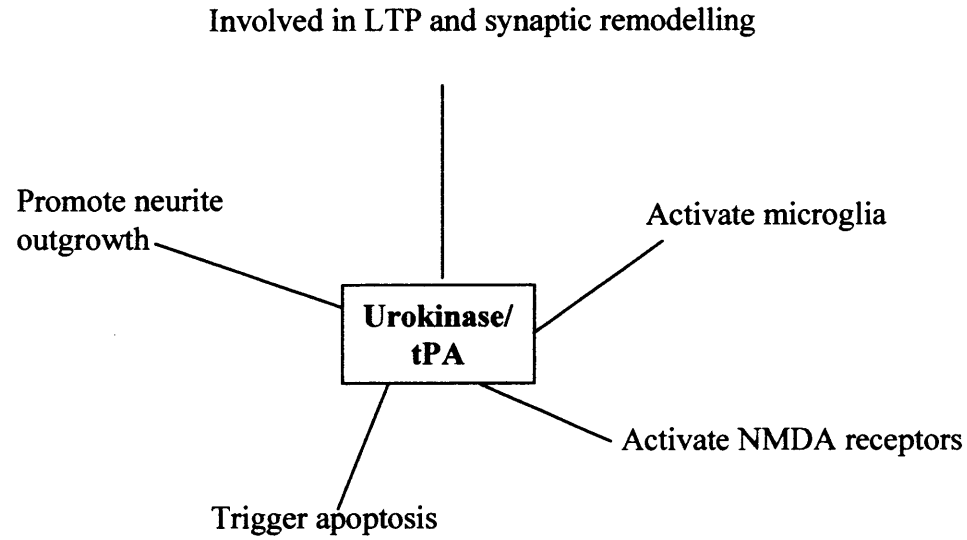
1995). Thus, many of the *in vitro* effects of serine proteases are indicative of an important role during nervous system development. The continuing expression of these molecules in the adult CNS may contribute to functions such as synaptic plasticity and long-term potentiation, and the maintenance of tissue homeostasis and tPA has been suggested to participate in several related processes including synaptic remodelling in long term potentiation (Monard 1988; Baranes *et al.* 1998; Qian *et al.* 1993).

Serine proteases and CNS injury

Many studies have suggested that serine proteases play a role in maintaining CNS homeostasis. As such, disruption of the balance of expression and activity of these molecules following injury might be expected to contribute to CNS pathology. It has been suggested that serpins in the CNS may act to neutralise potential harmful increases in serine protease levels, but that their capacity to maintain this balance might be reduced in injury situations which result in sudden high levels of serine protease activity (Choi *et al.* 1990; Vaughan and Cunningham 1993).There could be multiple causes of such disruption, including excessive extravasation of serine proteases, their activators or inhibitors, from the blood, or increased endogenous expression of these molecules in response to injury. In support of this suggestion, endogenous levels of serine proteases have been demonstrated to be altered after injury and in some neuropathological disease states (Wagner *et al.* 1989; Rao *et al.* 1993; Vaughan *et al.* 1994; Turgeon *et al.* 2000; Riek-Burchardt *et al.* 2002; Masos and Miskin 1997). mRNA levels of prothrombin, PAR-1 and plasminogen activators have all been demonstrated to be up-regulated after controlled contusion spinal cord injury in the rat (Citron *et al.* 2000b). There have also been reports of altered serpin expression after injury, while thrombomodulin expression has also been demonstrated to increase in astrocytes after injury (Nitsch *et al.* 1993; Pindon *et al.* 2000). The plasminogen activators and thrombin have been the most prominent of the serine proteases in studies into CNS injury responses.

Plasminogen activators and CNS injury

The plasminogen activators tPA and urokinase have been suggested to have a number of deleterious effects in the CNS (Fig 1.11). This is of particular importance in brain injuries such as stroke, in which tPA is often used as therapeutic agent to promote the dissolution of blood clots (del Zoppo 1998). The mechanisms by which these factors cause damage are not fully determined but may involve the conversion of plasminogen



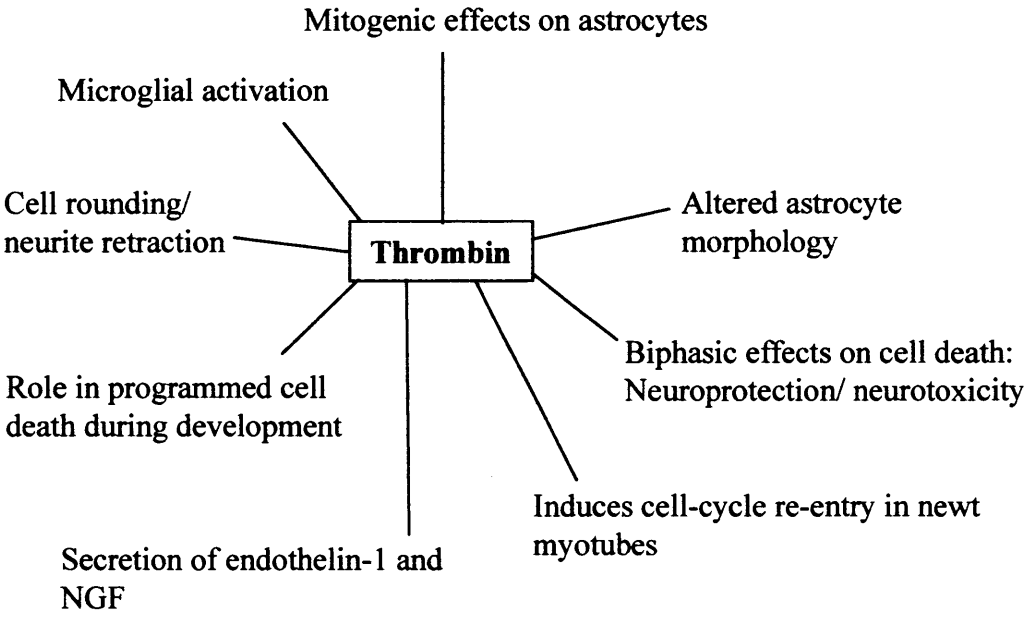


Fig 1.11. Selected experimental effects of serine proteases suggesting roles in nervous system development and pathology.

to plasmin and the initiation of a proteolytic cascade which results in cell death (Chen and Strickland 1997; Tsirka *et al.* 1997; Melchor *et al.* 2003). In addition, tPA has been linked to both activation of NMDA receptors, providing a mechanism by which this factor might exacerbate excitotoxicity, and to microglial activation, suggesting that it may also modulate the inflammatory response (Tsirka *et al.* 1995; Nicole *et al.* 2001). In support of this, mice in which the tPA gene has been removed have been shown to be resistant to excitotoxicity and to have reduced damage in models of stroke (Tsirka *et al.* 1997; Wang *et al.* 1998). Similarly, urokinase-/- mice demonstrated less damage in a brain stab wound model (Kataoka *et al.* 2000). Furthermore, administration of tPA itself can exacerbate damage in models of ischaemia or stroke and inhibition of tPA using antibodies or the tPA inhibitor, tPA STOP reduces the induction of neuronal apoptosis by activated microglia (Wang *et al.* 1998; Flavin *et al.* 2000). Importantly, tPA knock out mice have also been demonstrated to have reduced neuronal damage after spinal cord injury (Abe *et al.* 2003). Co-injection of tPA with thrombin into the brain has been demonstrated to increase injury beyond levels seen with either serine protease alone, suggesting that these two factors may potentiate each others action (Figueroa *et al.* 1998). On the other hand, up-regulation of urokinase and tPA in the PNS has been suggested to contribute to axonal re-growth and mice lacking these proteins have reduced recovery after PNS injury (Siconolfi and Seeds 2001a; Siconolfi and Seeds 2001b).

Thrombin in CNS injury

Several studies have highlighted potentially damaging effects of thrombin on neurons and glia while others suggest that thrombin might promote regeneration (Fig 1.11). Some of the effects of thrombin associated with nervous system development, such as changes in neurite outgrowth, might have potentially detrimental influences on CNS repair after injury, by preventing axonal regeneration. Importantly, thrombin might also have significant effects on neuronal cell survival and apoptosis.

In vitro, thrombin appears to have a bi-phasic effect on cell death, providing protection for neurons and glia against metabolic insults at lower concentrations but promoting apoptosis at higher concentrations (Vaughan *et al.* 1995; Smith-Swintosky *et al.* 1995; Pike *et al.* 1996). Pre-treatment with low doses of thrombin even seems to protect cells against subsequent larger doses of thrombin, via a receptor-mediated mechanism (Jiang

et al. 2002). Similarly *in vivo*, the effects of thrombin on cell death in models of ischemia are dose-dependent and low doses of thrombin have been shown to produce a 'pre-conditioning' protective effect against subsequent injury or larger doses of thrombin (Xi *et al.* 1999; Striggow *et al.* 2000; Masada *et al.* 2000). Inhibition of thrombin by PN-1 protects sciatic neurones from cell death after axotomy (Houenou *et al.* 1995). Injection of thrombin into the brain has been demonstrated to result in oedema, cell death and inflammation, although it is debatable whether the large quantities of thrombin injected were comparable with likely amounts of thrombin which would enter the brain during an injury (Nishino *et al.* 1993; Lee *et al.* 1997; Xue and Del Bigio 2001).

Some effects of thrombin on neuronal cells may be a direct result of proteolytic action of this enzyme on cell components. Thrombin can also cause apoptosis directly via its interaction with the PAR-1 receptor and downstream caspase-dependent mechanisms, as demonstrated in cultures of hippocampal neurons and embryonic chick spinal motorneurons (Donovan *et al.* 1997; Turgeon *et al.* 1998; Turgeon *et al.* 1999; Smirnova *et al.* 1998). Similarly, many of the neuroprotective effects of thrombin have been demonstrated to involve PAR-1 signalling (Pike *et al.* 1996). Interestingly both the neuroprotective and apoptosis-promoting effects of thrombin appear to involve the activation of Rho GTPase (Donovan *et al.* 1997; Donovan and Cunningham 1998). The mechanisms underlying the 'pre-conditioning' effect of low dose thrombin treatment are not well understood, although activation of thrombin receptors has been reported to be important, as has up-regulation of thrombin inhibitors such as PAI-1 and heat shock proteins (Jiang *et al.* 2002; Xi *et al.* 2003a; Xi *et al.* 2003b). Thrombin may promote cell survival through its effects on the synthesis and secretion of survival related factors such as endothelin-1 and NGF (Neveu *et al.* 1993; Ehrenreich *et al.* 1993).

In addition to its direct effects in apoptotic pathways, thrombin may also act as a pro-inflammatory factor in the CNS and may contribute to the formation of the glial scar, primarily through effects on the activation of microglia and astrocytes (Cavanaugh *et al.* 1990; Pindon *et al.* 2000; Suo *et al.* 2003; Suo *et al.* 2002). However, one recent paper has suggested that the reported effects of thrombin on microglial cytokine secretion are not mediated via thrombin activity but by the action of an associated higher molecular weight protein in the preparations used (Hanisch *et al.* 2004). As outlined in an earlier

section, the effects of inflammation after CNS injury may have both a beneficial and a harmful component.

One way in which thrombin might benefit regeneration is demonstrated by its actions after peripheral nervous system injury. Both prothrombin and pN-1 have been demonstrated to be up-regulated by Schwann cells after injury (Smirnova *et al.* 1996). It is believed that the balance between these two factors may help to control neurite outgrowth to ensure correct axonal targeting and synapse formation by the regenerating neurons.

Overall, the complexity of the effects of thrombin on CNS cells suggests a number of different, possibly interacting roles after injury. Physiological concentrations of thrombin in the CNS remain to be determined and it is therefore not established whether the effects of thrombin after injury are likely to be primarily beneficial or harmful. It should be noted that the concentrations of thrombin required to produce cell death may be significantly reduced in cells which are undergoing metabolic stress (Xi *et al.* 2003a). Therefore, from these previous findings it is certainly possible that increased concentration of CNS thrombin might contribute to increased cell death after injury.

Thrombin and regenerative capacity

In light of the complex roles of thrombin which have been suggested by previous *in vitro* and *in vivo* studies it would be of interest therefore to establish whether changes in thrombin expression or activity might contribute to differences in regenerative capacity in different systems or species. Although increased expression of components of the thrombin signalling pathway are a feature of non-regenerative spinal cord injury models, very few such studies have been carried out in regenerative systems.

The correlation between increased apoptotic response in the non-regenerating chick spinal cord and increased haemorrhage provides the framework for the hypothesis that increased thrombin activity in the post-E13 chick might be responsible for the lack of regeneration. In support of this suggestion, a study was conducted in which thrombin activity was compared between two regenerating systems, the fish optic nerve and the rat sciatic nerve, and a non-regenerating system, the rat optic nerve. This study showed that a large (40-fold) increase in thrombin activity in the rat optic nerve at one day after

injury was not matched by the smaller increase observed in either of the regenerative systems (Friedmann *et al.* 1999). The authors suggest that the smaller increase in thrombin activity in the sciatic nerve or in the fish optic nerve, in which thrombin activity was up-regulated later and for a longer duration, might actually be beneficial for regeneration while the larger increase results in toxicity, although this proposal was not tested.

Although much of what is known about thrombin suggests potentially deleterious roles in the injured nervous system, some other studies carried out in regenerative species suggest that thrombin might also have a directly positive effect in promoting regeneration. Thrombin has been highlighted as a key influence in the regeneration of both the newt limb and lens. Thrombin has been shown to regulate the cell-cycle re-entry which occurs in cultured newt myotubes following stimulation with serum, mimicking a key process which takes place during limb regeneration (Tanaka *et al.* 1999). Furthermore, *in vivo* studies of lens and limb regeneration in the newt have demonstrated an increase in thrombin activity specifically in regions corresponding to the location of cells which contribute to regeneration via dedifferentiation and proliferation (Imokawa and Brockes 2003). Lens regeneration was inhibited by injection of thrombin inhibitors. This thrombin activity was not observed in cell types that do not contribute to the regenerating lens or in non-regenerative species such anuran amphibians. The effects of thrombin on newt myotubes are not mediated via direct activation of PAR-1 but seem to involve cleavage of a serum protein, which has recently been partially characterised and demonstrated to be a glycoprotein (Straube *et al.* 2004). The authors of these papers suggest that the specific effects of thrombin in these regenerating systems may be due to the presence of a membrane tissue factor found on newt myotubes or dorsal iris cells which activates thrombin (Imokawa and Brockes 2003; Maden 2003).

1.7. Cell replacement after spinal cord injury

1.7.1. The contribution of cell replacement to regenerative capacity

One factor, which remains constant in all systems and species, is that, regardless of the success of neuroprotective or axonal regeneration strategies, there will inevitably be a

degree of cell loss after spinal cord injury. For this reason, the ability to replace lost and damaged cells by recapitulating the developmental process of neurogenesis could represent a key factor in determining regenerative success. A number of studies have investigated whether populations of progenitor cells exist in the spinal cord of different species and the response of these populations to injury. These studies have demonstrated an apparent correlation between the capacity for endogenous progenitor cells to generate new neurons and the regenerative capacity of the spinal cord.

In lower vertebrates, such as the urodele amphibians, cellular replacement is a key element of the regenerative response. Spinal cord regeneration is associated with the proliferation of progenitor cells in the ependyma, and their subsequent differentiation into neurons and glia (Benraiss *et al.* 1999). This is particularly evident in the case of urodele tail amputation, in which the entire missing section of spinal cord is completely rebuilt, but also plays an important role after transection injury. One major issue which remains unresolved is the relative contribution of such undifferentiated progenitor populations versus the dedifferentiation or transdifferentiation of already differentiated cell types. This has been addressed in cell-lineage tracing experiments with varying results (Echeverri and Tanaka 2002; Gargioli and Slack 2004). Both transdifferentiation and dedifferentiation have been demonstrated to occur during the regeneration of organs including the limb and the eye (Brockes 1998). However, in a number of regenerative systems it has not yet been fully established whether these mechanisms predominate over stem cell recruitment and it is likely that each individual situation may rely on different processes for regeneration (Ferretti 2004). One unique feature of the lower vertebrate spinal cord that differs from that of less regenerative species is the persistent presence in the adult spinal cord of a type of ependymal cell that has a radial glia like morphology and long processes which extend to the pial surface (Holder *et al.* 1990). These ependymal cells express the astrocytic marker GFAP together with other intermediate filaments such as nestin and vimentin and up-regulate expression of these molecules after injury (O'Hara *et al.* 1992; Walder *et al.* 2003). These cells are thought to play a major role in regeneration by proliferating and differentiating into neurons (Nordlander and Singer 1978; Benraiss *et al.* 1999).

Very little is known about the relative contribution of cell replacement strategies to repair in higher regeneration-competent species such as the opossum or chick. Most

studies in these species have focused exclusively on axonal regeneration. It would be of particular interest to determine whether axon re-growth alone is sufficient to provide these systems with regenerative ability or whether there is also a role for cell replacement, as this may have implications for the treatment of human spinal cord injury. In the developing opossum, a proliferative response to injury has been observed. However subsequent generation of neurons has not been demonstrated (Terman *et al.* 2000). In the chick spinal cord no studies so far have investigated this issue. As these models represent a midpoint between the highly regenerative lower vertebrates and the non-regenerative adult mammal it is of particular interest to determine the importance of cell replacement for regeneration in these species.

For many years it was commonly believed that adult mammalian CNS neurons were post-mitotic and that, once lost due to injury or disease, there was no endogenous population of cells available for replacement. More recently, this has been challenged by a number of *in vitro* studies which have demonstrated that neuronal precursors, both stem cells and more restricted progenitors, exist in multiple regions of the adult mammalian CNS including the spinal cord (Gage 2000; Reynolds and Weiss 1992; Weiss *et al.* 1996; Morshead *et al.* 1994; Nunes *et al.* 2003). Furthermore, neurogenesis has been demonstrated to be an ongoing process in at least two regions of the brain. Neural progenitors arising in the subventricular zone close to the lateral ventricle migrate to the olfactory bulb via the rostral medullary stream (RMS) and undergo neuronal differentiation in a continuous process (Lois and Alvarez-Buylla 1993; Luskin 1993). Secondly, the subgranular zone in the hippocampus contains neural stem cells which give rise to neurons that migrate to the granular layer of the dentate gyrus (van Praag *et al.* 2002).

In the adult mammalian spinal cord, neural stem cells have been suggested to be present in the ependymal region surrounding the central canal and in the parenchyma (Johansson *et al.* 1999; Horner *et al.* 2000; Yamamoto *et al.* 2001). However, although these cells have been reported to proliferate in response to injury, and up-regulate markers such as nestin, neurogenesis has not been observed and in most cases only glial cell types are generated (Frisen *et al.* 1995; Johansson *et al.* 1999; Namiki and Tator 1999; Horner *et al.* 2000; Yamamoto *et al.* 2001; Shibuya *et al.* 2002; Takahashi *et al.* 2003).

This comparison of different species illustrates that a fundamental difference between the adult mammalian nervous system and that of regenerative lower vertebrate species is that endogenous progenitor cells are not recruited in response to injury to generate new neurons. The reasons for this difference are unknown but may include intrinsic differences in the size or properties of progenitor populations, or differences in the environmental cues that might be responsible for recruitment. It is also clear that, with the exception of clear-cut examples such as urodele spinal cord amputation, the actual contribution of such newly generated neurons to spinal cord repair has not been examined fully in all regenerative species. One interesting proposition, outlined by Holder and Clarke, 1988, is that, in systems in which endogenous neurogenesis persists into adulthood it may be the case that the molecular cues necessary for axonal growth and cellular migration are retained, allowing axonal regeneration to take place. Whether this has a greater impact than cell replacement is unknown; however in regeneration following tail amputation it is apparent that proliferation must make a major contribution to repair. The importance of the endogenous progenitor cell response for regenerative capacity therefore remains to be determined. This issue is of great importance when considering whether therapeutic strategies aimed at restoring this function are likely to bring functional benefit. These questions may ultimately be answered by future studies in which such processes may be stimulated in the adult mammalian spinal cord; however, it my be possible to gauge the likely outcome of such treatment by establishing whether endogenous progenitor cell recruitment does indeed correspond to regenerative capacity in species such as the chick.

1.7.2. Strategies for identifying progenitors and measuring neurogenesis

As described above, a number of studies have attempted to assess the relative contribution of progenitor cells and neurogenesis to regenerative capacity. However this type of study is complicated by difficulties associated with the identification of neural stem cells and in measuring neurogenesis.

Several different types of neural progenitor cells are present in the embryonic CNS. These can be defined in terms of their developmental potential and include true neural stem cells which can both self renew by dividing to produce at least one new stem cell and differentiate into every cell type present in the CNS (McKay 1997). More restricted

progenitor cell populations also exist. With the growing realisation that neural progenitor cell populations are also active in the adult CNS there has been increasing interest in the study of these cells and their response to injury. Protocols have been developed to allow these cells to be studied extensively *in vitro*, where it is possible to examine factors such as the potential of a cell to self-renew and the types of cells it generates. However, *in vivo* investigations into the role of endogenous progenitor cells in the central nervous system are hampered by the lack of suitable markers for the various stages of progenitor cell development.

Dividing cells can be identified *in vivo* by a number of different strategies (Ming and Song 2005). These include methods for labelling cells undergoing DNA synthesis by incorporation of labelled nucleotides such as ^{3}[H]-thymidine or BrdU, a synthetic thymidine analogue. ^{3}[H]-thymidine is detected by autoradiography, whereas BrdU can be detected using a specific monoclonal antibody (Gratzner 1982; Sidman *et al.* 1959). Both labels are retained and passed down to subsequent generations during rounds of cell division, allowing the fate of dividing cells to be traced, although the label is gradually diluted with each division. It is important to note that incorporation of BrdU or ^{3}[H]-thymidine is an indication of DNA synthesis rather than cell division, and there is therefore a theoretical possibility that mechanisms such as DNA repair, or abortive cell cycle re-entry might make little contribution to the overall labelling which is observed using these techniques (Ming and Song 2005; Kuan *et al.* 2004; Selden *et al.* 1993). Alternative methods include labelling dividing cells using retroviruses, which, when incorporated into the host genome, express detectable proteins such as Lac Z or green fluorescent protein (GFP). In order to determine whether the labelled dividing cells go on to adopt a neuronal fate it is necessary to couple this labelling with cell specific markers. Using BrdU this is achievable by double immunostaining techniques. However, these methods have a number of caveats and restrictions; these include the fact that they cannot generally be used to study neurogenesis in the human *in vivo*, due to the requirement to administer BrdU, which is potentially carcinogenic, to living humans, although in rare circumstances this has been carried out (Eriksson *et al*, 1998). For human studies the only real option to identify newly born neurons is to examine tissue post-mortem for proteins which are expressed during various stages of neurogenesis. To this end, a number of different markers have been proposed. However

to date there is no available unequivocal marker that labels dividing cells that are destined to become neurons.

The intermediate filament (IF) protein family has provided a number of useful markers that are commonly used to label different cell types in the CNS. As shown in Fig. 1.12, several different IF proteins including vimentin, nestin, peripherin, neurofilaments and GFAP are sequentially expressed as a cell progresses from a stem cell to a differentiated neuron or glial cell (Lendahl *et al.* 1990). One of the most widely used markers for neural stem cells is nestin, yet this protein is also expressed by a number of other cell types including reactive astrocytes and radial glia (Lendahl *et al.* 1990; Dahlstrand *et al.* 1995). In the chick, a nestin-like protein, transitin, has been identified which is thought to be similarly expressed in progenitor cells and radial glia (McCabe and Cole 1992; McCabe *et al.* 1992; Cole and Lee 1997; Lee and Cole 2000). Thus, so far, no single protein has been identified which is uniquely expressed by a neural stem cell, which means that unequivocal identification of these cells is impossible and most studies rely on a combination of imperfect markers.

The identity of neural stem cells has been complicated further by recent revelations about the role of radial glial cells and astrocytes in neurogenesis. Radial glial cells develop from the neuroepithelium during embryogenesis and are present in the CNS transiently during periods of neurogenesis, where they have been suggested to have a role in the guidance of migrating neurons (Hatten 1999). They were classified as glial cell types due to the expression of molecules typically found in astrocytes such as glial fibrillary acid protein (GFAP) or an astrocytic glutamate transporter (GLAST) and it was believed previously that, after the completion of neurogenesis, these cells differentiated solely into astrocytes (Levitt and Rakic 1980; Hartfuss *et al.* 2001). However, recent *in vitro* studies have demonstrated that radial glia can generate neurons and may be a major source of neuronal progenitors in the embryo (Malatesta *et al.* 2000; Noctor *et al.* 2002). Furthermore, studies suggest that radial glial cells are a heterogeneous population of cells within which specific sub-populations may be destined to differentiate into different cell types (Hartfuss *et al.* 2001; Kriegstein and Gotz 2003). Recent studies have suggested that GFAP-positive cells present in the embryonic and early postnatal subventricular zone and the dentate gyrus can also act as

neuronal precursors although the identity of these cells is unclear (Doetsch *et al.* 1999; Seri *et al.* 2001).

Once a progenitor cell becomes committed to a neuronal fate, a number of different neuron-specific proteins are expressed and are commonly used to identify neurons by immunohistochemistry. These include β3-tubulin, poly-sialylated-neural cell adhesion molecule (PSA-NCAM), collaspin response-mediated protein 4 (CRMP) and HuC/D, which are expressed in relatively immature neurons, and NeuN, which is expressed at a slightly later stage after cell cycle exit (Fig 1.12). However, many of these proteins continue to be expressed throughout the life of a neuron and are therefore unsuitable for studies of neurogenesis, except in conjunction with a mitotic marker such as BrdU. The ideal marker of a newly born neuron would need to be only transiently expressed in order to identify a newly born neuron. A protein that has been reported to have these properties, at least in the adult CNS, is the microtubule associated protein doublecortin.

1.7.3. Doublecortin

In the quest for a unique marker of neurogenesis, doublecortin appears to be a promising candidate. Several studies have demonstrated that, in neurogenic regions of the adult brain, doublecortin is expressed early and transiently during neuronal development, with expression levels which correlate well with neurogenesis assessed by other methods (Brown *et al.* 2003; Couillard-Despres *et al.* 2005; Gleeson *et al.* 1999; Hannan *et al.* 1999; Nacher *et al.* 2001; Rao M.S. and Shetty A.K. 2004). Doublecortin is a microtubule associated protein which has come to attention mainly due to its essential role in cortical development (Bai *et al.* 2003). Mutations in doublecortin are associated with x-linked lissencephaly, a condition in which correct lamination of the cortex fails to occur, presumably due to abnormal neuronal migration (Francis *et al.* 1999; Gleeson *et al.* 1999; Sossey-Alaoui *et al.* 1998; Gleeson *et al.* 1998; des Portes *et al.* 1998). In addition to its potential as a specific neurogenic marker, doublecortin itself has been demonstrated to have a number of properties that suggest that this protein might actually have an interesting role in spinal cord regeneration. It is apparent that a number of the responses of spinal cord cells to injury involve substantial rearrangements of the cytoskeleton. Doublecortin has been demonstrated to have a role in regulating the

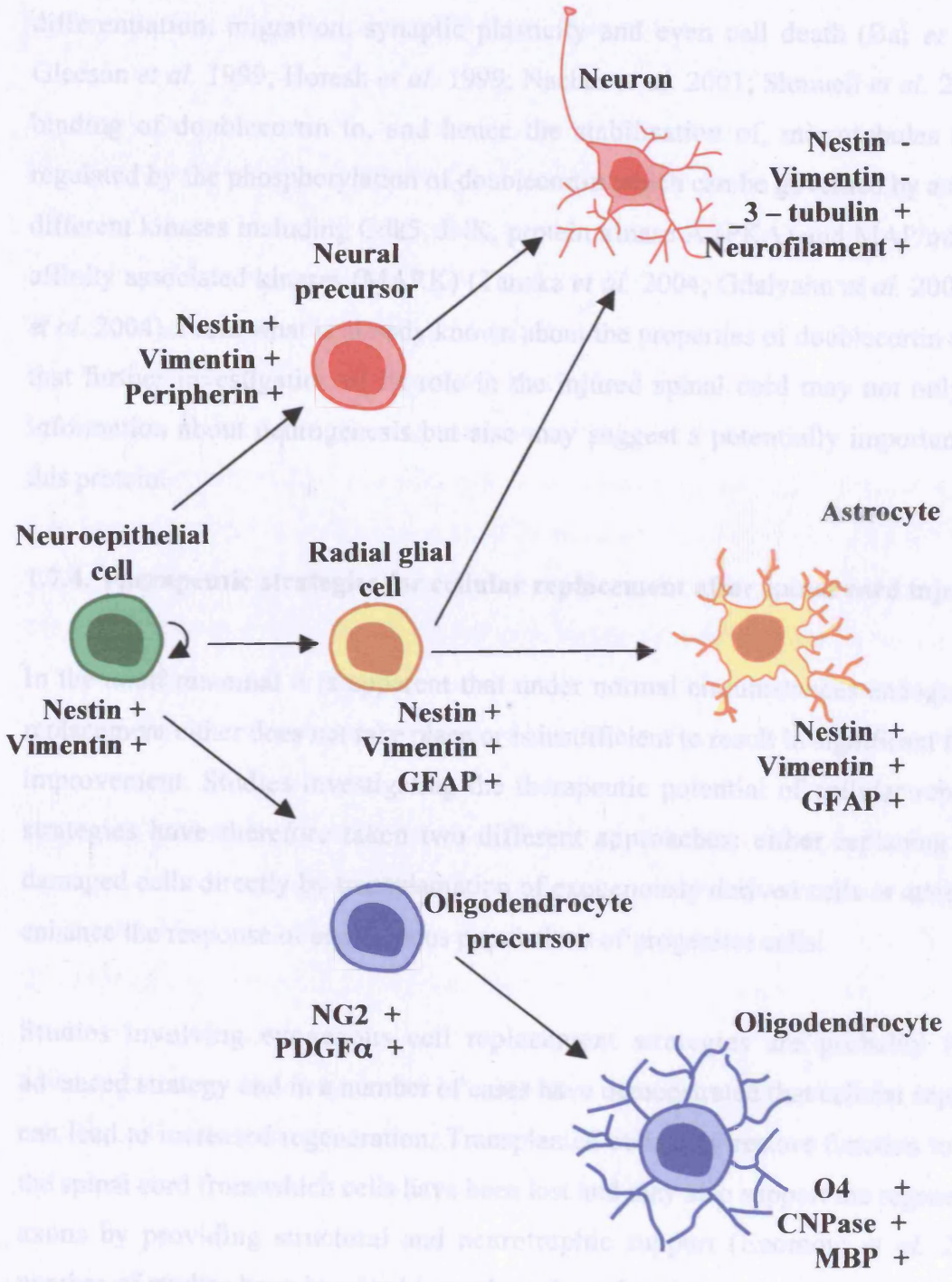


Fig 1.12 Sequential expression of markers during nervous system development. Figure illustrates the principal pathways of development of neurons, astrocytes and oligodendrocytes from neuroepithelial cells. Some of the many specific antigenic markers present at each stage are indicated. Illustrations adapted from http://www0.gsf.de/idg/groups/adult_neurogenesis/start.html.

stability of microtubules, a function which could be of importance for cell differentiation, migration, synaptic plasticity and even cell death (Bai *et al.* 2003; Gleeson *et al.* 1999; Horesh *et al.* 1999; Nacher *et al.* 2001; Shmueli *et al.* 2001). The binding of doublecortin to, and hence the stabilisation of, microtubules is strictly regulated by the phosphorylation of doublecortin which can be governed by a number of different kinases including Cdk5, JNK, protein kinase A (PKA) and MAP/microtubule affinity associated kinases (MARK) (Tanaka *et al.* 2004; Gdalyahu *et al.* 2004; Schaar *et al.* 2004). From what is already known about the properties of doublecortin it is likely that further investigation of its role in the injured spinal cord may not only provide information about neurogenesis but also may suggest a potentially important role for this protein.

1.7.4. Therapeutic strategies for cellular replacement after spinal cord injury

In the adult mammal it is apparent that under normal circumstances endogenous cell replacement either does not take place or is insufficient to result in significant functional improvement. Studies investigating the therapeutic potential of cellular replacement strategies have therefore taken two different approaches; either replacing lost and damaged cells directly by transplantation of exogenously derived cells or attempting to enhance the response of endogenous populations of progenitor cells.

Studies involving exogenous cell replacement strategies are probably the more advanced strategy and in a number of cases have demonstrated that cellular replacement can lead to increased regeneration. Transplanted cells may restore function to areas of the spinal cord from which cells have been lost and may also support the regeneration of axons by providing structural and neurotrophic support (Enomoto *et al.* 2003). A number of studies have involved transplantation of various types of neuronal precursor cell into the rodent spinal cord and have monitored the subsequent differentiation of these cells using labelling with markers such as GFP or BrdU (Reier *et al.* 1992; Cao *et al.* 2001; Enomoto *et al.* 2003; Mothe *et al.* 2005; Blits *et al.* 2005). These studies have so far demonstrated little evidence of neuronal differentiation within the spinal cord. One of the most important factors to consider when contemplating stem cell transplantation is the source of the cells. A number of studies have indicated that region specific stem cells exist, while other studies have suggested that the environment of the

transplant is sufficient to re-direct cells from an alternative region towards the appropriate fate (Shihabuddin *et al.* 2000).

In addition to the transplantation of neural stem or progenitor cells, a number of studies have suggested that transplantation of alternative cell types, including fibroblasts, olfactory ensheathing cells and Schwann cells, may enhance regeneration, mainly by improving the permissiveness of the spinal cord environment for regenerating axons (Bunge 1994; Li *et al.* 1998; Xu *et al.* 1999; Liu *et al.* 2000; Ramon-Cueto *et al.* 2000; Raisman 2001). Cells may be transplanted by direct injection into the injury site or by seeding the cells onto a bio-engineered scaffold, which may be designed to provide structural support and to provide, via slow-release mechanisms, factors which will enhance cell survival, proliferation or differentiation (Geller and Fawcett 2002). These scaffolds may also be of great benefit in providing a mechanism for bridging regions of the spinal cord that are difficult for axons to cross, either due to the presence of a large cavity or the formation of a glial scar. As technological advances continue to improve these scaffolds they are likely to become a key part of transplantation strategies.

At present, transplantation appears to be one of the most advanced and promising therapeutic avenues under investigation and clinical trials of some of these transplants have been initiated (Tsai and Tator 2005). However, there are a number of caveats to this approach that do need to be considered. An exogenous source of cells raises ethical issues to regarding the use of embryonic stem cells and may also have practical difficulties associated with immune rejection of transplants. Finally, due to a lack of long-term studies it has not been established fully that cells transplanted in this way will not result in the formation of tumours.

As an alternative approach, therapeutic strategies might be developed to enhance the endogenous capability for repair, which would alleviate many of the ethical and practical complications of using exogenous sources of cells for replacement, although safety issues may remain. Despite these advantages and in contrast to the number of studies involving transplantation of exogenously-derived cells, there have been relatively few studies investigating the therapeutic potential of stimulating endogenous precursors. As outlined above, although proliferation and migration of precursor cells has been demonstrated in the mammalian spinal cord in response to injury, this does not

result in neurogenesis (Mothe and Tator 2005; Namiki and Tator 1999; Yamamoto *et al.* 2001). An understanding of the factors, lacking in the adult mammal, responsible for 'switching on' neurogenesis is essential if endogenous progenitor cell stimulation is to be achieved. It is known that neurogenesis in the hippocampus can be affected by a number of different stimuli, including stress, physical activity and certain growth factors (Gould *et al.* 1997; Jin *et al.* 2002; Kempermann *et al.* 1997). Similarly, a number of different neurotrophic and growth factors including EGF, FGF, NGF and VEGF have been demonstrated to influence proliferation and differentiation of neural stem cells *in vitro* (Reynolds and Weiss 1992; Cattaneo and McKay 1990; Ray *et al.* 1993; Jin *et al.* 2002). This has prompted a limited selection of studies to attempt treatment of the spinal cord with neurotrophic factors. Administration of EGF and FGF2 has been shown to increase the proliferation and migration of spinal cord ependymal cells (Kojima and Tator 2002; Martens *et al.* 2002). It is also notable that FGF2 upregulation is associated with the urodele spinal cord regeneration, although its role in this process is not fully understood (Zhang *et al.* 2000). Expression of Notch-1 may also be important since this molecule is associated with tail regeneration in *Xenopus* larvae (Beck *et al.* 2003). Supposing that the correct signals to stimulate proliferation are identified, it may also be necessary to recapitulate some of the signalling associated with embryonic neuronal development to stimulate differentiation and correctly pattern newly born cells. Unfortunately, the factors which might be responsible for these processes are even less well understood.

To a large extent, the potential for stimulation of endogenous progenitors has been under-exploited due to our limited understanding about the factors necessary to achieve such stimulation. These strategies would therefore benefit greatly from a better understanding of the mechanisms underlying stimulation of endogenous progenitor populations in regenerative species. To date, the contribution of proliferation and neurogenesis to the regenerative capacity of the chick spinal cord has not been examined. Establishing the extent to which these processes are important in higher regenerative species such as the chick might be the key to determining whether such stimulation is likely to be effective in the adult mammal.

1.8. Objectives of this study

From this overview of the literature it is apparent that an enormous variation in spinal cord regenerative capacity exists between different species and that a multitude of different factors might contribute to this variability. It is also clear that many of these factors are likely to be interrelated and that a full understanding of their relative contributions to the determination of regenerative capacity would be of benefit when trying to identify factors which can be translated to the therapeutic treatment of the non-regenerative spinal cord. In this thesis the embryonic chick spinal cord will be used as a model to investigate the potential contribution of some of these factors. The chick spinal cord has a number of advantages over other available models of spinal cord injury and regeneration. The window of time during which regenerative capacity is lost in this model is well defined and relatively rapid, allowing this model to be used to examine both regeneration competent and incompetent states. The similarities between the pathological features observed in the post-E15 chick and those in adult mammalian species are striking, and suggest that findings in this model are likely to be of greater relevance to the human spinal cord than those in lower vertebrates. Finally, access to the chick embryo while *in ovo* improves ease of experimental manipulations in comparison to mammalian embryonic models such as the opossum.

Research using this model has in the past largely concentrated on factors affecting the permissiveness of the spinal cord environment for axonal re-growth. While this issue is of key importance, it is evident that a number of other factors may make an equal and complementary contribution to regenerative capacity in this model. In particular early responses to injury, including those which lead to cell death and those which promote cell proliferation and replacement may affect the number of viable cells which remain in the spinal cord after injury. Fig 1.13 summarises current knowledge about the differences in the early response of the chick spinal cord to injury at regenerating and non-regenerating stages and illustrates some of the questions that remain to be answered.

Changes in cell death and survival responses to injury have been demonstrated to correlate with the loss of regenerative capacity in the chick spinal cord (O'Neill, 2002, PhD thesis). In this thesis these findings will be investigated further, with a particular

concentration on the potential role of haemorrhage and factors brought into the spinal cord by blood. The potential for endogenous or exogenous serine proteases, particularly thrombin, to contribute to the apoptotic response after injury will be investigated.

Endogenous cell replacement strategies are currently the subject of a number of studies in the adult mammal. In order to understand whether such strategies are likely to be of benefit, it is of interest to determine the contribution of such mechanisms to recovery in regeneration competent systems. In this thesis the extent to which neurogenesis contributes to the success of regeneration in the E11 spinal cord will be examined.

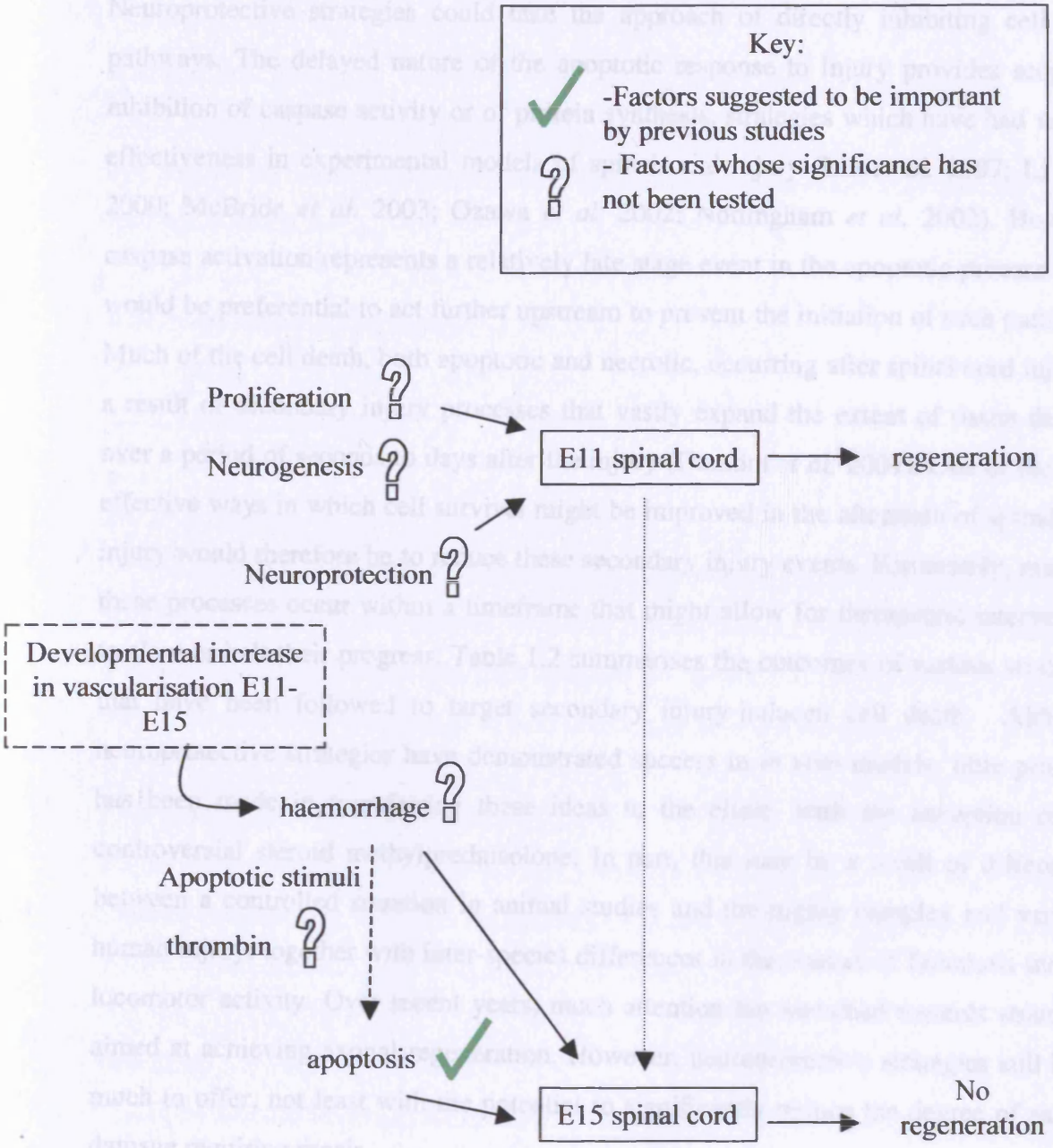


Fig. 1.13 Summary of current understanding about the early response to injury in the chick spinal cord. Figure illustrates a number factors which might contribute to the loss of regenerative capacity between E11 and E15, which will be investigated in this thesis.

1.6.2. Neuroprotective strategies after spinal cord injury

Neuroprotective strategies could take the approach of directly inhibiting cell death pathways. The delayed nature of the apoptotic response to injury provides scope for inhibition of caspase activity or of protein synthesis, strategies which have had varying effectiveness in experimental models of spinal cord injury (Liu *et al.* 1997; Li *et al.* 2000; McBride *et al.* 2003; Ozawa *et al.* 2002; Nottingham *et al.* 2002). However, caspase activation represents a relatively late stage event in the apoptotic process and it would be preferential to act further upstream to prevent the initiation of such pathways. Much of the cell death, both apoptotic and necrotic, occurring after spinal cord injury is a result of secondary injury processes that vastly expand the extent of tissue damage over a period of seconds to days after the injury (Dumont *et al.* 2001). One of the most effective ways in which cell survival might be improved in the aftermath of spinal cord injury would therefore be to reduce these secondary injury events. Fortunately, many of these processes occur within a timeframe that might allow for therapeutic intervention to slow or halt their progress. Table 1.2 summarises the outcomes of various strategies that have been followed to target secondary injury-induced cell death. Although neuroprotective strategies have demonstrated success in *in vivo* models, little progress has been made in transferring these ideas to the clinic, with the exception of the controversial steroid methylprednisolone. In part, this may be a result of differences between a controlled situation in animal studies and the highly complex and variable human injury, together with inter-species differences in the control of functions such as locomotor activity. Over recent years, much attention has switched towards strategies aimed at achieving axonal regeneration. However, neuroprotective strategies still have much to offer, not least with the potential to significantly reduce the degree of axonal damage requiring repair.

1.6.3. Relationship between haemorrhage and apoptosis

There is evidence to suggest that one of the earliest pathophysiological events to occur after spinal cord injury, haemorrhage, makes an important contribution to subsequent tissue damage and cell death. This hypothesis is supported by the observation that regions of haemorrhage found early after injury correspond to the later formation of large cavities.

Chapter 2. Materials

2.1. General Reagents

All general laboratory reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.2. Preparation of Solutions

2.2.1. General Solutions

EDTA

186.1g of disodiumethylenediametetracetate.2 H_2O was dissolved in 800ml of distilled H_20 (dH_20). The pH was adjusted to 8.0 by adding sodium hydroxide pellets until the powder dissolved. The pH was then adjusted to 7.4 with concentrated HCl if required, before making the volume up to 1l with dH_2O. The solution was autoclaved before use.

Phosphate Buffered Saline (PBS)

1 PBS tablet (Oxoid, Basingstoke, UK) was dissolved in 100ml of dH_20 to give a solution containing 0.16M NaCl, 0.003M KCl, 0.008M Na_2HPO_4 and 0.001M KH_2PO_4. Where required, the solution was autoclaved before use.

Tris (1M)

121.1g of Trizma base was dissolved in 800ml dH_2O. The pH was adjusted as required by adding concentrated HCl. After cooling to room temperature final adjustments to the pH were made. The volume was made up to 1l with dH_2O and the solution was autoclaved before use.

2.2.2. Solutions for histology

Paraformaldehyde (PFA)

4% PFA was prepared by dissolving 16g of PFA in 400ml PBS and heating at 70°C in a water bath until dissolved. After cooling, aliquots were stored at -20°C until required.

Cryoprotectant solution

A cryoprotectant solution for the preservation of frozen floating cryosections was prepared by mixing 150ml ethylene glycol, 100ml glycerol and 250ml PBS. The mixture was stored at +4°C until required.

Acid Alcohol

An acid alcohol solution was prepared by mixing 70ml EtOH, 1ml concentrated HCl and 29ml dH_2O.

2.2.3. Solutions for immunohistochemistry

Citric Acid buffer

2.1g of citric acid monohydrate was dissolved in 800ml dH_2O. The pH of the solution was adjusted to 6.0 by adding sodium hydroxide pellets. The solution was made up to 1l with dH_2O. The solution was prepared fresh for each use.

Sodium Borate (0.1M)

19g sodium borate was dissolved in 500ml dH_2O. The pH of the solution was adjusted to 8.5 by adding concentrated HCl. The solution was stored at room temperature

2.2.4. Solutions for TUNEL

Proteinase K permeabilisation solution

A permeabilisation solution was prepared by combining 915µl dH_2O, 50µl Tris (1M), 25µl EDTA (200mM) and 10µl proteinase K (1mg/ml). The solution was prepared fresh each time it was used and was pre-heated for 30 minutes at 37°C before use.

2.2.5. Solutions for RT-PCR

For all procedures involving RNA manipulation reagents were prepared with water treated with 1ml diethylpyrocarbonate (DEPC) per litre overnight at 37°C followed by autoclaving to inactivate the DEPC.

10x MOPS buffer

41.85g of MOPS, 6.81g sodium acetate trihydrate and 3.76g EDTA were dissolved in DEPC-treated dH_2O to give a total volume of 1l. The buffer was stored in the dark at room temperature.

RNA denaturing buffer

An RNA denaturing buffer was prepared by combining 24mg orange G, 300µl glycerol, 450µl 10x MOPS buffer, 720µl formaldehyde and 2.25ml formamide. The solution was aliquoted and stored at -20 °C

5 x Tris Borate-EDTA (TBE) buffer

54g Trizma base, 27.5g boric acid and 10ml 0.5M EDTA (pH 8.0) were dissolved in dH_2O to give a total volume of 1l. The solution was diluted 10x before use to give a 0.5x working solution.

TE Buffer

TE Buffer contained 10mM Tris and 1mM EDTA. The solution was adjusted to pH 8.0 by adding concentrated HCl.

2x PCR buffer for semi-quantitative PCR

A 2x PCR buffer was prepared, containing the reagents listed in Table 2.1. Aliquots were stored at -20°C.

Orange G loading buffer

A 5x DNA loading buffer was prepared by combining 10g sucrose, 50mg orange G, 5mg sodium azide and 20ml DEPC-treated dH_2O.

Table 2.1. 2x PCR buffer preparation

Reagent	Supplier	Stock concentration	Final concentration in PCR reaction	Volume (in µl) for 1ml
PCR Buffer	Invitrogen	10x	1x	200
$MgCl_2$	Invitrogen	50mM	2.5mM	100
dNTPs	Amersham	10mM each	0.2mM each	40
W-1	Invitrogen	1%	0.05%	100
Distilled H_2O	-	-	-	560

2.2.6. Solutions for Western blotting

10% Sodium dodecyl sulphate (SDS)

5g of SDS was dissolved in dH_2O to give a total volume of 50ml.

Protein extraction buffer

Protein extraction buffer was prepared by combining the following reagents:

3M NaCl	5ml
Nonidet P-40	1ml
10% SDS	1ml
1M Tris pH 8.0	5ml
Distilled H_2O	88ml

The buffer was stored at +4°C. Immediately prior to use, proteinase inhibitor cocktail was added from a 25x stock solution.

Protein Loading buffer

A 2x loading buffer was prepared from the following reagents:

1M Tris pH 6.8	0.5ml
Glycerol	1ml
2-mercaptoethanol	0.05ml
10% SDS	0.5ml
H_2O	2.9ml
Bromophenol blue	a few crystals

The buffer was stored at 4°C until used.

10x Running buffer

30g Trizma base, 144g glycine and 10g SDS were dissolved in dH_2O to give a final volume of 1l.

Transfer buffer

Transfer buffer was prepared by combining 100ml 10x Running Buffer, 400ml methanol and 1500ml dH_2O.

10x Tris Buffered Saline (TBS)

80g NaCl, 2g KCl and 30g Trizma base were dissolved in 800ml distilled H_2O. The pH was adjusted to 8.0 by adding concentrated HCl. The solution was made up to a final volume of 1l and autoclaved.

Blocking buffer

Blocking buffer was prepared fresh before each use by dissolving 5% non-fat milk in TBS with 0.05% Tween 20.

Wash buffer

Wash buffer was prepared by adding Tween 20 to TBS to a concentration of 0.5%.

2.2.7. Solutions for thrombin activity assay

Thrombin activity assay buffer

Activity assays buffer contained 0.05M Tris and 0.15M sodium chloride. The pH of the solution was adjusted to 8.3 by adding concentrated HCl.

2.3. Source of tissues

2.3.1. Chicken tissues

Fertilised White Leghorn chicken eggs were obtained from Henry Stewart & Co. Ltd., (Lincolnshire, UK). Humidified force flow incubators were supplied by Curfew Ltd. (Essex, UK).

2.3.2. Mouse tissues

Tissue sections from 129/Sv mice were donated by members of the Developmental Biology Unit, ICH.

2.3.3. Human tissues

Pre-embedded paraformaldehyde fixed tissue sections of human material were obtained from the MRC and Wellcome Trust funded Human Developmental Biology Resource.

2.4. Drugs

Tricaine

A 0.75g/l solution of ethyl 3-aminobenzoate methanesulfonate (tricaine) was prepared fresh on each occasion by dissolving 0.15g tricaine in 20 ml PBS.

Desmopressin

[deamino-Cys^{1},D-Arg^{8}]-Vasopressin (desmopressin) was dissolved in PBS to a concentration of 150μg/ ml.

Caspase inhibitor II

Biotin-X-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-CH_2F (Biotin-DEVD-FMK) was obtained from Calbiochem (San Diego, CA). A 10mM stock solution was prepared in dimethyl sulphoxide (DMSO). The solution was aliquoted and stored at -20°C.

Bromodeoxyuridine (BrdU)

A 10mg/ml solution of 5-Bromo-2'-deoxyuridine was prepared in PBS and stored at +4°C for up to 1 week.

2.5. Tissue culture media and equipment

Tissue culture plates, flasks and dishes were supplied by TPP (Trasadingen, Switzerland). Sterile strippettes were supplied by Corning (Corning, NY). Cultures were maintained in incubators supplied by Sanyo for cell culture (Bensenville, IL) and

Heraeus for slice cultures (Brentwood, UK). Gases were supplied by Cryoservice Ltd. (Worcester, UK). Low melting point agarose was supplied by Invitrogen (Paisley, UK).

2.5.1. Culture medium

Dulbecco's modified essential medium (D-MEM), containing 4500mg/l glucose, 4mM l-glutamine and 110mg/l sodium pyruvate, and Leibovitz's L-15 medium were supplied by Gibco (Paisley, UK) and stored at +4°C. L-glutamine (200mM), 100x antibiotic-antimycotic solution containing 10,000U/ml penicillin, 10,000µg/ml streptomycin and 25µg/ml amphotericin B, and Trypsin-EDTA were supplied by Gibco. Heat-inactivated fetal calf serum was obtained from sigma. All reagents were aliquoted under sterile conditions and stored at -20°C.

2.5.2. Thrombin and thrombin inhibitors

A 2000U/ml (~10µM) solution of bovine thrombin was prepared in 0.1% bovine serum albumin (BSA) and aliquots were stored at -20°C until required. Hirudin was dissolved to a concentration of 2000U/ml in 0.1% BSA and stored in aliquots at -20°C. D-Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Calbiochem (San Diego, CA). A 10mM solution of PPACK was prepared in distilled water and stored at +4°C.

2.5.3. Apoptotic Agents

A stock solution of 5mg/ml nocodazole was prepared in DMSO. A stock solution of 0.5mg/ml rotenone was prepared in DMSO. Roscovitine was obtained from Calbiochem (San Diego, CA) and was dissolved to 20mM in DMSO. All apoptotic agents were stored in aliquots at -20°C.

2.5.4. Reagents for detecting apoptosis in cell culture

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine (JC-1), was obtained from Molecular Probes (Paisley, UK). A 5mg/ml stock of JC-1 was prepared

in DMSO and aliquots were stored at -20°C. Stock solutions of ethidium bromide and acridine orange (1mg/ml) were prepared in dH_2O and stored at +4°C.

2.5.5. Antisense oligonucleotides

Unmodified antisense oligonucleotides against doublecortin were custom synthesized by Sigma-Genosys. Sequences for antisense and sense controls are shown in Table 2.2.

Table 2.2. Doublecortin antisense oligonucleotide sequences

	Oligonucleotide sequence
Doublecortin antisense	5' CCAAAATCAAGTTCCAT 3'
Doublecortin sense	5' ATGGAACTTGATTTTGG 3'

2.6. Histology equipment and reagents

Histoclear was supplied by National Diagnostics (Atlanta, GA). Paraffin wax and plastic moulds for wax embedding were obtained from Raymond Lamb (Eastbourne, UK). A Microm HM330 microtome was used to section wax blocks (Bicester, UK). OCT compound was obtained from BDH (Poole, UK). A Bright 5040 cryostat was used to cut frozen sections (Huntington, UK). Dispase was obtained from Gibco (Paisley, UK). The cytospin, filter cards and funnels were supplied by Thermo Shandon (Hemel Hempstead, UK). Ehrlich's Haemotoxylin was obtained from BDH and Eosin was supplied by Raymond Lamb. Glass slides were supplied by BDH. Poly-L-lysine and TESPA were obtained from Sigma. Glass coverslips in a variety of sizes were obtained from Raymond Lamb.

2.7. Immunohistochemistry Reagents

2.7.1. Primary Antibodies

For details of primary antibodies used in this study see Table 2.3.

2.7.2. Secondary Antibodies

All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA) and are summarised in Table 2.4. Secondary antibodies were reconstituted in dH_2O, diluted 50% in glycerol and stored in aliquots at -20°C

Table 2.3. Primary Antibodies used in this study

Antibody	Description of Antigen	Supplier	Dilution	Species	Antigen retrieval
RM-270	C-terminal domain of 160kDa neurofilament-M	Molecular Probes	1: 2000	Mouse	Yes
BrdU	Bromo-deoxyuridine, incorporated into nuclei of proliferating cells	Oxford Biotechnology	1:100	Rat	Yes
NeuN	Uncharacterised antigen found in nuclei of most post mitotic neuronal cell types	Chemicon	1:100	Mouse	Yes
β3 Tubulin	Labels cell bodies, axons and dendrites of post-mitotic neurons	Promega	1:2000	Mouse	Yes
HuC/D	Neuron specific RNA-binding proteins HuC, HuD and He-N1. Labels the cell body of neurons shortly after exit from mitotic cycle	Molecular Probes	1: 100	Mouse	Yes
Doublecortin (DCX)	Microtubule associated phosphoprotein located in migrating neurons and neuroblasts	Santa Cruz	1:200 (IHC) 1:500 (WB)	Goat	No
EAP3	Transitin, a intermediate filament protein, with some similarity to nestin	Developmental Studies Hybridoma Bank, Iowa	1:10 – 1:100	Mouse	Yes
3CB2	Radial glia	DSHB, Iowa	1:10	Mouse	Yes
GFAP	Intermediate filament protein specific for astrocytes	DAKO	1:100	Rabbit	Yes

Table 2.4. Secondary antibodies used in this study

Secondary Antibody	Dilution
Fluorescein (FITC)-conjugated affinipure goat anti-rat IgG	1:50
Fluorescein (FITC)-conjugated affinipure goat anti-mouse IgG	1:50
Cy™3-conjugated affinipure goat anti-mouse IgG	1:50
Cy™3-conjugated affinipure rabbit anti-goat IgG	1:50
Rhodamine conjugated affinipure donkey anti-rabbit IgG	1:50
HRP-conjugated affinipure rabbit anti-goat IgG	1:2000 (ELISA) 1:20,000 (WB)

2.7.3. Other reagents

Declere™ antigen retrieval solution was obtained from Cell Marque (Hot Springs, AR, USA). Hydrophobic pens for isolating sections were obtained from Sigma. Citifluor mounting medium was supplied by Citifluor products (London, UK). Hoechst 33258 was diluted to 1.2mg/ml in dH_2O. Aliquots were stored at -20°C and diluted 1:500 before use. Propidium Iodide was diluted to 1mg/ml in dH_2O and stored at +4°C. To-Pro-3-isomerase was supplied by Molecular Probes at a concentration of 1mM in DMSO. The solution was aliquoted and stored at -20°C.

2.8. TUNEL

TUNEL staining was carried out using the In-Situ Cell Death Detection Kit - Fluorescein from Roche (Mannheim, Germany).

2.9. Microscopes

For dissections, a Zeiss Stemi SV6 microscope was used (Carl Zeiss, Oberkochen, Germany). For light and fluorescent microscopy a Zeiss Axioplan 2 microscope was used. Cell cultures were observed on a Zeiss Axiovert 135M. Images were captured using a ProgRes C14 digital camera from Jenoptik (Jena, Germany). Confocal microscopy was carried out using a Leica TCS SP2 laser scanning spectral confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.10. Imaging software

Images were captured using Openlab software (Improvision, Coventry, UK). Further image preparation and analysis was carried out using Volocity software (Improvision) and Adobe Photoshop Elements 3.0 (Adobe, Edinburgh, UK). Confocal images were obtained and manipulated using Leica Confocal Software (LCS) and LCS lite software (Leica Microsystems, Wetzlar, Germany).

2.11. Molecular biology reagents and equipment

2.11.1. RNA extraction and reverse transcription

TRI reagent was obtained from Sigma. RNA was quantified using a GeneQuant spectrophotometer (Amersham Biosciences, Little Chalfont, UK). Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase (2.5U/μl), 10x PCR Buffer II and $MgCl_2$ (25mM) were obtained from Applied Biosystems (Foster City, CA). $Pn(N)_6$ random hexamers were obtained from Amersham Biosciences and were diluted to 1μM in TE buffer. The stock solution was further diluted in dH_2O to 50μM for reverse transcription. dATP, dCTP, dGTP and dTTP were obtained from Amersham Biosciences at a concentration of 100mM each. These were combined and diluted with dH_2O to give a final concentration of 10mM each. The stock solution was further diluted to 2.5mM each for reverse transcription. RNaseOUT recombinant ribonuclease inhibitor (40U/μl) was supplied by Invitrogen and was diluted to 20U/μl for reverse transcription.

2.11.2. Semi-quantitative RT-PCR

Taq DNA Polymerase (5U/μl), $MgCl_2$ (50mM), 10x PCR Buffer and 1% W-1 were obtained from Invitrogen. Primers were designed using Primer 3 software (Rosen & Skaletsky, 2000) and were obtained from Sigma-Genosys (Table 2.5). Primers were reconstituted to 100μM in sterile dH_2O. Aliquots were stored at -20°C. Before use, the primers were further diluted to 50pmole/μl. PCR was carried out on a programmable

thermal cycler (MJ Research Inc, Boston, MA). Thin walled PCR tubes were obtained from Elkay Ltd (Basingstoke, UK).

Table 2.5. Oligonucleotide sequences used for semi-quantitative PCR

Gene	Forward Primer Sequence	Reverse Primer Sequence	product size
Chick GAPDH	5'CAGTGAGAAAGTCG GAGTCA 3'	5'GACACCCATCACAAAC ATGG 3'	400 bp
Chick Doublecortin	5'GAACGTCAATCCCA ACTGGT 3'	5'CCTGTGCATAGCGGAAT TTT 3'	364 bp

2.11.3. Nucleic acid gel electrophoresis reagents and equipment

Agarose was obtained from BDH (Poole UK). RNA and DNA Gels were prepared and run using equipment supplied by Gibco. A 0.24-9.5kB RNA ladder was obtained from Invitrogen. DNA ladders were supplied by Bioline (London, UK). Ethidium bromide was diluted to 1mg/ml and stored at room temperature in the dark. Gel Imaging was performed using a UVdoc system (Topac, Hingham, MA) and LabImage software was used to investigate the density of bands (Halle, Germany)

2.11.4. Real time PCR

Primers and probes for chick thrombin, urokinase and GAPDH were custom designed and synthesised by Applied Biosystems from supplied sequences (Table 2.6). These were provided as a 20x assay mix, containing primers at 18μM and probes at 5μM. TaqMan Universal PCR master mix was obtained from Applied Biosystems. Optical plates and lids were obtained from Applied Biosystems. PCR was carried out on an Applied Biosystems 7000 real time PCR machine.

Table 2.6. Primers and probes used for real-time PCR

Gene	Forward primer sequence	Reverse primer sequence	Probe sequence
Chick GAPDH	5'GGGCACGCCATCAC TATCTTC 3'	5'ACCTGCATCTGCCC ATTTGAT 3'	5'CAGGAGCGTGACC CC 3'
Chick Thrombin	5'CCTCCTTGTGTTCA TGCTAAGCT 3'	5'TGGTGCTGCTAGCT ATTGTGATG 3'	5'AACAGCACCTGAAT CCA 3'
Chick Urokinase	5'GCCCTGCAGCACCA TAGA 3'	5'GTTTTGGAAGATGC CAGCTATCC 3'	5'CATGTACGCTCACA CTTC 3'

2.11.5. Western Blotting

Protein concentrations were determined using the BCA™ Protein Assay Kit from Pierce (Rockford, IL). A microplate reader was supplied by Dynex technologies (Worthing, UK) Proteinase inhibitors were supplied by Roche and dissolved to a 25x stock in dH_2O (Mannheim, Germany). Calf intestinal alkaline phosphatase (20U/µl) and 10x alkaline phosphatase reaction buffer were obtained from Promega (Southampton, UK). SDS-PAGE and Western transfer were performed using a BioRad electrophoresis system and tank transfer system (Hercules, CA). Hybond C Nitrocellulose membranes, enhanced chemoluminescent (ECL) reagents and Hyperfilm™ECL™ were supplied by Amersham Biosciences.

2.12. Centrifuges

To centrifuge samples of up to 1.5ml volume at +4°C, a Heraeus Biofuge 13R refrigerated centrifuge was used. For tissue culture, a Heraeus Labofuge 400 was used to centrifuge tubes up to a volume of 50ml. For all other experiments, a Heraeus Biofuge pico, capable of spinning volumes up to 1.5ml was used.

2.13. Activity assay reagents

The thrombin-specific chromogenic substrate CS-01(38) was obtained from Quadratech (Epsom, UK). The substrate was diluted fresh each time it was used to a concentration of 1mg/ml in assay buffer. A stock solution of bovine thrombin was prepared at 100U/ml in assay buffer. Aliquots were stored at -20°C. Recombinant hirudin from yeast was dissolved to 2492U/ml in activity assay buffer and aliquots were stored at -20°C.

Chapter 3. Methods

3.1. Animals

All procedures involving animals were carried out under Home Office Licensing approval and in accordance with the Animals (Scientific Procedures) Act 1986. Fertilised White Leghorn chicken eggs were incubated on their sides in a humidified forced flow incubator at 37°C, taking the first day of incubation as embryonic day 0 (E0). For surgical and other manipulations, a window, approximately $2cm^2$, was opened on the upper side of the egg on or before E7, taking care not disturb the underlying membranes and vessels. This procedure was aided by piercing a hole in the wide end of the egg two or three days before windowing to encourage the membranes to drop away from the egg surface. The window was covered and sealed with clear tape and the eggs re-incubated until the required stage.

3.2. Surgery

Spinal cord transection was carried out under a dissecting microscope at E11 or E15. The protocol used was an adaptation of that described by Shimuzu *et al*, 1990. At E15 the embryos were anaesthetised by administration of 0.5ml of 0.75g/l tricaine in PBS prior to surgery. A pair of fine forceps was used to break a small hole in the chorioallantoic membrane, carefully avoiding major blood vessels. A glass hook, prepared by heating and bending a glass pipette, was pushed through the hole and used to lift the embryo gently towards the window, with the dorsal side facing upwards (Fig 3.1.). Fine forceps were placed on either side of the spinal column in the cervical region and closed for a few seconds, to perform the transection, before gently lowering the embryo. Following surgery, the window was re-sealed with tape and the eggs re-incubated for up to 4 days.

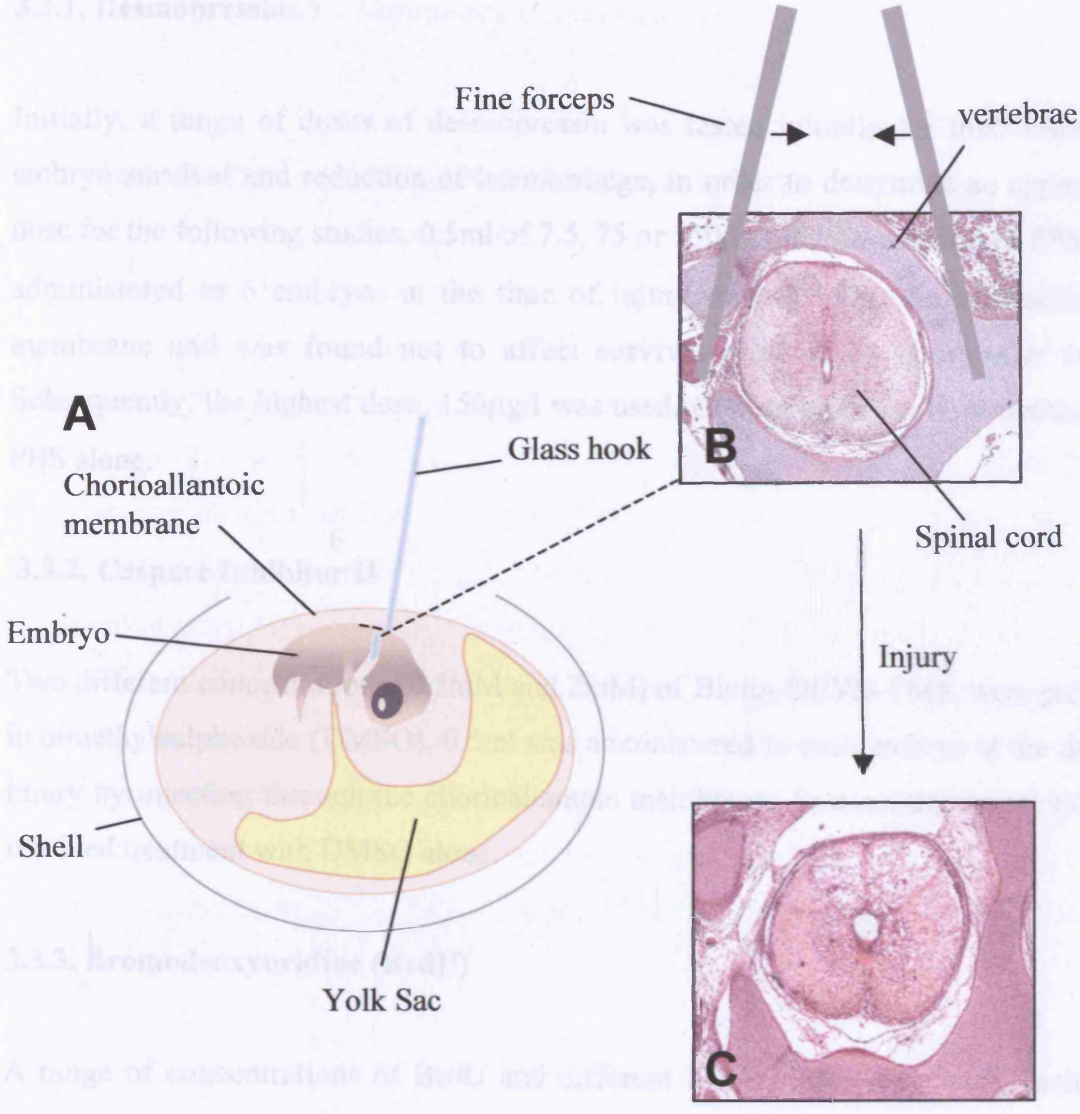


Fig. 3.1. Spinal cord injury in the chick embryo. Schematic diagram illustrating the process used to injure the chick embryo spinal cord. **A)** A small hole was made in the chorioallantoic membrane and a glass hook was used to bring the embryo close to a window made in the shell and stabilise the embryo during the injury. **B)** Fine forceps were inserted either side of the dorsal column and were closed to produce a transection/ crush injury. **C)** Haemotoxylin and Eosin stained section showing the typical morphology after the injury.

3.3. Drug and BrdU treatment.

3.3.1. Desmopressin

Initially, a range of doses of desmopressin was tested initially for their effects on embryo survival and reduction of haemorrhage, in order to determine an appropriate dose for the following studies. 0.5ml of 7.5, 75 or 150μg/ml desmopressin in PBS were administered to 6 embryos at the time of injury directly onto the chorioallantoic membrane and was found not to affect survival rates at 24 hours after injury. Subsequently, the highest dose, 150μg/l was used. Control embryos were treated with PBS alone.

3.3.2. Caspase Inhibitor II

Two different concentrations (0.5mM and 2mM) of Biotin-DEVD-FMK were prepared in dimethylsulphoxide (DMSO). 0.5ml was administered to each embryo at the time of injury by injecting through the chorioallantoic membrane. As a control some embryos received treatment with DMSO alone.

3.3.3. Bromodeoxyuridine (BrdU)

A range of concentrations of BrdU and different administration methods, including injecting onto and under the chorioallantoic membrane and sub-cutaneous injections were evaluated. For the final experiments, 100μl of 10mg/ml BrdU was administered directly onto the chorioallantoic membrane. In the experiments in which injury was carried out, BrdU was administered at the time of injury and this dose was repeated at 24-hour intervals throughout the incubation period following surgery.

3.4. Organotypic spinal cord slice culture

3.4.1. Preparation and maintenance of slice cultures

A protocol for the preparation of organotypic slice cultures was adapted from one kindly provided Dr. Laurent Nguyen (MRC-NIMR, London, UK). In the preparation of slice cultures as many steps as possible were carried out in a tissue culture hood. 3% low melting point agarose was prepared in L-15 medium and maintained at 40°C until required. The cervical spinal cord was dissected out from E11 or E15 embryos and embedded in the agarose, using a 6-well tissue culture plate as a mould (Fig. 3.2.). The agarose was placed on ice to set, and then blocks containing the spinal cord were trimmed and attached to the vibratome plate. 500μm slices were using autoclaved blades and collected in chilled L-15. The slices were transferred to the lid of a small tissue culture dish, allowing two slices per lid. A 50μl drop of medium was placed on top of each slice and then the dish was inverted to produce a 'hanging drop' in which the slice was suspended. Slices were cultured in humidified trays in at 37°C in 5%CO_2 for 24 hours. Slices were then collected, rinsed in PBS and fixed immediately with 4% PFA overnight at +4°C. They were then dehydrated through an ascending series of alcohol concentrations (50%, 70%, 85% and 95% EtOH) and stored in 100% EtOH at -20°C until required.

3.4.2. Treatment of slice cultures with low serum conditions

In order to test the effect of low serum conditions on apoptosis in slice cultures, slices were maintained for 24 hours in medium containing either 1% or 10% fetal calf serum.

3.4.3. Treatment of slice cultures with thrombin and thrombin inhibitors

To test the effects of thrombin on apoptosis in the slice cultures, bovine thrombin, at a final concentration of 100nM or 1μM, was added to the culture medium for 24 hours. In some cases, slices were incubated in medium containing 1% serum and 1μM thrombin. In others, the thrombin inhibitors hirudin (final concentration 40U/ml) or PPACK (final concentration 40nM) were added.

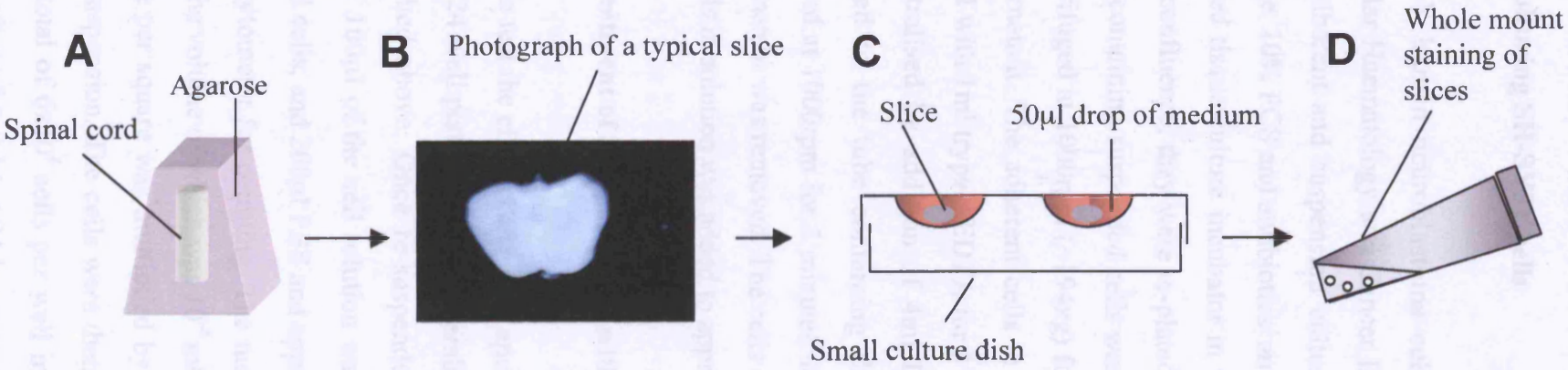


Fig. 3.2. Organotypic spinal cord slice culture. Schematic diagram illustrating the methods used to culture spinlal cord slices. **A)** Dissected spinal cords were embedded in agarose blocks. **B)** 500µm slices were cut using the vibratome **C)** Slices were cultured in 50µl hanging drops of medium. **D)** Slices were subsequently fixed and stained to detect apoptosis.

3.5. Cell culture

3.5.1. Culturing SH-SY5Y cells

SH-SY5Y human neuroblastoma cells were kindly donated by Dr. Arturo Sala (Molecular Haematology and Cancer Biology Unit, ICH). The cells were grown as a mixed adherent and suspension culture in $75cm^3$ flasks in DMEM with added l-glutamine, 10% FCS and antibiotics/ antimycotics. Cultures were maintained in a 37°C, humidified tissue culture incubator in 5% CO_2. Every 2-3 days, when the cells were close to confluence, they were re-plated after splitting by 50%. In order to do this, the medium containing suspended cells was transferred from the flask to a centrifuge tube and centrifuged at 1000rpm (~194xg) for 5 minutes to pellet the cells before removing the supernatant. The adherent cells in the flask were washed with 10ml PBS, then incubated with 1ml trypsin/EDTA for 3 minutes at 37°C to detach the cells. The trypsin was neutralised by addition of 4ml DMEM with 10% serum and the cells were transferred to the tube containing the pelleted suspension cells. The tube was centrifuged at 1000rpm for 5 minutes to collect the cells at the bottom of the tube and the supernatant was removed. The cells were re-suspended in 5ml fresh medium. 2.5ml of the cells in solution was added to approximately 8 ml fresh medium in a clean flask.

3.5.2. Treatment of SH-SY5Y cells with apoptotic agents

In order to test the effects of various apoptotic agents on SH-SY5Y cells, the cells were plated in 24 well plates on glass coverslips. This was achieved by trypsinising the cells as described above. Once re-suspended, an aliquot of the cells was removed for counting. 100µl of the cell solution was mixed with 200µl tryphan blue solution, to label dead cells, and 200µl PBS and approximately 18µl of the mixture was loaded into a haemocytometer for counting. The number of live cells was counted within 8 large squares, the volume of which was 10^{-4} ml each, using an inverted microscope. The mean cell count per square was multiplied by $5x10^4$ to give the number of cells/ ml in the original suspension. The cells were then plated at approximately $3x10^4$ cells per cm^2, giving a total of $6x10^4$ cells per well in a 24 well plate. The cells were cultured in normal medium for at least 24 hours to allow adherence. A range of different doses of

thrombin, rotenone or nocodazole was added to the cultures for up to 24 hours. An inverted microscope was used to monitor the progress of apoptosis and images were captured at various time intervals.

3.5.3. Treatment of SH-SY5Y cells with roscovitine

In order to determine the effect of inhibition of Cdk5 on cell death, SH-SY5Y cells were treated with the Cdk5 selective inhibitor, roscovitine. Cells were plated at a density of 3 x 10^4 cells per cm^2 in 10mm diameter tissue culture plates and grown for 48 hours to allow the cells to adhere. Roscovitine was added to the medium at a final concentration of 10μM or 20μM. The cells were incubated for a further 24 hours before observation using an inverted microscope.

3.5.4. Detection of apoptotic cells with Ethidium Bromide and Acridine Orange

In order to identify apoptotic cells after treatment with thrombin, rotenone or nocodazole, cells were stained simultaneously with two different nuclear dyes, ethidium bromide and acridine orange (Duke 2004). Ethidium bromide is only taken up by dying or dead cells, and stains DNA orange. In contrast, acridine orange is taken up by all cells and stains DNA green. Apoptotic cells can be identified by their condensed, abnormal chromatin. This assay therefore allows for the detection of four different cell states: 1) living, healthy cells (green, normal nucleus), 2) live, apoptotic cells (green, abnormal nucleus), 3) dead, necrotic cells (orange, normal nucleus) and 4) dead, apoptotic cells (orange, abnormal nucleus).

A working solution was prepared by combining 200μl each of acridine orange and ethidium bromide stock solutions (100μg/ ml) and 1600μl PBS. 40μl of this solution was added to the cells in 500μl medium in a 24 well plate and the samples were incubated at 37°C for 10 minutes before being observed using an inverted fluorescent microscope.

3.5.5. Detection of apoptotic cells with JC-1 dye

As an early indicator of apoptosis in SH-SY5Y cells the mitochondrial dye JC-1 was used. JC-1 accumulates in mitochondria in a membrane potential dependent manner (Reers *et al.* 1991). In healthy cells, JC-1 enters the mitochondria where, at high concentrations, it precipitates to form a red fluorescent stain. However, in cells in which mitochondrial membrane potential has been lost, an early indicator of apoptosis, JC-1 remains in the cytoplasm where, at low concentrations it stains the cells green. JC-1 dye was diluted in medium to a final concentration of 20μg/ ml. The dye does not dissolve well in aqueous solutions and the mixture was therefore vortexed vigorously for 5 minutes before being filtered to remove aggregates of dye. 500μl of the mixture was incubated with cells in a 24 well plate for up to 1 hour before observation on an inverted fluorescent microscope.

3.5.6. Antisense treatment of SH-SY5Y cells

To knockdown doublecortin mRNA expression, the cells were treated with antisense oligodeoxynucleotides, using sequences reported by Bai *et al*, 2003 (Section 2.5.5). SH-SY5Y cells were plated at a density of 10^4 cells per well in a 96 well plate. The cells were grown for 24 hours to allow them to adhere to the plastic. Antisense or sense oligodeoxynucleotides were added to the cells at concentrations ranging from 1μM to 20μM. In control cultures, an equivalent volume of sterile distilled H_2O was added. Since the oligodeoxynucleotides used in this study were unprotected and would therefore be degraded relatively quickly in culture, antisense was added to the cells every 12 hours for 3 days.

3.6. Tissue collection and processing for histology and immunohistochemistry

3.6.1. Dissection and fixation

At the appropriate time points following surgery the embryos were sacrificed and the entire thoracic/ cervical region of the spinal column dissected out on ice and rinsed in cold PBS. To prevent tissue degradation and stabilise cellular morphology, fixation was

carried out immediately by immersion in 4% paraformaldehyde (PFA) overnight at 4°C. After fixation, PFA was removed by rinsing for at least 1 hour in PBS at 4°C.

3.6.2. Decalcification

In order to permit sectioning through the vertebrae in embryos older than E13, the vertebrae were decalcified by immersion in 0.5M EDTA pH 7.4 at 4°C for 3-4 days, replacing the solution every 1-2 days.

3.6.3. Wax Embedding

For most immunohistochemistry experiments, samples were embedded in paraffin wax to support the tissue during sectioning and to preserve cellular morphology. As wax will not readily enter the tissue due to its high water content, the fixed samples were first passed through an ascending series of ethanol (EtOH) solutions to gradually dehydrate the tissue. The samples were passed through changes of 50% EtOH, 70% EtOH, 85% EtOH, 95% EtOH and 2 changes of 100% EtOH. The duration of each step varied according to the tissue size, but was typically 1 hour. The tissue was then passed through 3 changes of a synthetic solvent, Histoclear, at room temperature. This was followed by immersion in a mixture of 50% Histoclear: 50% wax in a 70°C oven, and, finally, 3 changes of 100% wax at 70°C. The tissue was carefully oriented in labelled plastic moulds for either longitudinal or transverse sectioning and embedded in clean wax. The blocks were cooled before removal from the moulds and sectioning.

3.6.4. Cryo Embedding

For a number of experiments samples were frozen before sectioning. Following fixation and rinsing as outlined above, tissues were immersed in 20% sucrose solution overnight at 4°C to preserve tissue morphology during freezing. After rinsing in PBS for 1 hour at 4°C, the tissue was then immersed in OCT compound in foil moulds for 30 minutes at 4°C to promote penetration of the OCT. The tissue was oriented within the mould using a fine needle and the blocks were rapidly frozen in isopentane chilled with dry ice. Blocks were stored at -80°C and then brought up to the cryostat temperature before sectioning.

3.6.5. Coating slides

Clean glass slides were coated with either poly-L-lysine or 3-aminopropyltriethoxy-silane (TESPA), to ensure adhesion of tissue sections to the slides. Although poly-L-lysine coating was sufficient for all wax sectioning it was noted that TESPA-coated slides provided better adhesion of cryosections.

For poly-L-lysine coating, clean glass slides were arranged in slide racks and immersed in a 10% solution of poly-L-lysine for 5 minutes before drying overnight at 37°C.

For TESPA-coating the slides were arranged in glass slide racks and cleaned by dipping in successive troughs containing 10% hydrochloric acid/ 70% ethanol, distilled H_2O and 95% ethanol for a minimum of 30 seconds each and then drying overnight at 60°C. The slides were then dipped for 30 seconds in a trough containing 2% TESPA in acetone, followed by dipping for 30 seconds each in two troughs containing acetone and finally they were rinsed in distilled H_2O. The slides were then dried overnight at 37°C.

3.6.6. Wax Sectioning

7μm sections of wax embedded tissue were cut using a Microm HM330 rotary microtome and floated on pools of distilled H_2O on labelled slides. The slides were gently warmed at approximately 37°C on a horizontal slide heater to flatten the sections. Excess water was removed using a plastic pipette and the slides were dried horizontally for at least 30 minutes before arranging in slide racks and drying in a 30°C oven for 24-48 hours.

3.6.7 Cryostat sectioning

10 or 30μm sections of frozen tissue were cut using a Bright 5040 cryostat. 10μm sections were transferred to TESPA-coated slides, air dried for 1-2 hours and stored at -80°C until required. 30μm sections were transferred, using a fine tipped paintbrush, to 24 well tissue culture plates containing PBS. These were stored at 4°C before staining,

or for longer term storage the sections were placed in a cryoprotectant solution and stored at -20°C.

3.6.8 Cytospin preparation

In order to produce slides for immunostaining with clear single-cell resolution, a cytospin preparation of dissociated spinal cord cells was used. Spinal cord tissue (approximately 6mm in length taken from the cervical spinal cord region) was diced and collected in L-15. The samples were centrifuged in 1.5ml eppendorf tubes briefly at 8000rpm (~6080xg) to collect the tissue in the bottom of the tube and the L-15 was removed. The tissue was dissociated by adding approximately 0.5ml of dispase (20U/ml) and incubating at 37°C for at least 20 minutes. During the incubation, the tissue was gently fragmented by pipetting up and down with a 1ml Gilson pipette. Once no visible tissue fragments could be seen the samples were centrifuged at 8000rpm for 2 minutes to collect the cells in a pellet. The dispase was removed and the cells were re-suspended in 0.5ml DMEM-F-12. The cells were diluted a further 10 times with DMEM-F-12. The cytospin was set up by fitting a TESPA coated slide, filter card and a clean cytospin funnel in each clip and loading these into the machine. 200µl of the cell suspension was added to each funnel and the cells were spun at 500rpm for 5 minutes onto the slides. The cell preparations were air-dried for 15 minutes to ensure adherence of the cells, dipped briefly in PBS to rinse and fixed with 4% PFA for 10 minutes before continuing with the staining procedure.

3.7. Haemotoxylin and Eosin Staining

To examine tissue morphology, sections were stained with Haemotoxylin and Eosin (H&E). Haemotoxylin is a basic dye which has a high affinity for nucleic acids and which stains cell nuclei a dark blue colour. Eosin, an acidic dye, provides an effective counterstain by staining all cytoplasm and connective tissue pink.

Both dyes are water-soluble; wax sections therefore must be deparaffinised and rehydrated for staining to be successful. This was achieved by immersing the slides in Histoclear in a coplin jar for 2 changes of 5 minutes duration to remove the wax and then passing the sections through a descending series of alcohol solutions as follows; 2

x 5 minutes in 100% EtOH, 1 x 5 minutes in each of 95%, 85%, 70% EtOH and finally 1 x 5 minutes in dH_2O. The sections were immersed in Ehrlich's Haemotoxylin for 10 minutes, rinsed in dH_2O for 1 minute and transferred to a running tap water bath for 5 minutes. This procedure produces 'over-staining' of the sections with Haemotoxylin which was then corrected by dipping in an acid alcohol solution for 3-6 seconds to remove some of the stain. The sections were returned to the tap water bath and then immersed in 1% Eosin for 10 minutes before a final rinse for 5 minutes in tap water. Finally, the sections were dehydrated by immersing in 95% EtOH for 1 minute, 100% EtOH for 3 minutes and Histoclear for 5 minutes. Sections were mounted and coverslipped using DPX.

3.8. Immunostaining

In order to investigate the localisation of various antigens and cell type markers in tissue sections and dissociated cell cytospin preparations a two-step indirect immunostaining procedure was used. Using this method a primary antibody directed against the antigen of interest first binds to the antigen. A secondary antibody, conjugated to a fluorescent label and directed against the species in which the primary antibody is raised, then reacts with the primary antibody. This step provides amplification of the signal as several secondary antibodies may react with a single primary antibody. The localisation of the antigen can then be visualised using a fluorescent microscope. The localisation of two different antigens can be visualised in a single tissue section by using secondary antibodies labelled with different fluorescent molecules (Fig 3.3).

3.8.1. Basic immunostaining protocol

Preparation of sections

In order to allow penetration and activity of the various water-soluble reagents used for immunostaining, wax sections were deparaffinised and rehydrated as described previously (Section 3.7.). Frozen sections were warmed up to room temperature and then immersed in PBS for 10 minutes to remove OCT compound.

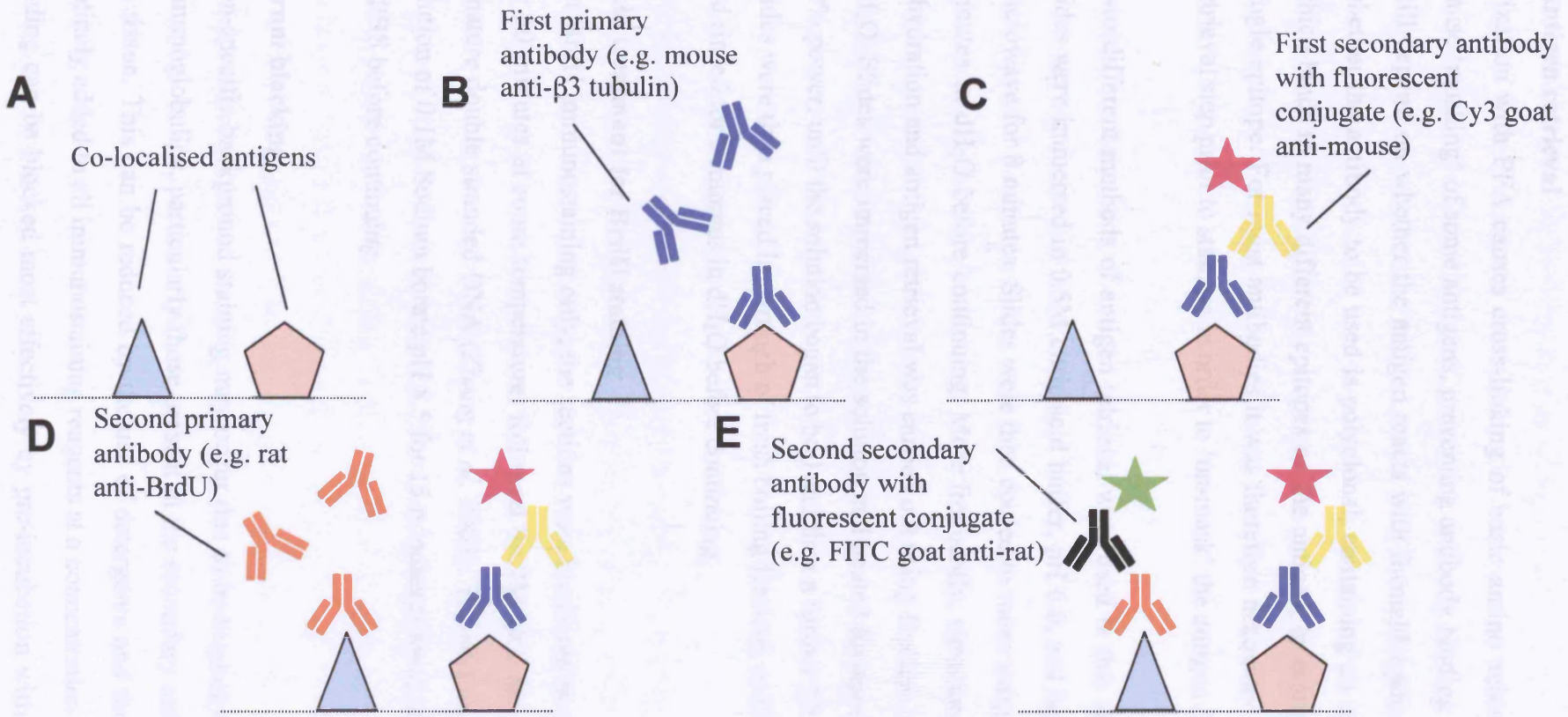


Fig. 3.3. Detection of co-localisation of two antigens by immunohistochemistry. Schematic diagram illustrating the steps taken to detect two independent antigens **(A)** by double immunostaining. **B)** Incubation with an excess of the primary antibody against the first antigen of interest is followed by incubation with a fluorescently-conjugated secondary antibody against the species in which the first primary was raised **(C). D)** Incubation with a second primary antibody, raised in an unrelated species is the followed by incubation with a second secondary, conjugated to a different fluorescent dye **(E).**

Antigen retrieval

Fixation with PFA causes cross-linking of basic amino acids in proteins which can cause 'masking' of some antigens, preventing antibody binding. The degree of masking will depend on whether the antigen reacts with formaldehyde and is also affected by whether the antibody to be used is polyclonal, containing an immunoglobulin mixture which binds to many different epitopes on the antigen, or monoclonal, raised against a single epitope. For most antibodies it was therefore necessary to carry out an antigen retrieval step prior to staining in order to 'un-mask' the antigen (Table 2.3, page 90).

Two different methods of antigen retrieval were used in this study. In some cases the slides were immersed in 0.5M citric acid buffer, pH 6.0, and heated at 60% power in a microwave for 8 minutes. Slides were then cooled to room temperature and rinsed for 5 minutes in dH_2O before continuing. More frequently, simultaneous deparaffinisation, rehydration and antigen retrieval was carried out using Declere solution, diluted 1:20 in dH_2O. Slides were immersed in the solution and heated for approximately 4 minutes at 80% power, until the solution began to boil and then a further 20 minutes at 30% power. Slides were then placed in a trough of fresh boiling Declere, cooled to room temperature and rinsed for 5 minutes in dH_2O before continuing.

Acid treatment for BrdU staining

For BrdU immunostaining only, the sections were incubated in a solution of 0.1M HCl for 30 minutes at room temperature, followed by 2M HCl for 30 minutes at 37°C to denature double stranded DNA (Zhang *et al.* 2003). The sections were neutralised in a solution of 0.1M Sodium borate pH 8.5 for 15 minutes at room temperature, then rinsed in PBS before continuing.

Serum blocking

Non-specific background staining may occur due to hydrophobic interactions between immunoglobulins, particularly those present in the secondary antibody, and proteins in the tissue. This can be reduced by the use of detergents and therefore Tween 20 was routinely added to all immunostaining reagents at a concentration of 5ml/l. Non-specific binding can be blocked most effectively by pre-incubation with a solution containing proteins, such as serum which will compete for hydrophobic binding sites in the tissue.

The sections were isolated by drawing a ring using a hydrophobic PAP pen to prevent reagents from running off the slide, and approximately 200µl serum per slide was added, covering the sections. In most cases a solution of 10% Fetal Calf Serum (FCS)/ 1% Bovine Serum Albumin (BSA) in PBS was used; for others a 10% solution of serum from the species donating the secondary antibody was prepared. These treatments appear to be interchangeable as no difference in the degree of non-specific background was observed. The sections were incubated in serum for 1 hour at room temperature in a humidity chamber.

Primary Antibodies

Following blocking, the serum was tipped off and replaced with the primary antibody made up to the required dilution (see Table 2.3, page 90, for dilutions and suppliers) in the blocking serum. For all new antibodies the optimum dilution was first established by carrying out a titration experiment in which staining intensity and background staining with different dilutions were compared. Sections were usually incubated overnight in primary antibody at 4°C in a humidity chamber.

Secondary Antibodies

Following incubation with the primary antibody, the sections were rinsed with 3 changes of PBS, before application of a secondary antibody, directed against the species in which the primary antibody was raised and conjugated to a fluorescent dye (Table 2.4, page 91). All secondary antibodies were used at a 1:50 dilution. Usually, the secondary antibody solution also contained Hoechst 33258 dye at a 1:500 dilution, which stains all cell nuclei, although this staining was found not to be effective after acid-treatment for BrdU-staining. In other cases, propidium iodide at a 1:1000 dilution was used to stain cell nuclei. The sections were incubated with the secondary antibody for 45 minutes at room temperature, before further rinsing with PBS and were mounted using a PBS/Glycerol mounting medium, Citifluor, to preserved fluorescence. Coverslips were applied and sealed with nail varnish to prevent the slides from drying.

Controls

A number of different controls were carried out to ensure the validity of the immunostaining procedure with each antibody. These included negative controls, in which no primary antibody was used, which gives an indication of the specificity of the

secondary antibody. In other cases, the primary antibody was replaced by non-immune serum of the same subclass and species as that of the primary antibody. This gives an indication of the non-specific binding of that antibody type in the tissue. In cases in which the peptide antigen was available this was used to pre-absorb the primary antibody by incubating the primary antibody in an excess of the peptide for 30 minutes before staining. These controls helped to ensure the specificity of the primary antibody.

3.8.2. Double immunostaining

In order to visualise more than one antigen in a tissue section at a time, double-immunostaining with two different primary antibodies was carried out. Since cross-reactivity between several of the antibodies was found to occur, it was necessary to incubate with each antibody separately.

The procedure was carried out as described above, including the acid treatment steps, and incubating with a first primary antibody which was usually a cell type marker antibody (NeuN, β3-tubulin, HuC/D, Transitin or GFAP) overnight at 4°C. Following incubation with the appropriate secondary antibody, the sections were rinsed 3 times with PBS and a second primary antibody (usually anti-BrdU) was applied and incubated for 2 hours at room temperature. This was followed by incubation with the appropriate secondary antibody, conjugated to a different fluorescent dye, before mounting. In order to minimise cross-reactivity between the second secondary antibody and the first primary antibody it was found necessary to use an antibody which was pre-absorbed against the species in question (usually mouse). Throughout the second round of the staining it was important to protect the sections from light as far as possible, to minimise bleaching of the fluorescent staining.

3.8.3 Immunostaining floating sections

For immunostaining floating sections the steps were essentially the same as those described above. Floating sections were stained in 24 well plates, with up to 4 sections in each well. 200µl of each solution was used per well and solutions were changed using a fine-tipped micro-pasteur pipette. All incubations were carried out with gentle shaking to ensure adequate penetration of the solutions. After staining, sections were

removed from the wells using a 'hockey stick', formed from a glass Pasteur pipette heated and bent to form an L-shape, and were placed on TESPA-coated slides. The sections were briefly air-dried to ensure good adherence, and were mounted using Citifluor and a glass coverslip.

3.8.4. Immunostaining cytospin preparations

For cytospin preparations, the immunostaining procedure was carried out as detailed above, however all incubation times were shorter. For BrdU staining the slides were incubated in 2M HCl for 10 minutes at 37°C and for 10 minutes in 0.1M Sodium Borate at room temperature. Non-specific binding was blocked by incubating the slides for 20 minutes in serum at room temperature. One hour incubations in primary antibodies and 30 minute incubations in secondary antibodies were carried out at room temperature.

3.8.5. Immunostaining cell cultures

For immunostaining cells cultured on glass coverslips the cells were first fixed for 20 minutes in PFA. The coverslips were removed from the dish using a needle and forceps and were rested on inverted micro tube lids throughout the immunostaining procedure. The steps were as detailed above, however all incubation times were shorter; non-specific binding was blocked by incubating in serum for 30 minutes at room temperature, whereas primary antibodies were incubated for 2 hours and secondary antibodies for 1 hour at room temperature.

3.9. Enzyme-linked immunosorbent assay (ELISA)

In order to quantify doublecortin protein levels in SH-SY5Y cells an adaptation of the standard ELISA protocol was used (Parnas and Linial 1998; Corcoran and Ferretti 1997). In this method cells are immunostained for doublecortin. In order to quantify the staining, a secondary antibody is used which is conjugated to an enzyme, horseradish peroxidase (HRP). Application of a chromogenic substrate for HRP then produces a measurable colour change in the solution which is related to the amount of antigen present allowing the determination of relative protein levels.

Cells were grown in 96 well plates and at the end of the experiment were fixed with 4% PFA for 20 minutes. Following fixation the cells were rinsed once in PBS for 5 minutes. In order to improve penetration for the reagents and allow access to the antigen, the cells were next rinsed in PBS containing a detergent, 0.1% triton-X 100. Endogenous peroxidase activity was quenched by incubating with 2% hydrogen peroxide for 5 minutes. The cells were rinsed again in PBS with 0.1% triton-X 100. Non-specific background staining was prevented by incubating the cells in 10% FCS/ 1% BSA for 1 hour at room temperature. A primary antibody against the antigen, doublecortin (Table 2.3, page 90) was diluted 1:200 in serum block and incubated with the cells for 2 hours at room temperature. The cells were rinsed thoroughly with PBS and then incubated with an HRP-conjugated rabbit anti-goat secondary antibody (Table 2.4, page 91), diluted 1: 2000 in serum block, for 45 minutes. The cells were then rinsed again with PBS. 100µl of a soluble chromogenic substrate o- phenylenediamine dihydrochloride (OPD) was added to each well and the plate was covered with foil and incubated on a plate shaker for 30 minutes at room temperature. A microplate reader was used to measure the optical density (OD) at 450nm.

3.10. TdT-mediated dUTP nick end labelling (TUNEL)

3.10.1. Principles of TUNEL staining

Apoptotic cells were detected using TUNEL staining. This assay is based on the detection of single strand breaks, or 'nicks' which are characteristic of the apoptotic process. These 'nicks' leave free 3'-OH DNA ends, to which a labelled nucleotide, fluorescein-dUTP is attached during the TUNEL procedure. The attachment of fluorescein-dUTP is catalysed by Terminal deoxynucleotodyl transferase enzyme (TdT) (Gavrieli *et al.* 1992).

3.10.2. TUNEL staining on wax sections

Wax sections were deparaffinised and rehydrated as described previously (see section 3.5), isolated using a PAP pen, and placed in a humidity chamber. In order to allow the TUNEL reagents to reach their target DNA inside cells the sections were permeabilised by incubating in proteinase K permeabilisation solution for 15 minutes at room

temperature. The sections were then rinsed 3 times in PBS. TUNEL reaction mixture was prepared on ice immediately before use by mixing TdT enzyme solution and labelling solution containing fluorescein-dUTP in a 1:10 ratio. 50µl of this reaction mixture was used to cover each section. As a control, some sections were incubated with 50µl labelling solution with no enzyme added. The sections were incubated with the reaction mixture for 1 hour at 37°C to promote enzyme activity. After rinsing again in PBS the sections were incubated with a 1:500 solution of Hoescht 33258 for 10 minutes at room temperature, to allow nuclear morphology to be assessed. The sections were mounted and coverslipped with Citifluor.

3.10.3. Whole-mount TUNEL staining/ Immunostaining

Apoptosis was assessed in 500µm spinal cord slices by whole-mount TUNEL staining, using an adaptation of the protocol described by Yamamoto & Henderson, 1999. The staining was carried out in 1.5ml micro-tubes. The slices were removed from storage in 100% ethanol at -20°C and incubated in a mix of ethanol: DMSO: H2O (4:1:1) for 5-10 hours at room temperature to aid permeabilisation of the tissue. They were then rehydrated and further permeabilised by incubating in 50% ethanol for 20mins followed by 3 changes of PBS, containing 0.5%Triton X-100 for 15 minutes each. Following this, the TUNEL mixture was prepared as described above, allowing 75µl total volume for 3-4 slices and the slices were incubated in the reaction mixture overnight at 4°C and then for 2 hours at 37 °C. This was followed by three 15 minute rinses with PBS containing 0.5% Triton X-100.

In order to assess neuronal apoptosis in the slices, whole-mount immunostaining was carried out following TUNEL staining. The slices were incubated in serum block containing 10%FCS/ 1%BSA and 0.5% Triton X-100 in PBS for 2 hours at room temperature. The serum block was then replaced by the primary antibody, anti NeuN, diluted 1:20, and incubated for 48 hours at 4 °C. After 3 rinses with PBS containing 0.5% Triton X-100, an appropriate secondary antibody was diluted 1:50 in PBS and incubated overnight with the slices. A nuclear dye, To-Pro-3 iodide was added to the secondary antibody to visualise all nuclei. After rinsing, the slices were mounted on slides with Citifluor.

3.11. Image Analysis

3.11.1. Microscopy

Light and fluorescence microscopy was carried out using a Zeiss Axioplan 2 microscope and digital images were captured using a ProgRes C14 camera, directly into Openlab software. Alterations to brightness and contrast were primarily made using Adobe Photoshop, and were kept constant within each experiment.

Confocal microscopy was carried out using a Leica TCS SP2 laser scanning spectral confocal microscope and Leica Confocal Software, using the settings shown in Table. 3.1. In order to avoid crossover between the emissions from different dyes, such as fluorescein and Cy3, each channel was captured separately using the sequence function. To visualise cells in 3 dimensions, z series were captured with steps of between 0.2-10μm. Subsequent manipulations of the data, including averaging, separations and cross-sectional analysis were carried our using Leica Confocal Software Lite.

Table 3.1. Confocal microscope settings for different fluorescent dyes

Dye	Laser	Min emission	Max emission
FITC	488nm	497nm	524nm
Cy3	543nm	561nm	634nm
To-Pro-3-iodide	633nm	662nm	802nm

3.11.2. Quantification

Measurement of haemorrhage and cavitation in the spinal cord

To quantify haemorrhage within the spinal cord, sections stained with H&E were imaged by light microscopy. A series of overlapping images were generated and used to create a composite of longitudinal sections of spinal cord at a level close to the centre of the injury. The extent of haemorrhage was quantified using these images by measuring the distance between the site of injury and the point where bleeding was no longer observed. Similarly, the size of visible cavities and tissue damage were measured from the site of injury.

Quantification of TUNEL labelling in longitudinal sections of spinal cord

To quantify TUNEL labelling in longitudinal sections of spinal cord, fluorescent microscopy was used to generate a series of overlapping images along the length of the cord. A scoring system was used to quantify the intensity of TUNEL staining. At each 1mm interval from the injury site a score from 0 (no staining) – 4 (intense staining) was given (Fig. 4.7, page 149). For each data point TUNEL scores from 3-6 animals were averaged. The maximum distance from the injury site at which apoptotic cells were observed was also recorded.

Quantification of TUNEL labelling in slice cultures

To quantify TUNEL labelling in the organotypic slice cultures, confocal microscopy was used to capture images every 10μm through the slice. Volocity software was used to measure the intensity of the TUNEL signal in the first 5 images collected from each slice. Each image was opened in the software and the region of interest (ROI) tool was used to outline the area of the slice. The measurements module was used to measure the total green intensity and the total area of the slice. To control for background fluorescence, which might show variation between experiments, an area of each image showing no positive TUNEL staining was outlined and the total green intensity in this area was also measured. The TUNEL intensity was expressed relative to the area of the ROI and the background fluorescence was deducted from each measurement. Data from at least 6 slices were obtained per experimental group.

BrdU cell counting

In order to generate a quantitative measure of proliferation, BrdU labelled cells in 30μm sections located 300 μm from the injury site were imaged on the confocal and average projections were generated using the Leica Confocal Software. Cells were counted manually using Openlab software. Each image was opened individually in Openlab. The ependymal region, grey matter and white matter were determined by eye and outlined using the region of interest (ROI) tool (see Fig 7.1). The area of each region was measured automatically by the software. All BrdU labelled cells in each area were counted by hand using the point measurement tool and were expressed as number of cells per μm2. For each data point, cell counts from 3-6 animals were averaged, and up to two sections were measured for each animal.

Analysis of double labelling

To identify cells double labelled with BrdU and a neuronal marker in 30_m spinal cord sections, confocal microscopy was used to generate a z-series of images captured through the section. Using the Leica Confocal Software, each stack was then separated into 4 stacks, in order to visualise the cells at a higher magnification. The cross-section tool was used to investigate each stack for double labelled cells. This enabled each cell in the stack to be visualised in 3 dimensions. In order for a cell to be identified as double-labelled with 2 markers, overlap of the signal in all dimensions was necessary. The number of double labelled cells per stack was determined.

In order to quantify double labelling in cytospin preparations, each slide was observed using a fluorescent microscope with a 40x objective. The numbers of BrdU labelled cells, and cells labelled with neuronal markers were counted blind in each of 5 randomly selected fields of view. The number of double-labelled cells was expressed as a percentage of the total number of BrdU labelled cells. For each data point, cell counts from at least 2 different animals were averaged.

3.12. RT-PCR

3.12.1. Principles of RT-PCR

To examine the expression of a particular gene it is necessary to be able to detect and quantify specific mRNA molecules from within the mixture of mRNAs found in a cell or tissue. In order to do this, the principle of the polymerase chain reaction is employed (Saiki *et al.* 1985).

The polymerase chain reaction allows a precise DNA fragment to be amplified from a relatively small amount of starting material, termed the template. This is achieved by using two oligonucleotide primers which bind to each DNA strand, just up-stream of the sequence to be amplified, and a DNA polymerase enzyme which uses these primers to generate new DNA molecules complementary to those of the target sequence. During the first step of a PCR reaction, the target sequence is exposed by denaturing the DNA using a temperature of around 95°C (Fig 3.4). The reaction is then rapidly cooled to a

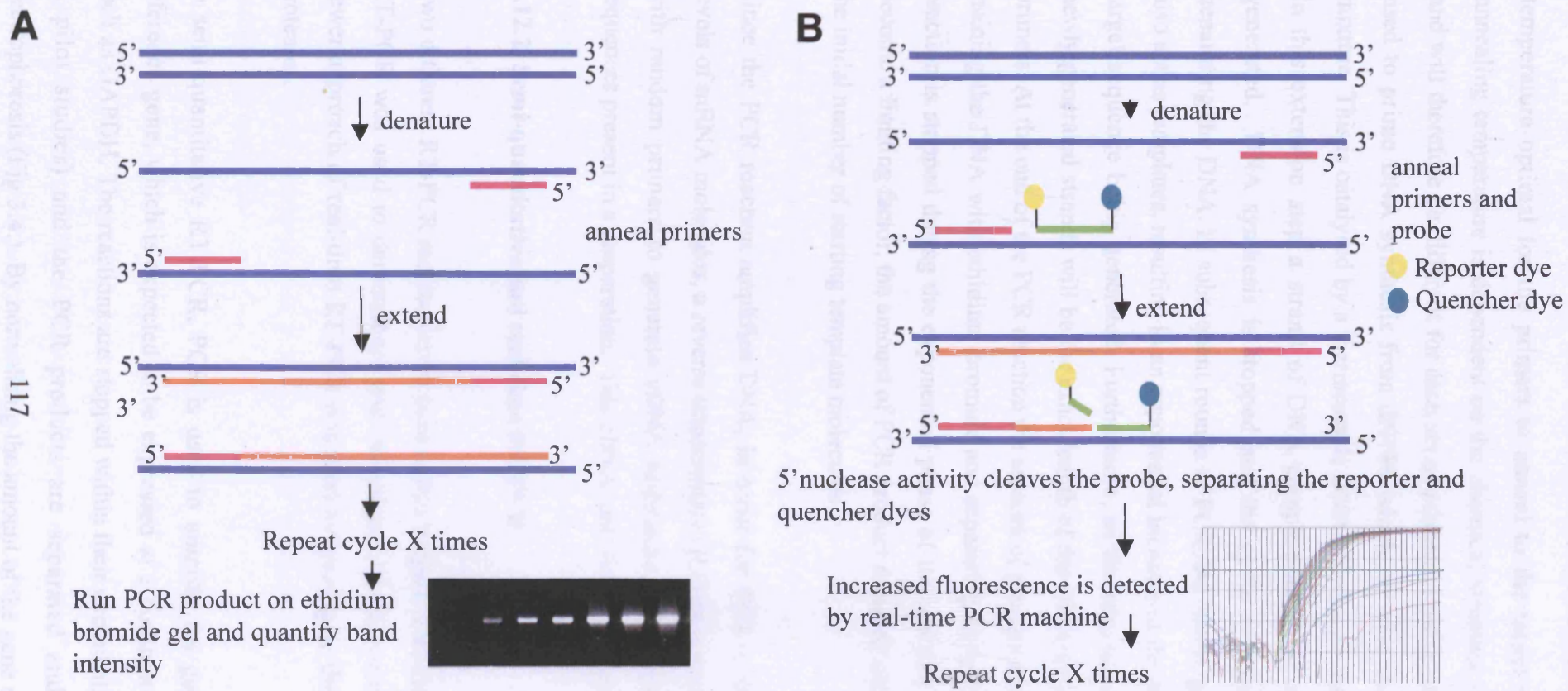


Fig 3.4. Principles of semi-quantitative (endpoint) and real time PCR methods. A) During PCR, double stranded DNA is denatured, by heating at a high temperature, to separate the strands. Sequence specific primers anneal to the target region, at lower temperatures. Extension of DNA strands is catalysed by DNA polymerase, giving rise to two new strands of the target DNA. As the process is repeated the amount of target DNA increases exponentially while reagents are available but slows as reagents are used up. **B)** Real-time PCR using the 5'nuclease assay involves the binding of specific primers and a special TaqMan Probe. The probe is labelled with a reporter fluorescent dye and a quencher dye, which prevents fluorescence being emitted while the probe is intact. During the extension phase of each PCR cycle, the probe is displaced by the activity of AmpliTaq® polymerase and the reporter dye is freed, causing fluorescence to be emitted. The real time PCR machine measures the increase in fluorescence over time. (adapted from Lewin, 1997 and Applied Biosystems TaqMan protocol)

temperature optimal for the primers to anneal to the target sequence. The precise annealing temperature is dependent on the chemical structure of the oligonucleotides and will therefore be different for each set of primers. Finally, the oligonucleotides are used to prime DNA synthesis from dNTPs which are also included in the reaction mixture. This is catalysed by a thermostable DNA polymerase such as Taq Polymerase. In this extension step a strand of DNA complementary to the target sequence is generated. DNA synthesis is stopped and the cycle of reactions starts again by denaturing the DNA. In subsequent rounds of PCR, the newly generated DNA strands also act as templates, resulting in an exponential increase in the number of copies of the target sequence being generated. Furthermore, as the reaction continues most of the newly generated strands will be the exact length of the DNA sequence between the two primers. At the end of the PCR reaction the amount of product produced is visualised by staining the DNA with ethidium bromide and separating by gel electrophoresis. If the reaction is stopped during the exponential phase of the reaction, before reagents have become a limiting factor, the amount of PCR product detected will be directly related to the initial number of starting template molecules.

Since the PCR reaction amplifies DNA, in order for PCR to be employed to detect levels of mRNA molecules, a reverse transcriptase (RT) enzyme is used in conjunction with random primers to generate cDNA sequences complementary to the mRNA sequences present in a preparation. This cDNA can then be analysed by PCR.

3.12.2. Semi-quantitative and real-time RT-PCR

Two different RT-PCR methods were used in this project. Traditional semi-quantitative RT-PCR was used to demonstrate and investigate doublecortin expression and the newer approach of real-time RT-PCR was used to investigate the expression of serine proteases.

In semi-quantitative RT-PCR, PCR is used to amplify the gene of interest and a reference gene, which is expected to be expressed at a constant level in all samples, such as GAPDH. The reactions are stopped within their exponential phase (established in pilot studies) and the PCR products are separated and quantified by gel electrophoresis (Fig 3.4.). By normalising the amount of the gene of interest detected to

the amount of GAPDH it is possible to control for differences in the starting quantities of RNA between samples. Semi-quantitative RT-PCR has been extensively used to quantify gene expression. The main limitations of this approach are relatively low sensitivity and the fact that it is not possible to be certain that a given reaction is during exponential amplification, which might lead to imprecise results.

In real-time PCR, the accumulation of PCR products over the course of the reaction is directly observed. This may be achieved by means of incorporation of a fluorescent dye into the newly synthesised DNA or, as was the case in the present study, by using a combination of a special Taq polymerase enzyme and fluorescent resonance energy transfer (FRET) (Morrison *et al.* 1998; Williams *et al.* 1996). In this approach, in addition to two oligonucleotide primers, a third oligonucleotide, or probe, is designed which binds to the target sequence in between the primers (Fig 3.4). As the Taq polymerase enzyme catalyses DNA synthesis it simultaneously cleaves the probe which is positioned in the way of the extending DNA strand. The probe is conjugated to both a fluorescent reporter molecule, which emits energy when excited by light, and a quencher molecule. When the probe is intact, these two molecules are in close proximity to each other and energy is transferred from the reporter to the quencher via FRET, preventing energy emission. However, as the PCR reaction proceeds and the probe is cleaved this will separate the reporter and quencher leading to an increase in energy emission from the reporter. The fluorescence emitted from the reporter dye therefore reflects the amount of PCR product generated. During real-time PCR this emission is measured throughout the reaction. The advantage of real-time PCR over semi-quantitative PCR is that it allows detection of the accumulation of PCR products over the course of the reaction. This means that it is possible to determine the exponential phase of the reaction more precisely making it more likely that the measurements taken will reflect the starting quantities of template. However the major disadvantage of real-time PCR is the cost which is much greater than traditional semi-quantitative methods.

3.12.3. Tissue Collection

Spinal cords were collected from normal embryos at E3, E7, E11, and E15. For injured animals, at the required time point after injury (2 hours or 24 hours), spinal cord tissue

was collected from a 6mm region around the injury site, and two 3mm regions either side of the injury site, as illustrated in Fig 3.5. Briefly, the embryo was sacrificed and rapidly dissected on ice. The skin was removed to expose the cervical/ thoracic spinal column which was dissected out. A pair of curved forceps was used to squeeze the spinal cord tissue out. This was rinsed in cold DEPC-treated PBS, transferred to sterile eppendorf tubes and quickly frozen on dry ice. Samples from 3 animals were pooled in each tube, and stored at –80°C.

3.12.4 RNA Extraction

RNA extraction was performed using an adaptation of the single step RNA isolation protocol. Spinal cord tissue was homogenised in 0.5 ml TRI Reagent, which contains guanidine thiocyanate to lyse cells and denature proteins, and an organic solvent, phenol. The mixture was left to stand for 5 minutes at room temperature. 0.1 ml chloroform was added to each tube and the tubes were shaken for 15 seconds, before standing at room temperature for 5 minutes. During this step the mixture separates into two phases, an organic phase and an aqueous phase. Denatured proteins and DNA cannot remain water soluble under these conditions and enter the organic phase of the solution, while RNA remains in the aqueous phase. The samples were centrifuged at 13,000rpm (~11,000xg) for 15 minutes at 4°C to separate the phases. The upper, aqueous phase was transferred to a sterile eppendorf and 0.25 ml isopropanol was added to each tube. This was placed at -20°C overnight to precipitate the RNA. The tubes were then centrifuged at 13,000rpm for 30 minutes at 4°C. The supernatant was removed and the RNA pellet was washed in 75% ethanol followed by centrifugation at 7,500g for 5 minutes at 4°C. Finally the supernatant was removed and the pellet allowed to air dry before re-suspending in 20µl DEPC-H_20. The RNA concentration was determined by measuring the absorbance at 260nm using a spectrophotometer. In order to confirm that the RNA was not contaminated with protein the ratio between absorbance at 260nm and 280nm was measured. This should be close to 1.8 for a pure RNA preparation.

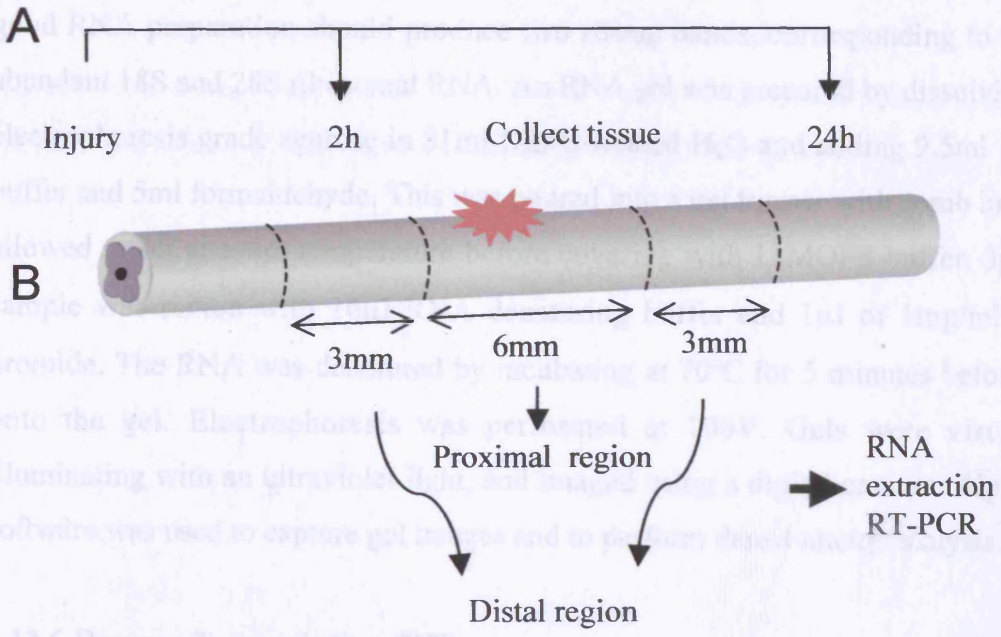


Fig 3.5 Tissue collection strategy for RT-PCR after spinal cord injury A). A 12 mm region of cervical spinal cord was dissected at 2 hour or 24 hours after spinal cord injury.**B)** The tissue was divided into a 6mm proximal region around the injury site (the proximal region) and two 3mm distal regions which were pooled for further analysis.

3.12.5 RNA Gel Electrophoresis

To check the integrity of the RNA, samples were separated by gel electrophoresis. A good RNA preparation should produce two strong bands, corresponding to the highly abundant 18S and 28S ribosomal RNA. An RNA gel was prepared by dissolving 0.95g electrophoresis grade agarose in 81ml DEPC-treated H_2O and adding 9.5ml 10xMOPS buffer and 5ml formaldehyde. This was poured into a gel former with comb in place and allowed to set at room temperature before covering with 1xMOPS buffer. 3µl of each sample was mixed with 16µl RNA denaturing buffer and 1µl of 1mg/ml ethidium bromide. The RNA was denatured by incubating at 70°C for 5 minutes before loading onto the gel. Electrophoresis was performed at 100V. Gels were visualised by illuminating with an ultraviolet light, and imaged using a digital camera. Alpha imager software was used to capture gel images and to perform densitometry analysis.

3.12.6 Reverse Transcription (RT)

Complementary DNA (cDNA) was prepared from the extracted RNA using M-MLV (Murine-Moloney Leukaemia Virus) Reverse Transcriptase. 1µg total RNA was used per reaction and was made up to a volume of 10µl with DEPC-H_2O. 2µl of random hexamers (50µM), 2µl of dNTPs (2.5mM each) and 1µl of RNaseOUT recombinant ribonuclease inhibitor (20U/µl) was added and the mixture was heated at 70°C for 10 minutes to denature the RNA, followed by immediate cooling on ice. Finally, 2µl of 2x PCR Buffer II, 2µl of $MgCl_2$ (2.5mM) and 1µl of MLV-transcriptase (50U/µl) was added and the mixture incubated at 42°C for 1 hour, using a PTC-100 Programmable Thermal Controller. cDNA samples were stored until required at –20°C.

3.12.7. Semi-quantitative PCR analysis of doublecortin expression

Each PCR reaction was carried out in 20µl total volume containing the following reagents:

5.9µl H_2O
10µl 2x PCR buffer

0.5µl each primer (Table 2.5, page 93)
0.1µl Taq DNA Polymerase
3µl cDNA

PCR was carried out using a PTC-100 Programmable Thermal Controller (MJ Research Inc, Boston, USA). The programme contained the following steps:

1. 94°C for 3 minutes
2. 94°C for 30 seconds
3. 56°C for 1 minute
4. 72°C for 1 minute
5. Go to step 2 x times
6. 72°C for 10 minutes
7. End

Where x = number of cycles. The number of cycles required to remain within the linear range was determined in a separate experiment for each primer set. For quantification GAPDH reactions were run for 21 cycles and doublecortin reactions for 26 cycles. PCR products were stored at 4°C before being separated by gel electrophoresis.

3.12.8. DNA Gel electrophoresis

A 1.5% agarose gel was prepared by dissolving 1.5g agarose in 100ml 1xTBE. Ethidium bromide (4µl) was added and the gel poured into a gel former with comb in place. 16µl of each sample was combined with 4µl of 5x orange G loading buffer. 16µl of the mixture was then loaded into each well. 5µl of Hyperladder IV was loaded into the first lane. Electrophoresis was performed at 90V for approximately 2 hours. Gels were visualised as described previously.

3.12.9. Semi-quantitative PCR data analysis

For all experiments 3 different pools of tissue samples were run in triplicate PCR reactions. The density of each band was measured and recorded using LabImage

software. Background density was subtracted from each band. Doublecortin bands were normalised to GAPDH bands for each sample by expressing as a ratio to GAPDH levels. The 3 replicates for each sample were averaged and the mean value for each developmental stage or experimental condition was determined.

3.12.10. Real-Time PCR analysis of serine protease expression

Primers and probes for chick thrombin, urokinase and GAPDH were custom designed and synthesised by Applied Biosystems and were supplied as a pre-mixed 20x'assay-mix'. The sequences of each primer and probe set are given in Table 2.6 (page 93). For each PCR reaction, the following reagents were combined:

1.25µl 20 x assay mix
12.5µl 2x Taqman universal PCR master mix
10.25µl H_2O
1µl cDNA prepared as in section 3.11.6

Reactions were carried out in a 96 well microplate, sealed with a clear plastic optical lid using an Applied Biosystems 7000 real time PCR machine. The programme consisted of the following steps:

1. 50°C for 2 minutes
2. 95°C for 10 minutes
3. 95 °C for 15 seconds
4. 60 °C for 10 seconds
5. Repeat steps 3 and 4 40-45 times

For each sample, 2 wells were prepared with target primers and probes and 2 with reference (GAPDH) primers and probes. Two different negative controls were run; no cDNA (water), and RNA only (noRT).

3.12.11 Real time PCR data analysis

Following the PCR reaction the amplification curves for each sample were examined. Fig. 3.6 shows the typical shape of an amplification curve, together with some examples of the curves obtained in this study. As shown in Fig 3.6.A, the real-time PCR machine provides a measurement of ΔR_n, which is the emission intensity of the reporter dye normalised to a passive reference dye incorporated in each reaction mix to control for loading variations. It was first necessary to calculate which cycles should be considered to represent the baseline value for the background signal. This was achieved by determining when the first sample appeared to emerge above the baseline, and the default settings were adjusted accordingly. For each experiment and each gene it was next necessary to set the threshold ΔR_n level (see green lines in Fig 3.6.B and C), which was used to determine the cycle number at which each sample is amplified significantly above baseline. This was set within the exponential phase of the reaction, above background. The Applied Biosystems software then calculated the C_T value for each well, which is the cycle number at which each sample reaches the threshold. The C_T value is related to the starting quantity of mRNA as outlined in section 3.11.12. A reference gene, GAPDH, was used in order to normalise the values obtained according to the starting cDNA concentration.

Gene expression levels were compared using the comparative C_T method (ABI User Bulletein #2 docs.appliedbiosystems.com/pebiodocs/04303859.pdf). The derivation of this method is outlined below. Briefly, for each experiment three separate pools of samples were used, and two replicate wells were run per sample. The C_T of each sample (see above) was normalised to GAPDH levels by subtracting the C_T of that sample with the reference (endogenous control, GAPDH) gene from the C_T with the target gene, to give a value which is termed ΔC_T (equation 6 below). The samples were then each normalised relative to a 'calibrator' sample (the calibrator was the mean ΔC_T of one of the experimental groups, such as the earliest developmental stage or the sham operated group) by subtracting the ΔC_T of each sample from the ΔC_T of the calibrator to give a value which is termed $\Delta\Delta C_T$ (see equation 8 below). Each sample was then expressed individually as 'relative expression' (relative to the calibrator)) using the following equation:

$$\text{Relative expression} = 2^{\Delta\Delta C_T}$$

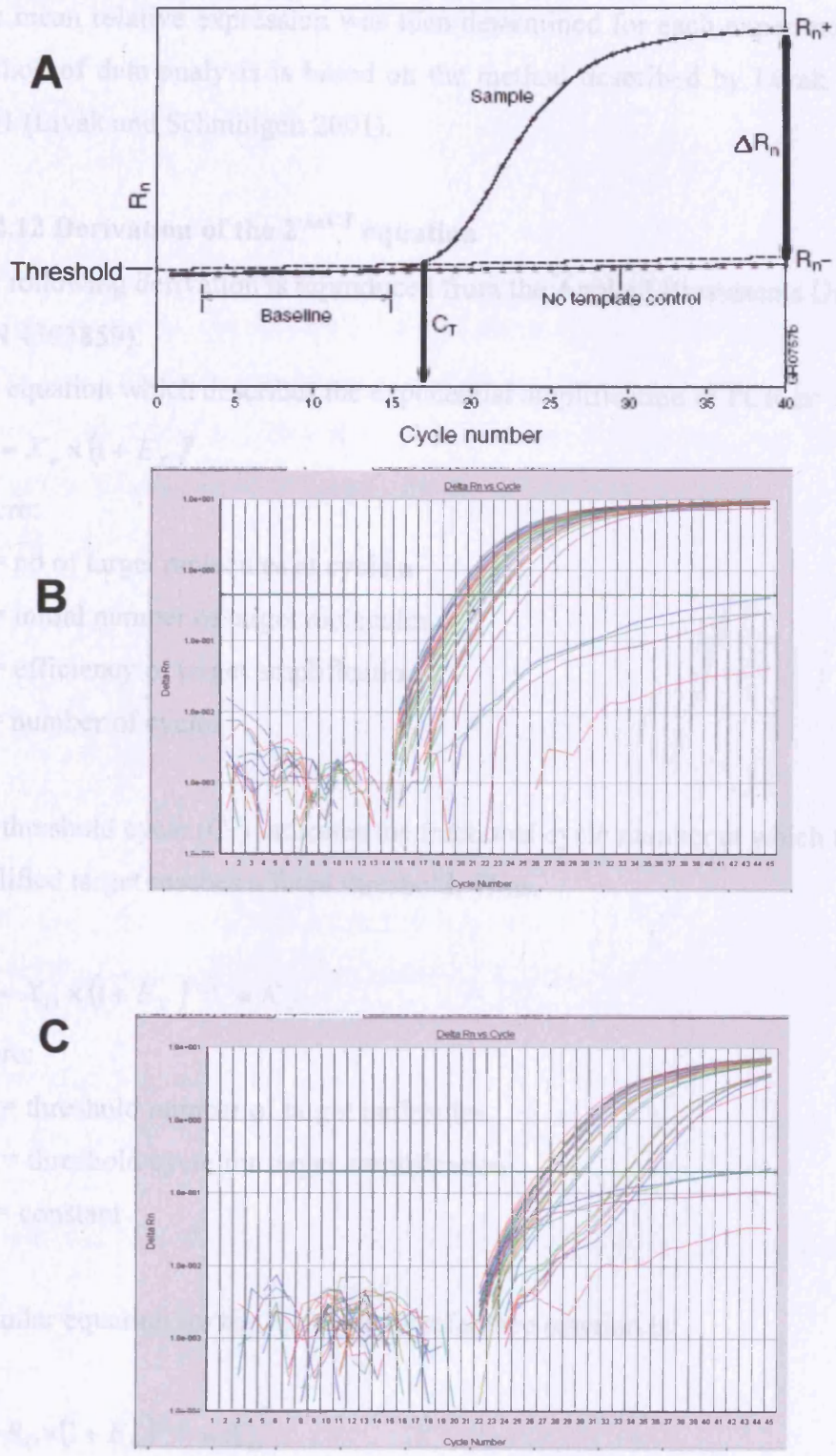


Fig. 3.6. Real-time PCR amplification curves. A) Schematic diagram illustrating the key features of a typical real-time PCR amplification curve (adapted from Applied Biosystems TaqMan protocol). **B)** and **C)** Representative examples of graphs plotting cycle number against ΔRn obtained during a real-time PCR run with GAPDH (B) and Urokinase (C)

.

The mean relative expression was then determined for each experimental group. This method of data analysis is based on the method described by Livak and Schmittgen, 2001 (Livak and Schmittgen 2001).

3.12.12 Derivation of the $2^{-\Delta\Delta CT}$ equation

The following derivation is reproduced from the Applied Biosystems User Bulletin 2 (P/N 4303859).

The equation which describes the exponential amplification of PCR is:

$$X_n = X_o \times (1 + E_X)^n \qquad \text{[equation 1]}$$

Where:

X_n = no of target molecules at cycle n

X_o = initial number of target molecules

E_X = efficiency of target amplification

n = number of cycles

The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_O \times (1 + E_X)^{C_{T,X}} = K_X \qquad \text{[equation 2]}$$

Where:

X_T = threshold number of target molecules

$C_{T,X}$ = threshold cycle for target amplification

K_X = constant

A similar equation for the endogenous reference reaction is:

$$R_T = R_O \times (1 + E_R)^{C_{T,R}} = K_R \qquad \text{[equation 3]}$$

Where:

R_T = threshold number of reference molecules

R_O = initial number of reference molecules

E_R = efficiency of reference amplification

$C_{T,R}$ = threshold cycle for reference amplification

K_R = constant

Dividing X_T by R_T gives the following expression

$$\frac{X_T}{R_T} = \frac{X_O \times (1+E_X)^{C_{T,X}}}{R_O \times (1+E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K \qquad \text{[equation 4]}$$

The exact values of X_T and R_T depend on a number of factors including the reported dye used in the probe, the sequence context effects on the fluorescence properties of the probe, the efficiency of probe cleavage, purity of the probe and setting of the fluorescence threshold. Therefore, the constant K does not have to be equal to 1. Assuming efficiencies of the target and the reference are the same:

$E_X = E_R = E$

$$\frac{X_O}{R_O} \times (1+E)^{C_{T,X} - C_{T,R}} = K \qquad \text{[equation 5]}$$

Or

$$X_N \times (1+E)^{\Delta C_T} = K \qquad \text{[equation 6]}$$

Where:
$X_N = X_O/R_O$, the normalised amount of target

$\Delta C_T = C_{T,X} - C_{T,R}$, the difference in threshold cycles for target and reference

Rearranging gives the following expression:

$$X_N = K \times (1+E)^{-\Delta C_T} \qquad \text{[equation 7]}$$

The final step is to divide the X_N for any sample q by the X_N for the calibrator (cb)

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1+E)^{-\Delta C_{T,q}}}{K \times (1+E)^{-\Delta C_{T,cb}}} = (1+E)^{-\Delta\Delta C_T} \qquad \text{[equation 8]}$$

Where:
$\Delta\Delta C_T = \Delta C_{T,q} - \Delta C_{T,cb}$

For amplicons designed to be less than 150bp and for which the primer and Mg^{2+} concentrations have been optimised, the efficiency is close to 1. Therefore, the amount of target, normalised to an endogenous reference and relative to a calibrator is given by:

$$2^{\Delta\Delta C_T}$$

3.13. Western blotting

3.13.1. Protein extraction for Western blotting

For Western blotting, tissues were collected and rapidly frozen using dry ice and stored at -80°C until required. Tissues were homogenised in 1ml ice-cold protein extraction buffer with added proteinase inhibitors to prevent protein degradation, by passing repeatedly through progressively smaller gauge needles. The homogenates were placed on ice for 30 minutes then centrifuged at 12,000g for 30 minutes at 4°C. The supernatants, containing the protein extract, were collected and stored in aliquots at -80 °C until required.

3.13.2. Protein Assay

Protein concentration was determined using a BCA^{TM} Protein Assay Kit (Pierce, Rockford, IL), which is based on an adaptation of the biuret method in which Cu^{2+} is reduced to Cu^{1+} by protein (Smith *et al.* 1985). The formation of Cu^{1+} is detected by the formation of a soluble purple-coloured complex when Cu^{1+} reacts with a reagent containing bicinchoninic acid (BCA). The formation of the reaction product can be determined by absorbance at approximately 562nm.

A series of protein standards ranging from 0-2mg/ml were prepared by diluting albumin stock solution (2mg/ml) in protein extraction buffer. Samples were generally diluted 1:5 with dH_2O for the assay. 25µl of standards or diluted samples was added to individual wells of a 96-well tissue culture plate in duplicate. Reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1M sodium hydroxide) and Reagent B (4% cupric sulphate), were combined at ratio of 50 parts A to 1 part B. 200µl of this mixture was added to each well and the plate was placed on a horizontal shaker for 30 seconds to ensure adequate mixing, before incubation at 37°C for 30 minutes. After cooling to room temperature, which reduces the rate of reaction to negligible levels, the absorbance at 560nm was determined using a microplate reader. Background absorbance was corrected for by subtracting the OD of the 'blank' wells from each of the standard or sample values. A standard curve was produced by plotting the average

OD of replicates against BSA concentration. The standard curve was then used to determine protein concentration of the unknown samples.

3.13.3. Alkaline Phosphatase treatment of protein samples

To remove phosphate groups from proteins several samples were treated with alkaline phosphatase. 10µl of protein (2µg/µl) was mixed with 2µl 10x alkaline phosphatase reaction buffer, 7µl distilled H_2O and 1µl calf intestinal alkaline phosphatase (20U/µl). The samples were incubated at 37°C for 30 minutes before proceeding with gel electrophoresis. Control samples were treated as above but had the alkaline phosphatase replaced with water.

3.13.4. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to investigate the protein samples for the levels of individual proteins, they were separated by denaturing gel electrophoresis. In this procedure, total protein extracts are denatured by boiling in a loading buffer which contains SDS and β-mercaptoethanol. In addition, SDS causes the proteins to be 'coated' with a negative charge that is directly proportional to the molecular weight of the protein. In combination, this means that, when run under denaturing conditions, to prevent secondary structures from reforming, proteins migrate down a gel towards a positive electrode, with the rate of migration being determined solely by the molecular weight of the protein.

Gel casting

A gel cast was prepared by placing two clean glass plates, one large and one slightly smaller, on top of each other, separated by 1mm thick gel spacers at either side. This was fastened into a gel casting frame and dH_2O was poured in to ensure that the mould was properly sealed. The water was then tipped out of the mould. A 12% resolving gel was prepared using the following reagents:

Distilled H_2O	3.3ml
30% acrylamide	4.0ml

1.5M Tris pH 8.8	2.5ml
10% SDS	0.1ml
10% ammonium persulphate	0.1ml
TEMED	0.004ml

The gel was mixed briefly and then poured into the mould, filling to a level approximately 4 cm below the top. A layer of isopropanol was poured on top of the gel. This helps to flatten the surface of the gel and prevents air reaching the gel, which aids setting. The gel was allowed to set for at least 30 minutes and the isopropanol was then poured off and the gel was rinsed with distilled H_2O. A 5% stacking gel was prepared from the following reagents:

Distilled H_2O	1.4ml
30% acrylamide	0.33ml
1.0M Tris pH 6.8	0.25ml
10% SDS	0.02ml
10% ammonium persulphate	0.02ml
TEMED	0.02ml

The stacking gel was poured into the mould, on top of the resolving gel. A 1mm thick gel comb was inserted into the stacking gel and the level of the gel was topped up to the top of the mould. The stacking gel was allowed to set for a further 30 minutes.

Sample preparation

The protein samples were diluted to 1μg/μl in distilled H_2O. The samples were then mixed 1:1 with protein loading buffer and heated at 100°C for 10 minutes to denature the proteins. The samples were then placed on ice until ready for loading.

Electrophoresis

Once the gels were set, the casting mould was attached to a gel frame and placed in an electrophoresis tank. The comb was gently removed and distilled H_2O was used to rinse any bubbles out of the wells. The central and outer reservoirs of the tank were filled with 1x running buffer. 10μl of protein standards was loaded into the first well and 15μl

of each sample was loaded into the remaining wells. The gel was run at 120V for approximately 2 hours.

3.13.5. Western transfer and blotting

Following SDS page, the separated proteins were transferred to a nitrocellulose membrane by Western blotting. In this process, the proteins become attached strongly to the membrane according to their precise position in the gel. The membrane can then be probed using antibodies specific to the protein of interest in order to visualise protein levels.

Western transfer

After removal from the electrophoresis apparatus the gel was placed into transfer buffer. The gel was then placed on top of two sheets of filter paper, and the nitrocellulose membrane was placed on top of the gel. Both the filter paper and the membrane were pre-soaked in transfer buffer. Two more sheets of filter paper were placed on top of the membrane and then the paper, gel and membrane were sandwiched between the two electrodes, so that the gel was towards the anode and the membrane towards the cathode. This was placed into the tank, filled with transfer buffer. The proteins were transferred for 2 hours at 100-200 mA at 4°C. Following transfer the apparatus was disassembled and the membrane was marked, by cutting off one corner, to provide orientation. In order to determine equal protein transfer, the membrane was incubated with a general protein stain, Ponceau S solution, for 10 minutes. The membrane was rinsed briefly in TBS, wrapped in clean Saran Wrap and an image was captured using a flat bed scanner.

Probing the blot

The membrane was rinsed in TBS to remove the staining and then incubated in blocking buffer overnight at 4°C to block hydrophobic binding sites on the membrane. After briefly washing in TBS with 0.05% Tween, the membrane was sealed in a small plastic bag with 2ml primary antibody (Table 2.3, page 90), which was diluted in blocking buffer. In some controls, the primary antibody was pre-incubated with equivalent amounts of a blocking peptide before being applied to the membrane. The membrane was incubated for 2 hours at room temperature on a horizontal shaker. Non-specifically

bound primary antibody was removed by washing for six 10 min. changes in wash buffer. The membrane was then incubated with an HRP-linked secondary antibody (Table 2.4, page 91), diluted in blocking buffer for 1 hour at room temperature. Non-specifically bound secondary antibody was removed with six 10 min. washes in wash buffer, followed by two 5 min. washes in TBS. To visualise antibody binding the membrane was incubated with a chemoluminescent substrate, which emits light when oxidised in a reaction catalysed by HRP. The membrane was placed in a clean petri dish. A 1:1 mix of ECL components was prepared and poured over the membrane, ensuring the whole surface was covered. After 1 min., the membrane was removed, blotted to remove excess reagents, and wrapped in cling film. In a dark room, photographic film was exposed to the membrane for intervals of up to 10min. before developing and fixing with a film processor.

3.14. Thrombin Activity Assay

3.14.1. Protein extraction for activity assay

For activity assays tissues were collected and rapidly frozen using dry ice and stored at -80°C until required. They were then homogenised on ice in 400ul assay buffer by drawing the solution in and out of progressively smaller gauge needles. The homogenates were centrifuged at 12,000g for 30 minutes at 4°C and the supernatants, containing the protein extract, were collected and stored in aliquots at -80 °C until required. Protein concentration was determined as described in section 3.12.2.

3.14.2. Activity assay

The activity of thrombin was measured using a chromogenic substrate specific to thrombin, D-Phe-Pip-Arg-p-nitroanilide (CS-01(38)). When cleaved by thrombin this substrate releases a coloured reaction product, p-nitroanilide (pNA), producing a change in absorbance which can be measured and is proportional to thrombin activity (Fig 3.7). The protocol for this assay was adapted from that described by Smirnova *et al*, 1996.

In order to confirm the linearity of the reaction, a standard curve of bovine thrombin ranging from 0.00002U/μl to 0.001U/μl was prepared in assay buffer and run alongside

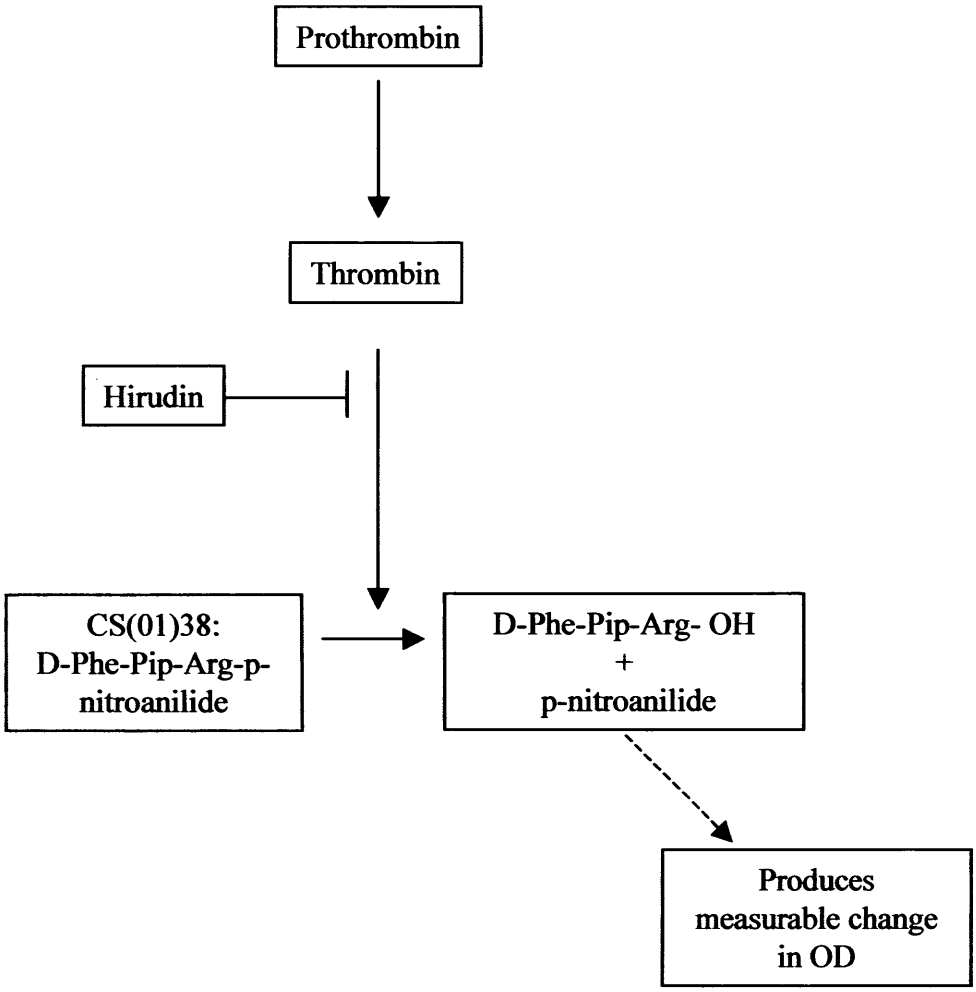


Fig. 3.7. Principles of thrombin activity assay. The semi-specific thrombin substrate CS(01)38 releases p-nitroanilide on cleavage by thrombin. This results in a measurable change in the optical density of the solution. The highly specific thrombin inhibitor, hirudin, can be used to block thrombin activity, allowing the determination of non-specific cleavage of the substrate by other proteases.

the experimental samples. The assay was carried out by incubating 10μl of the standards or 30μl of samples (approx 150μg/ well) with 50μl of the substrate (1mg/ml) in a total volume of 200μl for 30 minutes at 37°C in a 96 well plate. Following the incubation period the absorbance at 410nm was measured on a microplate reader. Blank wells contained the substrate only, and were subtracted from all other values. To control for non-thrombin specific activity, in some wells each sample was incubated with 30μl hirudin, a thrombin specific inhibitor. The absorbance recorded in these wells was subtracted from the relevant sample values to give a final measure of thrombin specific activity.

3.15. Statistical Analysis

All statistical analysis was performed using SPSS version 12 (SPSS, Woking, UK). The statistical support service at the Centre for Paediatric Epidemiology and Biostatistics, ICH also provided helpful assistance in the choice of statistical tests. Since sample sizes were necessarily small in most of the experiments described in this study, it was not possible to estimate the distribution of the population from which the samples were taken. Because it was not possible to assume a normal distribution on the basis of these sample sizes, non-parametric statistical analyses were used wherever possible. For all statistical tests significance was set at a p value of 0.05 or less. For analysis of 1 variable between 2 independent experimental groups, the data were compared using Mann Whitney Tests (Mann and Whitney 1947). For analysis of 1 variable between several groups, a Kruskal-Wallis test was performed (Kruskal and Wallis 1952). Following the Kruskal-Wallis test, in order to test the significance of differences between individual pairs of experimental groups, Mann Whitney Tests were performed. The p value was then multiplied by the total number of possible pairs which could be compared, according to the Bonferroni adjustment, which takes into account the increased probability of finding a difference between a pair of groups when multiple comparisons are being made.

Chapter 4. Pharmacological reduction of haemorrhage after injury in the non-regenerating chick spinal cord

4.1 Introduction

A major focus of much spinal cord injury research has been to understand the mechanisms which directly promote or impair axonal regeneration in the injured spinal cord (Sandvig *et al.* 2004). However, successful axonal regeneration also depends upon the presence in the spinal cord of surviving neurons that can participate in this re-growth. It is clear, therefore, that the extent of cell death and the capacity of spinal cord cells to survive an insult are likely to be important contributors to the success or failure of any such regeneration. How changes in these factors contribute to loss of regenerative capacity in the chick spinal cord has not been investigated in detail and may provide information about the contribution of such mechanisms to the response of non-regenerative systems, such as the adult human spinal cord.

Following primary mechanical damage to the spinal cord, tissue damage and cell death are progressively expanded by various pathological events which contribute to the secondary injury (Dumont *et al.* 2001). One of the earliest secondary injury events is haemorrhage within the spinal cord, which occurs as a direct result of the mechanical damage to the delicate blood vessel network of the spinal cord (Tator and Fehlings 1991; Mautes *et al.* 2000). The importance of such haemorrhage to spinal cord damage is indicated by the formation, at later stages, of cavitation within the cord which closely matches the early spread of haemorrhage (Noble and Wrathall 1989b; Noble and Wrathall 1989a; O'Neill 2002; Velardo *et al.* 2000). In addition to the loss of spinal cord cells, the formation of such cavities is itself a physical inhibitor to axonal re-growth. Haemorrhage within the cord leads to ischemic damage and consequent cell death by both necrotic and apoptotic mechanisms and exposes neurons and glia to a range of potentially toxic molecules and cell types which are normally excluded by the intact blood brain barrier (Dumont *et al.* 2001; Tator and Fehlings 1991).

Previous work in this laboratory has demonstrated an association between the loss of regenerative capacity in the chick at around E13 and an increase in secondary injury damage and apoptosis in the spinal cord (O'Neill 2002; Whalley *et al.* 2006). This

increase in cell death and tissue damage is likely to be an important contributor to reduced regenerative capacity in the post-E13 spinal cord. In particular, an increase in haemorrhage within the spinal cord after injury was observed to correlate with the developmental change from regeneration competent to incompetent stages. A dramatic increase in the vascularisation of the spinal cord was demonstrated to occur between E11 and E15, which is likely to account for the increased haemorrhagic response, and therefore may be a key factor in the loss of regenerative ability. There are evidently numerous additional changes occurring within this developmental window that might contribute to increased cell death. However, the link between haemorrhage and apoptosis was strengthened further by the observation that diazepam administration, which increased the extent of haemorrhage within the cord by unknown mechanisms, produced a large apoptotic response in the E11 spinal cord after injury; a situation in which cell death is normally low (O'Neill 2002; Whalley *et al.* 2006).

In order to validate the experimental model to be used and to check for any variations in surgical technique between myself and other investigators this project began with an initial attempt to replicate some of these results, before further investigation of the underlying mechanisms. The previous findings were highly suggestive of a causal link between haemorrhage and apoptosis but did not address the effects of reducing haemorrhage on consequent tissue damage and cell death in a system in which apoptosis is normally high. The work described in this chapter therefore aimed to continue the study by devising a pharmacological method to reduce haemorrhage at regeneration-incompetent stages and to assess its effects on the apoptotic response and the extent of spinal cord damage. To this end, a haemostatic drug, the synthetic vasopressin analogue, desmopressin, which is used in the treatment of haemophilia, was selected for use in these experiments (Kaufmann and Vischer 2003; Mannucci, 2000). Next, the molecular pathways involved in the apoptotic response at non-regenerating stages were explored by investigating the effects of an irreversible caspase-3 inhibitor on apoptosis after injury.

4.2. Results

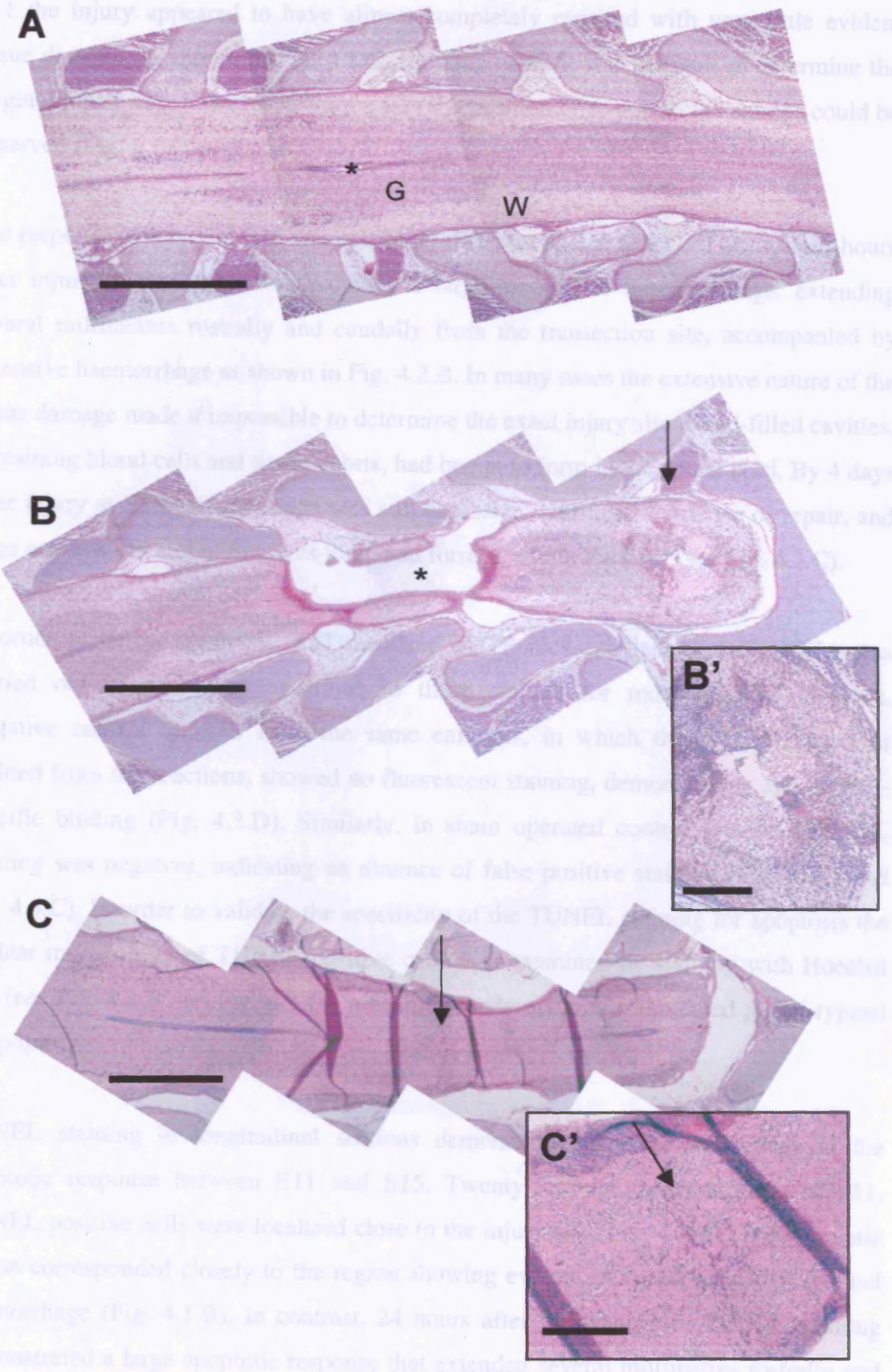
4.2.1. Comparison of the response of the chick spinal cord to injury at E11 and E15

The surgical techniques which are used in this study are an adaptation of those described by Shimizu and colleagues in studies in which the initial observations of loss of regenerative capacity were made (Shimizu *et al.* 1990). In previous work in our laboratory, a detailed study of the morphological changes occurring after transection injury at E11 and E15 was carried out (O'Neill 2002). As the current study aimed to follow on from these experiments, it was first necessary to confirm that the responses observed after injury were the same as those previously described.

Spinal cord transections were carried out at either E11 or E15, and tissues were collected at either 24 hours or 4 days after injury. Sham operated controls consisted of embryos in which the egg was windowed and all manipulations were identical to the injured embryos except the injury itself. It was found, throughout the course of this study that survival rates after injury were typically lower at E11 than at E15. Survival was also reduced at 4 days after injury at both stages in comparison to earlier time points. These observations are in agreement with findings by other groups and in our own laboratory (O'Neill 2002; Shimizu *et al.* 1990).

Longitudinal sections were stained with haematoxylin and eosin (H&E) in order to examine morphological changes after injury. Twenty-four hours after injury to the spinal cord at E11, a relatively small and localised injury was typically observed, extending less then 500 μm rostrally and caudally from the injury site, as shown in Fig. 4.1.B.

Fig. 4.1 (next page) **Analysis of morphological changes after injury at E11.** Longitudinal sections of spinal cords collected at various time points after transection injury at E11, stained with haematoxylin and eosin (H&E) to illustrate morphology. **A)** Sham operated control spinal cord at E12. * - ependymal region around the central canal, G – grey matter, W – white matter **B)** Spinal cord twenty four hours after injury. The injury site is shown by the arrow. Asterisk indicates a sectioning artefact. **B')** High magnification view of the injury site at 24 hours. **C)** H&E staining at four days after injury. The injury site is shown by the arrow. **C')** High magnification view of the injury site at 4 days. The injury site is shown by the arrow. Scale bars: A,B,C = 1 mm. B', C' =250 μm


A
*
G
W
B
*
B'
C
C'

A small amount of haemorrhage, close to the injury site could be observed (Fig. 4.1.B'); however there was little evidence of cavitation at this stage. By 4 days after injury at E11 the injury appeared to have almost completely repaired with very little evident tissue damage, as shown in Fig. 4.1.C. In many cases it was difficult to determine the original injury site; however in some cases the position of re-connected stumps could be observed (Fig. 4.1.C').

The response to injury at E15 was markedly different to that at E11. Twenty-four hours after injury at E15 there was typically a large amount of tissue damage, extending several millimetres rostrally and caudally from the transection site, accompanied by extensive haemorrhage as shown in Fig. 4.2.B. In many cases the extensive nature of the tissue damage made it impossible to determine the exact injury site. Fluid-filled cavities, containing blood cells and tissue debris, had begun to form in the spinal cord. By 4 days after injury at E15 tissue damage was still extensive, with little evidence of repair, and large cavities, several millimetres long, had formed within the cord (see Fig. 4.2.C).

In order to detect apoptosis, TdT-mediated dUTP nick-end labelling (TUNEL) was carried out in consecutive sections to those stained for morphological analyses. Negative control sections from the same embryos, in which the TdT enzyme was omitted from the reactions, showed no fluorescent staining, demonstrating lack of non-specific binding (Fig. 4.3.D). Similarly, in sham operated control samples, TUNEL staining was negative, indicating an absence of false positive staining (Fig. 4.3.E and Fig. 4.4.C). In order to validate the specificity of the TUNEL staining for apoptosis the nuclear morphology of TUNEL positive cells was examined by staining with Hoechst dye (see Fig. 4.3.B and Fig. 4.3.C), revealing condensed and fragmented nuclei typical of apoptosis.

TUNEL staining in longitudinal sections demonstrated marked differences in the apoptotic response between E11 and E15. Twenty-four hours after injury at E11, TUNEL positive cells were localised close to the injury site (Fig. 4.3.A). The apoptotic region corresponded closely to the region showing evident morphological damage and haemorrhage (Fig. 4.1.B). In contrast, 24 hours after injury at E15, TUNEL staining demonstrated a large apoptotic response that extended several millimetres rostrally and caudally from the injury site (Fig 4.4.A and Fig. 4.4.B), again corresponding closely to

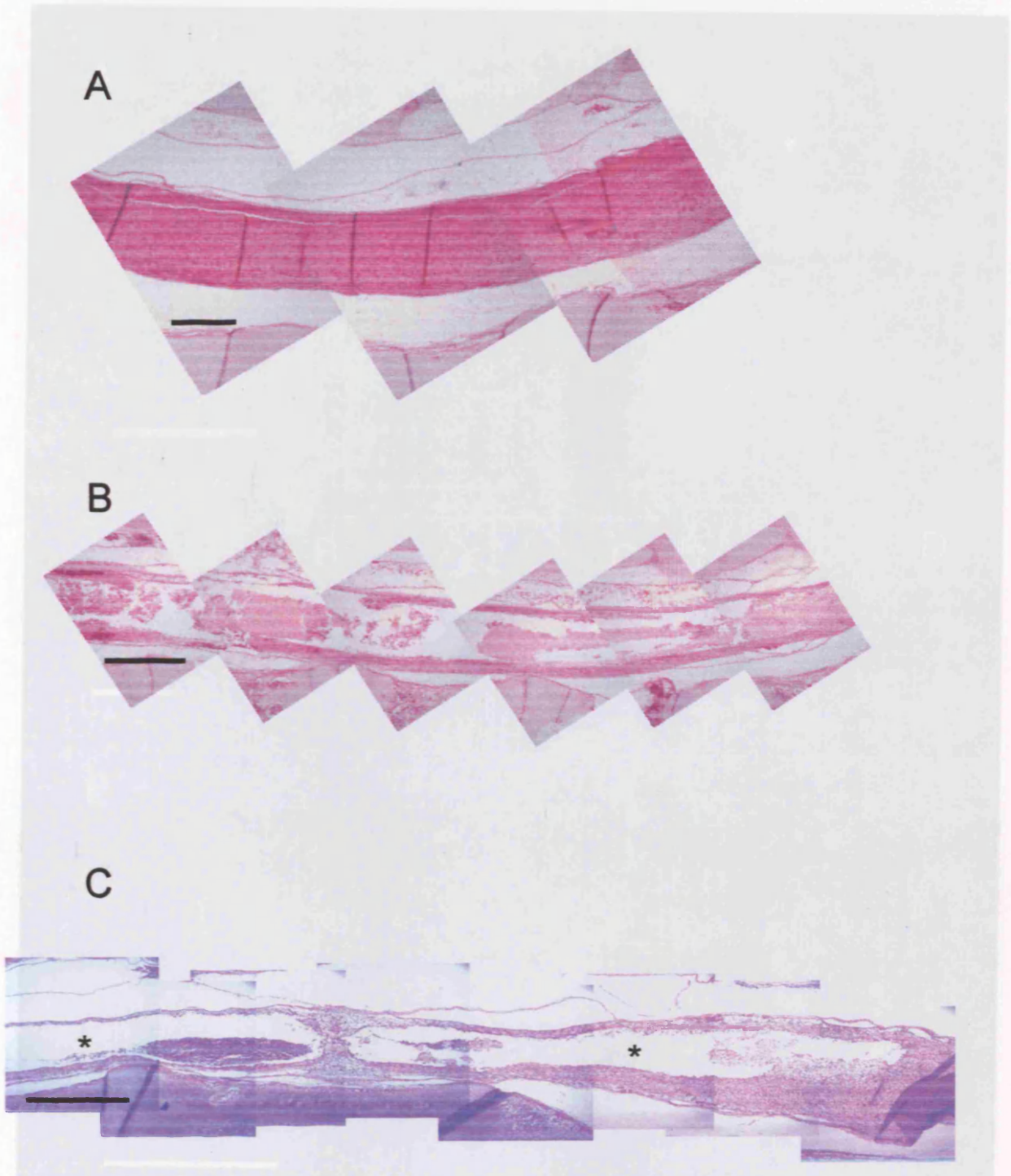


Fig. 4.2. Analysis of morphological changes after injury at E15. Longitudinal sections of spinal cords collected at various time points after transection injury at E15, stained with Haematoxylin and Eosin (H&E) to illustrate morphology. **A)** Sham operated control spinal cord at E16. **B)** Spinal cord 24 hours after injury. The precise injury site cannot be determined due to the extensive nature of the injury. **C)** Spinal cord 4 days after injury. Large cavities are indicated by asterisks Scale bars = 1 mm.

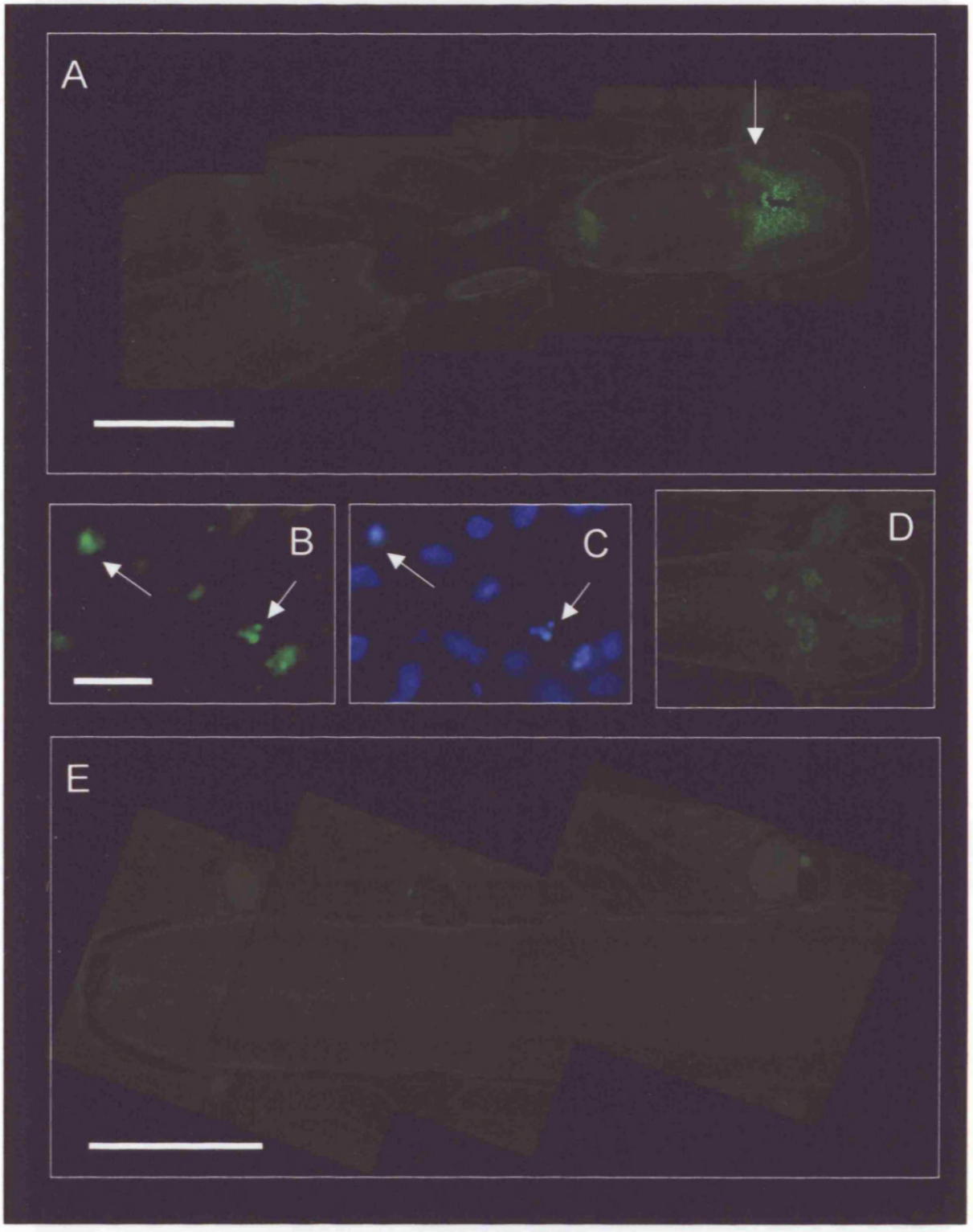


Fig. 4.3. Analysis of the apoptotic response to injury at E11. A) TUNEL staining (green) in a longitudinal section of spinal cord at 24 hours after transection injury at E11. The injury site is shown by the arrow. **B)** and **C)** nuclear morphology of apoptotic cells stained with TUNEL (B) and Hoechst (C) **D)** Negative control is a section from the same embryo as in A in which the TdT enzyme is omitted to control for non-specific labelling **E)** TUNEL staining in sham operated control spinal cord at E12. Scale bars A,D,E = 1 mm (D is the same magnification as A). B,C = 4μm (B and C are the same magnification.

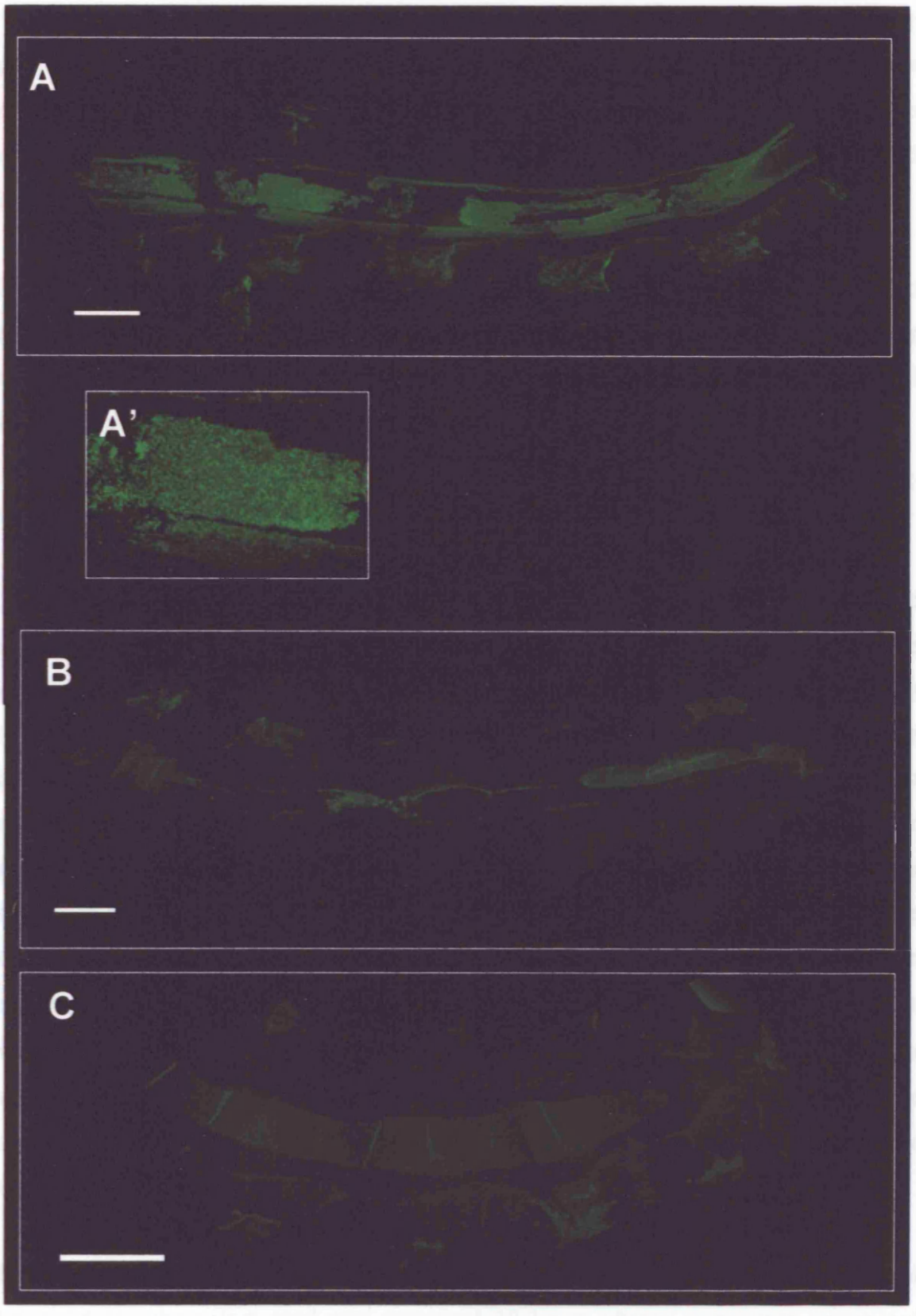


Fig. 4.4. (facing) **Analysis of the apoptotic response to injury at E15. A)** TUNEL staining (green) in a longitudinal section of spinal cord at 24 hours after transection injury at E15. **A')** Enlarged image showing TUNEL staining in A. **B)** TUNEL staining in a longitudinal section of spinal cord at 4 days after transection injury at E15. **C)** TUNEL staining in sham operated control spinal cord at E16. Scale bars = 1mm.

the extent of haemorrhage and tissue damage within the cord (Fig. 4.3.B). By 4 days after injury at E15 there was still evidence of apoptosis; however the apoptotic response was reduced as compared to 24 hours, with the most intense TUNEL staining observed in tissue 'islands' within the cavities (Fig 4.4.C).

The morphological and apoptotic responses to injury observed were therefore similar to those previously described, suggesting that the techniques were adequate for continuing this study, and supporting the previously recorded relationship between extent of haemorrhage after injury and the apoptotic response.

4.2.2. The effects of desmopressin on haemorrhage after injury at E15

To further investigate the previously described relationship between haemorrhage and apoptosis after injury a series of experiments was carried out to reduce haemorrhage after injury at E15, using the haemostatic compound desmopressin. Initially, it was necessary to determine whether desmopressin treatment could effectively reduce haemorrhage after injury without causing overt toxicity and to determine the most effective dose of the drug. Since desmopressin can be administered to haemophiliac patients via a nasal spray, it seemed likely that the drug would be readily absorbed by the blood vessels within the chorioallantoic membrane (Lethagen 2003). Haemorrhage occurs within the cord almost immediately after injury (O'Neill 2002). Therefore, the effects of three different doses of desmopressin, 7.5, 75 and 150 μg/ml, on haemorrhage were initially assessed at 2 hours after injury. A dose of 150 μg/ml desmopressin administered just prior to the injury appeared to be effective in reducing haemorrhage and was therefore used in all further experiments. No noticeable toxic effects of the drug were observed.

In order to quantitatively examine the effect of desmopressin on the extent of haemorrhage, the spinal cord was examined 12 hours after a transection injury and simultaneous treatment with desmopressin or its vehicle, phosphate buffered saline (PBS). This time point was selected as optimal for both the apoptotic response, which was previously demonstrated to begin at around 8 hours after injury, and in order to examine a relatively early time point within the course of the secondary injury response

when haemorrhage would still be present in the cord (O'Neill 2002). H&E staining was carried out on longitudinal sections of the spinal cord and composite images were created in order to examine morphology. Serial sections taken every 80 μm through the spinal cord were stained and sections corresponding to the centre of the injury were chosen for further analysis. Any samples for which the injury site could not be adequately determined due to an ineffective injury or a very extensive injury were excluded from measurements.

As illustrated in Fig 4.5.A, in PBS treated embryos the injury produced extensive haemorrhage and tissue disruption within the cord by 12 hours. Of the desmopressin treated group, 4 out of 6 of the embryos had noticeably reduced lesions, with very little damage in comparison to the PBS treated group (see Fig. 4.5.B and Fig. 4.5.C). In order to assess the extent of haemorrhage in injured spinal cords, the longitudinal extent to which haemorrhage extended in each direction from the injury site was measured (Fig. 4.5.A) and was found to be 4.96±0.53cm (mean ± SEM, n=3) in PBS treated cords at 12 hours after injury. Haemorrhage in the desmopressin treated group as a whole extended to 2.94±1.86cm (mean ± SEM n=6), which was not statistically different from the PBS treated group (p=0.197, Mann Whitney Test). However, in the smaller group consisting of the 4 embryos in which there was a noticeable difference in the extent of injury, haemorrhage was significantly reduced in comparison to controls (1.83±0.71, p=0.034, Mann Whitney Test). This sub-group was subsequently classified as 'desmopressin - reduced haemorrhage' for further analysis, in which the aim was to examine the effects of reduced haemorrhage on apoptosis.

4.2.3 The effects of desmopressin on the apoptotic response 12 hours after spinal cord injury at E15

To determine the effect of reduced haemorrhage on apoptosis, consecutive longitudinal sections to those stained for morphological analyses in section 4.2.2 were stained by TUNEL. TUNEL staining in the PBS treated group revealed extensive apoptosis extending several millimetres rostrally and caudally from the injury site at 12 hours after injury at E15, as shown in Fig. 4.6.A. In the reduced haemorrhage group, TUNEL staining was more localised to the injury site, as shown in Fig. 4.6.B and 4.6.C. In all

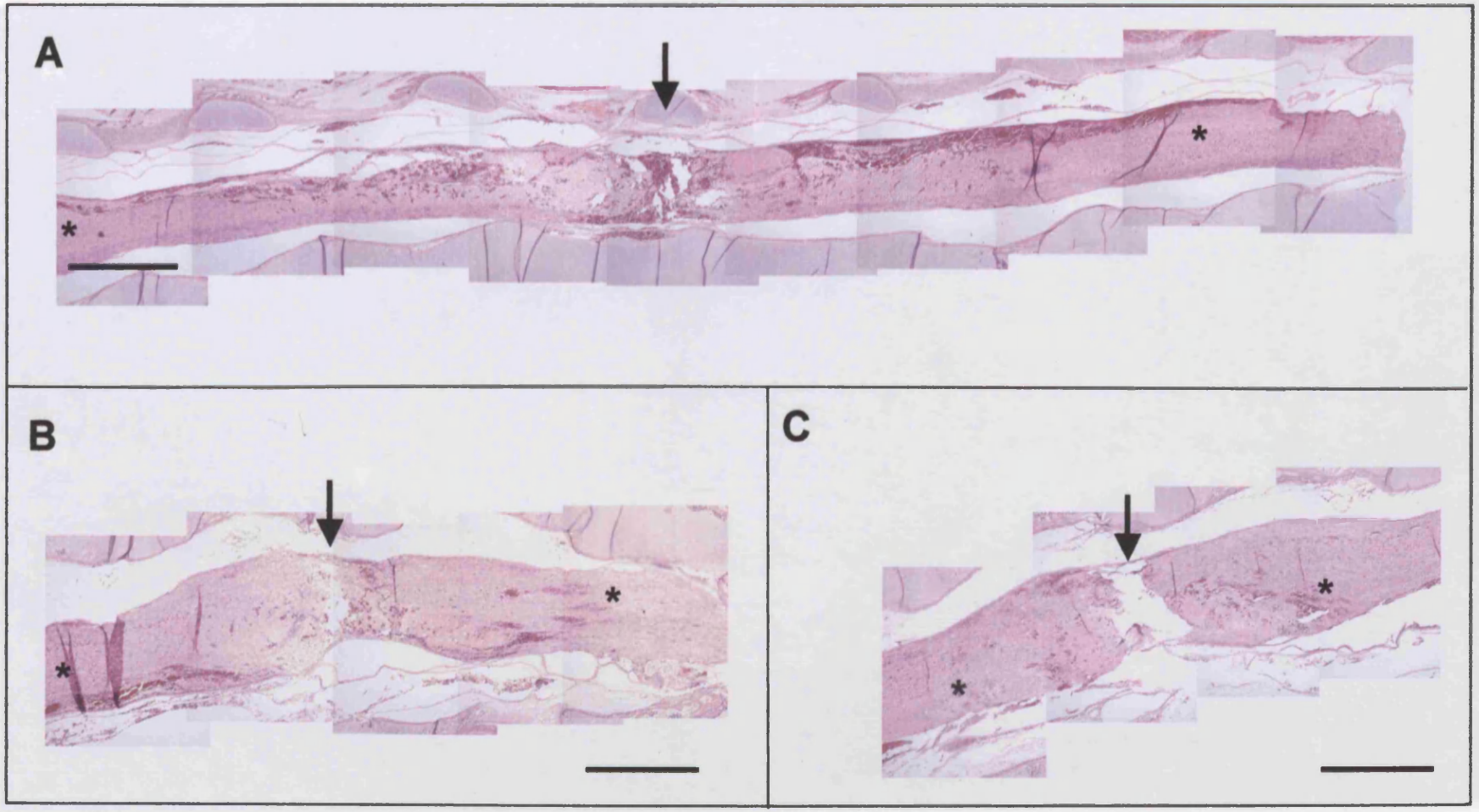


Fig 4.5. Analysis of the effects of desmopressin treatment on haemorrhage 12 hours after injury at E15. Longitudinal sections of spinal cord stained with Haematoxylin and Eosin, 12 hours after injury at E15. **A)** Section from embryo treated with vehicle. **B)** and **C)** Sections from embryos treated with desmopressin. The extent of haemorrhage within the spinal cord is reduced by desmopressin treatment. Injury sites are indicated by arrows. The cranial and caudal extent of haemorrhage in each case are indicated by asterisks. Scale bars = 1mm

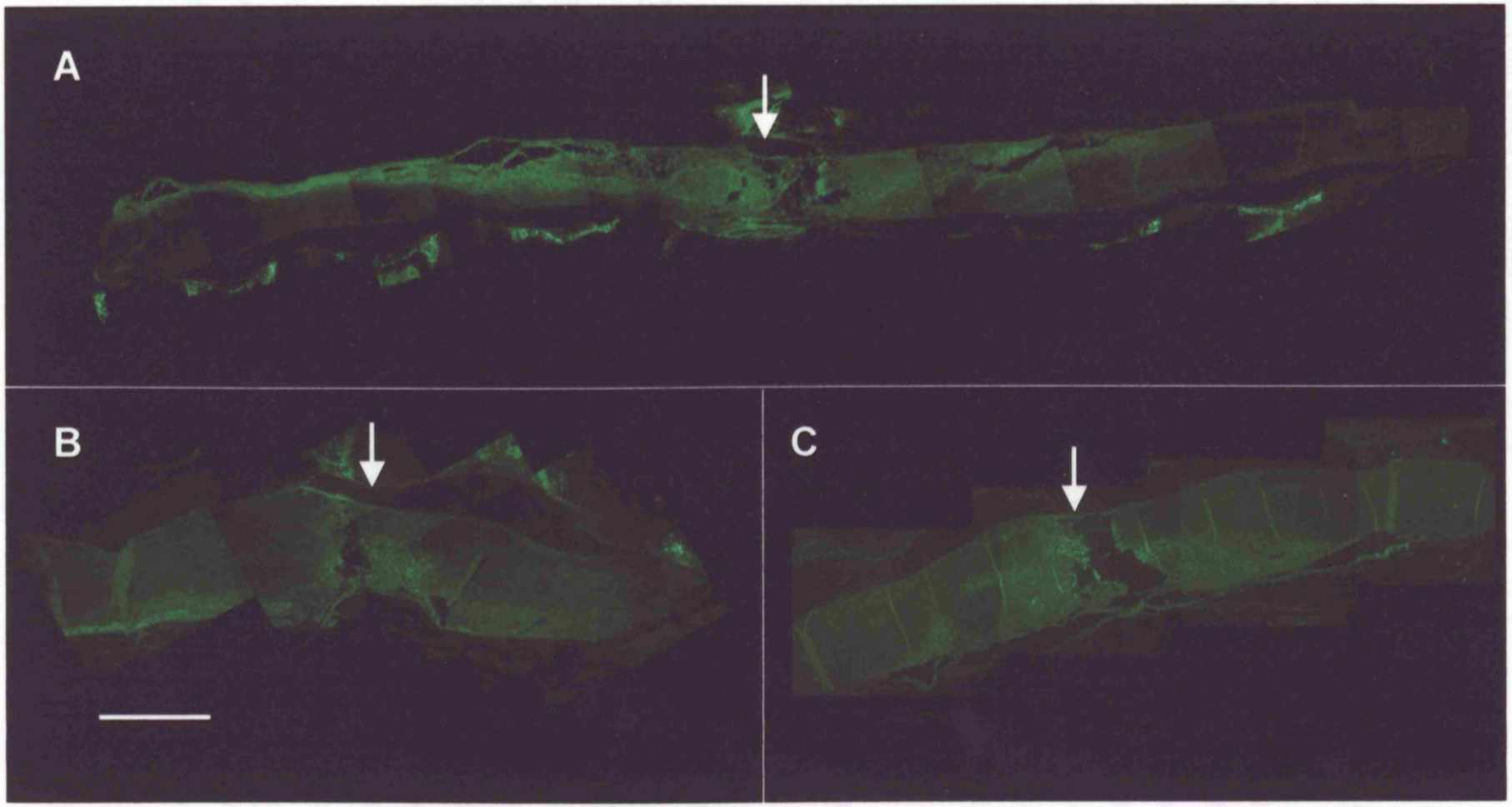


Fig 4.6. Analysis of the effects of desmopressin treatment on apoptosis 12 hours after injury at E15. Longitudinal sections of spinal cord stained by TUNEL for apoptotic cells, 12 hours after injury at E15. **A)** Section from embryo treated with vehicle **B)** and **C)** Section from embryo treated with desmopressin. The apoptotic response to injury was reduced by desmopressin treatment. Injury sites are indicated by arrows. Scale bars = 1mm

cases the extent of TUNEL staining closely corresponded to the extent of haemorrhage within the spinal cord.

In order to quantify changes in the apoptotic response, a scoring system, based on the intensity of TUNEL staining was devised, using the criteria shown in Fig. 4.7. TUNEL staining was scored at 1 mm intervals in both directions from the injury site in longitudinal sections of the spinal cord. Mean values for each cord were then averaged within each of the three groups; PBS treated, desmopressin treated and the 'desmopressin -reduced haemorrhage' sub-group. As shown in Table 4.1, although there was a reduced TUNEL score in the desmopressin treated group as compared to the PBS treated group this did not reach statistical significance. However the reduced haemorrhage sub-group had significantly lower TUNEL scores at 1, 2 and 4 mm from the injury site as compared to the PBS treated group.

4.2.4. The effects of desmopressin on cavitation and axonal damage 4 days after spinal cord injury at E15

To assess the effects of reduced haemorrhage on long-term survival and tissue damage, spinal cords were collected 4 days after a transection injury at E15 and treatment with desmopressin or PBS, and longitudinal sections were stained with H&E. As shown in Table 4.2, the survival rate at 4 days was 67% for desmopressin treated embryos as compared to 45% for PBS treated controls. At 4 days after injury, large cavities were present in many of the PBS treated controls (Fig. 4.8.A and 4.8.B). Although cavitation was also present in one sample from the desmopressin treated group, as shown in Fig. 4.8.C, overall there appeared to be reduced tissue damage in these samples, as shown in Fig. 4.8.D. Cavitation was measured as the total longitudinal length of the cavity in sections taken from approximately the centre of the injury. The number of embryos with a cavity at least 1mm in length was counted and was found to be reduced in the desmopressin treated group to 13% in comparison to 60% in controls (Table 4.2.).

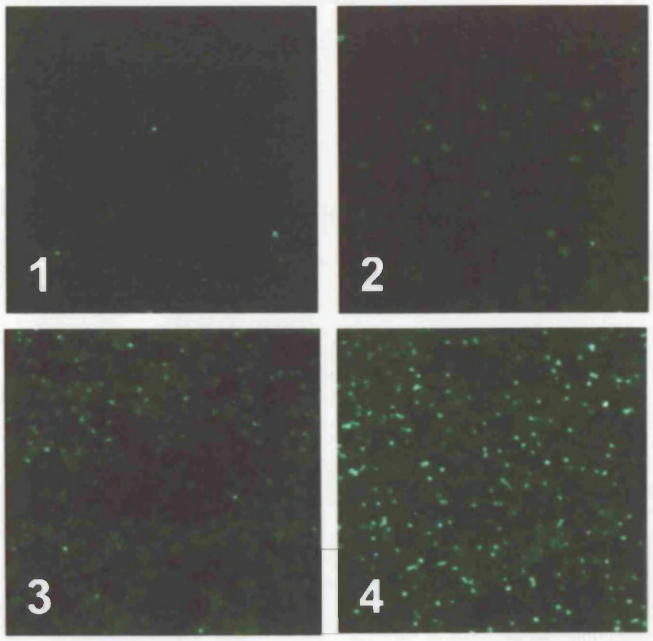


Fig. 4.7. Scoring criteria used for quantification of TUNEL staining. Panels represent the approximate density of TUNEL staining receiving each score in PBS and desmopressin treated spinal cords after injury.

Treatment group	TUNEL Score					
	Injury site	+1mm	+2mm	+3mm	+4mm	+5mm
PBS	4±0	4±0	3.7 ± 0.3	2.7± 0.9	3 ±0.6	1.7±0.3
Desmopressin	3.7±0.2	3±0.4	2.2±0.6	1.8±0.5	1.7±03	1.5±0.3
Desmopressin (reduced haemorrhage)	3.5±0.3	2.5±0.3 *	1.3±0.3 **	1.3±0.3	1.3±0.3 ***	1.3±0.3

Table 4.1. Quantification of apoptosis in spinal cord slices 12 hours after injury at E15 and treatment with PBS or desmopressin. Apoptosis is scored according the criteria illustrated in Fig. 4.7., below, at 1 mm intervals from the injury site. Data shown are mean apoptotic score ± SEM. Desmopressin treatment reduced haemorrhage in 4 out of 6 embryos, which are included in a sub-group named 'reduced haemorrhage' for further analysis. There was a significant reduction in apoptosis at 1mm, 2mm and 4mm from the injury site in the reduced haemorrhage group as compared to the PBS treated group. * p = 0.025, ** p=0.026, *** p=0.042 (Mann Whitney Test). All other p values were greater than 0.05.

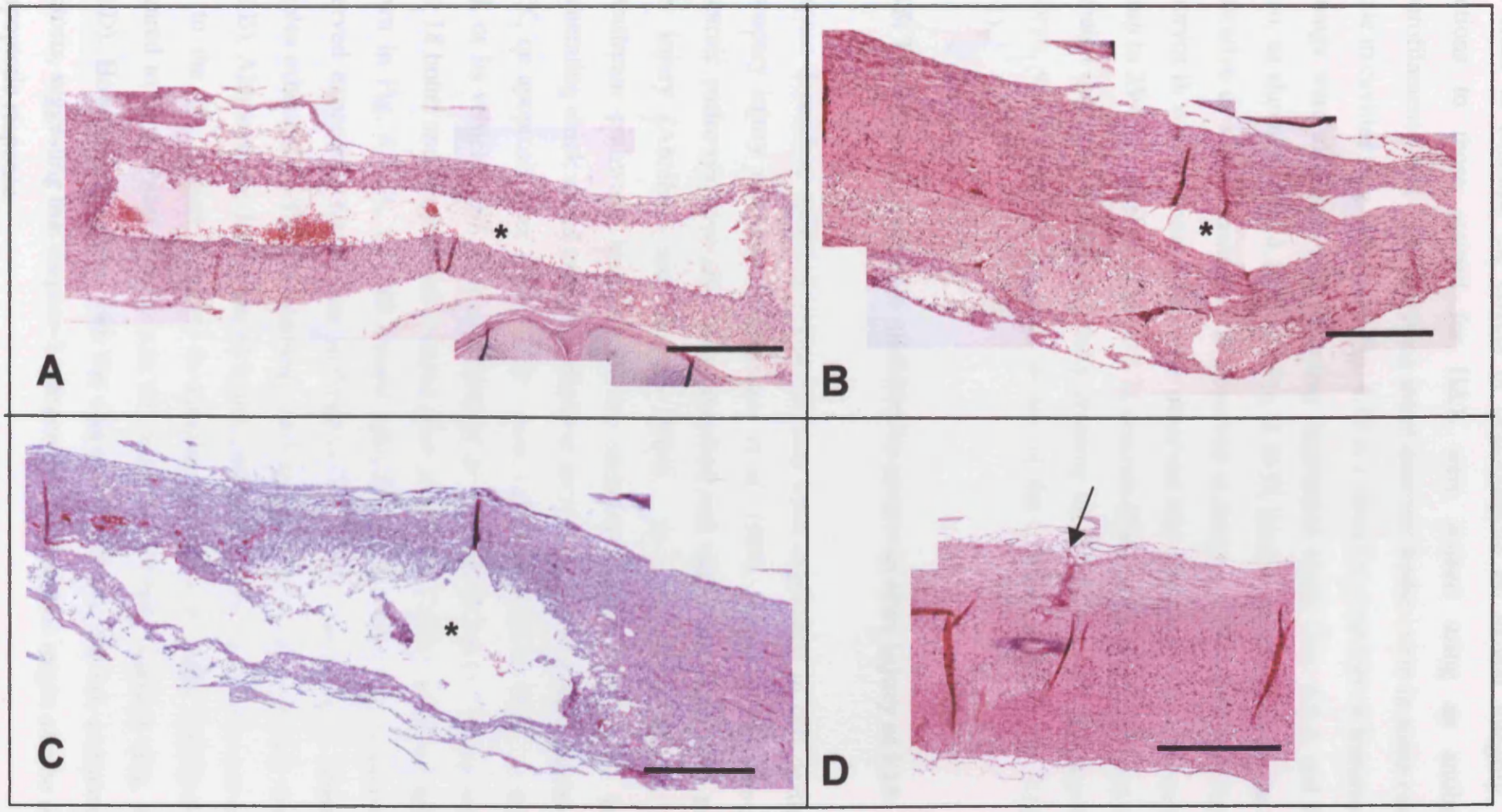


Fig. 4.8. Analysis of the effects of desmopressin treatment on cavitation and tissue damage 4 days after injury at E15. Longitudinal sections of spinal cord stained with Haematoxylin and Eosin 4 days after injury at E15. **A)** and **B)** Sections from embryos treated with vehicle. **C)** and **D)** Sections from embryos treated with desmopressin. The number of samples in which large cavitation was observed was reduced in desmopressin treated animals. Cavities are indicated by asterisks and in D) the injury site is indicated by an arrow. Scale bars = 500 μm

In order to examine the effects of desmopressin on axonal integrity, consecutive sections to those stained for H&E were stained using an antibody against neurofilaments, RM-270. Surviving intact neuronal bodies were in some cases observed close to cavities (Fig. 4.9.A and Fig. 4.10.A.), however a number of features of neuronal damage were also observed, including fragmented axons (Fig. 4.9.A and Fig. 4.10.B). Also, as shown in Fig. 4.10.C and Fig. 4.10.D, axonal blebbing within the spinal cord, indicative of axonal damage, was observed in several of the embryos. The number of embryos in which axonal blebs were observed was reduced in the desmopressin treated group to 25% in comparison to 60% in controls (Table 4.2). Neurofilament staining also revealed one case of axons partially crossing the injury site in a desmopressin treated embryo, which was not observed in any of the control embryos (Fig. 4.9.B and Fig. 4.11).

4.2.5. The effects of a caspase inhibitor on apoptosis after injury at E15

Caspase-dependent apoptotic pathways have been implicated in cell death caused by secondary injury mechanisms (Springer *et al.* 1999). However, caspase-independent apoptotic pathways have also been described and might also contribute to cell death after injury (Abraham and Shaham 2004). In order to investigate further the downstream pathways involved in the apoptotic response to injury in the non-regenerating chick spinal cord, the effects of an irreversible caspase-3 inhibitor, DEVD-FMK, on apoptosis after injury at E15 were examined. DEVD-FMK, at 0.5 mM or 2 mM, or its vehicle, PBS, was administered at the time of injury. Tissue was collected after 18 hours and longitudinal sections were stained by TUNEL to detect apoptosis. As shown in Fig. 4.12.A, in PBS treated control animals extensive apoptosis could be observed expanding for several millimetres within the spinal cord. Sham operated samples exhibited no TUNEL staining, confirming a lack of false positive staining (Fig. 4.12.B). Although the data obtained in this preliminary experiment was not quantitative due to the low sample number, in samples treated with 2mM DEVD-FMK there appeared to be a visible reduction in the density of TUNEL staining (Fig. 4.12.C and 4.12.D). However, treatment with the caspase-3 inhibitor did not completely inhibit apoptosis, suggesting that caspase-3 independent mechanisms might also be involved in the apoptotic response.

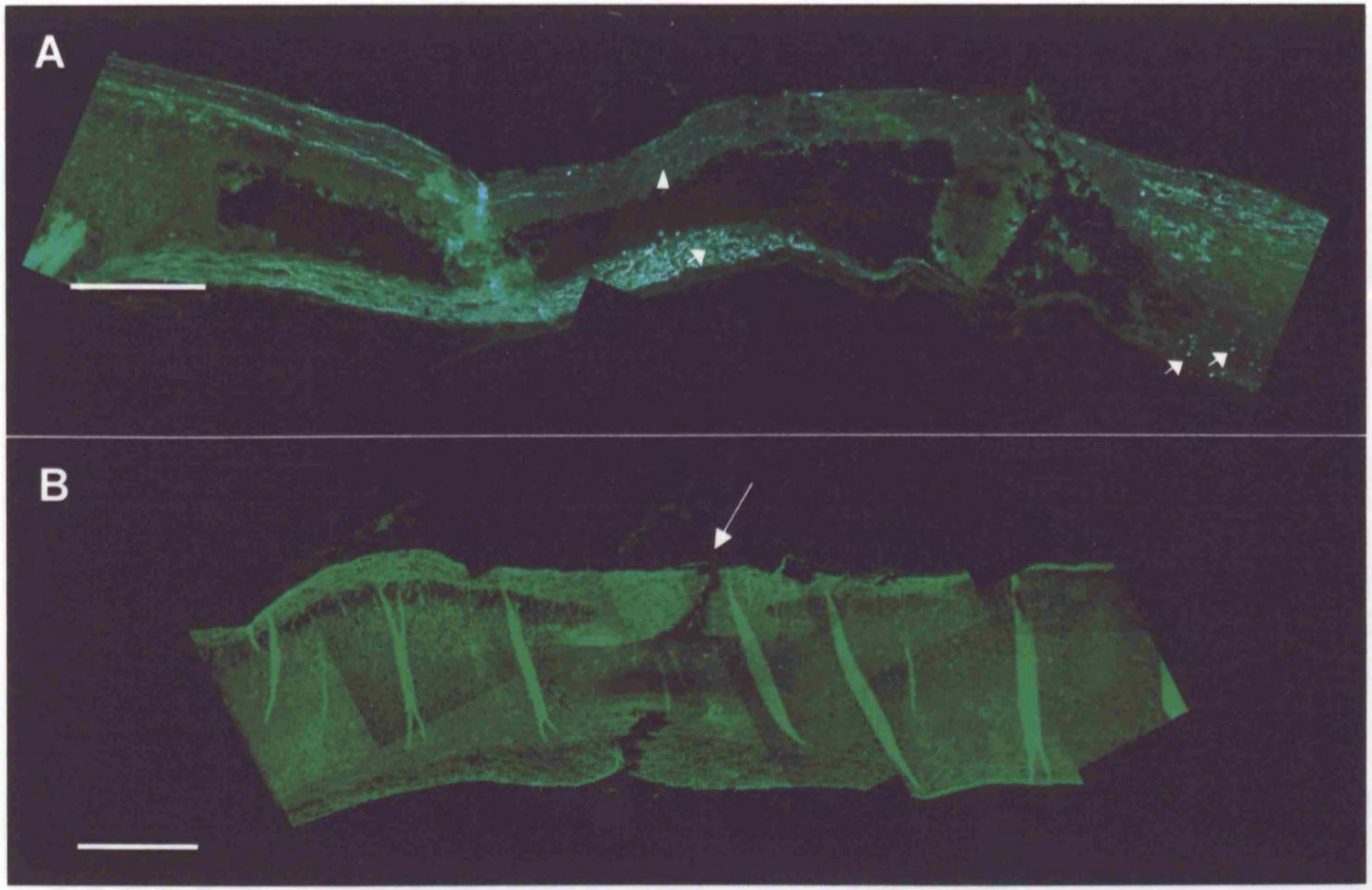


Fig. 4.9. Analysis of the effects of desmopressin treatment on axonal morphology 4 days after injury at E15. Longitudinal sections of spinal cord stained for neurofilaments (in green) 4 days after injury at E15. **A)** Spinal cord treated with vehicle at the time of injury. Axonal swelling can be observed close to the injury site (arrows) and interrupted axonal tracts are clearly visible running in the white matter next to the cavity (arrowheads). **B)** Spinal cord treated with desmopressin at the time of injury. The injury site is indicated by an arrow. Note the absence of axonal swellings in this spinal cord. Scale bars = 500μm

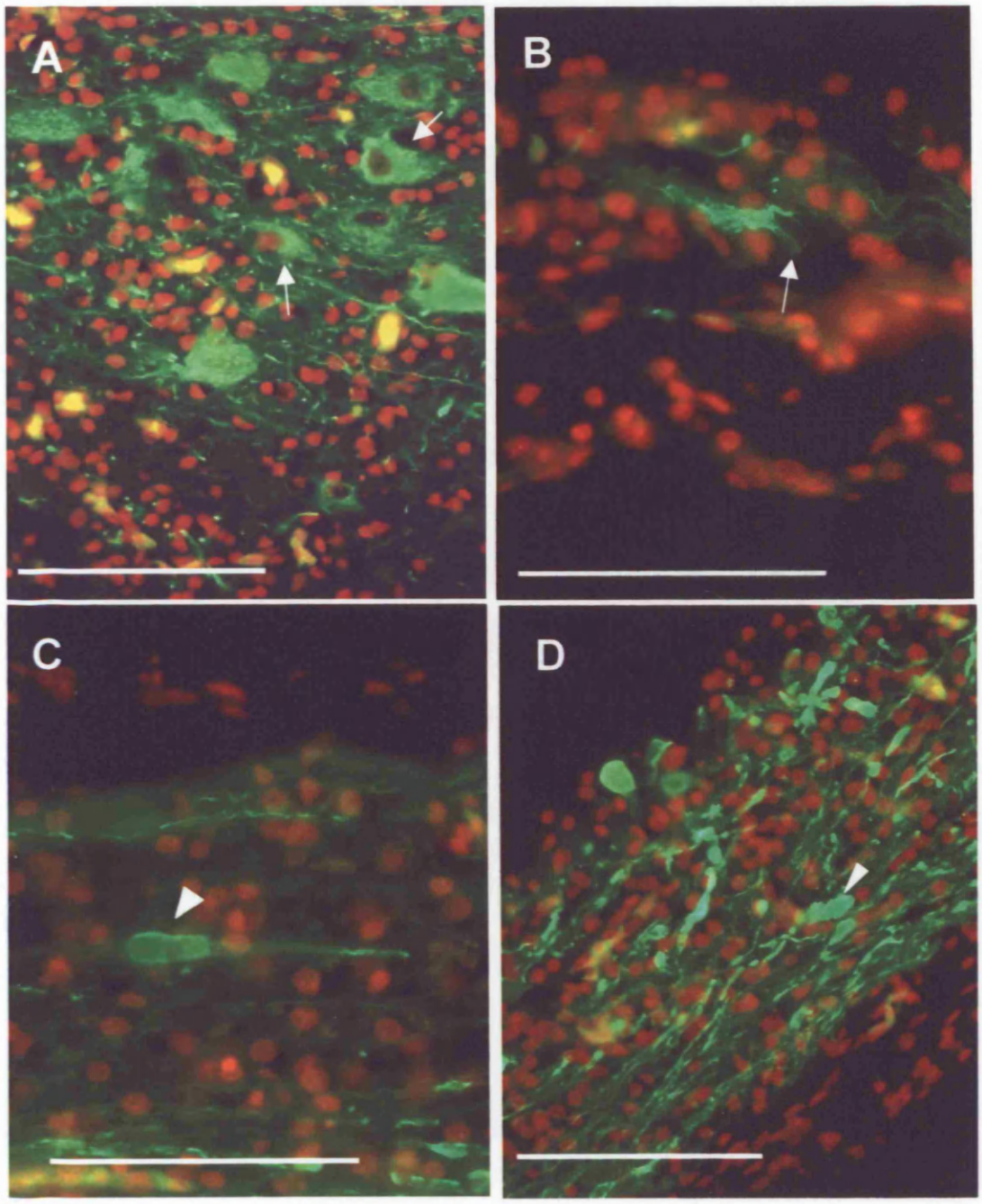


Fig. 4.10. Axonal damage four days after injury at E15. Neurofilament staining is shown in green and nuclear counterstaining in red in spinal cord sections 4 days after injury at E15 and treatment with PBS. **A)** Surviving neuronal cell bodies within the spinal cord indicated by arrows **B)** Fragmented axons were found close to cavities indicated by arrows **C)** and **D)** Examples of axonal swellings (arrowheads) indicating neuronal damage. Scale bars A,D = 100 μm, B,C = 80 μm.

	Survival rate (survived/ operated)	Injuries ≥ 1mm visible damage	Presence of axonal swellings in cord
PBS	5/11 (45%)	3/5 (60%)	3/5 (60%)
Desmopressin	8/12 (67%)	1/8 (13%)	2/8 (25%)

Table 4.2. Quantification of the effects of desmopressin treatment on outcome after injury at E15. The number of embryos surviving to four days after injury was expressed as a percentage of the total injured. The size of visible cavitation in longitudinal sections was measured and the number of embryos having a cavity larger than 1mm in length was expressed as a percentage of the total. Finally, neurofilament staining was assessed and the number of embryos in which axonal swellings were observed was expressed as a percentage of the total. Desmopressin treatment improved outcome by all three measures.

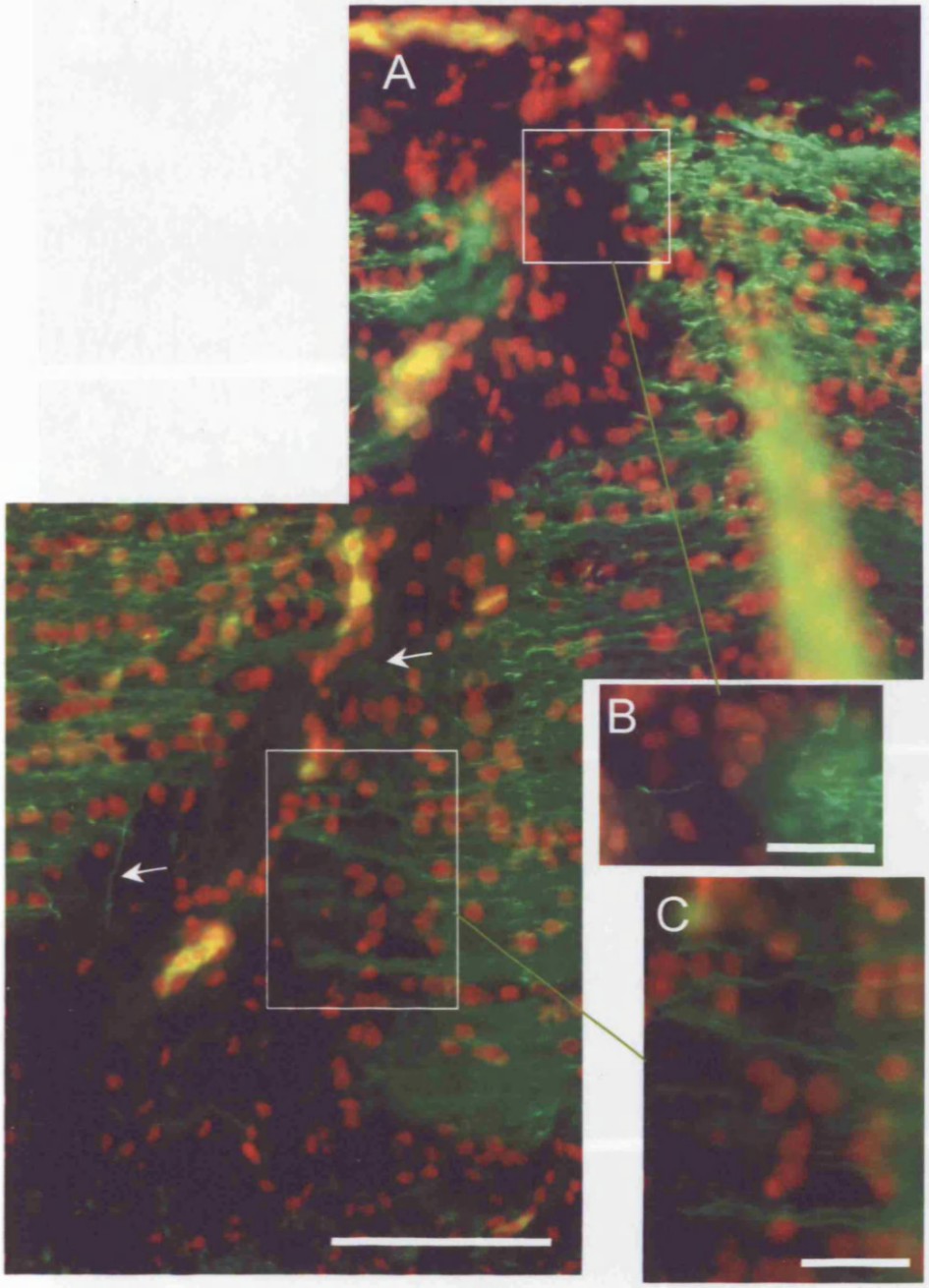


Fig. 4.11. Analysis of axonal integrity four days after injury at E15 and treatment with desmopressin. Neurofilament staining is shown in green and nuclear counterstaining in red in spinal cord sections 4 days after injury at E15 and treatment with desmopressin. **A)** Axons appeared to partially cross the injury site after desmopressin treatment (arrows) B-C) High magnification view of the areas boxed in A). Scale bars A = 125 μm, B,C = 40 μm.

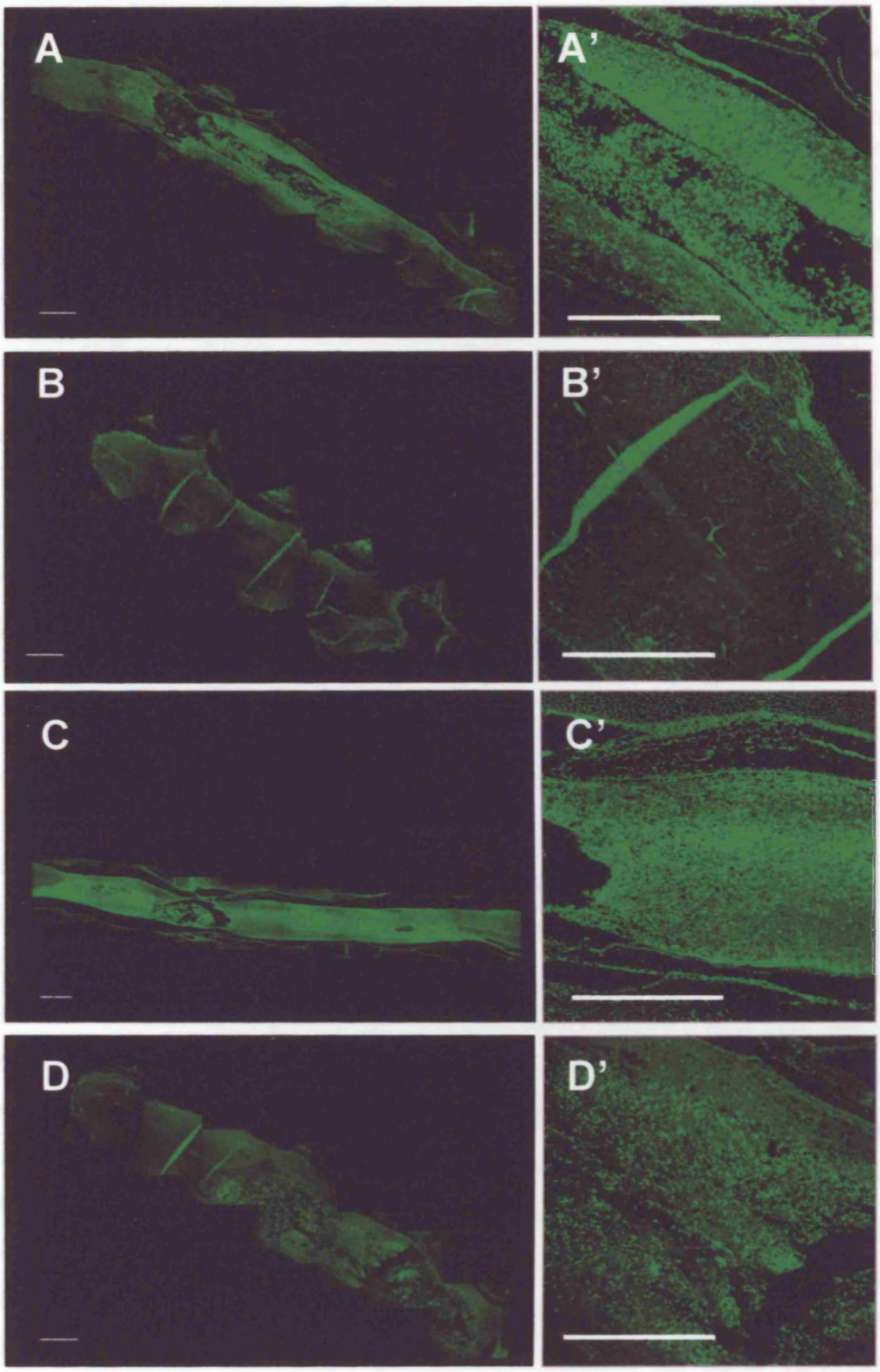

A
A'
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Fig. 4.12. (previous page) Analysis of the effects of a caspase inhibitor on apoptosis after injury at E15. Longitudinal sections of spinal cord stained by TUNEL for apoptotic cells 18 hours after injury at E15 and treatment with the caspase-3 inhibitor DEVD-Biotin-FMK. A) Section from PBS-treated injured embryo. B) Section from sham-operated control. C) Section from injured embryo treated with 0.5mM DEVD-FMK. D) Section from injured embryo treated with 2mM DEVD-Biotin-FMK. A'), B'), C') and D') High magnification images of the injury site in each case. Inhibition of caspase-3 partially reduced the apoptotic response to injury. Scale bars =500μm

4.3. Discussion

In this chapter a previously observed relationship between increased haemorrhage and increased apoptosis following injury at non-regenerating stages of chick spinal cord development was further investigated. The results provide support for a link between haemorrhage and apoptosis after spinal cord injury. Reduced haemorrhage was shown to correspond to reduced apoptosis and reduced cavitation after injury, both of which may significantly contribute to the loss of regenerative capacity in the chick spinal cord.

4.3.1. Verification of the model

Consistent with previous work carried out in our laboratory (O'Neill 2002; Whalley *et al.* 2006), the current results demonstrated several marked differences in the response of the chick spinal cord to injury at E11 and E15. Injury at E11 resulted in localised tissue damage and haemorrhage within the first day after injury and by 4 days after injury, the lesion site appeared to have returned to normal and the regenerative process seemed to be well advanced. By contrast, injuries at E15 resulted in much greater tissue damage and extensive haemorrhage and showed no indication of improvement within 4 days. The technique used produces an injury that combines elements of incomplete transection with a crush injury, thus mimicking some, but not all, types of human spinal cord injury. Several of the observed morphological changes after injury at E15, including cavitation and haemorrhage, are characteristic of those observed in mammalian species, including human and rat, adding further support to this system as a useful model for human spinal cord injury (Velardo *et al.* 2000). One potential disadvantage of this injury method is that there is variability in the severity of the injury between different embryos. Interestingly, survival rates for E15 injured embryos were consistently higher than those for E11 injured embryos, an observation which is

consistent with previous studies using this model (O'Neill, 2002, PhD thesis, Shimizu *et al*, 1990). This may be due to the smaller size of the embryo at E11, which makes it more likely that essential blood vessels will be damaged during the injury procedure.

The apoptotic response to injury was investigated throughout this study using TUNEL, which allows the detection of single strand breaks in genomic DNA that are characteristic of the apoptosis process. This technique is generally accepted as a specific method for identifying apoptosis *in situ* in tissue sections; however it is important to eliminate the possibility of false positive results, which could occur due to non-specific background labelling. This was achieved by the use of several controls, which demonstrated negative results in the absence of the TdT enzyme and in spinal cords in which no injury has taken place. It has been reported that treatment of sections with diethylpyrocarbonate (DEPC) can reduce any false positive staining which is due to endogenous nuclease activity (Stahelin *et al.* 1998). Previous studies in our laboratory demonstrated no change in labelling after such treatment using this protocol, suggesting that the labelling achieved by this method is accurate (O'Neill 2002). It has also been reported that, in some cases, TUNEL may stain necrotic cells, in which DNA fragmentation can occur, and it was therefore necessary also to examine the morphology of the nuclei for characteristic signs of apoptosis including chromatin condensation and fragmentation (Collins *et al.* 1992). This confirmed that the labelled cells were apoptotic rather than necrotic. A final caveat of this method is that some types of apoptosis have been described which do not involve DNA fragmentation, and hence may would not be detected by TUNEL, although these are thought to be rare (Cohen *et al.* 1992).

TUNEL staining confirmed the previous observation that there is a greatly increased apoptotic response to injury at non-regenerating stages compared to regenerating stages in the chick. Apoptotic cells were observed in both the grey and the white matter. Unfortunately, although double-labelling studies were attempted, it did not prove possible to identify the TUNEL labelled cells as neurons, glia or microglia by immunohistochemistry, possibly due to the actions of proteinase K, which was used to permeabilise the sections. It would be of interest to find a way to overcome this limitation, as previous reports have demonstrated age-related changes in the apoptotic response of glia to injury, which may be linked to the onset of myelination (McBride *et al.* 2003; Ludwin 1990) The extent of apoptosis correlated closely with the extent of

haemorrhage in the spinal cord, suggesting the possibility of a causal relationship between these two factors. Again, these results matched those of previous work carried out in our laboratory (O'Neill 2002; Whalley *et al.* 2006).

4.3.2. Correlation between haemorrhage and apoptosis

Although both the results presented here and previous work show a correlation between increased haemorrhage and apoptosis in the post-E13 chick spinal cord this does not necessarily mean that these factors are interrelated. There are undoubtedly many other developmental changes between E11 and E15 that might contribute to increased cell death at later developmental stages. It was therefore desirable to be able to manipulate the degree of haemorrhage at each time point in order to examine further the evidence for a causal effect.

An ideal and obvious approach to test this potential causative relationship directly would be to administer blood to the E11 spinal cord and examine the effects of such treatment on apoptosis. This approach is however complicated by difficulties with *in vivo* blood administration, since rapid clotting would necessitate the use of anticoagulants. This might mask some of the effects of blood coagulation factors such as thrombin, which might themselves be responsible for the effects on apoptosis (Smirnova *et al.* 1998; Turgeon *et al.* 1998). In previous work in this laboratory it was demonstrated that administration of diazepam at E11 resulted in both a large haemorrhagic response after injury, more characteristic of that usually seen at E15, and a greatly increased apoptotic response, demonstrating a correlation between the two effects (O'Neill 2002; Whalley *et al.* 2006). However, it still remained important to examine the effects of reduced haemorrhage in the E15 spinal cord on apoptosis, which is normally extensive at this stage.

In this chapter, this was addressed using a pharmacological method which was chosen to produce an effective reduction in haemorrhage without causing toxicity. The choice of agent presented a challenge since many pro-coagulant agents, such as thrombin, might be expected to cause excessive blood clotting or acute toxicity when administered at effective doses (Turgeon and Houenou 1997; Smirnova *et al.* 1998). The agent which was chosen for these experiments, desmopressin, is used in the treatment of

haemophilia, typically to reduce bleeding after surgery (Lethagen 2003; Villar *et al.* 2002). In human patients, desmopressin is commonly administered as a nasal spray. It was therefore expected to be relatively easily absorbed when administered to the chorioallantoic membrane and non-harmful. This proved to be the case and a non-toxic dose was established for further experiments.

The results presented here demonstrate that desmopressin is capable of significantly reducing haemorrhage at 12 hours after injury, although it appeared to be effective only in a sub-group of treated animals. This may be due to differences in bio-availability after administration as similar variability has been observed in the uptake of other compounds administered in this way, notably BrdU (see Chapter 7). However, since the aim of this study was to investigate the effects of a reduction in haemorrhage on apoptosis, it was decided to consider the sub-group in which an effective reduction in haemorrhage was achieved as a separate group for further analysis. Subsequently, TUNEL staining showed that there was significantly reduced apoptosis in this group of animals in which the haemorrhage was reduced by desmopressin treatment. Furthermore, 4 days after injury, desmopressin treatment resulted in a lower frequency of animals displaying features of tissue and neuronal damage, including large cavities within the spinal cord and axonal blebbing. Desmopressin treatment also resulted in a higher survival rate.

The mechanisms by which desmopressin exerts its haemostatic effects are not well understood, although it is known that desmopressin can specifically activate vasopressin V2 receptors (Kaufmann J.E. and Vischer U.M 2003). In humans, treatment with desmopressin produces a transient increase in the levels of Factor VIII, a key enzyme in the blood clotting cascade, as well as von Willebrand factor, each of which may contribute to increased blood clotting (Kaufmann J.E. and Vischer U.M 2003; Mannucci p 2000). It remains possible that desmopressin might have a direct or indirect effect on apoptosis unrelated to its role in haemostasis. However, to date no evidence exists showing that spinal cord cells express the V2 receptors or can respond directly to this compound (Phillips *et al.* 1990). In further support of these findings, in a compression model of spinal cord injury in the rat, treatment with activated protein C has been shown to reduce both haemorrhage and motor disturbances, although this

affect was attributed mainly to a reduction in cytokine production and subsequent inflammation (Taoka *et al.* 1998).

Haemorrhage within the spinal cord after injury could produce a number of detrimental effects which might result in cell death by apoptosis. Interruption of the blood supply to the spinal cord deprives the spinal cord of oxygen and glucose and leads rapidly to ischemia, which can result in cell death by excitotoxicity and the production of free radicals (Profyris *et al.* 2004; Dumont *et al.* 2001). The entry of blood into the spinal cord also exposes neurons and glia to a number of potentially toxic cell types and molecules normally excluded from the central nervous system by the tight junctions of the blood brain barrier. These include inflammatory cells and molecular components of the immune response such as cytokines which are considered to be both beneficial and harmful to the spinal cord after an injury (Bethea and Dietrich 2002; Kwon *et al.* 2004). There are additionally many other factors present in the blood which can be neurotoxic. In particular, several member of the serine protease family of enzymes, such as thrombin, well recognised for their essential roles in haemostasis, have been shown to have potentially detrimental effects on neurons and glia (del Zoppo 1998; Lee *et al.* 1997; Smirnova *et al.* 1998; Turgeon and Houenou 1997). Many of these enzymes, together with their inhibitory counterparts, the serpins, and their specific receptors are expressed within the CNS and may have a role during development and in normal CNS function (Turgeon and Houenou 1997; Gingrich and Traynelis 2000). The serine proteases are therefore good candidate molecules for providing a molecular link between haemorrhage and apoptosis and their role in the injured chick spinal cord will be explored further in Chapter 5.

4.3.3. Reduced haemorrhage and axonal regeneration

A reduction in the extent of cavitation and neuronal damage such as that observed in this study might be expected to improve the likelihood of successful axonal regeneration. Although a detailed analysis of axonal regeneration was not within the remit of this study, in one desmopressin treated embryo, axons were observed apparently crossing the injury site. However, it is not possible to determine from neurofilament staining alone whether this is the result of axonal re-growth or sprouting. In order to determine fully whether reduced haemorrhage alone could result in improved axonal regeneration and consequent behavioural recovery it would be necessary to look

at post-hatching stages and to use a retrograde axonal tracing method such as that used to demonstrate regeneration in the E11 spinal cord (Hasan *et al.* 1993; Sholomenko and Delaney 1998). However, on the basis of examination of sections at 4 days after injury, the current study showed little evidence of axonal regeneration, suggesting that other factors in addition to haemorrhage may require manipulation in order to promote axonal growth. This might include inhibition of inhibitory molecules present in damaged myelin or the glial scar, or provision of growth-promoting factors necessary to stimulate and encourage re-growth (Sandvig *et al.* 2004; Profyris *et al.* 2004). Nevertheless, a reduction in cavitation and cell death, such as that produced by desmopressin treatment would certainly make the process of axonal regeneration much easier to achieve. Even a small increase in the number of axons spared after an injury has been shown to have significant effects on recovery and as little as 7% axonal survival below the injury site can result in significant motor function in humans (Kwon *et al.* 2004).

4.3.4. Caspase-dependent and independent apoptosis after spinal cord injury in the chick

Although previous studies had clearly demonstrated an apoptotic response to injury in the chick spinal cord, when this study was initiated there had been no attempt to classify the apoptosis as caspase-dependent or independent. Both types of apoptosis have been demonstrated to occur following traumatic injury to the nervous system and it was therefore of interest to understand which type was prominent after spinal cord injury in the chick (Citron *et al.* 2000a; McBride *et al.* 2003; Springer *et al.* 1999). The results of this study suggest that both mechanisms may be involved in the cell death observed after transection injury in the chick. Although the numbers of samples used in the present study were too low to give quantitative results, apoptosis appeared to be reduced but not abolished by inhibition of the key effector caspase, caspase-3. During the course of these experiments, a study by Steeves and colleagues was published, which demonstrated that caspase dependent mechanisms are involved in the apoptotic response of the chick spinal cord to injury (McBride *et al.* 2003). The study by Steeves and colleagues differed from the present study in a number of ways, in particular by focusing on delayed (24 hours – 6 days after injury) apoptosis in oligodendrocytes at a greater distance from the injury site (up to 7mm from the injury site). Nevertheless these results support the preliminary findings outlined here; showing that inhibition of

caspase-1 and caspase-3 reduced but did not eliminate oligodendrocyte apoptosis. It was therefore decided not to follow up this preliminary experiment with more investigation into the role of caspases. Additionally, caspase activation is a relatively late stage event in the apoptotic cascade and it would be desirable to be able to manipulate the factors which are responsible for initiating this cascade, in order to provide neuroprotection. The next chapter will therefore concentrate on the identification of factors up-stream of caspase-dependent or independent mechanisms, which might provide a molecular link between haemorrhage and apoptosis.

4.3.5. Conclusions

Overall the results presented here provide further evidence for a link between the extent of haemorrhage in the spinal cord after injury and the extent of apoptosis and subsequent cavitation. Reducing haemorrhage or targeting the downstream molecular pathways which lead to apoptosis may result in increased neuroprotection and reduced tissue damage. This may therefore represent a useful therapeutic strategy to be used in conjunction with other therapeutic approaches such as inhibition of myelin related factors or cellular transplantation.

Chapter 5. Endogenous and exogenous serine proteases in the developing and injured spinal cord

5.1. Introduction

As described in the previous chapter, the loss of regenerative capacity in the developing chick spinal cord, which occurs around E13, corresponds to a dramatic change in the response of the spinal cord to injury. Previous work in our laboratory, together with the results described in Chapter 4, have highlighted the relationship between an increase in the extent of haemorrhage in the spinal cord after injury and increased apoptotic cell death at non-regenerating stages (O'Neill 2002; Whalley *et al.* 2006). These findings suggest that there may be factors present in the blood that are responsible for triggering an apoptotic response in spinal cord neurons and glia. Circulating blood carries a number of potentially neurotoxic substances that could be considered candidates for this role. Among these, the serine protease family of enzymes, and in particular thrombin, have attracted interest for a number of reasons.

As well as being present in the blood, thrombin, together with the PAR-1 receptor and several specific inhibitors, are found within the CNS and are thought to play key roles in nervous system development as well as in plasticity and homeostasis during adulthood (Dihanich *et al.* 1991; Deschepper *et al.* 1991; Mansuy *et al.* 1993; Niclou *et al.* 1994; Reinhard *et al.* 1994; Weinstein *et al.* 1995; Turgeon and Houenou 1997). Thrombin signalling has been implicated in nervous system pathology following various types of injury and in neurological diseases such as Alzheimer's disease and stroke (Vaughan *et al.* 1994; Riek-Burchardt *et al.* 2002). *In vitro*, thrombin has been demonstrated to provide protection from or promotion of neuronal cell death by apoptosis, depending on the dose and the experimental model used (Turgeon *et al.* 1998; Turgeon and Houenou 1999; Vaughan *et al.* 1995; Donovan *et al.* 1997; Pike *et al.* 1996; Smirnova *et al.* 1998; Smith-Swintosky *et al.* 1995). Changes in endogenous CNS levels of thrombin, its receptor or inhibitors, or the entry of high concentrations of exogenous thrombin from the blood after injury, could potentially disrupt the delicate regulatory balance between these molecules, leading to neuropathological effects.

The overall aim of the current study was to determine to what extent serine proteases might play a role in the increased apoptotic response observed in the non-regenerating chick spinal cord after injury. As outlined above, the available literature suggests that thrombin is likely to be a key serine protease involved in apoptosis in the chick spinal cord and this enzyme was therefore the primary focus of this study. In addition, an investigation into the plasminogen activator, urokinase, was initiated. Two parallel routes of investigation were followed, examining changes in endogenous CNS thrombin after injury and the potential of exogenous thrombin to cause apoptosis.

Endogenous expression of prothrombin, the inactive precursor of thrombin, and urokinase has been previously described in the developing mouse and rat (Dihanich *et al.* 1991; Sumi *et al.* 1992). However the expression of these molecules has not been studied extensively in the chick. The first part of this study therefore set out to investigate their expression in the developing chick spinal cord using real-time RT-PCR. Endogenous levels of prothrombin, the PAR-1 receptor and urokinase have been demonstrated previously to be altered after spinal cord injury in the rat (Citron *et al.* 2000b), a model which bears many morphological similarities to the chick spinal cord at E15. It was of interest to determine whether similar changes are observed in the non-regenerating chick spinal cord. Changes in the endogenous expression of prothrombin and urokinase mRNA after injury at E15 were therefore investigated using real-time RT-PCR. In addition to its regulation at the level of mRNA transcription, thrombin activity is also regulated by cleavage of its precursor protein, prothrombin, to active thrombin. Consistent with this, changes in the levels of thrombin activity have also been described after insults to the nervous system (Smirnova *et al.* 1996). Therefore, in the following experiments an assay for the measurement of endogenous thrombin activity in the spinal cord was developed and used to investigate changes in activity after injury at E15.

In parallel to these investigations, the possible effects of exogenous thrombin on apoptosis in the spinal cord were considered. After an injury, thrombin might enter the nervous tissue from the blood during haemorrhage. In order to investigate this possibility, an organotypic culture system, which would mimic as closely as possible the *in vivo* situation, was set up and used to test the potential effects of thrombin and thrombin inhibition on apoptosis.

5.2. Results

5.2.1. Real time PCR analysis of serine protease expression in the spinal cord

In order to examine the expression of prothrombin and urokinase, RT-PCR was carried out on pools of spinal cord samples. A preliminary analysis by semi-quantitative PCR showed that both prothrombin and urokinase mRNA, but not tPA mRNA, is expressed in the developing spinal cord. To obtain more accurate and quantifiable information of the expression levels of these molecules a real-time PCR strategy was employed. Primers and probes against chick prothrombin, urokinase and GAPDH were custom designed by Applied Biosystems. In order to use these primer sets for relative quantitative analysis using the comparative C_T method (see Chapter 3), it was first necessary to confirm that the efficiency of amplification of each set were approximately identical. Standard curves were set up for each set of primers in which 10 fold dilutions of cDNA were added to each reaction. Fig 5.1 shows the standard curves produced for each primer and probe set. As shown in Fig 5.1, the slopes of the standard curves for prothrombin, urokinase and GAPDH were approximately equal, which demonstrates that the efficiency of amplification was equivalent and that these primer sets were appropriate for determining relative gene expression by the comparative C_T method.

5.2.2. Analysis of the expression of prothrombin and urokinase in the developing chick spinal cord

Having verified the amplification efficiency of each probe set, the expression of prothrombin mRNA in the developing chick spinal cord was examined in three independent pools of samples at each time point and the comparative C_T method was employed in order to analyse the results (see Chapter 3 and Discussion). Briefly, the mean C_T for each sample was normalised to GAPDH levels and then the fold change in expression relative to the E3 group was determined. Fig 5.2 shows the relative expression levels of prothrombin mRNA, compared to the E3 group, at four different developmental stages. Prothrombin was found to be expressed throughout the developmental stages examined. Statistical analysis was used to compare the relative expression at each stage and showed that there was no significant change in expression

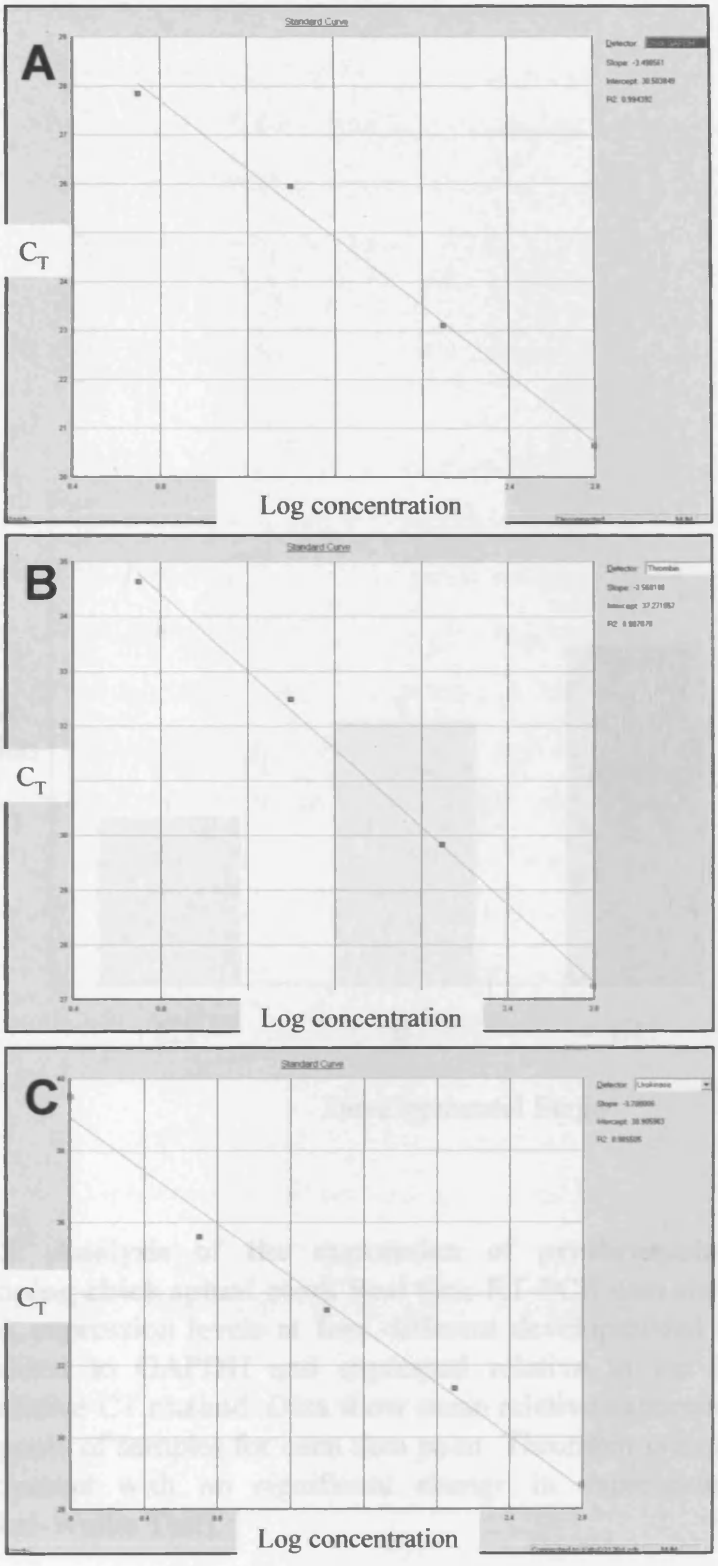

Fig. 5.1. Validation experiment for primers for real-time PCR. Standard curves are shown for primer and probe sets for chick GAPDH (A) prothrombin (B) and urokinase (C). Graphs show log [cDNA] against threshold cycle number (C_T) in each case (see Chapter 3). The slope of the standard curve is approximately equal in each case, demonstrating equal amplification efficiency.

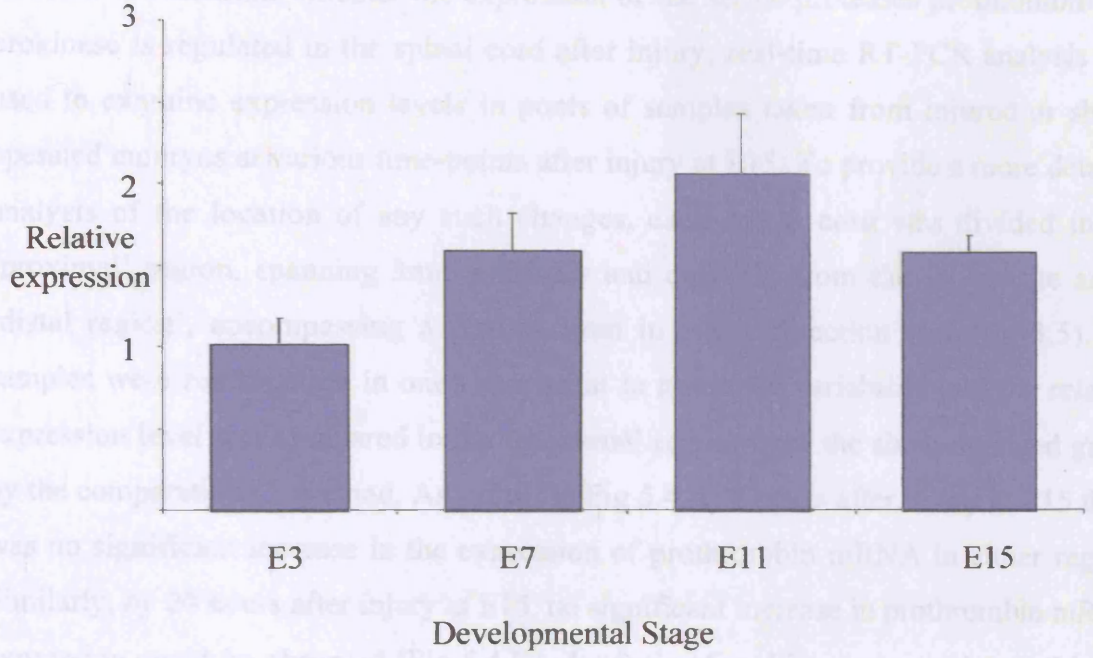

Fig.5.2. Analysis of the expression of prothrombin mRNA in the developing chick spinal cord. Real time RT-PCR data showing prothrombin mRNA expression levels at four different developmental stages. Levels are normalised to GAPDH and expressed relative to the E3 group by the comparative CT method. Data show mean relative expression level ± SEM in three pools of samples for each data point. Thrombin is expressed throughout development with no significant change in expression levels. P=0.070 (Kruskal-Wallis Test).

levels with development. Similarly, real-time RT-PCR analysis of the expression of urokinase mRNA showed that urokinase is expressed throughout development (Fig 5.3.). Expression was lowest at E3 and then increased by more than 15-fold by E11, although this increase did not prove statistically significant.

5.2.3. Analysis of the expression of prothrombin and urokinase after injury at E15

In order to determine whether the expression of the serine proteases prothrombin and urokinase is regulated in the spinal cord after injury, real-time RT-PCR analysis was used to examine expression levels in pools of samples taken from injured or sham-operated embryos at various time-points after injury at E15. To provide a more detailed analysis of the location of any such changes, each spinal cord was divided into a 'proximal' region, spanning 3mm cranially and caudally from the injury site and a 'distal region', encompassing a further 3mm in either direction (see Fig 3.5). All samples were run together in one experiment to minimise variability and the relative expression level was compared to the 'proximal' region from the sham operated group by the comparative C_T method. As shown in Fig 5.4.A, 2 hours after injury at E15 there was no significant increase in the expression of prothrombin mRNA in either region. Similarly, by 24 hours after injury at E15, no significant increase in prothrombin mRNA expression could be observed (Fig 5.4.B). Analysis of urokinase expression at 2 hours after injury at E15 showed no significant difference between injured and sham operated spinal cords in either the proximal or the distal region (Fig 5.5.A). Again, at 24 hours after injury at E15 there were no significant changes in urokinase mRNA expression (Fig 5.5.B).

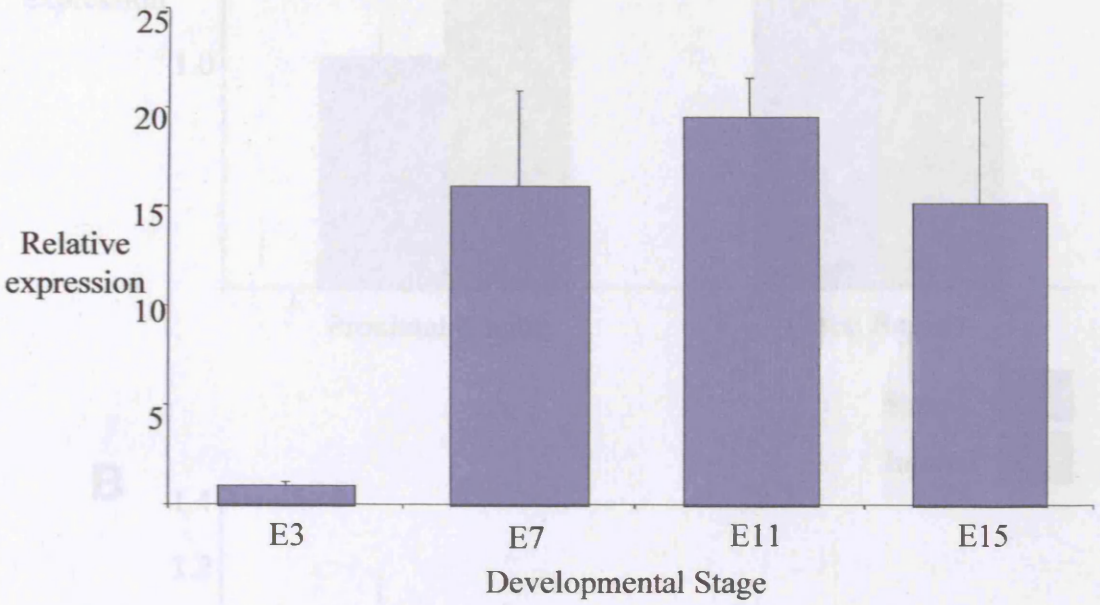

Fig. 5.3. Analysis of the expression of urokinase mRNA in the developing chick spinal cord. Real time RT-PCR data showing urokinase mRNA expression levels at different developmental stages. Levels are normalised to GAPDH and expressed relative to the E3 group by the comparative CT method. Data show mean relative expression level ± SEM in three pools of samples for each data point. Urokinase is expressed throughout development with no significant change in expression levels. P=0.086 (Kruskal-Wallis Test).

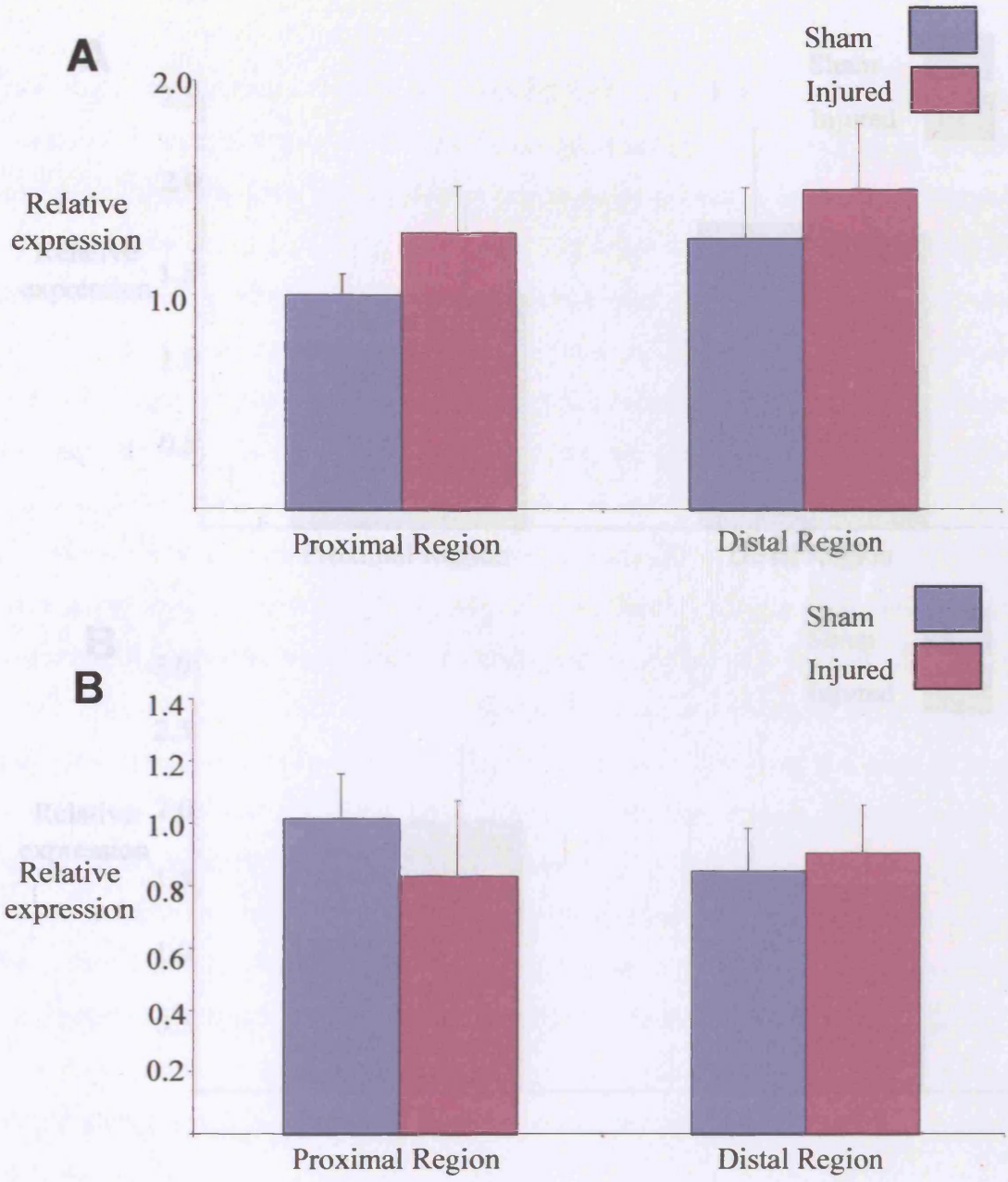

Fig 5.4. Analysis of prothrombin mRNA expression after injury to the spinal cord at E15. A) 2 hours after injury. **B)** 24 hours after injury. Real time RT-PCR data showing prothrombin mRNA expression within a region expanding 3mm rostrally and caudally from the injury site (the proximal region) and in a region between 3mm-6mm from the injury site (the distal region). Levels are normalised to GAPDH and expressed relative to the 'proximal sham' group by the comparative CT method (see chapter 3). Data show mean relative expression level ± SEM in three pools of samples for each data point. Prothrombin mRNA levels are not significantly altered by injury at this time point. A) p=0.275 for proximal region, p=0.513 for distal region B) p=0.513 for proximal region, p=0.827 (Mann Whitney Test)

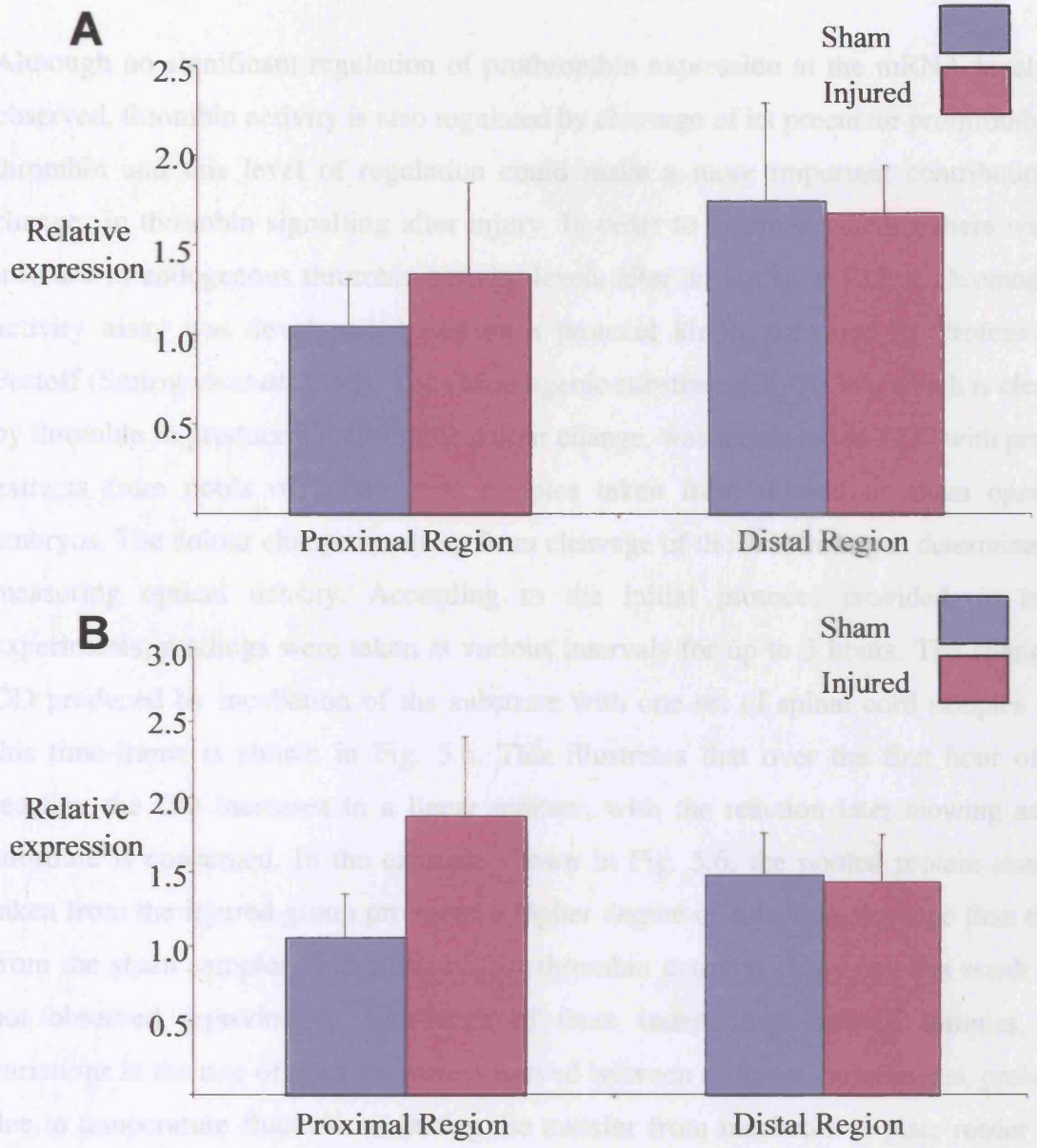

Fig 5.5. Analysis of urokinase mRNA expression after injury to the spinal cord at E15. A) 2 hours after injury. **B)** 24 hours after injury. Real time RT-PCR data showing mRNA expression levels within a region expanding 3mm rostrally and caudally from the injury site (the proximal region) and in a region between 3mm-6mm from the injury site (the distal region). Levels are normalised to GAPDH and expressed relative to the 'proximal sham' group by the comparative CT method (see chapter 3). Data show mean relative expression level ± SEM in three pools of samples for each data point. Urokinase mRNA levels are not significantly altered by injury at this time point. A) p=0.827 for proximal region, p=0.827 for distal region B) p=0.127 for proximal region, p=0.513 for distal region (Mann Whitney Test)

5.2.4. Analysis of thrombin activity after injury

Although no significant regulation of prothrombin expression at the mRNA level was observed, thrombin activity is also regulated by cleavage of its precursor prothrombin to thrombin and this level of regulation could make a more important contribution to changes in thrombin signalling after injury. In order to examine whether there was an increase in endogenous thrombin activity levels after an injury at E15, a chromogenic activity assay was developed, based on a protocol kindly provided by Professor B. Festoff (Smirnova *et al.* 1996). The chromogenic substrate, CS-01(38), which is cleaved by thrombin to produce a measurable colour change, was incubated at 37°C with protein extracts from pools of spinal cord samples taken from injured or sham operated embryos. The colour change resulting from cleavage of the substrate was determined by measuring optical density. According to the initial protocol provided, in initial experiments, readings were taken at various intervals for up to 3 hours. The change in OD produced by incubation of the substrate with one set of spinal cord samples over this time-frame is shown in Fig. 5.6. This illustrates that over the first hour of the reaction the OD increases in a linear manner, with the reaction later slowing as the substrate is consumed. In the example shown in Fig. 5.6, the pooled protein samples taken from the injured group produced a higher degree of substrate cleavage than those from the sham samples, indicating higher thrombin activity. However, this result was not observed reproducibly with each of three independent sets of samples, and variations in the rate of reaction were observed between different experiments, probably due to temperature fluctuations during the transfer from incubator to plate reader (see section 3.13.2).

In order to obtain results that could be statistically analysed, it was decided to take a single reading after 30 minutes incubation, during the linear part of the amplification and to run three independent sample pools for each group together in one reaction. In order to further confirm that the reaction was linear at this time point, a range of standard concentrations of pure bovine thrombin were incubated alongside the experimental samples. As shown in Fig. 5.7, after 30 minutes incubation there is a linear relationship (correlation co-efficient 0.99) between the concentration of active thrombin and the colour change. This also confirmed that, at this time point, availability of the substrate had not become a limiting factor, as even at very high concentrations of

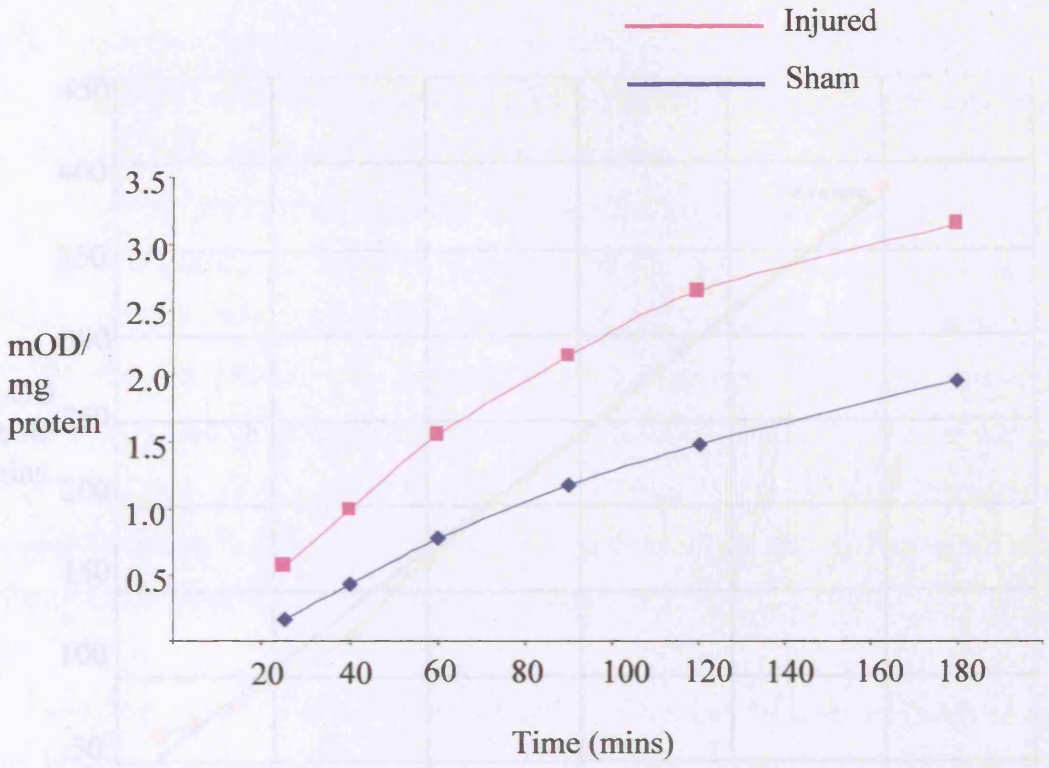

Fig. 5.6. Analysis of the cleavage of a chromogenic substrate by thrombin in spinal cord samples. Graph shows mOD measured at various intervals after incubation of the chromogenic substrate CS-01(38) with protein extracts from pools of samples from injured or sham operated spinal cord.

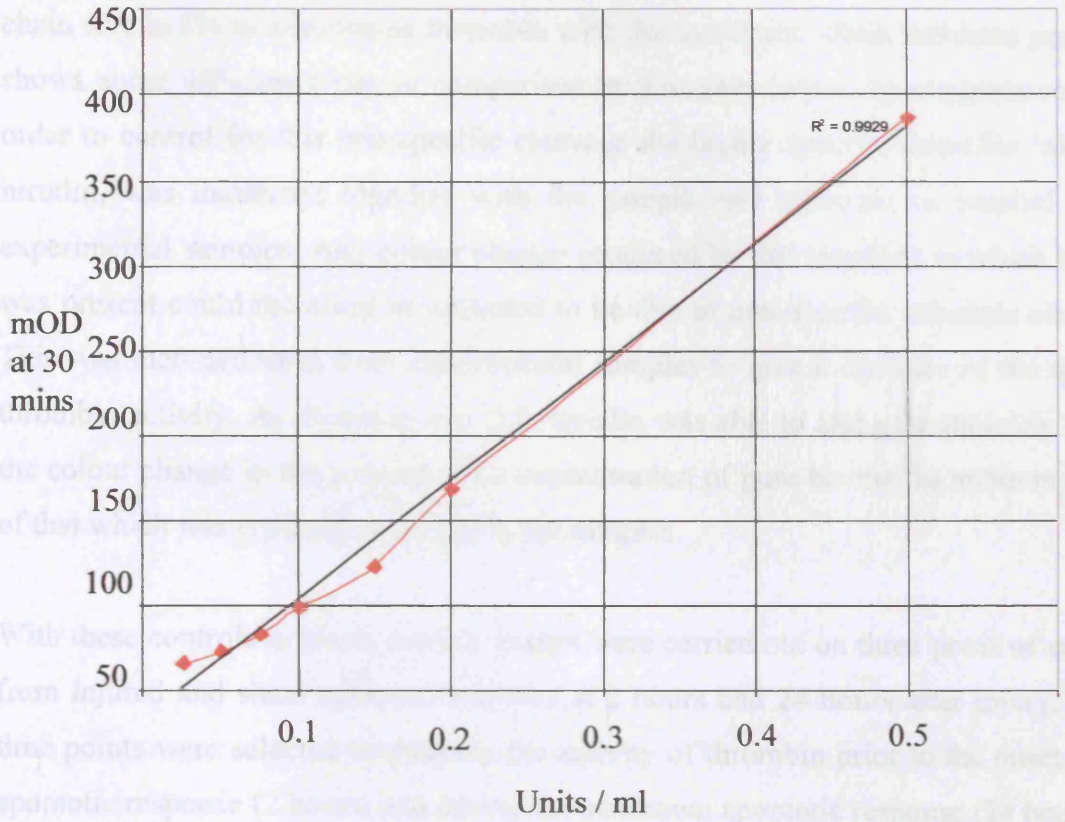

Fig. 5.7. Standard curve demonstrating relationship between thrombin activity and cleavage of CS-01(38) after 30 minutes incubation. Red line shows mOD measured 30 minutes after incubation of various concentrations of bovine thrombin with the chromogenic substrate CS-01(38). Correlation co-efficient: R=0.99 (trendline shown in black).

thrombin, in excess of that which was seen in our samples, the relationship remained linear. CS-01(38) is among the most specific substrates available for thrombin. However, cleavage by other serine proteases can also take place, although these have a greatly reduced affinity for the substrate in comparison to thrombin. For example, single chain tPA is 5% as reactive as thrombin with this substrate, while activated protein C shows about 40% reactivity in comparison to thrombin (www.chromogenix.com). In order to control for this non-specific cleavage the highly specific thrombin inhibitor, hirudin, was incubated together with the sample and substrate in parallel to the experimental samples. Any colour change produced by the reactions in which hirudin was present could therefore be assumed to be due to non-specific substrate cleavage. This was then deducted from experimental samples to give a measure of the specific thrombin activity. As shown in Fig. 5.8, hirudin was able to almost completely inhibit the colour change in the presence of a concentration of pure bovine thrombin in excess of that which was typically observed in the samples.

With these controls in place, activity assays were carried out on three pools of samples from injured and sham operated embryos at 2 hours and 24 hours after injury. These time points were selected to examine the activity of thrombin prior to the onset of the apoptotic response (2 hours) and during the maximum apoptotic response (24 hours), in order to provide an indication of the potential role of thrombin in this response. The protein concentration of each sample was determined prior to starting the assay and the total protein loaded in each case was approximately equal. However, in order to take into account variations in protein loading, the OD measured was normalised to total protein in each case. As shown in Fig. 5.9, at 2 hours after injury the total substrate cleavage after 30 minutes incubation was significantly higher in the injured than the sham operated samples, indicating an increase in overall protease activity. However, most of this activity was not inhibited by treatment with hirudin and there was no significant difference in specific thrombin activity detectable. Similarly, at 24 hours after injury, there was an overall significant increase in protease activity, but, although at this time point a trend towards an increase in thrombin-specific activity could be observed, this did not prove significant (Fig. 5.10). The magnitude of the non-specific activity increase was also noticeably greater at 24 hours when compared to 2 hours after injury.

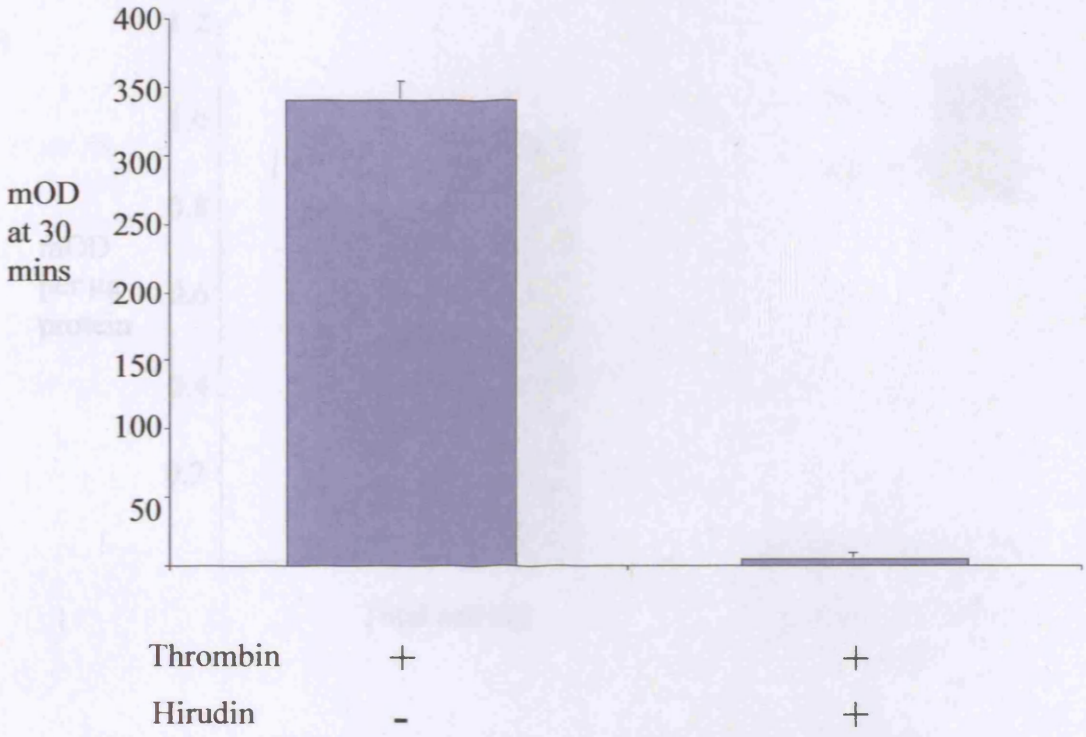

Fig 5.8. Determination of the inhibition of thrombin activity by hirudin. Graph shows OD measured 30 minutes after incubation of 1unit/ml of thrombin with the chromogenic substrate CS-01(38) with or without hirudin (2U/μl). The addition of hirudin almost completely eradicates thrombin activity.

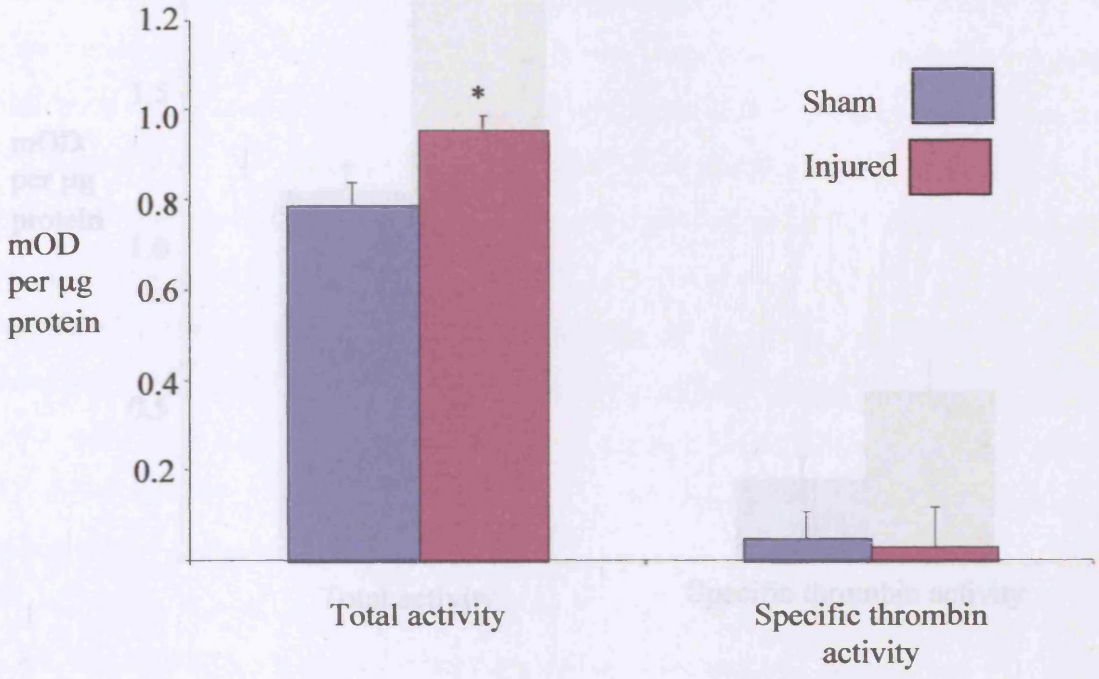

Fig. 5.9. Analysis of thrombin activity two hours after injury at E15. Graph shows mOD per µg protein after 30 minutes incubation with the chromogenic substrate CS-01(38). Specific activity is determined as the difference between total activity and that measured in reactions carried out in the presence of hirudin. Data shown are mean ± SEM for 3 pools of samples per group. There is a significant increase in total activity but no increase in specific thrombin activity at this time point. * p=0.050 for total activity, p=0.827 for specific activity (Mann Whitney Test)

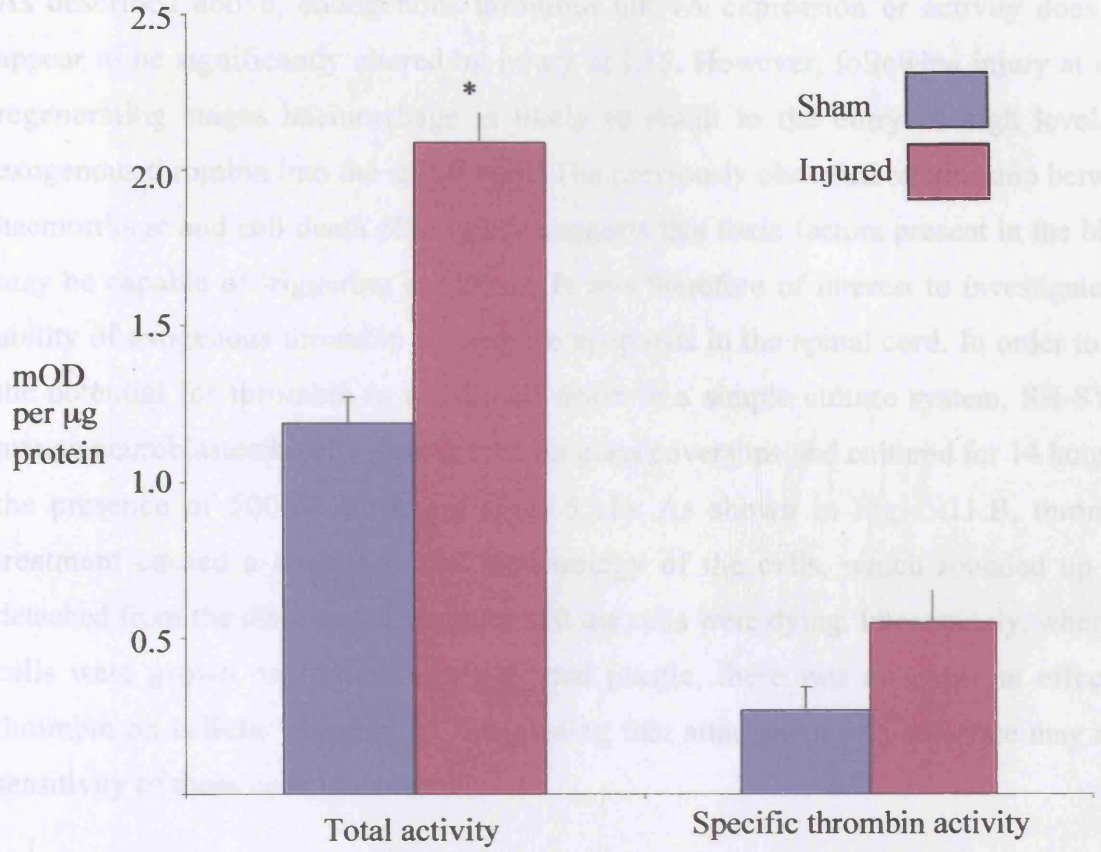

Fig. 5.10 Analysis of thrombin activity 24 hours after injury at E15. Graph shows mOD per µg protein after 30 minutes incubation with the chromogenic substrate CS-01(38). Specific activity is determined as the difference between total activity and that measured in reactions carried out in the presence of hirudin. Data shown is mean± SEM for three pools of samples per group. There is a significant increase in total activity but no significant increase in thrombin specific activity at this time point. * p = 0.050 for total activity, p=0.127 for specific activity (Mann Whitney Test).

5.2.5 Analysis of the apoptosis-inducing effects of thrombin.

As described above, endogenous thrombin mRNA expression or activity does not appear to be significantly altered by injury at E15. However, following injury at non-regenerating stages haemorrhage is likely to result in the entry of high levels of exogenous thrombin into the spinal cord. The previously observed relationship between haemorrhage and cell death after injury suggests that toxic factors present in the blood may be capable of triggering apoptosis. It was therefore of interest to investigate the ability of exogenous thrombin to increase apoptosis in the spinal cord. In order to test the potential for thrombin to cause cell death in a simple culture system, SH-SY5Y human neuroblastoma cells were grown on glass coverslips and cultured for 14 hours in the presence of 500nM thrombin (Fig. 5.11). As shown in Fig. 5.11.B, thrombin treatment caused a change in the morphology of the cells, which rounded up and detached from the dish, which suggests that the cells were dying. Interestingly, when the cells were grown on tissue culture treated plastic, there was no apparent effect of thrombin on cellular morphology, suggesting that attachment to a substrate may alter sensitivity of these cells to thrombin.

5.2.6. Establishing an organotypic spinal cord slice culture system.

The results obtained when neuroblastoma cells were cultured with thrombin confirmed the potential of this molecule to cause cell death, but also suggested that interactions with the cellular environment are likely to influence the sensitivity of cells to this factor. It was desirable to be able to test the effect of thrombin in an environment more similar to that which spinal cord cells would experience *in vivo*. Owing to the potential toxic effects of systemic thrombin treatment *in vivo*, it was decided that an *in vitro* organotypic culture system would provide the best method for investigating the specific effects of thrombin in the spinal cord. A slice culture system was chosen to maximise the number of cultures which could be obtained per embryo. Initially, several different methods of cutting fresh spinal cord slices were tested. Of these, cutting slices by eye with a small surgical scalpel blade, proved to be the quickest method but produced slices of uneven thickness, which might have an impact on the availability and effects of exogenous treatments. The final method chosen for consistency in slice thickness and

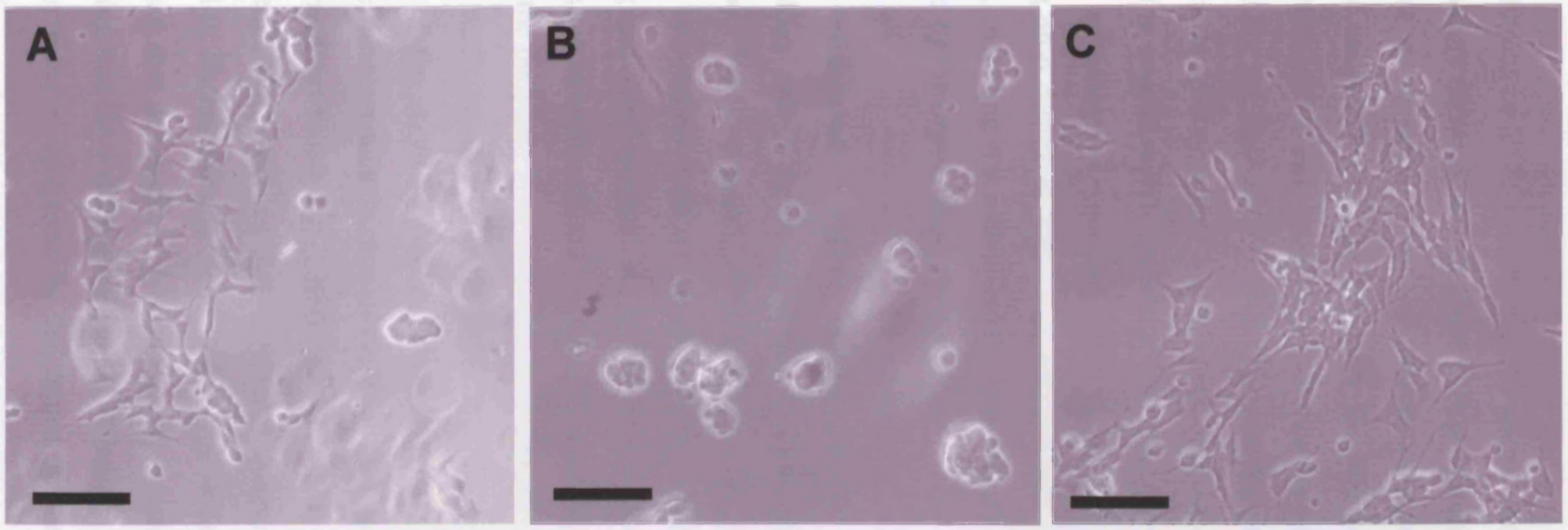

Fig. 5.11. Analysis of the effects of thrombin on human neuroblastoma cells. Images shown SH-SY5Y cells after culturing for 14 hours in three different conditions. A) cells cultured on glass coverslips in normal medium showing normal morphology. B) cells cultured on glass coverslips in the presence of 500nM thrombin. C) cells cultured on plastic in the presence of 500nM thrombin. Scale bars = 100 μm.

for reduced damage to the slices during cutting was therefore to use a vibratome to cut 500 μm slices of spinal cord embedded in agarose.

Two different methods of culturing the slices were tested. Culturing on filter paper in organ culture dishes produced slices with good morphology after 24 hours, in which the grey and white matter could be clearly distinguished and the nuclear morphology appeared normal (Fig 5.12A). However, the volume of medium required for this method would have added considerably to the expense of the pharmacological manipulations required for the following experiments, and therefore reduced the sample size. It was therefore decided to culture the slices in small hanging drop cultures. This method also resulted in a good morphology and little sign of tissue degradation over 24 hours, and had the advantage of requiring only a small volume of medium. Fig. 5.12.B shows a hanging drop culture in which it is still possible to distinguish the various spinal cord regions, including the central canal, although the morphology is not as good as that obtained by the organ culture dish method.

Having established a culture system capable of maintaining slices with good morphology over 24 hours a method for the detection of apoptosis in these slices was developed. Whole-mount TUNEL staining of the slices was used as this minimised the amount of reagents used and the time taken to process the samples in comparison to staining individual thin sections of the slices. Under normal culture conditions, TUNEL staining can be observed in all regions of the spinal cord and is particularly concentrated around the periphery (Fig 5.13). Confocal analysis allowed images to be captured up to 40 μm depth into the slice. TUNEL staining was always more intense towards the outside of the slice. To determine whether the observed apoptosis was occurring in neurons, whole-mount immunostaining for the neuron-specific nuclear antigen NeuN was carried out following TUNEL staining. Many TUNEL labelled nuclei co-localised with NeuN, demonstrating that neuronal apoptosis can also be observed in this model (Fig 5.14). Successful immunostaining up to at least 50 μm depth into the slices also confirmed that penetration of the slices by reagents was possible using this method, suggesting that the reduction in TUNEL labelling towards the centre of the slice is the result of reduced apoptosis in the centre, rather than decreased penetration of the reagents.

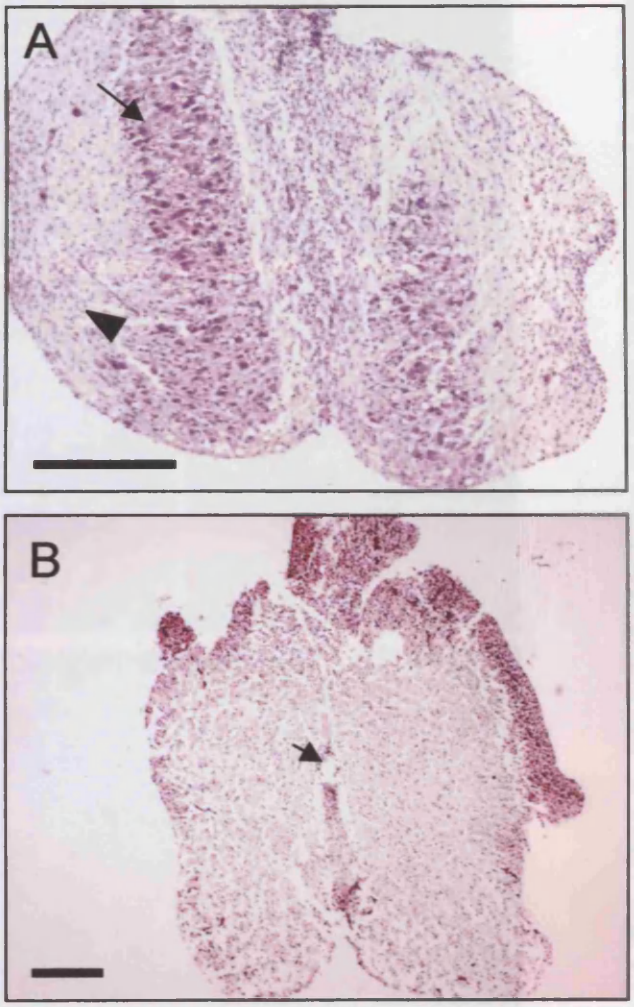

Fig 5.12. Comparison of the morphology of slice cultures prepared and cultured by different methods A) Haematoxylin and Eosin (H&E) staining of section taken from a slice cultured on filter paper in an organ culture dish for 24 hours shows excellent morphology with clearly distinguishable grey (arrow) and white (arrowhead) matter. B) H&E staining of section taken from a slice cultured in a hanging drop for twenty four hours shows good morphology, with the ependymal region being clearly identifiable (arrow). Scale bars = 200 μm.

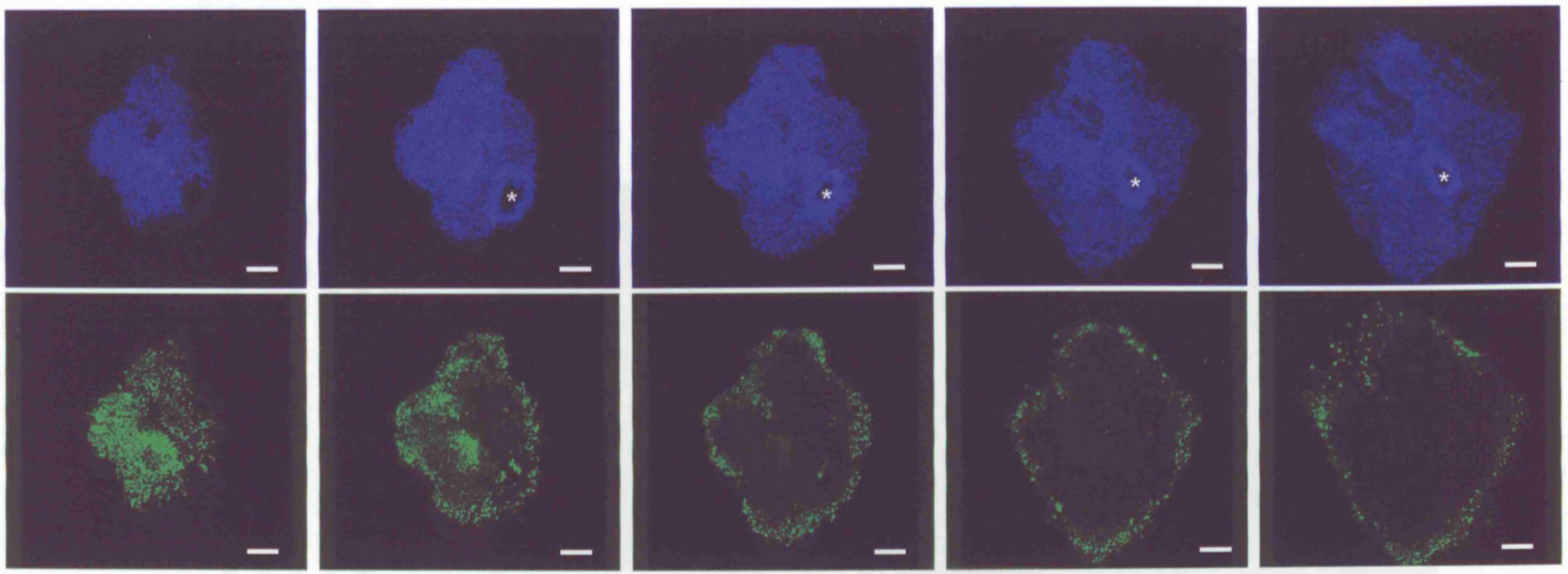

Fig. 5.13 Whole-mount TUNEL labelling of spinal cord slice cultures. 500 μm slice cultures of spinal cord maintained in hanging drop cultures for twenty four hours before whole-mount TUNEL staining for apoptosis (green). Nuclei are counterstained with To-Pro-3-isomerase (blue). Images show, from left to right, confocal scans taken every 10 μm through a typical slice culture. Asterisks indicate position of central canal. Scale bars = 80 μm.

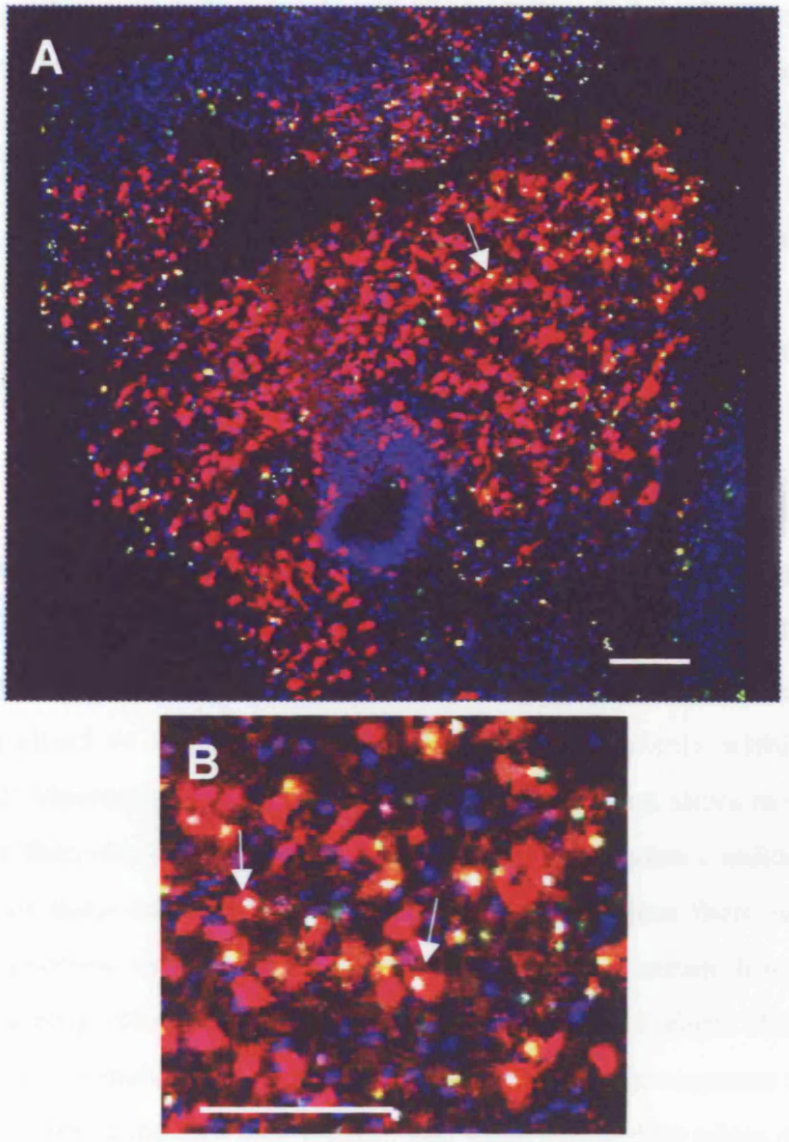

Fig. 5.14. Analysis of neuronal apoptosis in spinal cord slice cultures. A) 500 μm slice culture maintained in hanging drop cultures for 24 hours before whole-mount TUNEL (green) and immunostaining for NeuN (red). Nuclei are counterstained with To-Pro-3-isomerase (blue). **B)** High magnification view of grey matter shows apoptotic neuronal cells (arrows). Scale bars = 80 μm

Once a method for obtaining good TUNEL staining had been optimised it was necessary to confirm that quantifiable differences in apoptosis could be detected by this method. In order to do this, slices from E11 and E15 spinal cords were cultured in either 10% serum or 1% serum for 24 hours, and whole-mount TUNEL staining was carried out to detect apoptosis. In order to ensure that changes in apoptosis reflect the treatment rather than inter-embryo variability, in each experiment at least three different embryos were used and slices from each were randomly assigned to treatment groups. In E11 slices there was little difference in the amount of TUNEL staining in slices cultured in 1% or 10% serum (Fig. 5.15.A-D). In E15 slices, however, culturing in 1% serum produced an increased apoptotic response in comparison to culturing in 10% serum (Fig. 5.16.A-D).

In order to quantify the apoptosis, confocal images were captured every 10 μm through the first 40 μm of each slice from the cut surface. For each image, software was used to measure the total intensity of green fluorescence in the image. These measurements were normalised to the area, and the average intensity/area within each slice was determined. Measurements from a minimum of six different slices in each experimental group were then averaged. As shown in Fig. 5.17, low serum conditions did not affect the extent of apoptosis in E11 slices, whereas in E15 slices there was a significantly increased apoptotic response as a result of culturing in 1% serum. It was notable that the TUNEL staining intensity was always higher in the E11 slices than the E15 slices. However, this probably does not reflect a larger apoptotic response to culture at E11. The larger width of the E15 slices meant that some of the slice edges, where the greatest staining was usually found, were cut off from each image captured by the confocal, whereas at E11, the entire slice fit into the frame. This is likely to have contributed to the higher TUNEL measurements at E11.

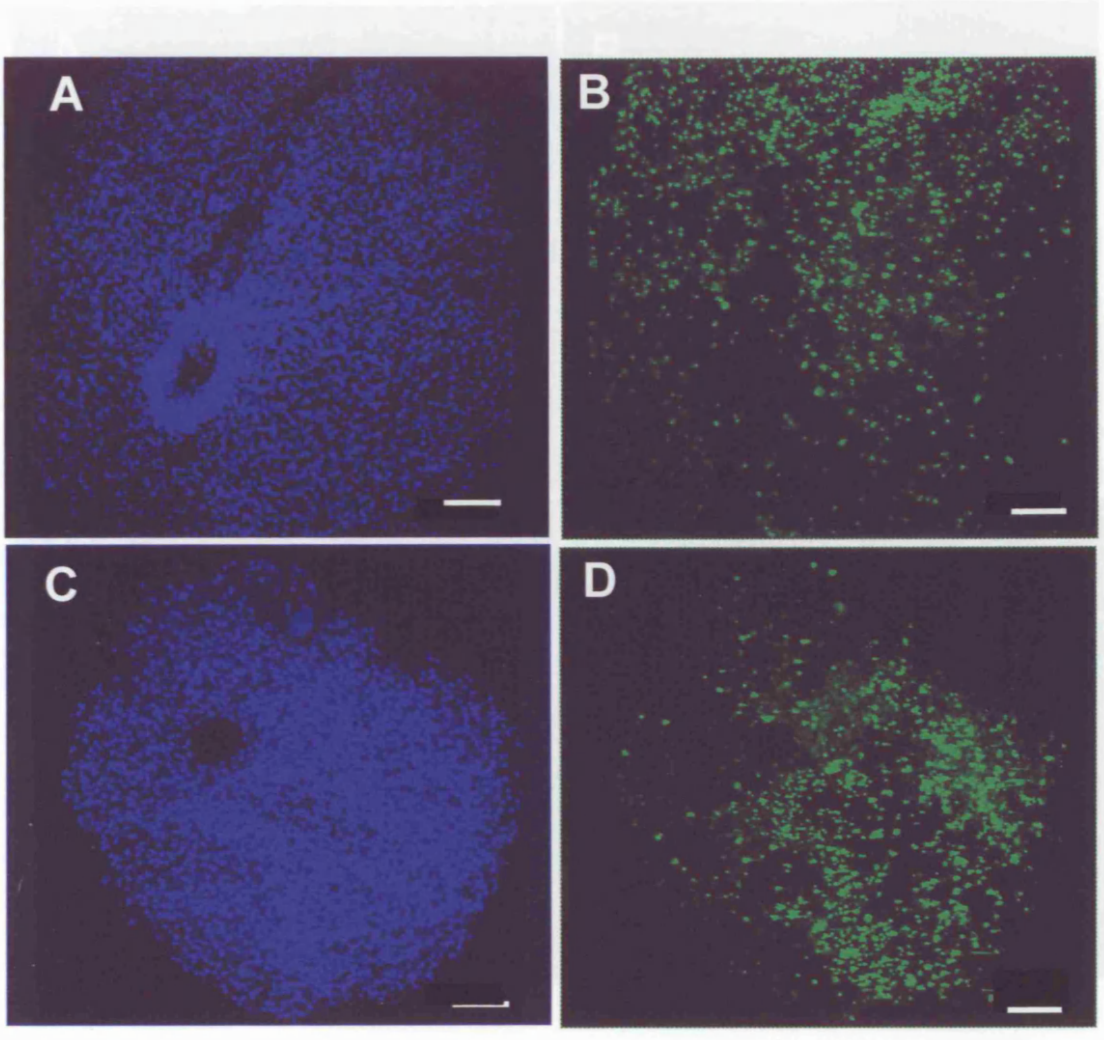

Fig. 5.15. Analysis of the effects of low serum on E11 spinal cord slice cultures. E11 slice culture maintained in 10% serum (A & B) or 1% serum (C & D) for 24 hours before whole mount TUNEL staining (B & D, green). Nuclei are counterstained with To-Pro-3-isomerase (A & C, blue). There is no noticeable difference in the extent of apoptosis between the two culture conditions. Scale bars = 80 μ m

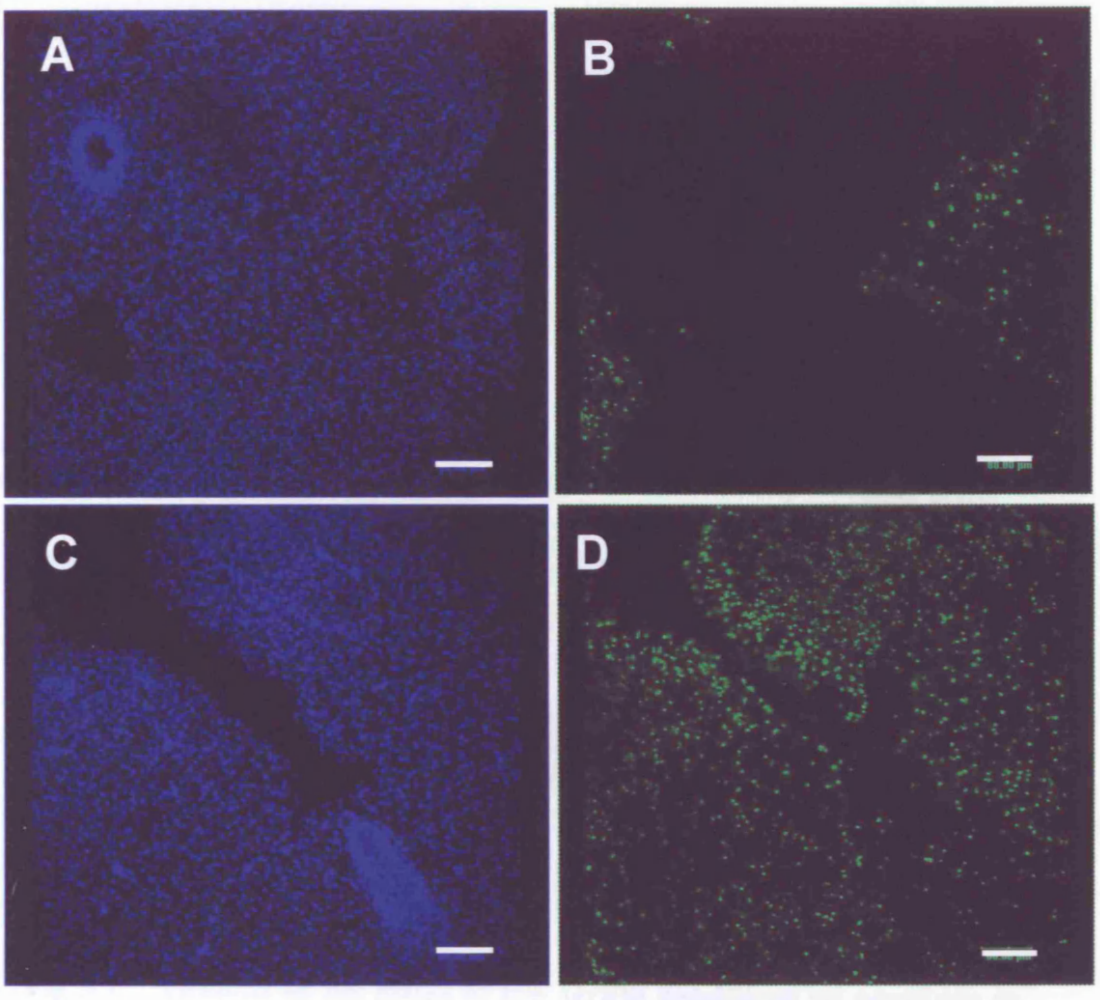

Fig. 5.16. Analysis of the effects of low serum on E15 spinal cord slice cultures. E15 slice culture maintained in 10% serum (A & B) or 1% serum (C & D) for 24 hours before whole mount TUNEL staining (B & D, green). Nuclei are counterstained with To-Pro-3-isomerase (A & C, blue). There is a noticeable increase in apoptosis in slices cultured in 1% serum. Scale bars = 80 μm

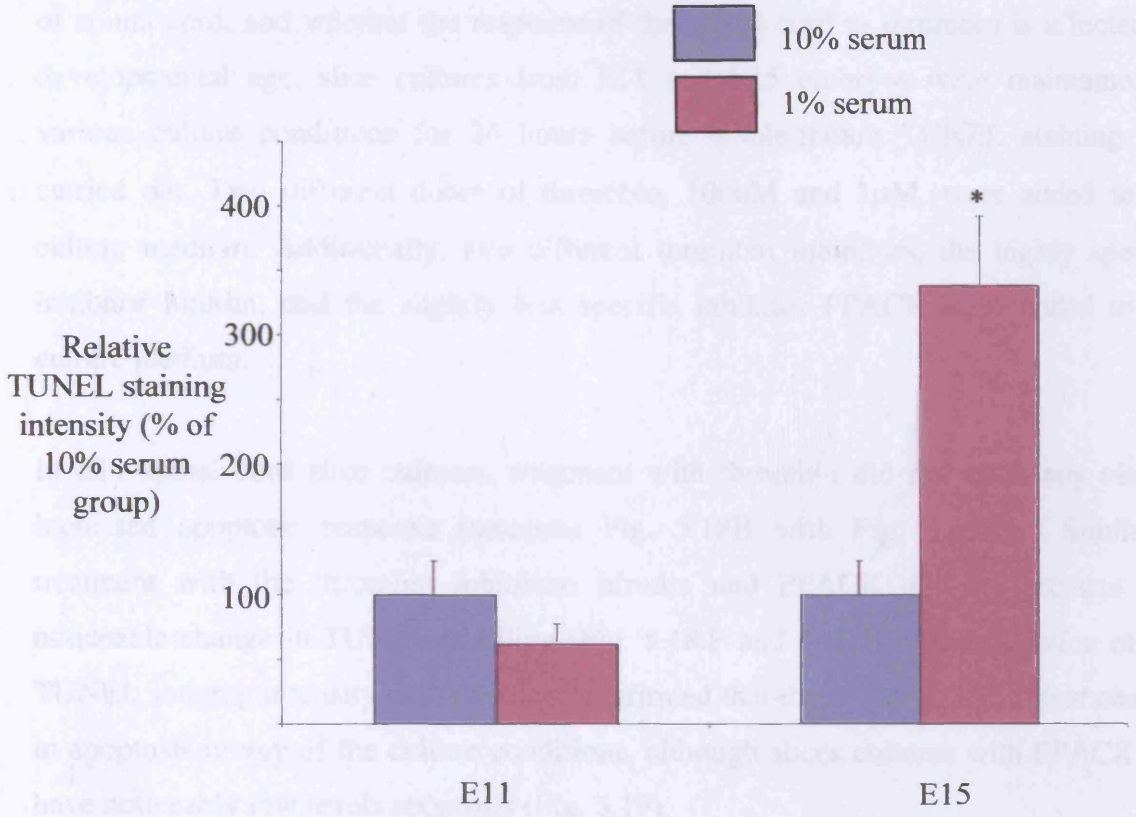

Fig 5.17. Analysis of the effect of low serum conditions on apoptosis in spinal cord slice cultures. Data show relative intensity of TUNEL staining for apoptotic cells in slice cultures of E11 or E15 spinal cords maintained for 24 hours in 10% or 1% serum. Levels of apoptosis were not affected by low serum levels in E11 slice cultures. However, apoptosis increased significantly in the presence of low serum concentrations in E15 slice cultures. $p = 0.551$ for E11, * $p<0.001$ for E15 (Mann Whitney Test)

5.2.6. Analysis of the effects of exogenous thrombin and thrombin inhibitors on apoptosis in E11 and E15 slice cultures

In order to determine whether thrombin could trigger apoptosis in organotypic cultures of spinal cord, and whether the response of the spinal cord to thrombin is affected by developmental age, slice cultures from E11 and E15 embryos were maintained in various culture conditions for 24 hours before whole-mount TUNEL staining was carried out. Two different doses of thrombin, 100nM and 1μM, were added to the culture medium. Additionally, two different thrombin inhibitors, the highly specific inhibitor hirudin, and the slightly less specific inhibitor PPACK were added to the culture medium.

In E11 spinal cord slice cultures, treatment with thrombin did not elicit any visibly increased apoptotic response (compare Fig. 5.18B with Fig. 5.15B). Similarly, treatment with the thrombin inhibitors hirudin and PPACK did not produce any noticeable change in TUNEL labelling (Fig. 5.18.F and 5.18.H). Quantification of the TUNEL staining intensity in these slices confirmed that there was no significant change in apoptosis in any of the culture conditions, although slices cultures with PPACK did have noticeably low levels apoptosis (Fig. 5.19).

Next, the effect of thrombin on E15 slice cultures was tested. As shown in Fig. 5.20B (compare with Fig 5.16.B) and Fig. 5.21, there was no increase in apoptosis in E15 slices after thrombin treatment. Similarly, the thrombin inhibitors hirudin and PPACK did not produce any significant changes in the apoptotic response as compared to the control (Compare Fig. 5.20F and Fig. 5.20H with Fig. 5.16.B).

In order to determine whether there was any interaction between the effects of low serum conditions and thrombin on apoptosis, the slices were also cultured in 1% serum with the addition of 1μM thrombin. In the E11 slices, treatment with both thrombin and low serum did not produce any increased apoptotic response (Fig. 5.18.D compared to Fig. 5.15.B, and Fig. 5.19). Similarly, treatment of the E15 cultures with thrombin and low serum conditions did not result in significantly increased apoptosis as compared to the control (Fig. 5.20.D compared to Fig. 5.16.B). This is confirmed by quantification

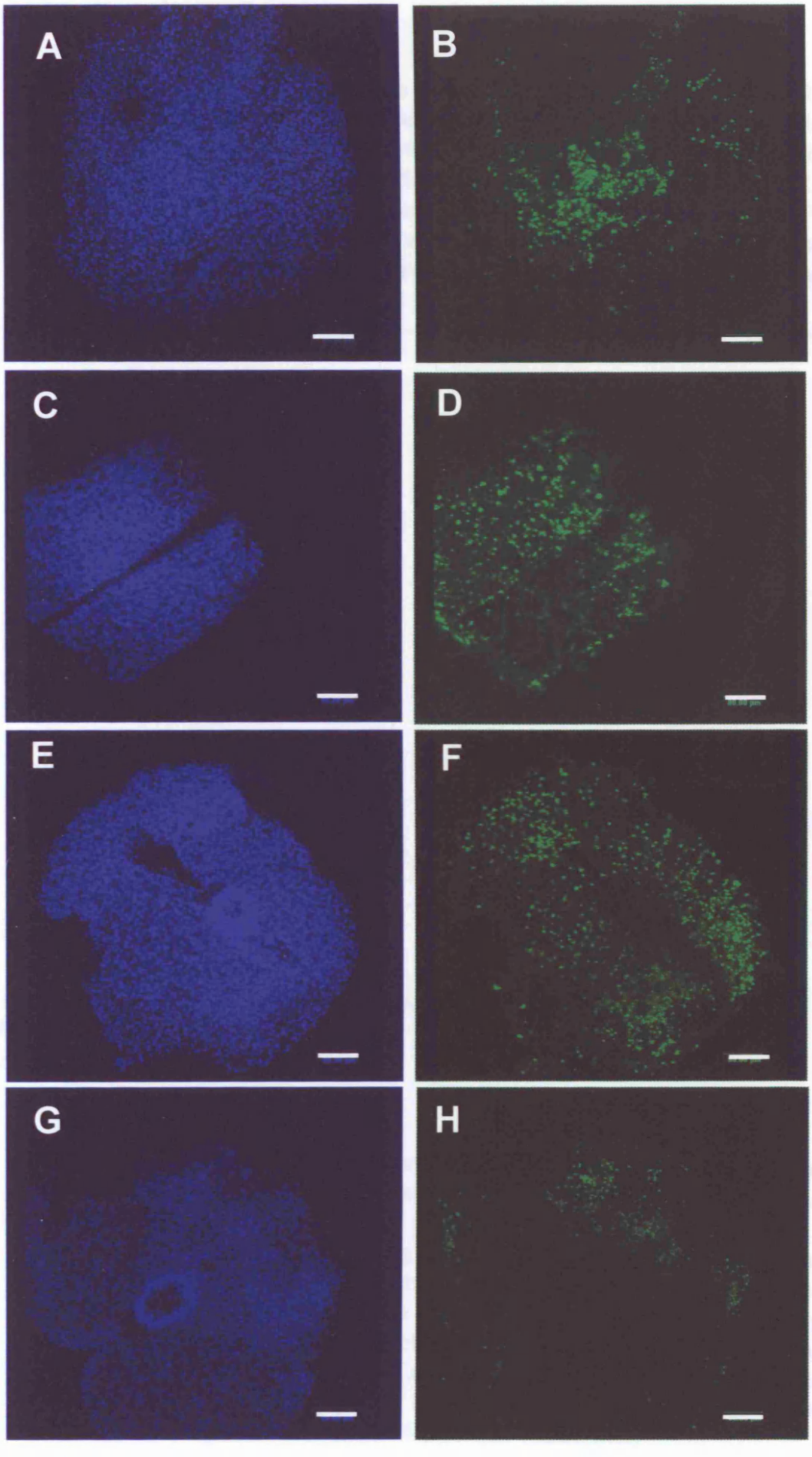
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Fig. 5.18 (previous page). Analysis of the effect of thrombin and thrombin inhibitors on apoptosis in E11 slice cultures. E11 slice culture maintained in medium containing 1μM thrombin with 10% serum (A & B), 1μM thrombin with 1% serum (C & D), hirudin with 10% serum (E & F) or PPACK with 10% serum (G & H) for 24 hours before whole mount TUNEL staining (B,D,F,H green). Nuclei are counterstained with To-Pro-3-isomerase (A,C,E,G blue). There is no noticeable increase in apoptosis above control (Fig. 5.16.B) in any culture condition. Scale bars = 80μm

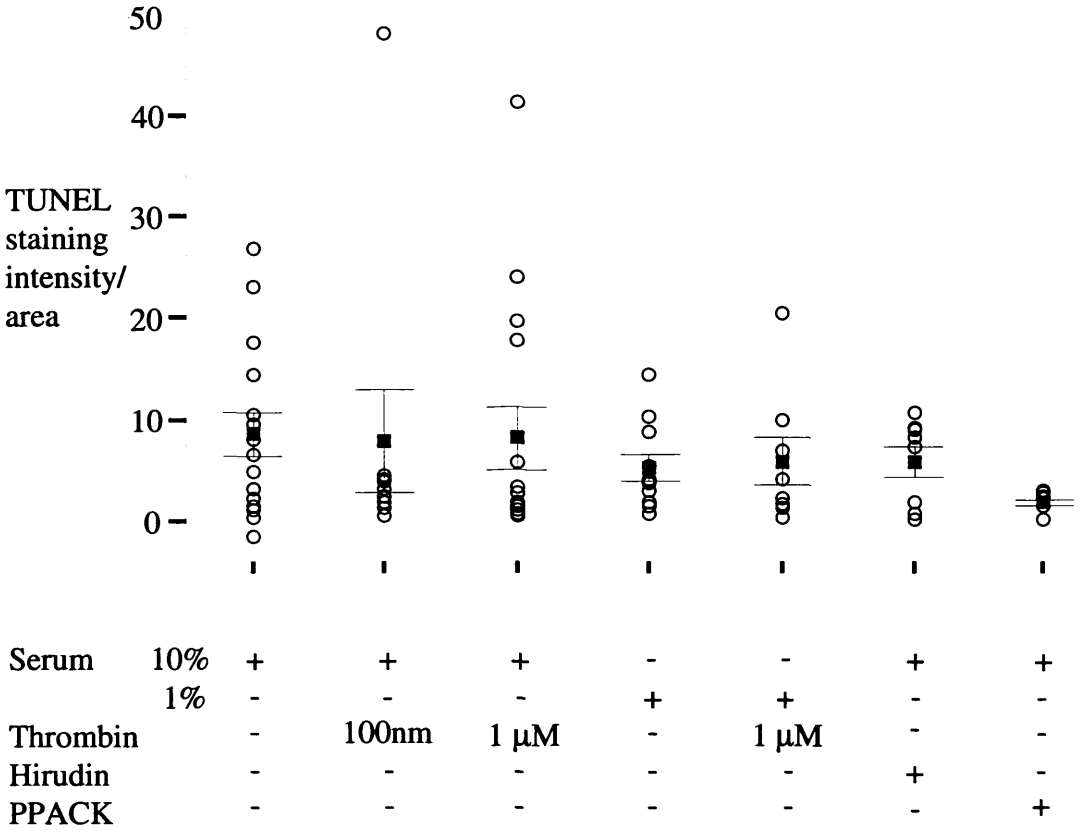

Fig. 5.19. Quantification of the effects of exogenous thrombin and thrombin inhibitors on apoptosis in E11 spinal cord slice cultures. Dot-plots showing TUNEL intensity in organotypic slice cultures of E11 spinal cord maintained for 24 hours in the presence of increasing concentrations of thrombin, the thrombin inhibitors hirudin and PPACK, thrombin in combination with low serum levels and low serum levels alone. Images are captured by confocal microscopy at 10 μm intervals through the first 40 μm of each slice after whole mount TUNEL staining and fluorescence intensity is measured and normalised to area. Data shows mean ± SEM for at least 6 samples per group. No significant change in TUNEL intensity is observed with any treatment. P=0.436 (Kruskal-Wallis Test)

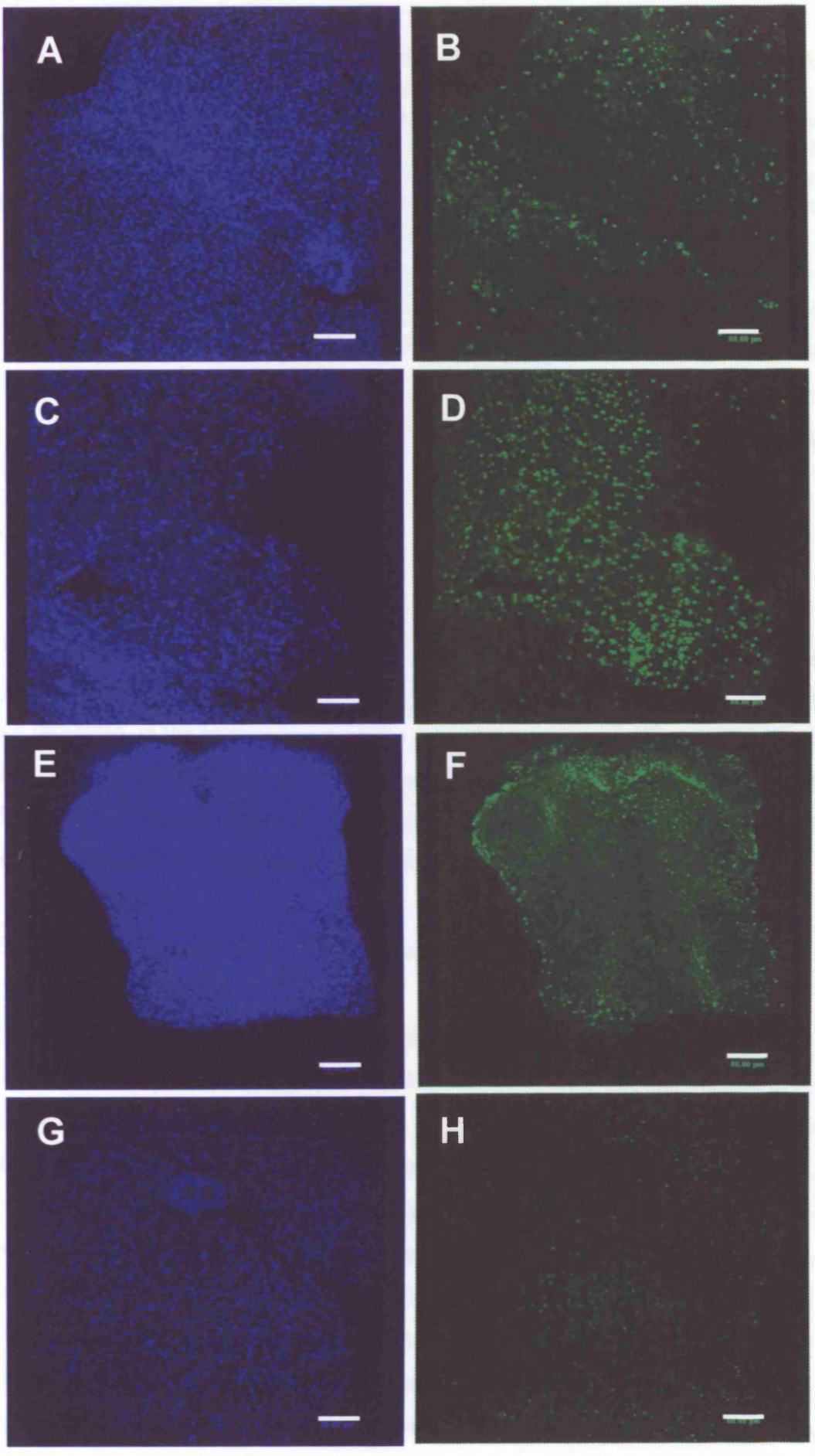
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Fig. 5.20. (previous page) Analysis of the effect of thrombin and thrombin inhibitors on apoptosis in E15 slice cultures. E15 slice culture maintained in medium containing 1μM thrombin with 10% serum (A & B), 1μM thrombin with 1% serum (C & D), hirudin with 10% serum (E & F) or PPACK with 10% serum (G & H) for 24 hours before whole mount TUNEL staining (B,D,F,H green). Nuclei are counterstained with To-Pro-3-isomerase (A,C,E,G blue). There is no noticeable increase in apoptosis above control (Fig. 5.17.B) in any culture condition. Scale bars = 80μm

of the TUNEL staining which reveals that there is no significant change in the apoptotic response in the presence of thrombin and low serum as compared to controls at E11 or E15 (Fig. 5.21). This suggests that the addition of thrombin to the medium containing 1% serum may to some extent rescue the E15 slice cultures from the increased apoptosis occurring in the presence of low serum concentrations (Fig. 5.20.B)

5.3. Discussion

In this chapter, two parallel sets of experiments were carried out to investigate the potential role of endogenous and exogenous serine proteases, with a particular emphasis on thrombin, in the response of the chick spinal cord to injury. The results demonstrated that, in contrast to reports in other species thrombin is not significantly regulated by injury in this model. However, the study indicated that further investigation of other serine protease family members might be of interest.

5.3.1. The use of real-time PCR for gene expression analyses

Following initial experiments in which expression of prothrombin and urokinase mRNA was confirmed by conventional RT-PCR, a real-time RT-PCR approach was chosen to study the endogenous expression levels of thrombin and urokinase in the spinal cord. This technology allows for the accurate determination of small changes in gene expression. For the purposes of these experiments, relative changes in gene expression between different samples are more relevant than absolute quantification and the comparative C_T method was therefore chosen for data analysis. A number of different factors must be taken into consideration when using this method. In particular the choice of housekeeping gene must be taken with care as a number of studies have highlighted the limitations of various commonly used genes (Bustin 2002; Dheda *et al.* 2005). In this study GAPDH was chosen as a housekeeping gene principally because it

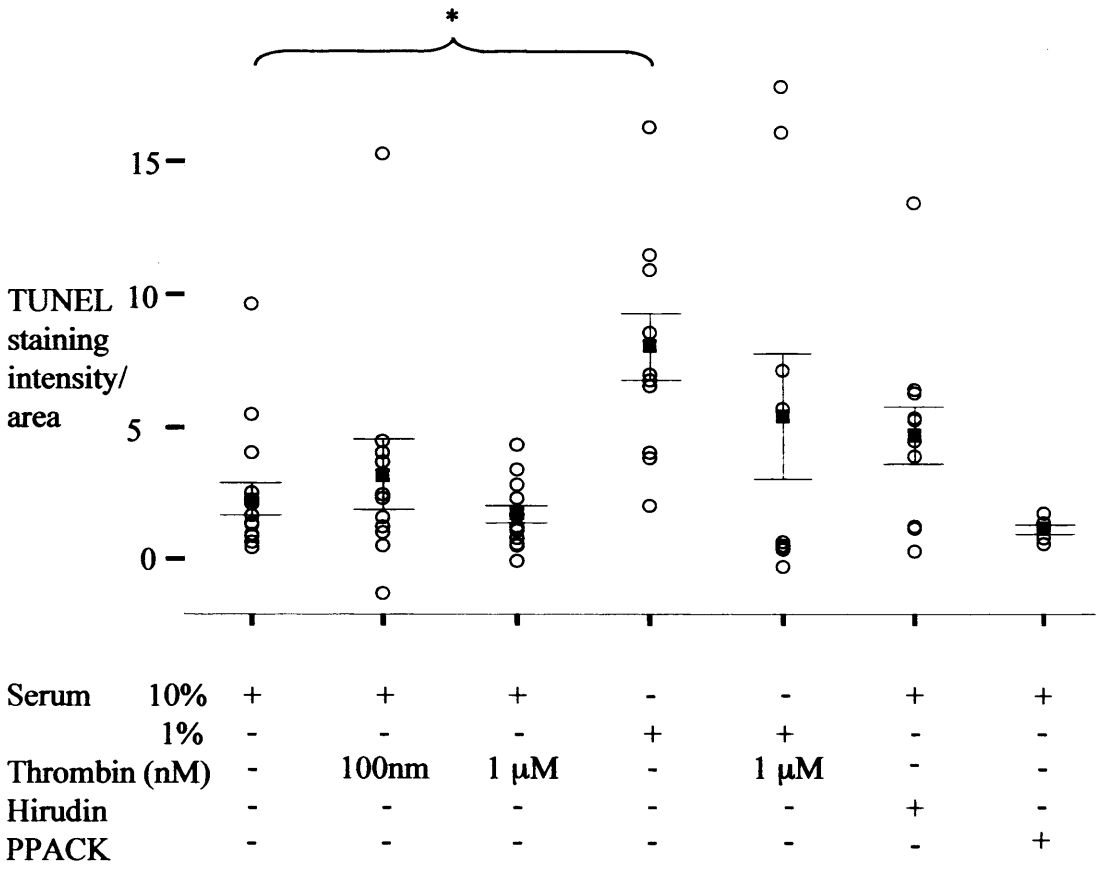


Fig. 5.21. Quantification of the effects of exogenous thrombin and thrombin inhibitors on apoptosis in E15 spinal cord slice cultures. Dot-plots showing TUNEL intensity in organotypic slice cultures of E15 spinal cord maintained for 24 hours in the presence of increasing concentrations of thrombin, the thrombin inhibitors hirudin and PPACK, thrombin in combination with low serum levels and low serum levels alone. Images are captured by confocal microscopy at 10 μm intervals through the first 40 μm of each slice after whole mount TUNEL staining and fluorescence intensity is measured and normalised to area. Data shows mean ± SEM for at least 6 samples per group. No significant change in TUNEL intensity is observed with thrombin or thrombin inhibitor treatment, although a significant increase in TUNEL intensity is observed in low serum conditions. P=0.002 (Kruskal-Wallis Test), *p<0.002 (Mann Whitney Test with Bonferroni adjustment).

has been used reliably in the past for this purpose. In this study no obvious changes in GAPDH expression were noted in different experimental conditions. Comparison of 'relative expression' values obtained using a different housekeeping gene, the 18S RNA, was carried out on one set of samples and did not reveal obviously different results to those using GAPDH. Nevertheless, finances permitting, ideally more than one housekeeping gene would be used in every experiment to validate the results. As described in Chapter 3, the comparative C_T method assumes that the primer sets used for both endogenous control and experimental genes have equivalent amplification efficiency. This was therefore validated for each set of primers to be used in the current experiments. One major disadvantage of real-time PCR is that, as a relatively new methodology, conventions on data analysis and in particular statistical analysis of results are still in the process of being established. The comparative C_T method involves a series of mathematical manipulations, starting with the C_T read-outs from the software, to produce a measurement of 'relative expression', in which all the samples are expressed relative to one sample which is nominated as a 'calibrator', as outlined in Chapter 3. However as yet there is no established convention on the best method of reporting these results or comparing them statistically, although a number of recent papers have attempted to address this issue (Ginzinger 2002; Livak and Schmittgen 2001). In the current experiments, each of the three individual pools of samples run for each experimental group was separately converted to 'relative expression' and statistical analysis was also performed at this stage, according to previously reported guidelines (Livak and Schmittgen, 2001). There may be other valid methods of carrying out the data analysis for these experiments. However, given that in this case the results did not indicate any large changes in gene expression, these would be unlikely to affect the overall outcome of these experiments.

It was noted in the course of these experiments that there was often some detection of amplification in negative control samples that contained RNA but did not undergo reverse transcription, although reactions containing only water in place of RNA were almost always negative. This suggests that any contamination must have been in the RNA samples themselves, despite all attempts to minimise this risk. These controls were, however, always amplified only at very high cycle numbers, suggesting that they would not significantly impair the experimental analyses.

5.3.2. Analysis of serine protease expression in the developing chick spinal cord.

The expression of serine proteases in the nervous system has been investigated to some extent in rodent embryo development (Dihanich *et al.* 1991; Sumi *et al.* 1992); however, their expression in the developing chick spinal cord has not been described. Therefore it was important first to establish whether these molecules are expressed in the chick spinal cord at relevant stages and whether any developmental changes in expression could be observed which might correspond to the loss of regenerative capacity. The results described in this chapter demonstrate that both prothrombin and urokinase are expressed in the chick spinal cord throughout development. There was no particular correlation between the levels of expression of either prothrombin or urokinase mRNA and the transitional period from E11 to E15, suggesting that regulation of these genes at the transcriptional level does not make an important contribution to the loss of regenerative capacity. In previous experiments in the rat, prothrombin mRNA was demonstrated to be expressed in the brain from E13 onwards, with a decrease in the level of expression postnatally, followed by an up-regulation during adulthood (Dihanich *et al.* 1991). The results of the current study show that prothrombin expression is present at slightly earlier stages in the chick than that reported in the rat and no significant changes in expression level are observed at later stages. Urokinase mRNA has been previously demonstrated to be expressed in the spinal cord at E12.5 but not at E10.5 in the rat, and to increase into early postnatal life (Sumi *et al.* 1992). These previous findings have pointed to a role for urokinase in axonal growth during development. The temporal expression pattern of urokinase mRNA in the chick demonstrated in the current study, with low levels of expression during early development and a trend towards high levels of expression at later stages, agrees with these previous findings, and also with preliminary results obtained using semi-quantitative PCR. The remit of the current study did not include an in depth analysis of the function of these serine proteases in spinal cord development in the chick; however it would be of interest in the future to determine the localisation of the expression of serine protease mRNA and protein in the developing chick spinal cord.

5.3.3. Analysis of serine protease expression and activity after injury

In previous experiments, in various animal models of injury or insult to the nervous system, endogenous levels of serine proteases have been shown to be altered after injury

(Friedmann *et al.* 1999). Of particular note, in a rat controlled weight drop model of spinal cord injury, thrombin, its receptor PAR-1 and its specific inhibitor PN-1 were all up regulated, as were tPA and urokinase (Citron *et al.* 2000b). However, the results presented in this chapter suggest that, in the developing chick spinal cord, alterations in endogenous prothrombin mRNA expression or thrombin activity levels are unlikely to play a major role after spinal cord injury.

No changes in gene expression were observed at either 2 hours or 24 hours after injury and activity levels of thrombin were unchanged at the same time points. These two time points were selected for investigation based on the timing of the apoptotic response in the chick, which has been shown to begin 8 hours after injury and to peak around 24 hours (O'Neill 2002). The experiments were therefore designed to examine the regulation of thrombin before and during the apoptotic response. These time points also correspond to previously reported changes in gene expression and thrombin activity in different experimental models (Citron *et al.* 2000b; Friedmann *et al.* 1999), and it is therefore possible that the time frame for any such changes in the developing chick might be different and therefore precluded from the current study.

The sample numbers used in these experiments were limited by both survival rates after injury and the number of samples which it was possible to run in any one experiment. This, together with inter-sample variability, means that a very small change in either the expression levels or activity of thrombin would not be detectable by these methods. Retrospective power calculations suggest that with these numbers a 4-fold change in gene expression could be detected with greater than 97% power. Similarly, a 3-fold change in specific thrombin activity could be detected with greater than 98% power. However, it is possible that changes smaller than this might be of biological significance and missed by this assay. For example, in the rat spinal cord a 1.4-fold increase in prothrombin mRNA levels was observed after injury using a Gene Filter hybrisisation technique while in a model of cerebral iscahemia prothrombin mRNA levels were increased by approximately 2.5-fold (Citron *et al.* 2000b; Riek-Burchardt *et al.* 2002). The sensitivity of the assay could be improved by increasing the sample numbers, although this would have to be balanced by the increase in variability likely if more than one plate is required. Nevertheless, these experiments do not provide any evidence for a large up-regulation of thrombin activity in the chick spinal cord after injury.

These results do not necessarily mean that endogenous thrombin does not contribute to tissue damage after injury. It is clear that active thrombin is present in the spinal cord at relevant stages of development and it is possible that changes in the expression of the thrombin receptor or the levels of thrombin inhibitors could result in a greater amount of thrombin signalling after an injury. Changes in the levels of PAR-1 and the serpin, protease nexin-1, have been observed in a number of models of traumatic injury to the nervous system (Niclou *et al.* 1998; Citron *et al.* 2000b; Striggow *et al.* 2001). Analysis of the expression levels of these molecules in the chick spinal cord after injury would be required to investigate this possibility. Additionally, it would be interesting to determine whether there are changes in the localisation of thrombin activity within the spinal cord after injury. Methods for investigating thrombin activity *in situ*, using membranes impregnated with a chromogenic thrombin substrate which can be placed over frozen sections, have been described (Imokawa and Brockes 2003). However, initial attempts to use this method in the current experiments were unsuccessful and the procedure would need to be fully optimised for this application.

Whereas significant changes in thrombin activity were not detected in this study, the results presented here do suggest that there may be a greater role for other endogenous serine proteases. The overall protease activity in the spinal cord was increased at 2 hours and even more so at 24 hours after injury at E15, as determined by the non-specific cleavage of the substrate CS-01(38). A number of other serine proteases are able to cleave this substrate including tPA, activated protein C and plasmin, although cleavage by urokinase has not been described (www.chromogenix.com). Other serine proteases which could be involved in tissue damage and cell death after spinal cord injury include the plasminogen activators tPA and urokinase. These molecules have been demonstrated to be up-regulated after spinal cord injury in the rat (Citron *et al.* 2000). Modulation of the activity of plasminogen activators has demonstrated the detrimental effects of these molecules in a number of models of nervous system damage including spinal cord injury (Abe *et al.* 2003; Chen and Strickland 1997; Tsirka *et al.* 1995; Wang *et al.* 1998). As shown in the results presented in this chapter, endogenous urokinase mRNA expression in the spinal cord was not increased after injury. However, changes in the specific activity of urokinase after injury were not determined in this experiment. The extent to which urokinase can cleave the substrate used in this study, CS-01(38), is unknown; however, specific chromogenic substrates for urokinase do

exist and these could be used to investigate this further. It would also be of interest to determine the duration of the elevated serine protease activity, by examining later time points after injury.

Although the study presented in this chapter was designed to investigate the role of serine proteases in the non-regenerating chick spinal cord, it might also be of interest to carry out similar analyses in the regenerating (E11) spinal cord. Although the potentially detrimental effects of thrombin after injury have been highlighted by a number of studies, other findings have pointed to a role for thrombin in the regenerative response of the newt limb and lens (Imokawa and Brockes 2003; Tanaka *et al.* 1999). It would therefore be interesting to determine whether this molecule might be involved in the regenerative response of the E11 spinal cord.

Gene expression changes after injury have been successfully investigated in both human and rodent tissue by micro array gene expression analyses. This type of investigation has the ability to assess a large number of potential candidate molecules for up or down-regulation after injury. Considering the vast array of genes expressed within a tissue such as the spinal cord, many of which have the potential to be involved in neuropathological situations, this approach could save both time and money in comparison to investigating each candidate gene individually. Unfortunately, at the time of commencement of the current investigations, gene chips useful for this type of analysis in the chick were not available and so this approach was not feasible. However, in more recent years this technology has become more widely used and such gene chips are now available. This therefore represents an excellent opportunity to determine the relative changes in expression of various genes, including a comprehensive analysis of the serine proteases, serpins and their receptors.

5.3.4. The effects of exogenous thrombin on apoptosis in slice cultures

In parallel to the investigation of changes in endogenous levels of serine proteases, the effects of exogenous serine proteases on the spinal cord were examined. Following a traumatic injury to the nervous system, haemorrhage and the disruption of the blood brain barrier expose the spinal cord to molecules carried in the blood, including thrombin, which are potentially neurotoxic. The *in vitro* effects of thrombin on neural

cell types suggest that the arrival of high concentrations of thrombin after an injury could have an effect on cell viability (Turgeon *et al.* 1998; Turgeon and Houenou 1999; Donovan *et al.* 1997; Pike *et al.* 1996; Smirnova *et al.* 1998; Smith-Swintosky *et al.* 1995). In the current study, the potentially toxic effects of thrombin were confirmed initially by examining the effects of thrombin treatment in neuroblastoma cells, in which an altered morphology, reminiscent of the cell death processes, was observed. Interestingly, cells which were attached to plastic rather than glass, appeared to be more resistant to the effects of thrombin. This finding is in agreement with previous studies which have shown that the susceptibility of neuroblastoma cells to toxic stimuli is regulated by integrin-mediated cell adhesion (Bozzo *et al.* 2004). The primary aim of this part of the study was therefore to determine whether exogenous thrombin treatment could similarly cause apoptosis in the developing chick spinal cord.

In order to investigate the effects of exogenous thrombin treatment on apoptosis it was decided to set up an organotypic culture system of the spinal cord. This overcomes a number of the problems associated with treating the spinal cord directly with thrombin, which is both technically difficult and likely to prove toxic, due to its potential to cause blood clotting. Also, by using slice cultures, it was possible to obtain several samples from each embryo, minimising the number of animals which needed to be used in order to produce a quantitative result. By culturing the slices in hanging drop cultures, the amount of medium and therefore pharmacological treatments required was minimised, again allowing the numbers of samples which could be tested to be increased. Finally, the organotypic culture system has advantages over a cell culture model, as it more closely represents the cellular environment which would be found *in vivo*. This is of particular importance given that the results obtained with cultured neuroblastoma cells strongly suggested that cellular attachment could modify the responsiveness to thrombin treatment.

The ability of the system to demonstrate changes in apoptosis was validated by comparing the effects of culturing in low serum conditions between regenerating and non-regenerating stages of the chick spinal cord. The E11 spinal cord was demonstrated to be resistant to low serum conditions, while, in contrast, the E15 spinal cord showed a large increase in apoptosis in low serum conditions. This developmental change in the response of the spinal cord cultures to low serum conditions mimics the *in vivo* response

to injury, in which there is a much greater apoptotic response at E15 than at E11, which suggests that this model appropriately represents the *in vivo* situation.

The effects of exogenous thrombin treatment on apoptosis at E11 and E15 were compared in the slice culture system. From these results, exogenous thrombin does not appear to cause an apoptotic response in the spinal cord at either developmental stage. These results therefore differ from reports in the literature demonstrating that exogenous thrombin can cause increased apoptosis in several *in vitro* systems (Donovan *et al.* 1997; Smirnova *et al.* 1998; Smith-Swintosky *et al.* 1995; Turgeon *et al.* 1998; Turgeon and Houenou 1999). Similarly, a number of previous studies have demonstrated the ability of thrombin inhibitors to reduce cell death in various *in vitro* models (Smith-Swintosky *et al.* 1995; Houenou *et al.* 1995). In contrast, the results of the current study show that inhibition of endogenous thrombin activity in the slices by two different thrombin inhibitors did not result in any significant changes in the apoptotic response, although the less specific inhibitor, PPACK, did show a trend towards lower apoptosis. Overall, these findings suggest that endogenous thrombin in not involved in the signalling pathways leading to apoptosis in these cultures.

The discrepancies between results obtained in the current experiment and those reported in the literature may to an extent be explained by the different apoptotic stimuli involved in the experiments. Additionally, there was a fairly large variability in results obtained from different slices, which meant that only large differences in the apoptotic response could be distinguished statistically. This may have been accentuated by the fact that the results were obtained from a series of experiments due to practical difficulties in handling large sample numbers. This problem might therefore be alleviated by carrying out this type of testing on a larger scale in the future. One probable explanation for the differences between the results of the current study and those of previous reports is that the slice culture method may result in better protection of neurons and glia from cell death than the dissociated systems used in most previous experiments. This could be due to the presence of protective factors or cell types, or the reduced accessibility of the drug to its target cells or receptors. Additionally, the interaction of cells with their neighbours and the extracellular substrate might provide a protective effect against the effects of thrombin, as was observed in the neuroblastoma cultures. Organotypic slice culture protocols have been shown in some cases to be less

susceptible to neurotoxic stimuli (Keynes *et al.* 2004). However, thrombin has been shown at equivalent doses to those used in the current experiment to cause cell death in organotypic slice cultures of rat hippocampus (Striggow *et al.* 2000). It is also possible that these differences represent either differences between species, developmental stages or the CNS region being studied, with the hippocampus being more sensitive to thrombin-induced cell death. In the chick spinal cord the blood brain barrier may not be completely formed by the stages being examined in this study (Wakai and Hirokawa 1978). This may necessitate higher levels of endogenous protection against blood-borne neurotoxins at these stages than in the adult. It is also important to note that the current study investigated only apoptotic cell death; whereas cell death in the hippocampal slices following thrombin treatment was not defined as apoptotic or necrotic. Nevertheless, these results suggest that neither an increased susceptibility to thrombin-induced cell death at E15, nor the increased exposure of the E15 spinal cord to thrombin due to increased haemorrhage, is likely to account for the increased apoptosis seen at non-regenerating stages in the chick after injury.

However, it is also worth considering the possibility that a combination of thrombin with other active molecules, which might be present in the blood which enters the spinal cord after an injury but which were missing from this model, may be required in order to induce apoptosis. In order to address this issue, attempts were made to determine whether whole blood could induce apoptosis in the slice cultures. Unfortunately due to the blood clotting rapidly, and the desire not to pre-treat the blood with anticoagulants which might affect key factors such as thrombin, this was not possible. Factors present in blood which might act together with thrombin might include other serine proteases. Indeed one previous report has demonstrated that the detrimental effects of thrombin in a brain injury model are increased by treatment with plasminogen activators (Figueroa *et al.* 1998). It is interesting to note that treatment of the slice cultures with the thrombin inhibitor PPACK produced an indication of reduced apoptosis, although this did not prove statistically significant. As PPACK is also thought to inhibit the activity of other serine proteases to a lesser extent (Weitz and Hirsh 2001), this suggests that inhibition of the activity of one or more serine proteases other than thrombin might be neuroprotective. It would be of great interest to assess the potentially neurotoxic effects of other serine proteases such as urokinase in this model.

Although the main focus of this study was to investigate the potentially neurotoxic effects of thrombin in the chick spinal cord, there are indications that thrombin may also be of benefit to the injured nervous system. Some previously reported studies have demonstrated protective effects of low concentrations of thrombin against apoptosis following various stimuli (Pike *et al.* 1996; Vaughan *et al.* 1995). In the current study, thrombin treatment did not reduce apoptosis under normal culture conditions in 10% serum. However, the results suggest a slight reduction in the increased apoptotic response to low serum conditions in the E15 slice cultures. This suggests that thrombin may be neuroprotective against the apoptotic response induced by low serum conditions. Furthermore, as mentioned above, thrombin has also been reported to promote regenerative responses in the newt limb and lens by stimulating dedifferentiation and proliferative responses (Tanaka *et al.* 1999; Imokawa and Brockes 2003). It would be of interest to determine whether thrombin activity is involved in these processes in the regenerating chick spinal cord.

5.3.5. Serine proteases and the injury response

In this chapter, the hypothesis that serine proteases, and in particular thrombin, might have a role in the increased apoptotic response of the non-regenerating chick spinal cord to injury has been investigated. The results presented here suggest that although thrombin may not play this role in this system as it does in other experimental models, perhaps due to differences in the developmental stage or species used, other serine proteases are likely to be of importance. Importantly, the results demonstrate an increase in total serine protease activity after injury, pointing to a potential role for other serine proteases which cleave the substrate CS-01(38). This was also supported by the trend towards a decrease in the apoptotic response in slice cultures treated with the relatively non-specific thrombin inhibitor PPACK. This study has concentrated on the apoptotic response to injury; however, thrombin may have other detrimental effects within the injured nervous system. For example, thrombin has been shown to cause neurite retraction, growth cone collapse and microglial activation (Gurwitz and Cunningham 1988; Jalink and Moolenaar 1992; Suidan *et al.* 1992; Fritsche *et al.* 1999). The organotypic culture system which was devised for these experiments may represent a good model for screening for the effects of potentially neurotoxic molecules, including other serine proteases such as urokinase.

Chapter 6. Expression and regulation of doublecortin in the normal and injured chick spinal cord

6.1. Introduction

In order for the spinal cord to be capable of regeneration after injury, a sufficient number of viable neurons must be present to re-establish functional neural networks. As described in Chapters 4 and 5, cell death following injury is a major contributor to neuronal loss and neuroprotective strategies represent one way to improve the ability of the spinal cord to recover from injury. Another therapeutic avenue which may be explored would be to enhance the endogenous repair responses of the spinal cord and this may include the replacement of lost or damaged cells with newly generated cells from pools of endogenous neural stem or progenitor cells. In assessing the likely success of this strategy, an understanding of the relative contribution of endogenous neurogenesis to repair in regenerative species would be of great benefit.

Endogenous stem cells capable of differentiating into neurons and glia, as well as cells with a more restricted potential, have been identified in the mammalian brain and spinal cord (Gage 2000; Horner *et al.* 2000; McKay 1997; Nunes *et al.* 2003; Shihabuddin *et al.* 2000; van der and Weiss 2000). A proliferative response to spinal cord injury has also been observed in the adult rat (Mothe and Tator 2005; Namiki and Tator 1999; Yamamoto *et al.* 2001). The presence of endogenous neural progenitors within the spinal cord does not result in significant regenerative capacity in the adult mammal but does suggest potential for the stimulation of these endogenous cells to produce replacement neurons and glia. In contrast to the situation in the adult mammal, in many regenerative lower vertebrate species proliferation and the subsequent generation of neural cells appears to make a significant contribution to the regenerative process (Benraiss *et al.* 1999; Nordlander and Singer 1978; Anderson *et al.* 1994). In species which undergo a developmental change in regenerative capacity, such as the opossum and the chick, the contribution of a proliferative response to the regenerative process is less clear and is of particular interest (Shimizu *et al.* 1990; Hasan *et al.* 1993; Saunders *et al.* 1998). In these species, most research to date has tended to focus on axonal regenerative capacity. However it is possible that a loss of an early ability to stimulate endogenous progenitors to replace lost cells may also be important. It is therefore of

interest to determine whether neurogenesis occurs after injury at regeneration-competent stages of chick development.

To this end, the identification of a marker of newly born neurons would be of great benefit and, in this study, the expression and regulation of doublecortin, a microtubule-associated protein which is expressed early during neuronal development and has been shown to accurately reflect levels of neurogenesis in the adult mammalian brain was investigated (Couillard-Despres *et al.* 2005; Francis *et al.* 1999; Gleeson *et al.* 1999; Rao and Shetty 2004; Sossey-Alaoui *et al.* 1998). Doublecortin has been shown to be down regulated as neurons mature, resulting in its transient expression in newly born neuron populations (Brown *et al.* 2003). Doublecortin may therefore be a potentially useful marker for newly generated neurons in the embryonic chick nervous system. In addition to its potential role as a marker of neurogenesis, doublecortin has a number of features that make it an interesting molecule to consider in the context of spinal cord regenerative capacity. Doublecortin contributes to the control of microtubule stability and is likely be involved in situations in which cytoskeletal reorganisation is of importance (Bai *et al.* 2003; Gleeson *et al.* 1999; Horesh *et al.* 1999; Nacher *et al.* 2001; Shmueli *et al.* 2001).

The current study initially considered whether doublecortin might prove to be a useful marker for neurogenesis in the E11 chick spinal cord. In order to be suitable for this purpose doublecortin expression would have to be otherwise absent or low at this stage, as in the adult mammalian brain (Nacher *et al.* 2001). Neurogenesis in the chick spinal cord has been reported to be complete by E9 and the formation of major descending neuronal tracts is complete around E11 (Fujita 1964; Okado and Oppenheim 1985). These experiments therefore set out to determine whether doublecortin expression is down-regulated with development in the chick in a manner which would be compatible with its use as a marker of neurogenesis. In subsequent experiments changes in the expression of doublecortin in the normal and injured spinal cord were investigated in order to determine whether they are compatible with a role in controlling the regenerative capacity of the chick spinal cord.

6.2. Results

6.2.1. Selection of doublecortin antibody for use in the chick.

The expression of doublecortin in the chick spinal cord was investigated using an antibody against the C-terminal region of human doublecortin. As this antibody has not been previously used in chick, it was necessary to confirm the specific recognition of doublecortin in the chick. Protein samples from E11 chick spinal cord and liver were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with either the doublecortin antibody or antibody which had been pre-incubated with a blocking peptide. As shown in Fig. 6.1, in E11 chick spinal cord samples a doublet was detected at approximately 40kDa. This corresponds to the previously reported size of chick doublecortin. The second band observed probably corresponds to a phosphorylated form of the protein, as previously described (Tanaka *et al.* 2004). No bands were observed in the liver sample, confirming the neuronal specificity of doublecortin. Likewise, no bands were observed when the antibody was pre-incubated with blocking peptide, which confirms that the antibody recognises the correct antigen.

6.2.2. Expression of doublecortin protein in the developing chick spinal cord

The expression of doublecortin in the chick spinal cord was investigated firstly by immunostaining sections taken from embryos at various developmental stages. As shown in Fig. 6.2.A and B, at E4, doublecortin is expressed in the ventral and lateral parts of the neural tube, where the post-mitotic neurons reside. No doublecortin staining was observed at the ventricular surface, suggesting that doublecortin is not expressed in proliferating neuronal precursors. At E8 and E11, doublecortin is expressed at low levels throughout the grey matter (Fig. 6.2.C-F). In the E13 and E15 spinal cord, the strength of doublecortin expression is much lower than at earlier stages, and appears to have become restricted to the dorsal horns of the grey matter (Fig. 6.3.A-D). In order to confirm that the staining observed was due to specific binding to doublecortin, some sections were incubated with antibody that had been pre-incubated with blocking peptide. This resulted in the complete absence of any staining; suggesting that the staining observed with this antibody is specific (Fig. 6.3.E and F.). These results showed that doublecortin is down-regulated as development progresses. However, there

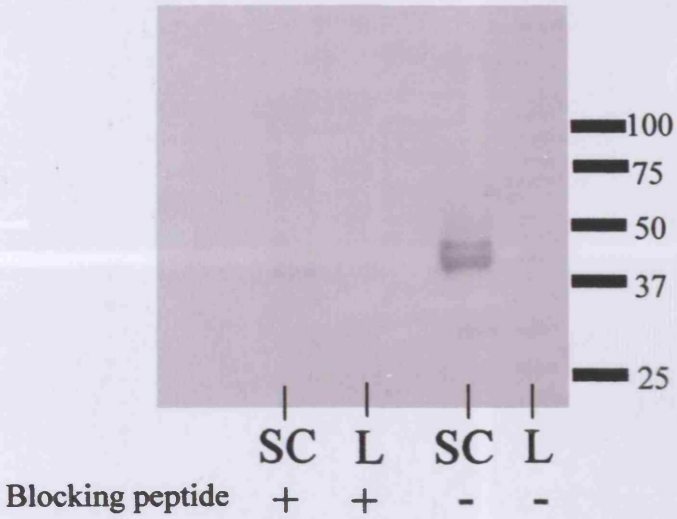


Fig. 6.1. Western blot showing specificity of doublecortin antibody in chick. Western blot showing binding of anti-doublecortin (C-18) in chick spinal cord (SC) and liver (L) samples. A 40kDa band is visible in the spinal cord samples but not in the liver. Half the samples were stained using the antibody after pre-incubation with blocking peptide to confirm specificity.

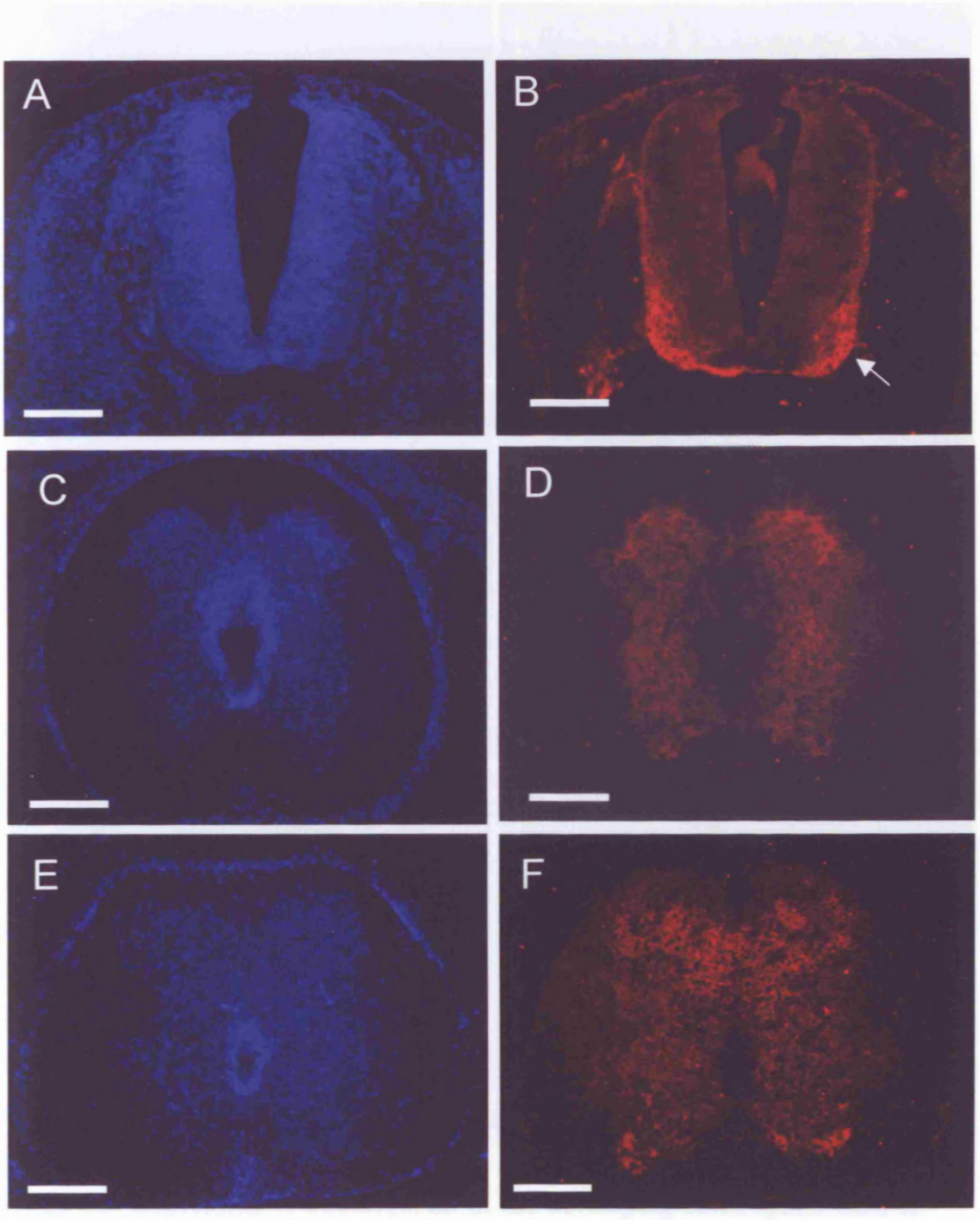


Fig. 6.2. Expression of doublecortin in the developing chick spinal cord I. Doublecortin immunostaining (red) and nuclear counterstaining with Hoechst (blue) in transverse sections of spinal cord at **A) and B)** E4 (arrow indicates doublecortin staining in the ventrolateral neural tube), **C) and D)** E8, and **E) and F)** E11. Scale bars: A,B-250 μm, B-F-500 μm. In all images dorsal is at the top of the picture.

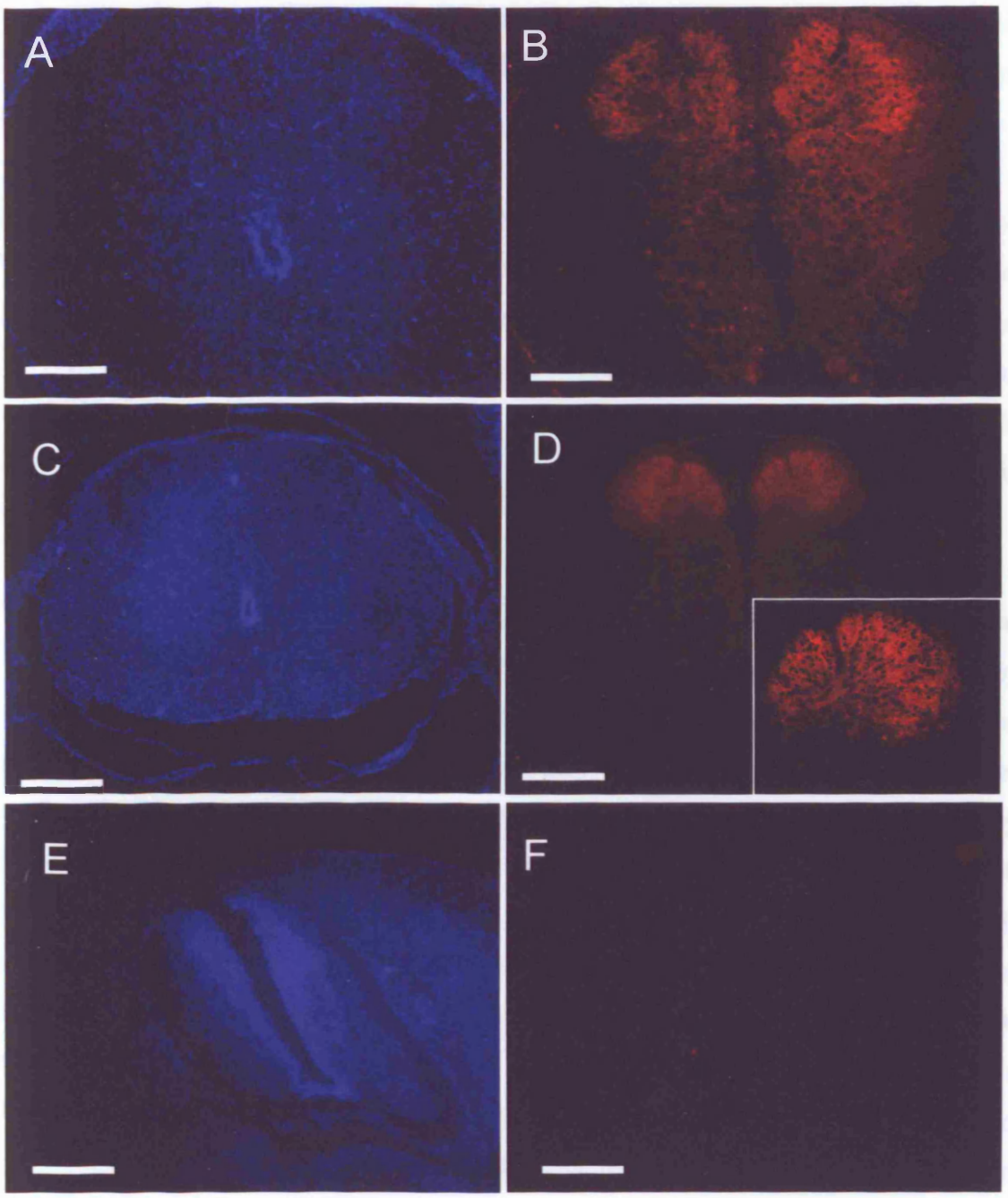


Fig. 6.3. Expression of doublecortin in the developing chick spinal cord II. Doublecortin immunostaining (red) and nuclear counterstaining with Hoechst (blue) in transverse sections of spinal cord at **A) and B)** E13, and **C) and D)** E15. Inset in D shows higher magnification view of doublecortin staining in the E15 dorsal horns. In order to confirm specificity of the antibody staining was carried out after pre-absorption with blocking peptide, producing a negative result as shown in **E and F**. Scale bars: A,B,E and F-500 μm and C and D-1000 μm. In all images dorsal is at the top of the picture.

is still a widespread low level expression of doublecortin throughout the grey matter at E11, suggesting that at this stage many neurons are still not fully mature. Doublecortin is therefore unlikely to be useful as a marker of neurogenesis in the spinal cord at this stage.

The results of the immunostaining study described above suggested a correlation between the level of doublecortin expression and developmental stage. In particular there appeared to be a reduction in the level and extent of doublecortin expression between E11 and E15. In order to confirm these results Western blotting was carried out on chick spinal cord samples collected at various developmental stages. As shown in Fig. 6.4, there was an overall reduction in the level of doublecortin protein present in spinal cord samples between E11 and E15.

As before, in some of the samples two bands were observed following blotting with the doublecortin antibody (Fig. 6.4.). Doublecortin has been shown to be regulated by phosphorylation and the upper band probably represents a phosphorylated form of the protein (Tanaka *et al.* 2004). In order to confirm this, half of the samples were pre-incubated with calf intestinal alkaline phosphatase (CIAP) before being separated by electrophoresis. Treatment with CIAP completely eliminated the upper band, confirming that this was a phosphorylated protein (Fig 6.4). In addition to the overall reduction in doublecortin levels with development, this analysis also revealed a reduction in the amount of the phosphorylated form of the protein. As shown in Fig. 6.4, at E8 and E11 the upper band representing the phosphorylated form of doublecortin is strong. However by E13, this band has been markedly down-regulated and by E15, no upper band can be observed. This demonstrates that the phosphorylation of doublecortin in the chick spinal cord is developmentally regulated over a time-period that corresponds to the loss of regenerative capacity.

6.2.3. Expression of doublecortin mRNA in the developing chick spinal cord

The results outlined above revealed that doublecortin protein is present at relatively high levels at fairly late stages of development in the chick spinal cord, and that this expression may be developmentally regulated. Doublecortin is thought to be expressed early during neuronal differentiation and migration and then down-regulated in mature

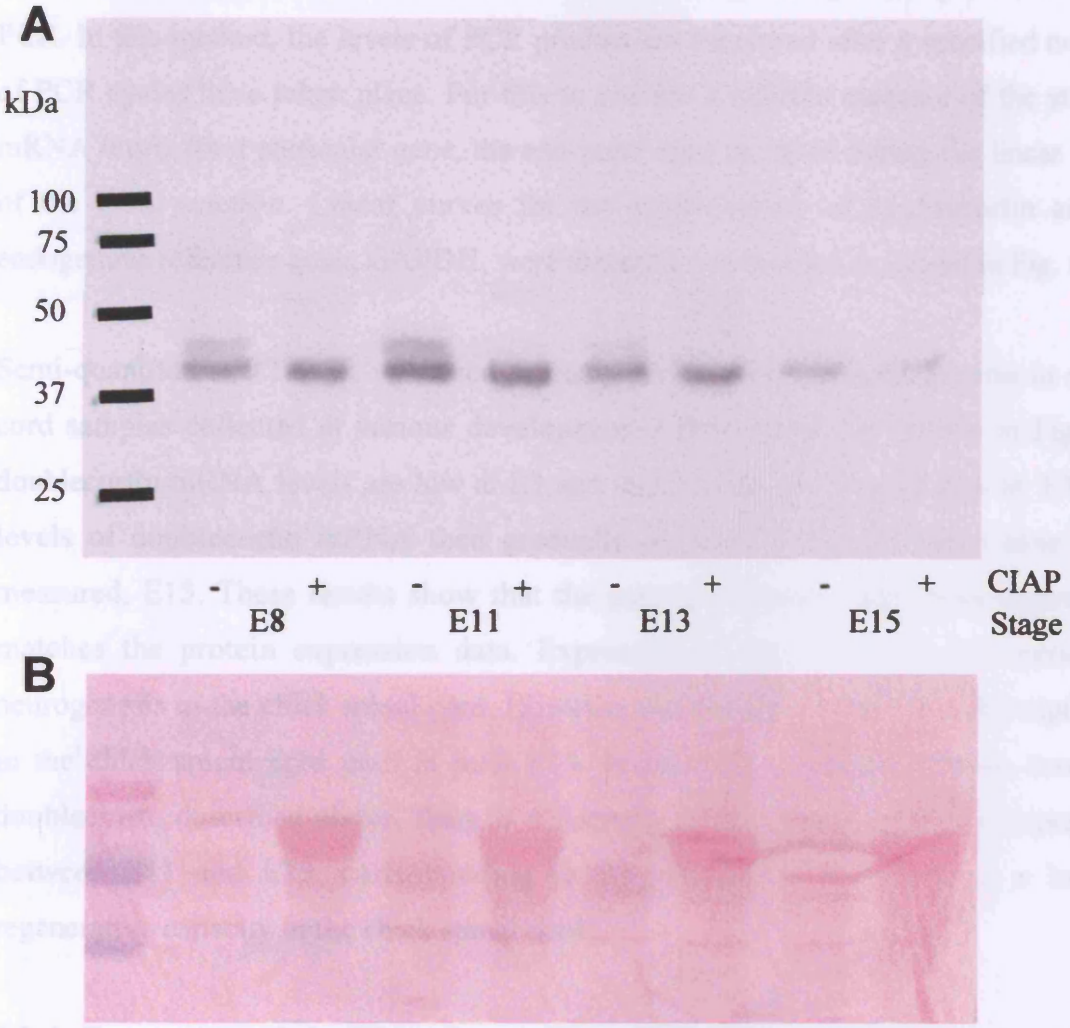


Fig 6.4. Doublecortin protein levels in the developing chick spinal cord A) Western blot data showing changes in doublecortin levels and phosphorylation at different developmental stages. Samples were incubated either with or without calf intestinal alkaline phosphatase (CIAP) before SDS-PAGE. **B)** Ponceau Red staining of the same blot to show protein loading.

neurons (Brown *et al.* 2003). In order to determine whether the presence of doublecortin in the chick spinal cord is the result of continued gene expression or persistence of the protein, the mRNA levels of doublecortin were investigated by semi-quantitative RT-PCR. In this method, the levels of PCR product are measured after a specified number of PCR cycles have taken place. For this to provide a reliable measure of the starting mRNA levels for a particular gene, the end-point must be taken during the linear phase of the PCR reaction. Linear curves for the amplification of doublecortin and an endogenous reference gene, GAPDH, were therefore constructed as shown in Fig. 6.5.

Semi-quantitative RT-PCR was used to compare the levels of doublecortin in spinal cord samples collected at various developmental time points. As shown in Fig. 6.6, doublecortin mRNA levels are low at E3 and then increase to a maximum by E7. The levels of doublecortin mRNA then gradually decrease up to the latest time point measured, E15. These results show that the pattern of doublecortin gene expression matches the protein expression data. Expression is highest during the period of neurogenesis in the chick spinal cord. However doublecortin continues to be expressed in the chick spinal cord until at least E15. In agreement with the protein levels of doublecortin described above, there is a decrease in doublecortin mRNA expression between E11 and E15, corresponding to the period in which there is a loss of regenerative capacity in the chick spinal cord.

6.2.4. Expression of doublecortin protein in the mouse and human developing spinal cord

Although doublecortin expression in the developing brain has been previously examined, its expression in the spinal cord has not been investigated to any great extent. In order to determine whether the expression pattern in chick spinal cord described above is conserved in other species, immunostaining was carried out in mouse and human spinal cord sections collected at various developmental stages.

In mouse embryos at E13.5 and E14.5, which corresponds to approximately E5-E7 in the chick, doublecortin expression was detected throughout the grey matter of the spinal cord (Fig. 6.7.A-D). However, in the adult mouse spinal cord, doublecortin expression

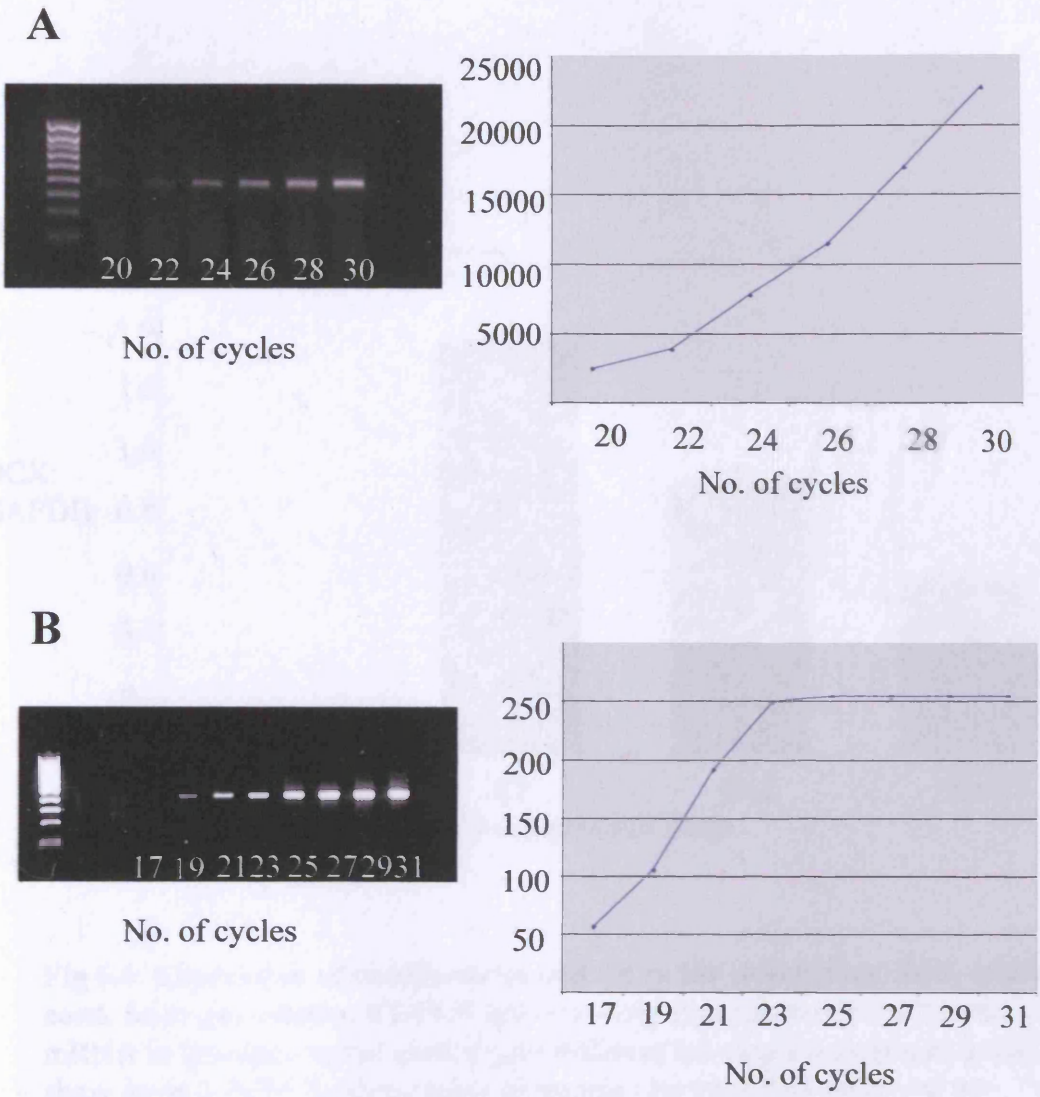


Fig. 6.5. Determination of the linear range for semi-quantitative RT-PCR. PCR reactions using **A)** doublecortin and **B)** GAPDH primers were stopped after various cycles and the PCR products were separated by gel electrophoresis. Band intensity was measured by densitometry and plotted to give an amplification curve. For following experiments a cycle number from within the linear part of the graph was chosen.

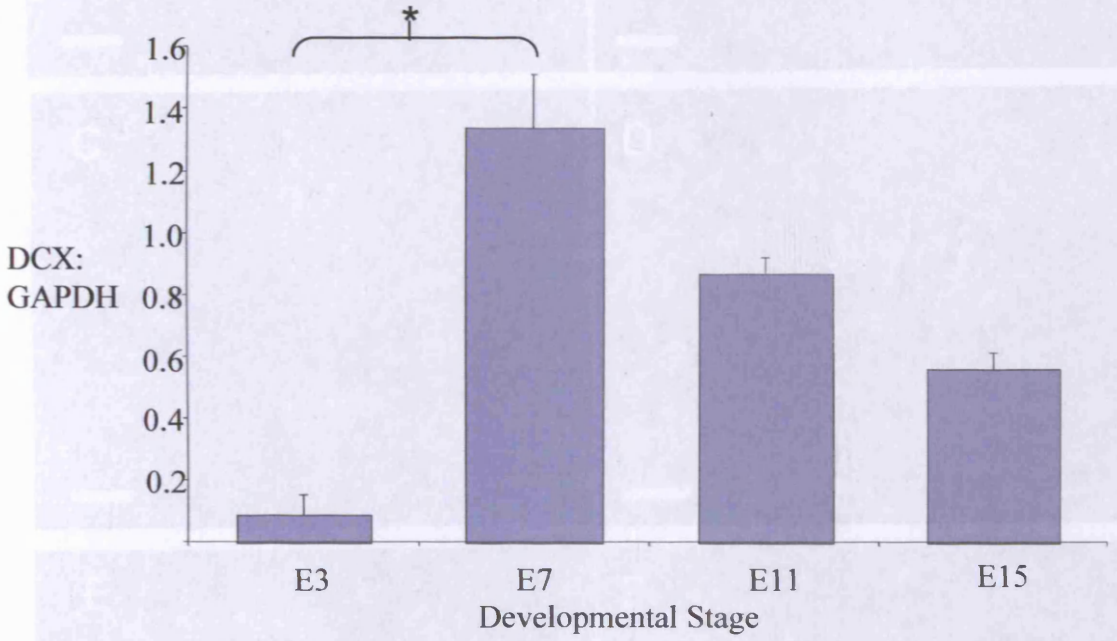


Fig 6.6. Expression of doublecortin mRNA in the developing chick spinal cord. Semi-quantitative RT-PCR data showing expression of doublecortin mRNA in the chick spinal cord at four different developmental stages. Data show mean ± SEM for three pools of samples for each data point and are normalised to GAPDH levels. Doublecortin mRNA is expressed throughout development, with the highest peak of expression around E7. * $p = 0.05$ (Kruskal Wallis test with post hoc paired comparisons).

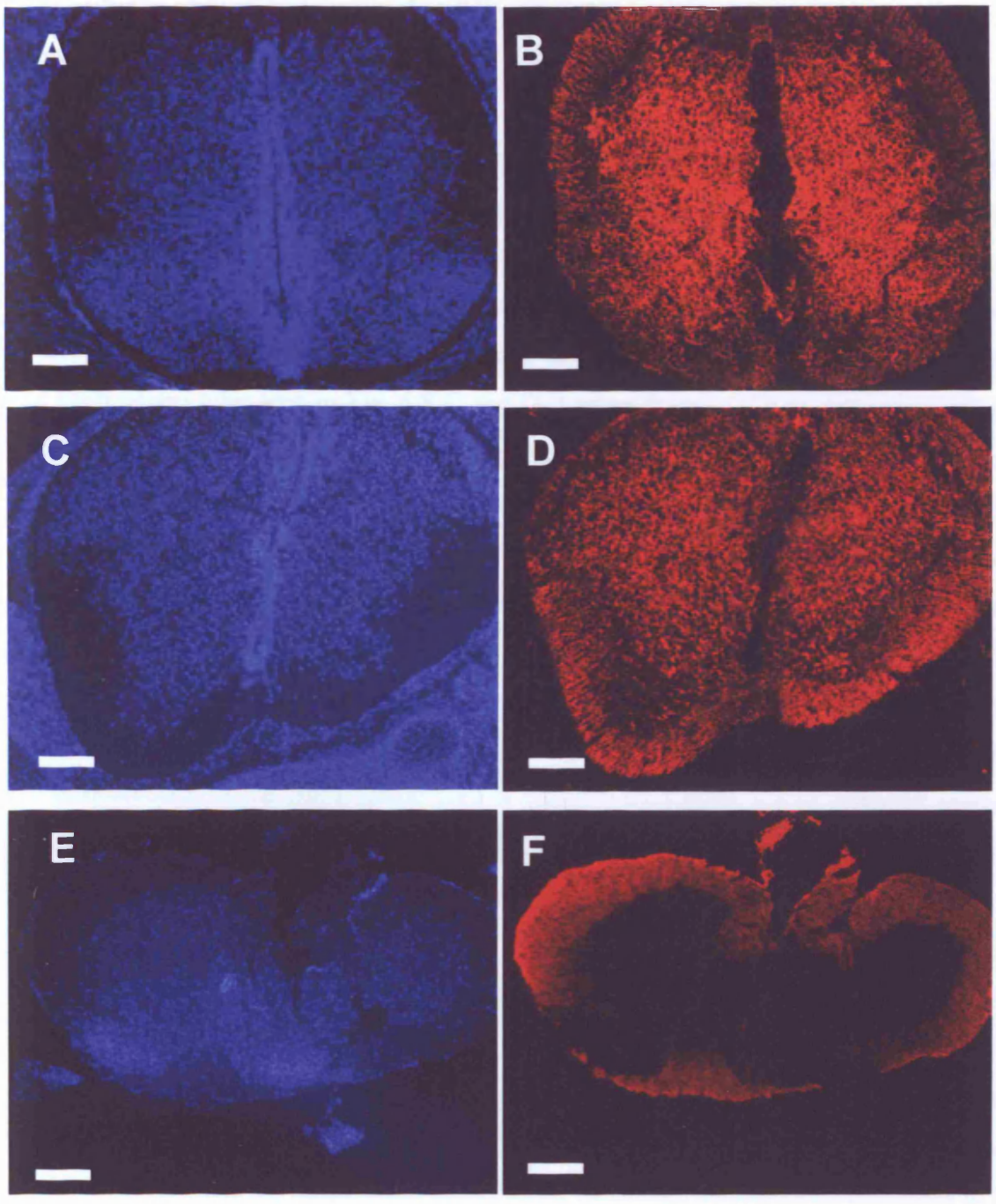


Fig. 6.7. Expression of doublecortin in the developing mouse spinal cord. DCX immunostaining is shown in red and nuclear counterstaining with Hoechst is shown in blue at **A) and B)** E13.5, **C) and D)** E14.5 and **E) and F)** adult mouse. Scale bars: A and B-200 μm, C-F – 100 μm. In all images dorsal is at the top of the picture.

was virtually absent, suggesting that neurons in the spinal cord are fully mature by this stage (Fig. 6.7.E and F).

Immunostaining for doublecortin in the human embryonic spinal cord revealed that in embryos between 32-39 days gestation, corresponding to approximately E3-E4 in the chick, doublecortin expression was found in the ventral and lateral neural tube and was also observed in the dorsal and ventral roots and dorsal root ganglia (Fig. 6.8.A-F). Although the precise rostral-caudal levels of these sections is unknown, it is likely that the open neural tube seen in Fig. 6.8.C and D is taken from a slightly different region of the spinal cord than those observed in Fig 6.8.A and B. By 44 days gestation, corresponding to around E5.5 in the chick, doublecortin expression was observed most strongly in the ventral horns of the spinal cord (Fig. 6.9.A and B). At 54 days gestation, corresponding to approximately E8 in the chick, doublecortin expression was absent from the spinal cord (Fig. 6.9.C and D). Similarly, by 9 weeks gestation, corresponding to around E11 in the chick, doublecortin expression could not be detected in the human spinal cord (Fig. 6.9.E and F). These results suggest that the expression pattern observed for doublecortin in the chick spinal cord is broadly conserved across different species, although doublecortin expression appears to be down-regulated slightly earlier in the human spinal cord.

6.2.5. Expression of doublecortin in a human neuroblastoma cell line

The results described above revealed a developmental regulation of doublecortin expression and post-translational modification, which appears to correspond to the loss of regenerative capacity in the chick spinal cord. Although doublecortin continues to be expressed throughout spinal cord development the expression level and, in particular, the level of the phosphorylated form of the protein, seems to correlate with a less mature neuronal state. In order to further confirm these findings, the expression of doublecortin in a neuroblastoma cell line, composed of immature neural precursors, was assessed. Doublecortin expression was investigated in the human neuroblastoma cell line, SH-SY5Y. Twenty four hours after plating, immunostaining showed that doublecortin is expressed in many SH-SY5Y cells (Fig 6.10.A-D). The specificity of the staining was confirmed by staining separate coverslips with the antibody after pre-incubation with

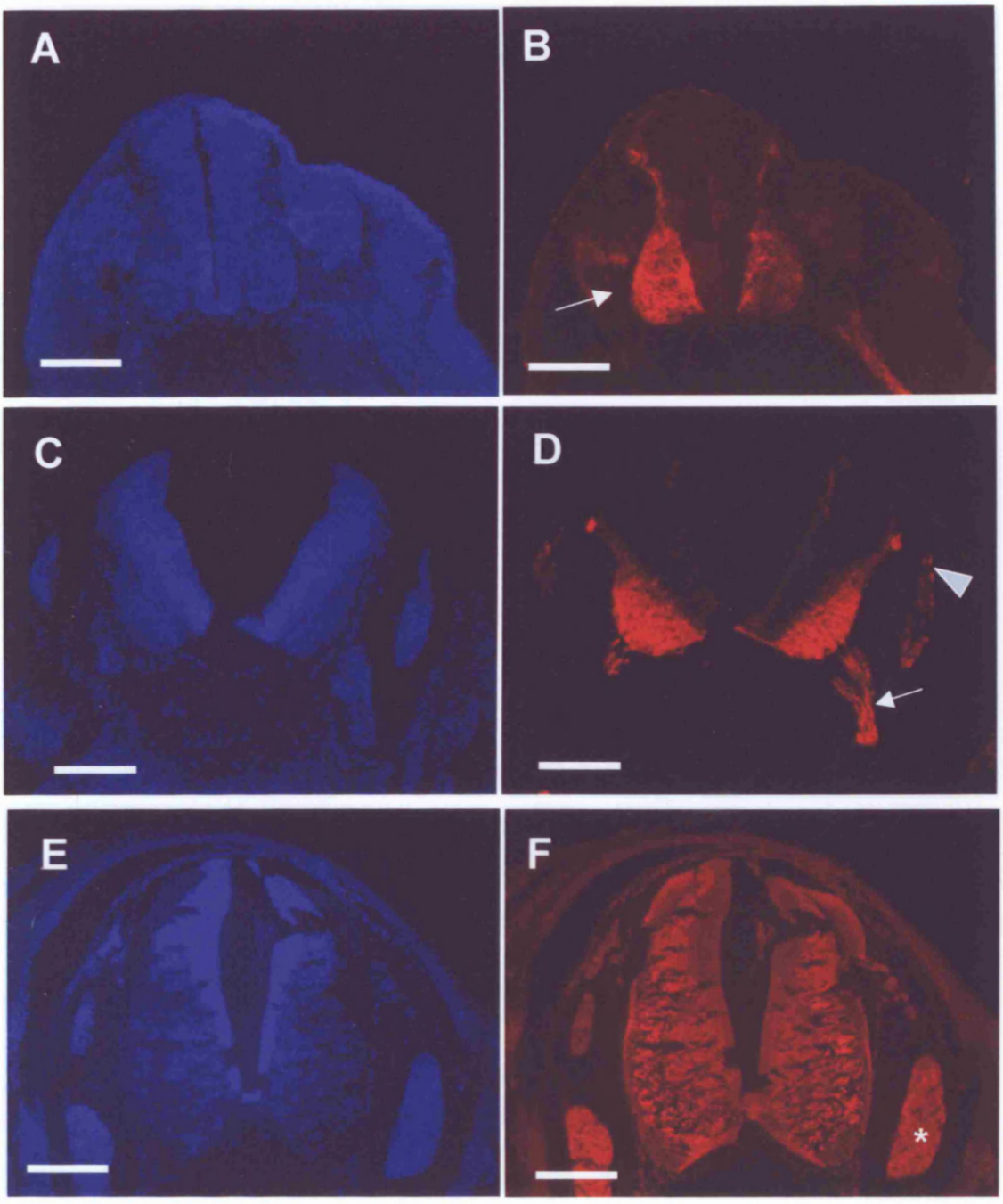


Fig. 6.8. Expression of doublecortin in the embryonic human spinal cord I. DCX immunostaining is shown in red and nuclear counterstaining with Hoechst is shown in blue at A) and B) 32 days (arrow indicates doublecortin staining in the ventrolateral neural tube) C) and D) 37days (arrows indicate staining in the dorsal (arrowhead) and ventral (arrow) roots) and E) and F) 39days (asterisks indicates staining in the dorsal root ganglia). Scale bars: A,B,E and F - 500 μm. C and D- 1000 μm

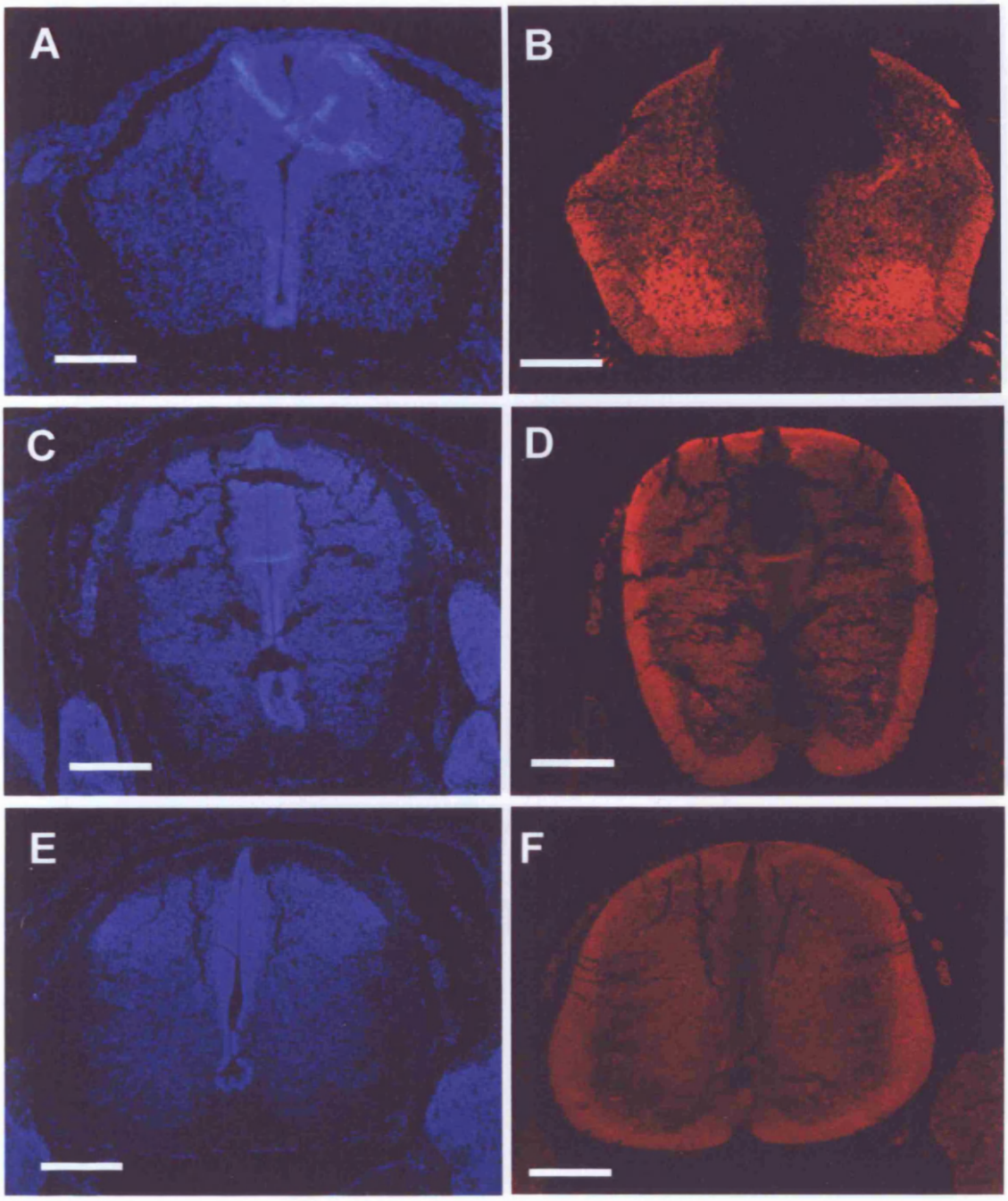


Fig. 6.9. Expression of doublecortin in the embryonic human spinal cord II. DCX immunostaining is shown in red and nuclear counterstaining with Hoechst is shown in blue at A) and B) 44 days C) and D) 54days and E) and F) 9 weeks. Scale bars: 500 μm.

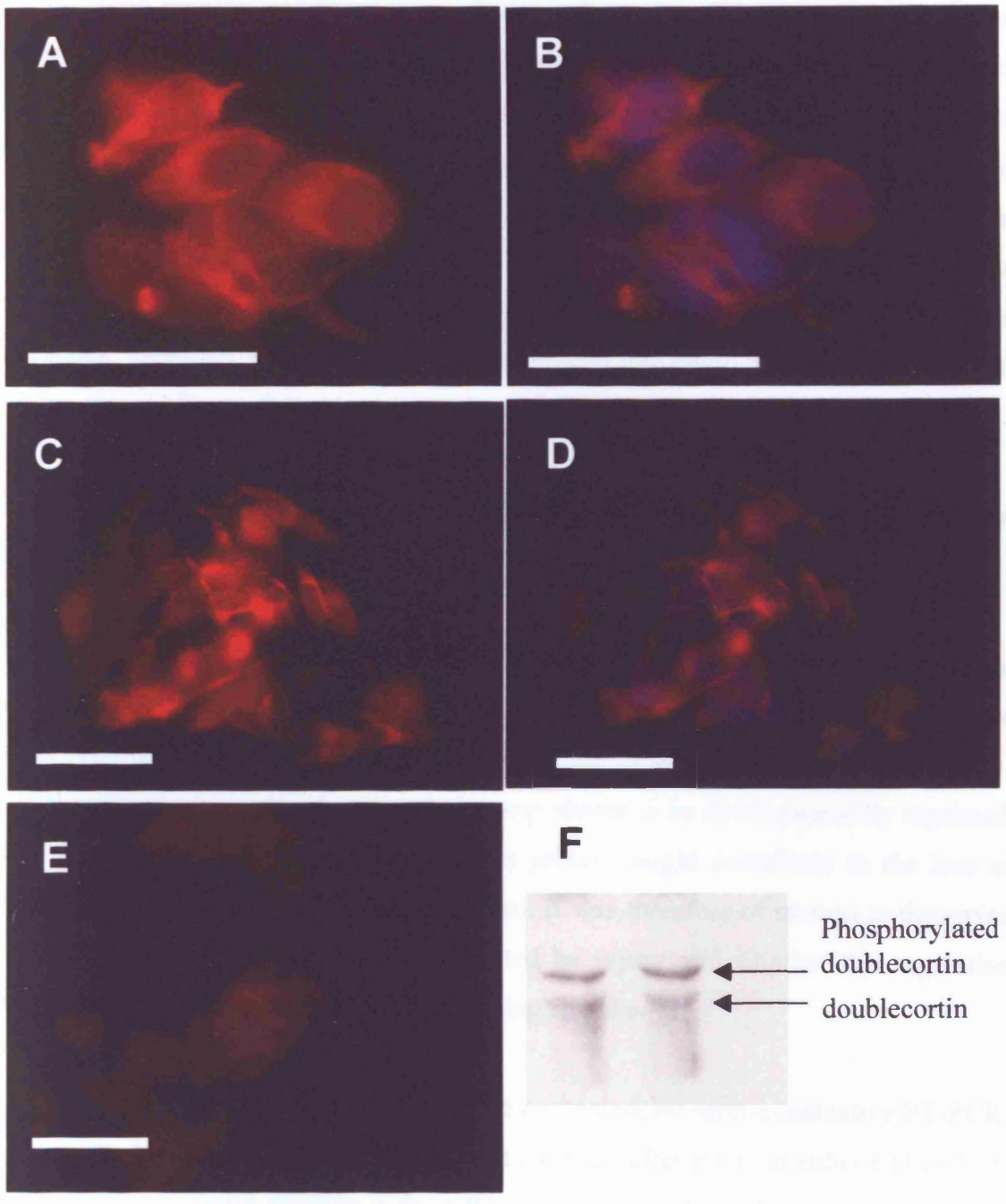


Fig. 6.10. Expression of doublecortin in a human neuroblastoma cell line. **A)-D)** Immunostaining for doublecortin (red, A,C) and nuclear counterstaining (blue, B,D) in SH-SY5Y cells. **E)** Staining using doublecortin antibody after pre-incubation with blocking peptide. **F)** Western blotting for doublecortin on SH-SY5Y cells. Scale bars = 50μm.

the blocking peptide, resulting in an absence of staining (Fig, 6.10.E). This further supports the specificity of the staining observed in human spinal cord sections, on which it was not possible to use the blocking peptide due to limited material being available. Western blotting of protein extracts from the cells demonstrated two bands at approximately 40kDa, revealing strong expression of the phosphorylated form of the protein in this cell line (Fig. 6.10.F). These results support the suggestion that expression of the phosphorylated form of doublecortin is a hallmark immature neuronal cell types. These results also indicated that this cell line could be useful to investigate the functional effects of doublecortin in future experiments.

6.2.6. Analysis of changes in the expression of doublecortin after injury in the chick spinal cord.

The results described above revealed that doublecortin is expressed in the chick spinal cord at relatively late developmental stages and that levels of doublecortin are reduced at developmental stages corresponding to the loss of regenerative capacity. Additionally, doublecortin phosphorylation was shown to be developmentally regulated. It is possible that down-regulation of this protein might contribute to the loss of regenerative capacity in the chick spinal cord. It was therefore of interest to determine whether doublecortin expression is regulated by injury and whether this regulation differs in the regenerating and non-regenerating spinal cord.

Initially, mRNA levels of doublecortin were compared, by semi-quantitative RT-PCR, in the injured and sham-operated spinal cord 24 hours after injury at either E11 or E15. There was a significant increase in the mRNA expression of doublecortin after injury at E11 (Fig 6.11A). Although, 24 hours after injury at E15, there appeared to be an increase in doublecortin mRNA expression, this did not prove to be statistically significant (Fig. 6.11.B). These results suggest that doublecortin up-regulation occurs after injury in the regenerating but not in the non-regenerating spinal cord. Next, doublecortin protein levels were investigated in sham-operated and injured spinal cords 24 hours after injury at E11 or E15 by Western blotting. There appeared to be an up-regulation in the overall protein level of doublecortin in injured spinal cord samples as compared to sham operated samples at 24 hours after injury at E11 (Fig 6.12).

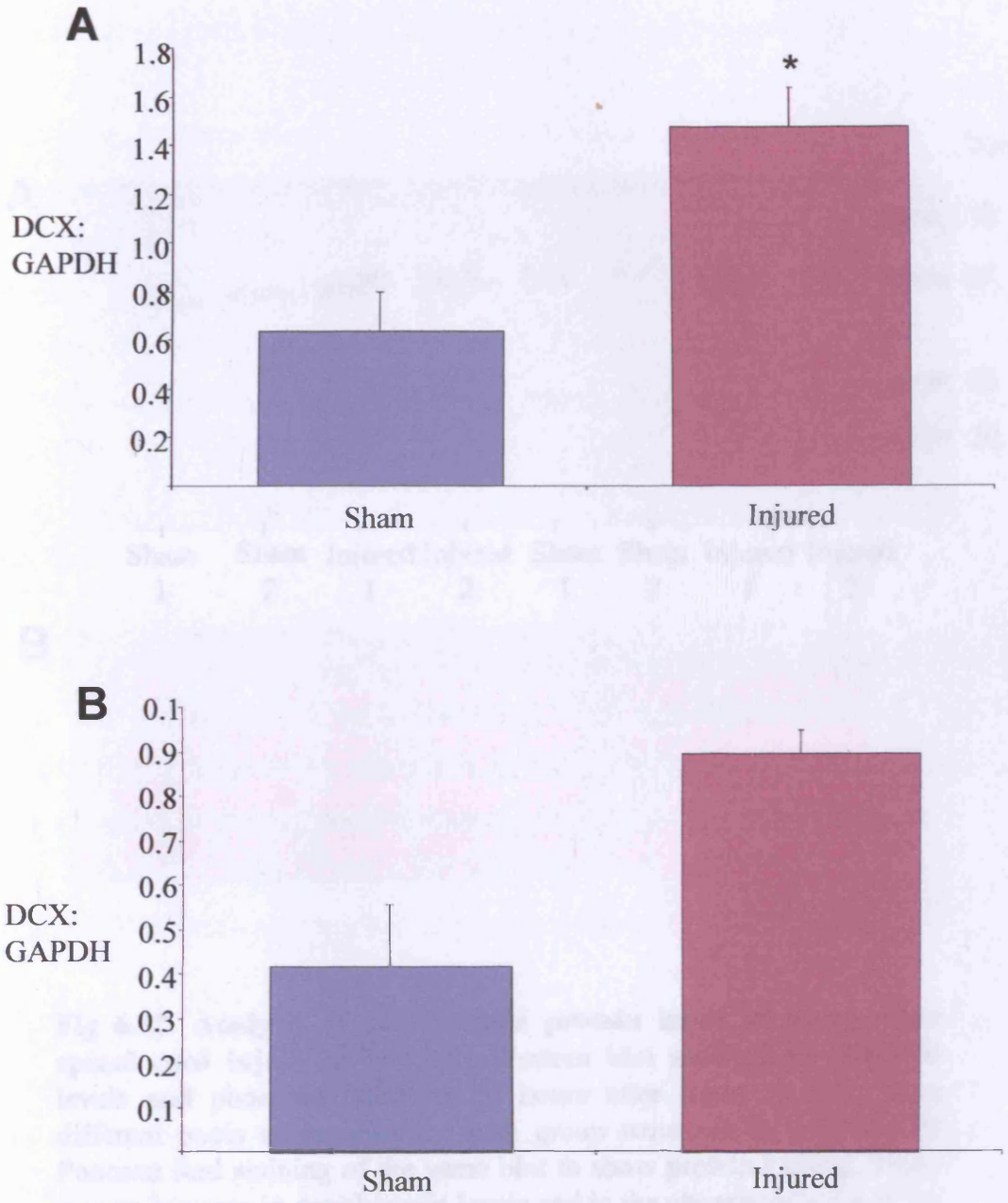


Fig. 6.11. Analysis of doublecortin mRNA expression after injury to the spinal cord. Semi-quantitative RT-PCR data showing expression of doublecortin mRNA 24 hours after injury at **A)** E11 and **B)** E15. Data show mean ± SEM for three pools of samples for each data point and are normalised to GAPDH levels. There was a significant increase in doublecortin mRNA expression after injury at E11 but not at E15. * p=0.05 for E11 (Mann-Whitney Test) , p=0.376 for E15 (Mann Whitney Test)

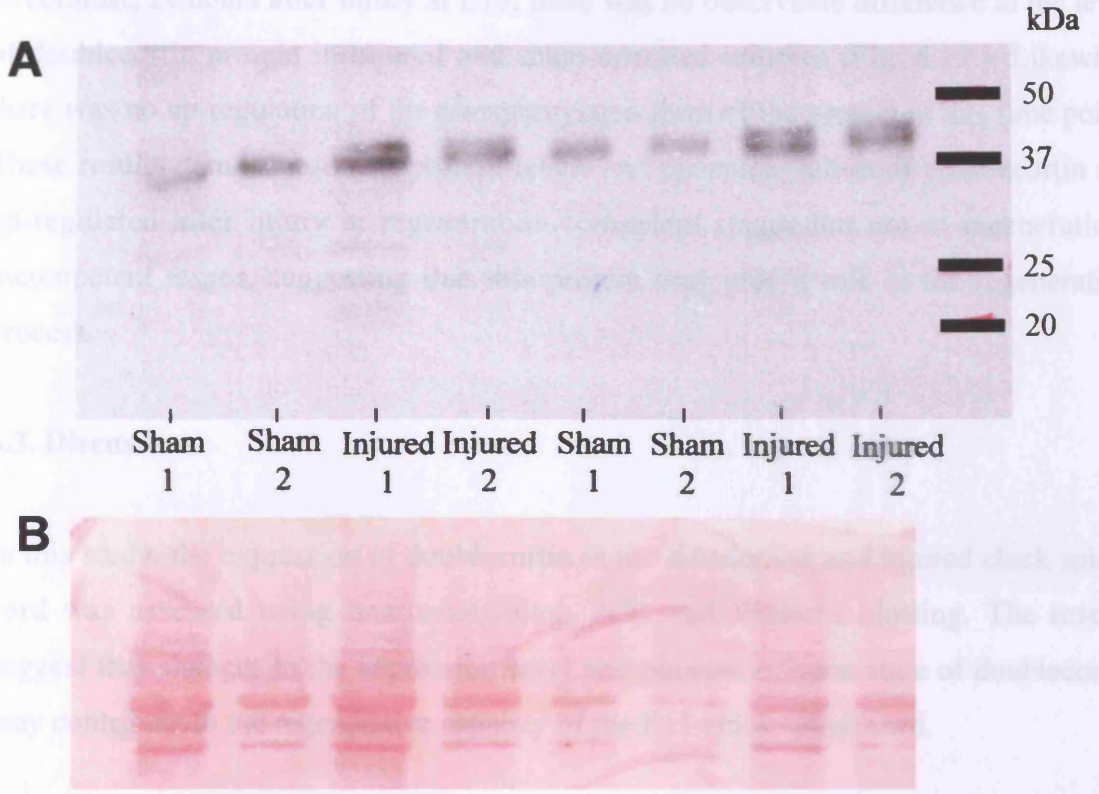


Fig 6.12. Analysis of doublecortin protein levels 24 hours after spinal cord injury at E11. A) Western blot showing doublecortin levels and phosphorylation at 24 hours after injury at E11. Two different pools of samples for each group were run in duplicate **B)** Ponceau Red staining of the same blot to show protein loading. There was an increase in doublecortin levels and in the phosphorylation of the protein in the injured samples as compared to the shams.

There was also a striking increase in the intensity of the upper band, representing the phosphorylated form of the protein, in injured samples at this time point.

In contrast, 24 hours after injury at E15, there was no observable difference in the level of doublecortin protein in injured and sham-operated samples (Fig. 6.13.). Likewise, there was no up-regulation of the phosphorylated form of the protein at this time point. These results demonstrate that protein levels and phosphorylation of doublecortin are up-regulated after injury at regeneration-competent stages but not at regeneration-incompetent stages, suggesting that this protein may play a role in the regeneration process.

6.3. Discussion

In this study, the expression of doublecortin in the developing and injured chick spinal cord was assessed using immunostaining, PCR and Western blotting. The results suggest that changes in the expression level and phosphorylation state of doublecortin may contribute to the regenerative capacity of the E11 chick spinal cord.

6.3.1. Doublecortin as a marker of neurogenesis in the chick spinal cord

The initial aim of these experiments was to determine whether doublecortin might act as a marker of neurogenesis in the embryonic chick spinal cord. The results described in this chapter demonstrate that there is a strong correlation between the expression of doublecortin and neurogenesis. However these results have also demonstrated the persistence of doublecortin gene and protein expression to developmental stages beyond the period of neurogenesis in the chick spinal cord. This means that doublecortin would not make an adequate marker for neurogenesis in this model, in contrast to findings in the adult mammalian brain (Brown *et al.* 2003; Couillard-Despres *et al.* 2005; Rao and Shetty 2004). Strikingly, doublecortin was downregulated at an earlier equivalent stage in the human spinal cord, suggesting that the human spinal cord is more mature than the chick spinal cord at similar gestational ages.

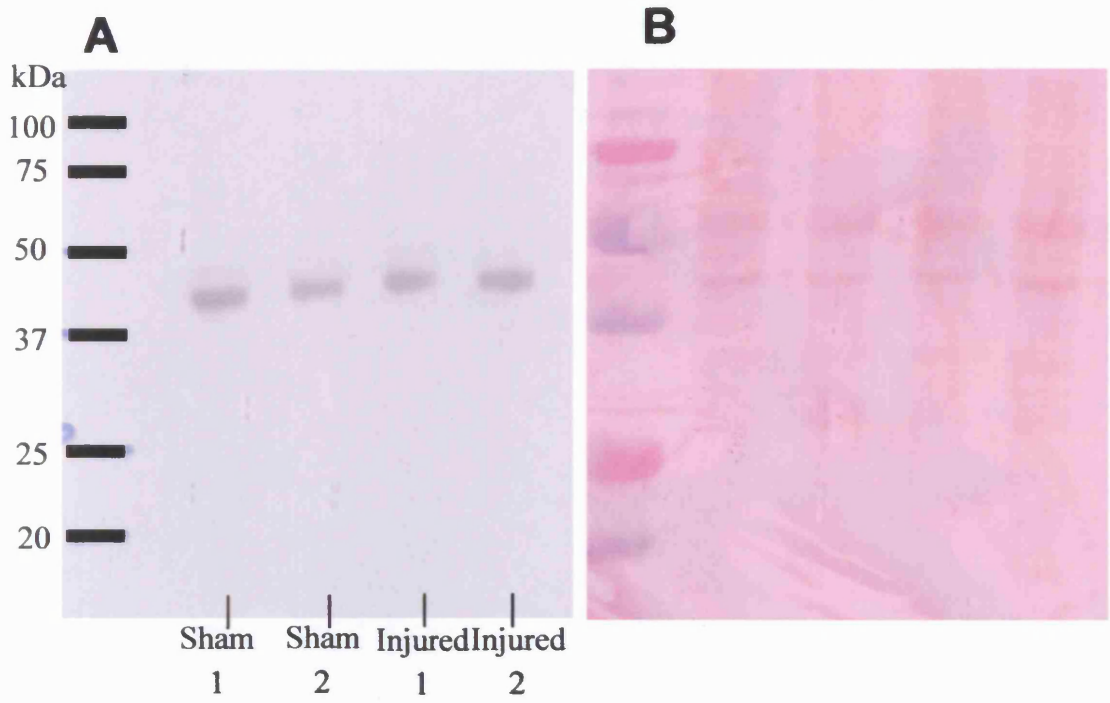


Fig 6.13. Analysis of doublecortin protein levels 24 hours after spinal cord injury at E15 A) Western blot showing doublecortin levels and phosphorylation at 24 hours after injury at E15. Two different pools of samples for each group were run. **B)** Ponceau Red staining of the same blot to show protein loading. There was no increase in doublecortin levels or phosphorylation of the protein after injury at this stage.

6.3.2. The role of doublecortin in neuronal development

By investigating the expression pattern of doublecortin in relation to known periods of neuronal development, the results of the current study provide some new insights into the potential role of doublecortin in the developing spinal cord. The expression of doublecortin was shown to increase at early developmental stages, coinciding with the major period of neurogenesis in the chick spinal cord which takes place between E3-E9 (Fujita 1964). Doublecortin expression is then reduced as development progresses, suggesting that expression of doublecortin begins to be lost as neuronal cells reach full maturity. The expression pattern of doublecortin in the chick spinal cord was shown to be closely matched by that in the mouse and human spinal cord, although the timing of the down-regulation of doublecortin appears to differ between species. These findings are in agreement with previous findings which have shown doublecortin expression during neuronal differentiation, migration and maturation followed by a down-regulation of doublecortin in the adult CNS, although only one previous study has included the spinal cord in this analysis (Gleeson *et al.* 1999; Hannan *et al.* 1999; Nacher *et al.* 2001).

There has been some controversy surrounding the timing of the earliest doublecortin expression during neuronal development with conflicting reports as to whether doublecortin is co-expressed with nestin, a marker of neural stem cells (Couillard-Despres *et al.* 2005; Rao and Shetty 2004; Kempermann *et al.* 2003; Kronenberg *et al.* 2003). This could not be tested in this model since there is no chick homologue of nestin. However, doublecortin was not expressed at the ventricular surface at any developmental age tested, indicating that this protein is not expressed in neural stem cells but begins to be expressed only once a neuron becomes a neuroblast. Indeed, most previous studies that have described an overlap of doublecortin and nestin expression have employed a nestin-GFP construct for this purpose rather than examining the endogenous protein, suggesting that what has been detected is GFP protein which has not yet been cleared from the cell.

Likewise, many studies have not attempted to determine the stage of neuronal development at which doublecortin is down-regulated. Previous studies have shown an almost complete absence of doublecortin expression in the adult brain, suggesting that it

is not expressed in mature neurons (Nacher *et al.* 2001). However, low levels of doublecortin expression have been observed in diverse brain regions in the adult and it has been suggested that this reflects a role in processes such as synapse formation and neurite outgrowth (Nacher *et al.* 2001). In the current study, doublecortin expression continues beyond the established period of neurogenesis and migration in the chick spinal cord. This expression was shown to be the result of ongoing gene expression and not merely the persistence of the protein in maturing neurons. Continued expression of doublecortin at stages beyond neurogenesis and migration suggests that this protein may have a functional role beyond its involvement in these processes. At later stages, doublecortin expression is seen to become restricted to the dorsal horns in the grey matter, which are known to be among the latest regions of the spinal cord to mature. Neurons in this region continue to receive and make new synaptic inputs from primary sensory neurons entering the spinal cord from the grey matter. The continued expression of doublecortin in these regions might therefore reflect a role in this process.

6.3.3. Doublecortin expression changes during the transition between regeneration-competent and incompetent developmental stages

This study has revealed a number of key differences in the expression and regulation of doublecortin between regeneration competent and incompetent stages of the spinal cord. Taken together, these findings suggest that doublecortin might make a contribution to the regenerative capacity of the chick spinal cord.

These results show that doublecortin expression levels in the chick spinal cord undergo a marked reduction during the period in which the spinal cord loses its ability to regenerate after injury. This reduction takes place at both the mRNA and the protein level. The immunostaining studies described in this chapter illustrate that, in the period between E11 and E15, doublecortin expression is reduced and becomes restricted to the dorsal horns of the spinal cord, with no expression observed in any other part of the spinal cord. The results of the Western blotting experiments further confirmed the dramatic reduction in protein levels between E11 and E15 and also provided some interesting findings about changes in the post-translational modification of doublecortin at these stages.

These results illustrated that at early, regeneration-competent stages doublecortin appears to be highly phosphorylated. However, by E15, virtually all the doublecortin present appears to be in the un-phosphorylated form. A highly phosphorylated form of doublecortin therefore appears to be a hallmark of less mature neurons and this was further confirmed by the presence of this form of doublecortin in human neuroblastoma cells. As will be described further below, the phosphorylation state of doublecortin is likely to affect its function and localisation and the disappearance of the phosphorylated form of the protein between E11 and E15 may therefore represent a key difference in the function of doublecortin at these two developmental stages. At the very least, the expression of phosphorylated doublecortin in the E11 spinal cord suggests that, at this stage, the spinal cord is still some way from full maturity. It is apparent that a less mature spinal cord may be better capable of responding to injury as any repair processes which would otherwise need to be up-regulated may already be ongoing at this stage.

6.3.4. Regulation of doublecortin in response to injury suggests a role in spinal cord regeneration

Investigation of the effects of injury on doublecortin expression revealed further differences between the E11 and E15 spinal cord, which correspond to the loss of regenerative capacity. Injury to the E11 spinal cord resulted in a significant increase in doublecortin mRNA levels. In contrast, after injury at E15, although there did appear to be an increase in doublecortin mRNA levels, this did not prove statistically significant. Nor was this increase mimicked by an increase at the protein level. While it is possible that with increased sample numbers this increase might be shown to be significant, it is clear that the greatest increase in doublecortin mRNA occurs at regenerating stages. At E11 this was accompanied by an increase in the level of doublecortin protein and, interestingly, a noticeable increase in the amount of phosphorylated doublecortin in the spinal cord. At E15 there was no such response at the protein level. This suggests that the up-regulation of doublecortin in the E11 spinal cord and an increase in the proportion of phosphorylated doublecortin might contribute to a favourable regenerative response after injury.

This study initially began with the consideration of doublecortin as a marker of neurogenesis. Although the extensive nature of doublecortin expression at this stage

means that detection of doublecortin alone is not sufficient to establish unequivocally new neuronal cell birth, it is certain that any up-regulation in neurogenesis after injury would ultimately result in increased doublecortin expression. Furthermore, from the results of the current study, newly born neurons are more likely to express the phosphorylated form of the protein. It is therefore possible that the up-regulation of phosphorylated doublecortin observed in the chick spinal cord after injury at E11 reflects an increase in neurogenesis. The results of these experiments are not sufficient to determine whether this is the case and further analysis of the potential increase in neurogenesis after injury at E11 is required. This will be addressed further in Chapter 7.

6.3.5. Potential mechanisms by which doublecortin might contribute to regenerative capacity

The results described above are suggestive of a role for doublecortin in determining the regenerative capacity of the chick spinal cord. If this were the case then it is possible that the presence of high levels of the phosphorylated form of doublecortin present at E11 itself contributes to the regenerative capacity of the chick spinal cord. Alternatively, up-regulation of doublecortin after injury at E11 may have a beneficial effect on the outcome of the injury. There are a number of mechanisms by which doublecortin might contribute to the regenerative capacity of the spinal cord.

Doublecortin has been demonstrated to be associated with microtubules and to have a role in the control of microtubule stability (Gleeson *et al.* 1999; Horesh *et al.* 1999). Doublecortin associates with microtubules via a conserved doublecortin domain (Kim *et al.* 2003b; Sapir *et al.* 2000; Taylor *et al.* 2000). In common with a number of other MAPs, the binding of doublecortin to microtubules is regulated in part by phosphorylation (Tanaka *et al.* 2004). Doublecortin has been demonstrated to be phosphorylated by Cdk5, JNK, protein kinase A (PKA) and MAP/ microtubule affinity regulatory kinase (MARK) (Schaar *et al.* 2004; Tanaka *et al.* 2004; Gdalyahu *et al.* 2004). These kinases act at a range of different phosphorylation sites and the different combinations of possible phosphorylation states add to the complexity of the regulation of doublecortin (Reiner *et al.* 2004). Phosphorylation of doublecortin by Cdk5, PKA or MARK reduces the affinity of doublecortin for microtubules and thus results in reduced microtubule stability. This is likely to be important in situations in which the

cytoskeleton undergoes dynamic changes and can explain the important role of doublecortin in neuronal migration during development. Furthermore, phosphorylation of doublecortin by Cdk5 may result in the preferential binding of doublecortin to microtubules in the perinuclear region, which has been suggested to have role in translocation of the cell soma during radial migration (Reiner *et al.* 2004; Tanaka *et al.* 2004). Phosphorylation of doublecortin by JNK has also been reported to enhance neurite outgrowth (Gdalyahu *et al.* 2004). In humans, mutations in doublecortin result in x-linked lissencephaly, a disorder linked to abnormal migration of cortical neurons during development (des Portes *et al.* 1998; Gleeson *et al.* 1998). Similarly, RNAi knockdown of doublecortin in the rat results in disrupted neuronal migration (Bai *et al.* 2003).

The role of doublecortin in microtubule stabilisation provides some suggestions as to a potential mechanism by which this protein might be involved in spinal cord regeneration. The high levels of the phosphorylated form of doublecortin present in the E11 spinal cord suggest that at this stage the cytoskeleton may be still in a fairly dynamic state and that the spinal cord may be fairly plastic. This would lead to more rapid responses to an injury than at later, less dynamic stages of development. After an injury, repair processes are likely to include neurite sprouting and outgrowth and the formation of new synapses. Neuronal migration may also take place in the re-organisation of the neuronal circuitry. These processes all involve re-organisation of the cytoskeleton and might therefore be expected to be accompanied by a decrease in microtubule stability. Increased phosphorylation of doublecortin after injury might therefore greatly facilitate these processes.

Another interesting possibility is that doublecortin might contribute to the survival of spinal cord neurons after injury. It has been demonstrated that overexpression of doublecortin can protect PC12 cells from apoptosis (Shmueli *et al.* 2001). As described in Chapter 4, a dramatic increase in the apoptotic response of the spinal cord to injury is likely to make an important contribution to the loss of regenerative capacity. It is therefore intriguing to consider the possibility that doublecortin might be in part responsible for the reduced apoptotic response observed at regeneration-competent stages. In support of this, inhibition of Cdk5 with roscovitine, which might result in reduced doublecortin phosphorylation, causes cell death in SH-SY5Y cells (Appendix

Fig. 1). It is not possible to determine from this experiment, however, whether this was due to reduced phosphorylation of doublecortin.

As an attempt to investigate this possibility further experiments have been initiated to investigate the effect of doublecortin knockdown, using an antisense approach, on apoptosis in SH-SY5Y neuroblastoma cells. As shown in this chapter, these cells have been shown to express doublecortin. Furthermore, apoptosis can be triggered in these cells by a number of different apoptotic agents including thrombin (Appendix Fig. 2 and Chapter 5, Fig 5.11). Experiments have been carried out to determine the threshold dose of these drugs which is required to cause apoptosis and methods for the measurement of apoptosis have been developed (Appendix Fig. 2 and Fig. 3). Finally, antisense oligodeoxynucleotides have been designed for the knockdown of doublecortin and experiments have been initiated to optimise the conditions required for successful knockdown (Appendix Fig. 4). Unfortunately, to date antisense treatment has not produced a consistent reduction in doublecortin levels, probably due to inefficient transfection. Nevertheless these preliminary experiments have set in place a viable method to assess whether knockdown of doublecortin increases the susceptibility of these cells to apoptosis-inducing agents.

6.3.6. Conclusions

In this chapter the potential role of a microtubule protein, doublecortin, in determining the regenerative capacity of the chick spinal cord has been explored. These results provide novel information about the regulation of doublecortin expression in the normal and injured chick spinal cord. Further work will be required to determine whether the up-regulation in phosphorylated doublecortin expression after injury at E11 reflects changes in neurogenesis (which will be explored in Chapter 7), cytoskeletal dynamics or a novel neuroprotective response.

Chapter 7. Analysis of neurogenesis in the regenerating chick spinal cord

7.1. Introduction

The previous chapter described attempts to establish the contribution of neurogenesis to spinal cord regenerative capacity in the chick, using the microtubule-associated protein, doublecortin, as a marker of newly born neurons. The results of these studies showed that, in the developing spinal cord, expression of doublecortin persists beyond the stages at which neurogenesis is expected to be complete, suggesting that this marker could not be used for this purpose. However the up-regulation of doublecortin expression and increased levels of the phosphorylated form of this protein after injury at E11 but not E15 might indicate the induction of neurogenesis in the regenerating chick spinal cord. In order to test this hypothesis, an alternative method for measuring neurogenesis was therefore sought.

Although no specific markers for neurogenesis are known, it is possible to assess the birth of neurons by labelling cells as they are born with a marker which is incorporated into DNA during synthesis, such as BrdU. BrdU is a synthetic analogue of thymidine that is incorporated into DNA in proliferating cells during S-phase. Double immunostaining for both BrdU and markers of neuronal differentiation can then provide evidence for neurogenesis and, therefore, this strategy was chosen for this study. Neurogenesis has been reported to be complete by E9 in the chick (Fujita 1964). Therefore, it was expected that no neurogenesis would be detected in the uninjured spinal cord at E11. This chapter describes the development and application of strategies to detect neurogenesis in the chick spinal cord in order to determine to what extent this process is re-instated after injury.

7.2. Results

7.2.1. Analysis of proliferation in the developing chick spinal cord

In order to investigate proliferation in the spinal cord, a method for labelling dividing cells *in ovo* with BrdU was developed. In preliminary experiments, several different methods of administering BrdU *in ovo*, were tested, including injecting under and on

top of the chorioallantoic membranes and injecting subcutaneously in the embryo. As these did not lead to a significantly different amount of labelling, it was decided that application of the BrdU onto the chorioallantoic membrane would be used for all subsequent experiments. BrdU labelling was carried out at several different developmental stages and spinal cords were collected after 24 hours and immunostained using an antibody to detect BrdU incorporation. As shown in Fig. 7.1, BrdU labelling was successful at all developmental stages tested. In this and subsequent experiments, attempts to use Hoechst dye for nuclear counterstaining with BrdU staining were unsuccessful, possibly due to the effects of the acid-treatment required for BrdU staining. BrdU labelling revealed that, at E4, proliferation is taking place throughout the neural tube (Fig. 7.1.A). Twenty-four hours after labelling at E10, BrdU labelling was observed in all regions of the spinal cord, with the highest concentration of DNA synthesis found in the ependymal region surrounding the central canal (Fig. 7.1.B, and B', see Fig. 7.1.D for illustration of the regions). At E16, 24 hours after labelling at E15, DNA synthesis was still observed in all regions of the spinal cord; however there were noticeably high levels of proliferation in the white matter at this stage, consistent with the birth of glial cells (Fig. 7.1.C). Therefore, these results indicated that a reduction in BrdU labelling in the grey matter corresponds to an increase in BrdU labelling in the white matter between E11 and E15, probably reflecting an increase in the birth of astrocytes. These results are in agreement with previous work in our laboratory in which proliferating cells were stained for PCNA or phosphorylated histone-3 (O'Neill 2002). It was noted in the course of these experiments that the BrdU labelling was never 100% effective; at all developmental time points tested some embryos were not labelled at all, suggesting that they had not effectively taken up the BrdU.

7.2.2. Analysis of the expression of transitin in the developing and injured chick spinal cord

The previous results demonstrated that high levels of proliferation are ongoing in the chick spinal cord at both E11 and E15, with a shift in the location of the highest concentration of BrdU positive cells from the grey to the white matter between these two time points. The aim of this study was to determine whether neurogenesis can be induced in the E11 spinal cord in response to injury. Therefore, it was of interest to determine whether these proliferating cells have the capacity to adopt a neuronal fate in

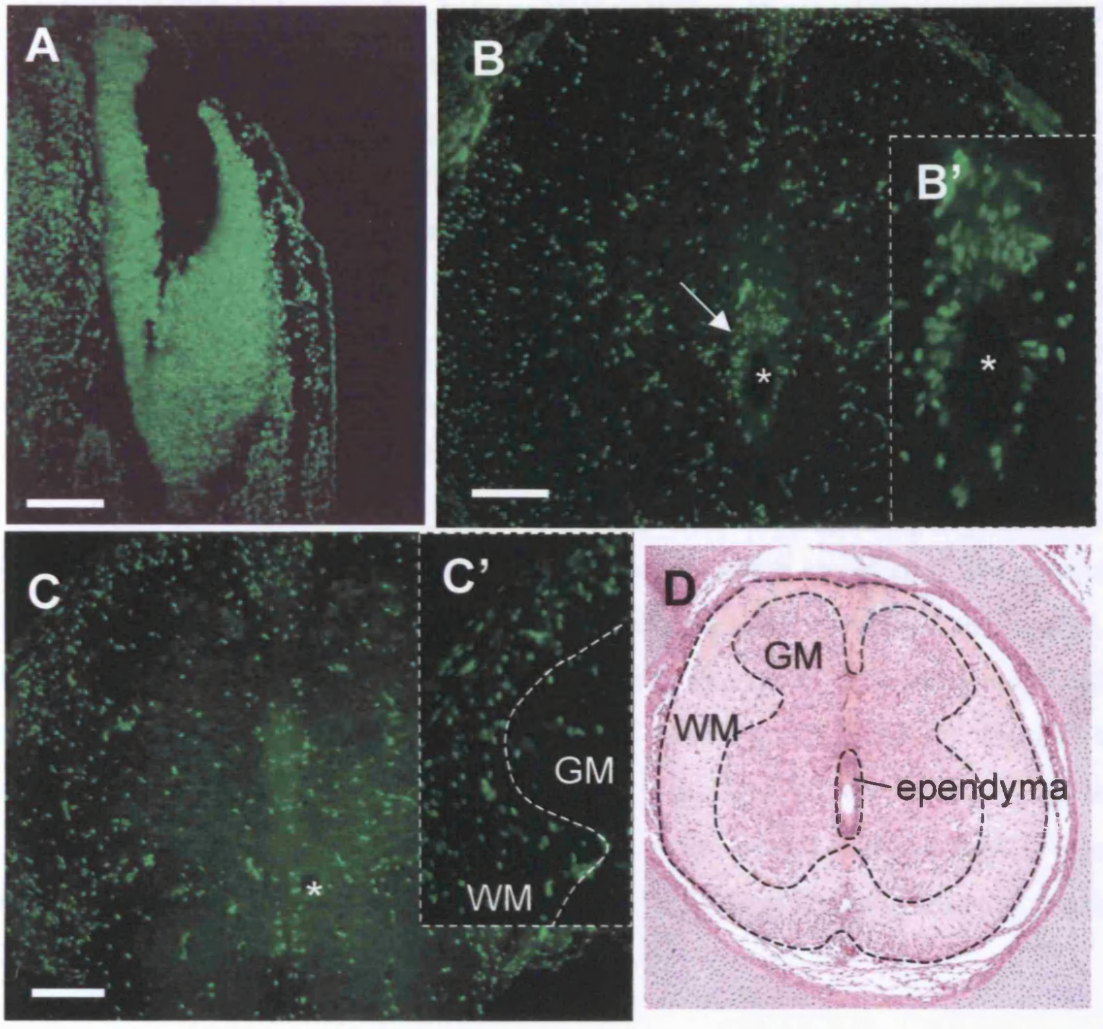


Fig. 7.1. Analysis of proliferation in the developing chick spinal cord by BrdU labelling. BrdU immunostaining (green) in transverse sections of spinal cord 24 hours after labelling with BrdU. **A)** Spinal cord labelled at E3 and collected at E4. **B)** Spinal cord labelled at E10 and collected at E11. High levels of proliferation in the ependymal region are indicated by the arrow. Asterisk indicates the central canal **B')** High magnification view of BrdU labelling in the ependymal region at E11. The central canal is marked by an asterisk. **C)** Spinal cord labelled at E15 and collected at E16. The central canal is indicated by an asterisk. **C')** High magnification view of BrdU labelling in the white matter at E16. Dashed line indicates the approximate boundary between the grey matter (GM) and the white matter (WM) **D)** Haemotoxylin and Eosin staining of transverse section of E12 spinal cord showing the three main regions assessed for BrdU labelling. Scale bars = 500 μm. In all images dorsal is at the top and ventral at the bottom of the picture.

response to injury. In a number of studies in mammalian species, the expression of the intermediate filament nestin has been used as a marker of neural precursors, including both neuroepithelial cells and radial glia, although it has also been shown that nestin is expressed by reactive astrocytes (Lendahl *et al.* 1990; Brewer 1999; Dahlstrand *et al.* 1995; Reynolds and Weiss 1992; Weiss *et al.* 1996). Although no chick homologue of nestin has yet been identified, transitin, a member of the same subgroup of intermediate filaments, has been suggested to be expressed in neural precursors as well as radial glia (Cole and Lee 1997; Lee and Cole 2000; Napier *et al.* 1999; Yuan *et al.* 1997). It was therefore of interest to determine whether a change in the expression of transitin and/ or double-labelling with BrdU and transitin occurs in the chick spinal cord after injury.

Initially, the expression of transitin in the developing chick spinal cord was examined. As shown in Fig. 7.2.A, at E5, transitin was shown to be expressed throughout the neural tube. At later stages (E7 and E9), transitin expression was most prominent in radial processes spanning the width of the spinal cord (Fig. 7.2.B and D). Transitin was also highly expressed along the dorsal midline (Fig. 7.2.C). From E11 onwards, most transitin expression became largely restricted to the dorsal midline, with low-level expression continuing in some radial fibres (Fig 7.2E). Expression of transitin remained in these regions until at least E15 (Fig. 7.2.E and F).

From these results, the expression pattern of transitin in the chick spinal cord corresponds closely to the developmental expression of nestin in other species (Takano and Becker 1997). Nestin has been reported to be up-regulated after spinal cord injury (Frisen *et al.* 1995; Namiki and Tator 1999; Shibuya *et al.* 2002) and it was therefore of interest to determine whether transitin expression is similarly up-regulated in the chick. Spinal cord injury was performed at E11 and the spinal cords were collected 24 hours after injury. 7μm sections, taken less than 500μm from the injury site were prepared and stained for transitin. As shown in Fig. 7.3, an increase in the expression of transitin in radial processes and scattered cells throughout the spinal cord was observed 24 hours after injury at E11. Some of the up-regulated transitin seemed to be localised in cells that extend radial fibres from the ependymal region towards the spinal cord surface, reminiscent of patterns of radial glia during development. Other staining appeared to be in scattered cells with short processes. In order to determine whether newly proliferating cells begin to express transitin after injury at E11, or whether transitin expression is

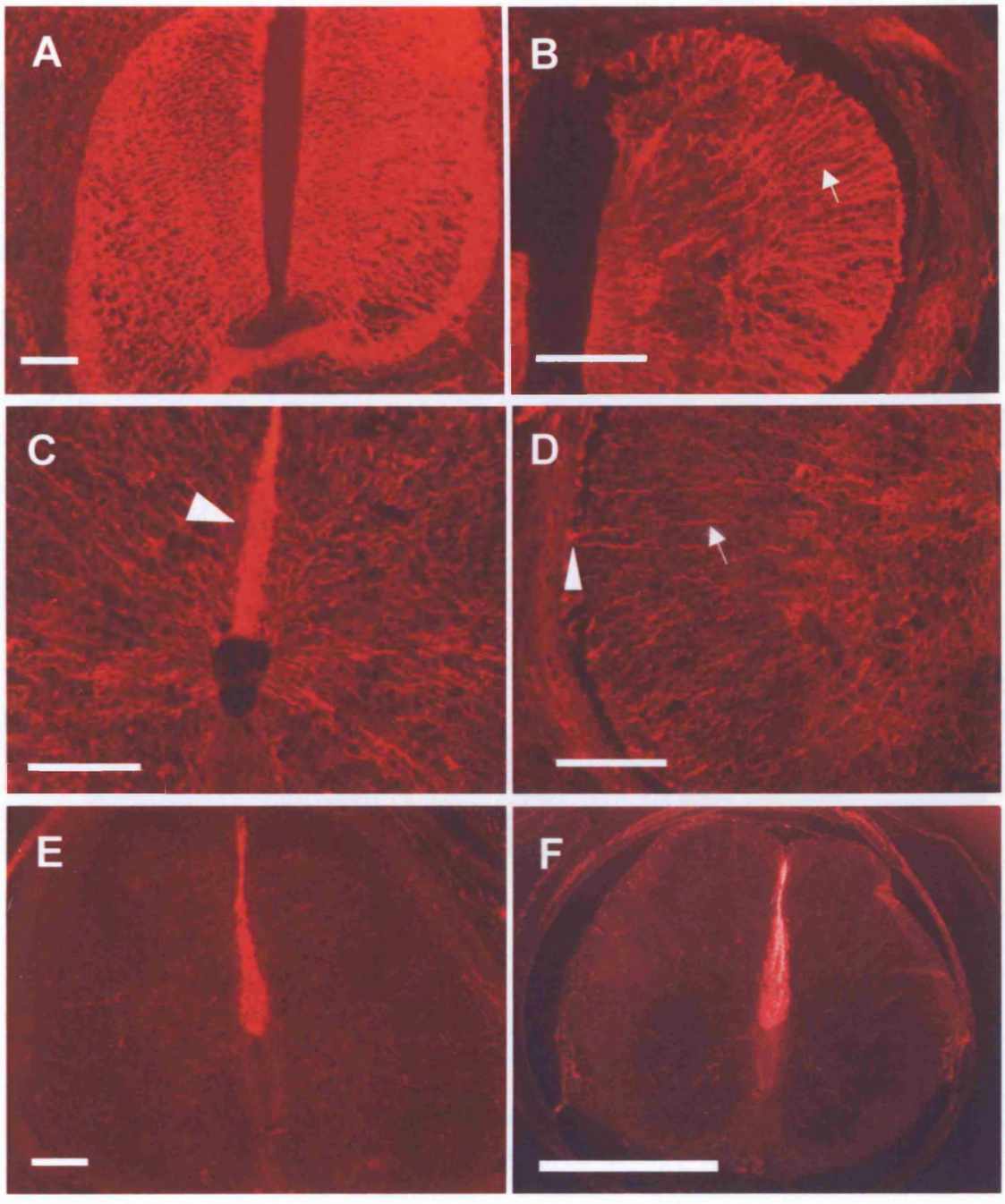


Fig.7.2. Expression of transitin in the developing chick spinal cord. Immunostaining for the chick nestin homologue, transitin (red), in transverse sections of the developing spinal cord. **A)** E5 spinal cord. Transitin is expressed throughout the early neural tube. **B)** E7 spinal cord. Transitin is expressed in radially oriented processes (arrow). **C)** and **D)** E9 spinal cord. Transitin is expressed in both the dorsal midline (arrowhead in C) and radial fibres (arrow in D) which extend to pial end feet at the spinal cord surface (arrowhead in D). **E)** E11 spinal cord. Transitin expression is restricted to the dorsal midline and transitin expression in radial fibres is absent. **F)** E15 spinal cord. Transitin continues to be expressed predominantly in the dorsal midline. Scale bars: 500μm.

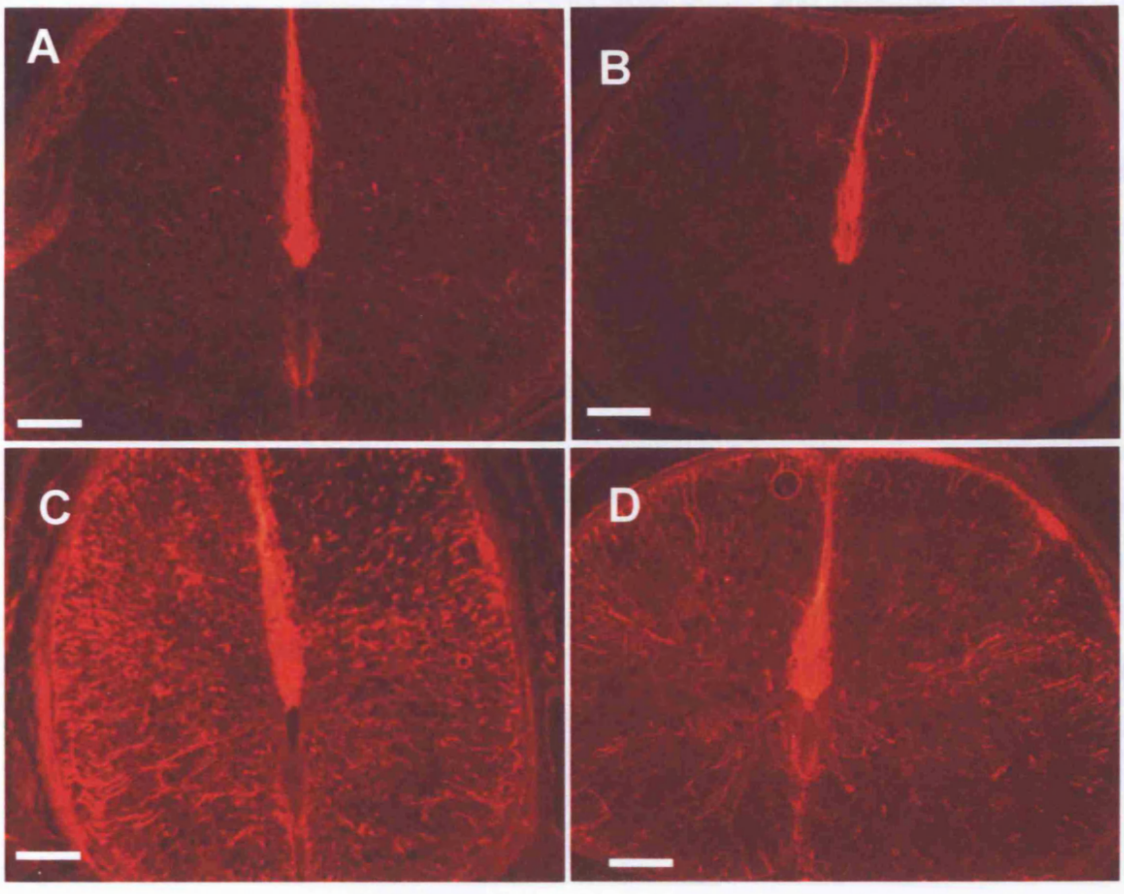


Fig. 7.3. Changes in transitin expression after injury at E11. Immunostaining for transitin (red) in transverse sections of spinal cord, 24 hours after injury at E11. **A) and B)** Sections from sham-operated embryos at E12. **C) and D)** Sections from injured embryos, 24 hours after injury at E11. There is a clear up-regulation of transitin expression in radial processes after injury. Scale bars = 500μm

up-regulated in existing differentiated cells, double labelling for transitin and BrdU was assessed in spinal cord sections, 24 hours after injury and labelling with BrdU (Fig. 7.4). The filamentous nature of the transitin staining made it difficult to assess co-localisation with any certainty. However, as shown in Fig. 7.4.B and Fig. 7.4.C, some cells in the injured spinal cord did appear to be labelled by both markers, including some in the grey matter. This suggests that the up-regulation of transitin after injury may be due, in part, to the expansion of progenitor cell populations.

In order to determine whether this up-regulation in transitin expression may contribute to the regenerative response at E11 it would be necessary to determine whether the same change in expression occurs after injury at non-regenerating stages. Transitin staining was therefore carried out in transverse sections of spinal cord 24 hours after injury at E15. As shown in Fig. 7.5, there was no obvious increase in transitin expression after injury at E15. Given the limited numbers of embryos that were available for this analysis (n=1), no firm conclusions can be drawn about the changes in transitin expression after injury at E15. However, these findings suggest that the increase in transitin expression may be specific to E11.

7.2.3. Analysis of the expression of 3CB2 in the developing and injured chick spinal cord

It has been suggested that, during development, radial glial cells may act as neural precursors (Gregg *et al.* 2002; Malatesta *et al.* 2000). Markers of radial glia have been demonstrated to be up-regulated after injury in the rat spinal cord (Namiki and Tator 1999; Shibuya *et al.* 2003). Furthermore transitin, like nestin in the mammal, has been reported to be expressed in radial glia and appeared in the current study to be up-regulated after injury at E11. It was therefore of interest to determine whether there are any changes in the number or localisation of radial glial cells after injury in the chick spinal cord. This was investigated by immunostaining using an antibody against radial glial cells, 3CB2 (Prada *et al.* 1995).

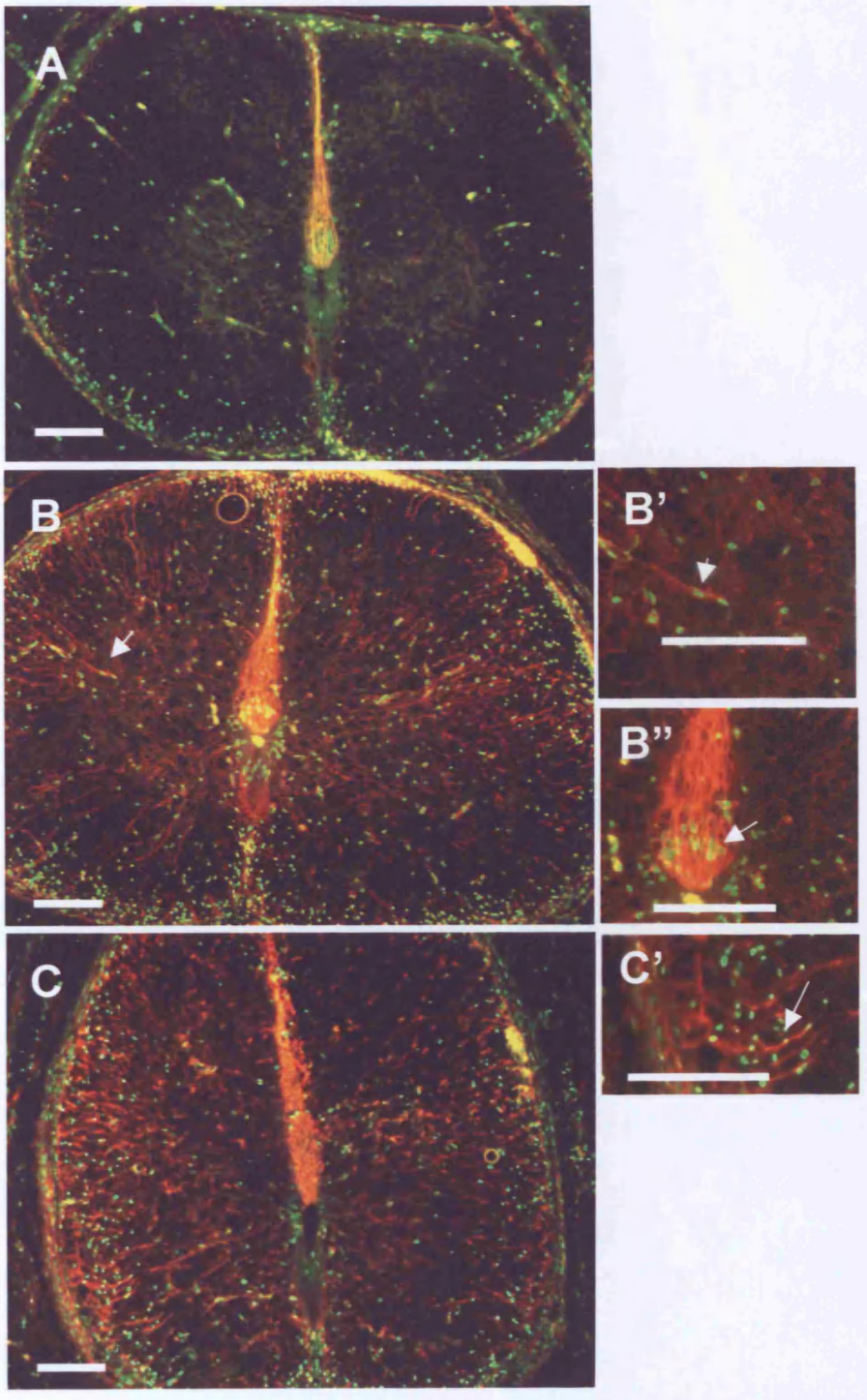


Fig. 7.4. Double labelling with BrdU and transitin after injury at E11. Immunostaining for BrdU (green) and transitin (red) in transverse sections of spinal cord 24 hours after injury and labelling with BrdU at E11. A) Sham-operated spinal cord at E12. B) and C) Injured spinal cord 24 hours after injury at E11. B', B'' and C' show high magnification view of potentially double-labelled cells (arrows) Scale bars = 500 μm.

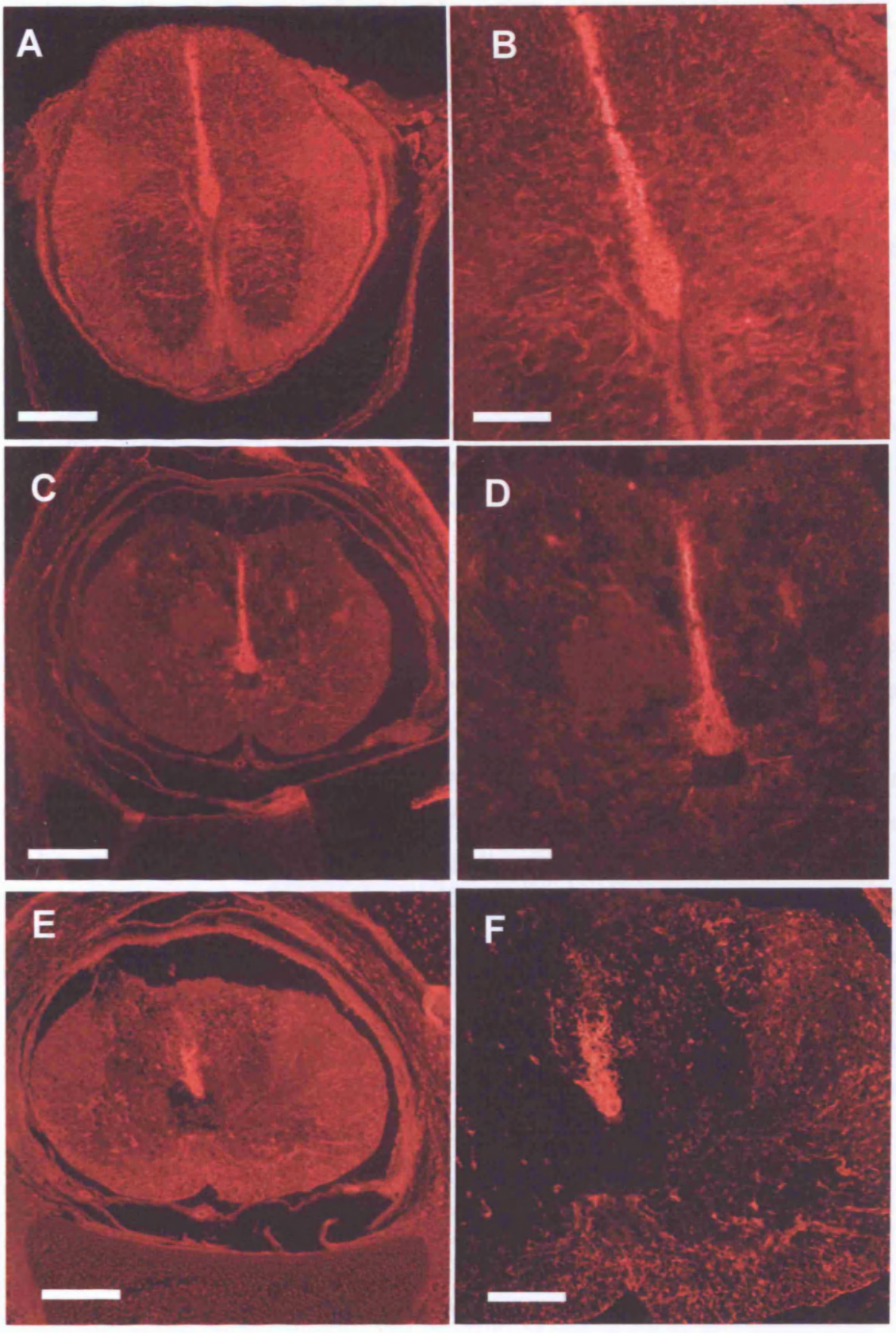


Fig. 7.5. Analysis of transitin expression after injury at E15. Immunostaining for transitin (red) in transverse sections of spinal cord, twenty four hours after injury at E15. **A) and B)** Section from a sham-operated embryo at E16. **C)-F)** Sections from an injured embryo, taken from two different levels, 24 hours after injury at E15. Scale bars A,C,E = 200 μm, B,D,F = 100 μm

Initially, the expression of 3CB2 was investigated in the developing chick spinal cord (Fig. 7.6). At E4, 3CB2 was expressed in cells lining the ventricular surface of the neural tube. By E5 3CB2 expression was observed in radial fibres projecting away from the ventricular zone and across the neural tube (Fig, 7.6.A and B). By E9, 3CB2 expression was more diffusely spread within the spinal cord; however the highest levels of expression remained in the cells surrounding the central canal (Fig. 7.6.C). By E11, the expression of 3CB2 was restricted mainly to the dorsal midline, spanning from the central canal to the dorsal surface of the spinal cord. Some expression was also observed in the ventral midline, close to the central canal (Fig. 7.6.D). This expression pattern was maintained until at least E16, with low levels of expression still visible in some radial fibres (Fig. 7.6.E and F).

The expression pattern of 3CB2 was consistent with previous reports and was noticeably similar to the expression pattern of transitin in the chick spinal cord. Consequently, it was of interest to determine whether 3CB2 is up-regulated after spinal cord injury in the same manner as transitin. In order to do this, 3CB2 expression was examined in transverse sections of the spinal cord. Twenty-four hours after injury at E11 (Fig. 7.7) there did not appear to be a significant up-regulation of 3CB2 expression. This suggests that 3CB2 positive radial glia were not up-regulated by injury in this model and that progenitor cells do not begin to express the 3CB2 antigen in response to injury. Similarly, after injury at E15, there was no noticeable increase in 3CB2 staining. In contrast, there appeared to be a decrease in the intensity of 3CB2 staining in the dorsal midline, although it was not possible to determine whether this was due to the extensive tissue damage occurring at E15 or represented a true down-regulation of protein expression (Fig. 7.8).

7.2.4. Selection of neuron-specific markers for the analysis of neurogenesis in the chick spinal cord

The experiments described above, showing changes in the expression of transitin, may be indicative of an up-regulation of neural precursors in the spinal cord after injury at regenerating stages. However, this does not necessarily mean that these cells will go on to produce new neurons. Thus, in order to investigate whether neurogenesis takes place in the regenerating chick spinal cord after injury cells in the spinal cord were assessed

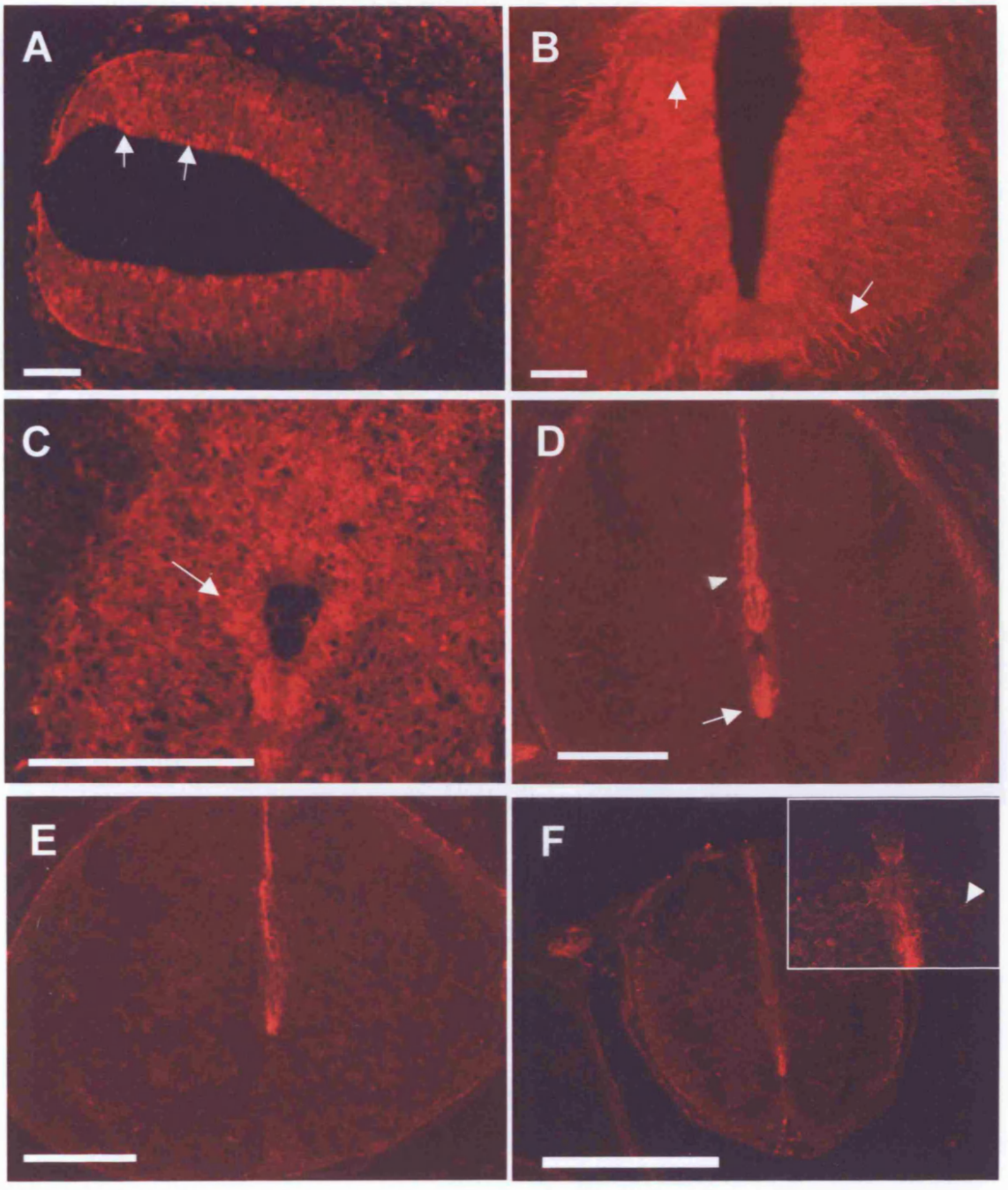


Fig 7.6. Expression of 3CB2 in the developing chick spinal cord. Immunostaining for a marker of radial glia, 3CB2 (red), in transverse sections of the developing spinal cord. **A)** E4 spinal cord. 3CB2 is expressed in cells lining the ventricular canal (arrows). **B)** E5 spinal cord. 3CB2 is expressed in radial fibres projecting from the ventricular zone of the early neural tube (arrows). **C)** E9 spinal cord. 3CB2 expression has become more diffuse but can still be observed around the ventricular zone (arrow in C). **D)** E11 spinal cord. 3CB2 is expressed in the dorsal midline (arrowhead) and extends into the ventral midline (arrow) **E)** E13 spinal cord. **F)** E16 spinal cord. Inset shows high magnification picture of 3CB2 staining in the ependyma and in radial processes (arrow). Scale bars: 500μm. In A) dorsal is to the left and ventral to the right. In all other pictures dorsal is at the top of the picture.

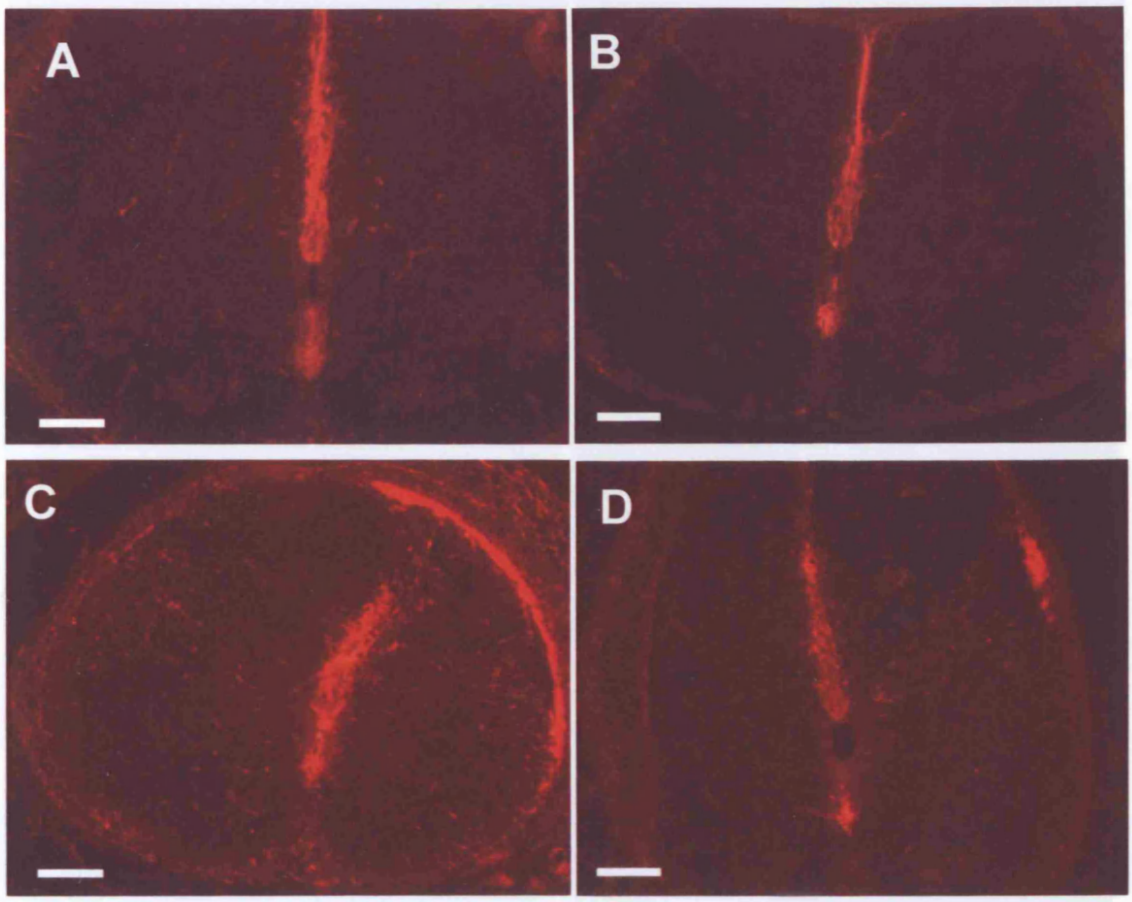


Fig. 7.7. Analysis of 3CB2 expression after injury at E11. Immunostaining for 3CB2 (red) in transverse sections of spinal cord, 24 hours after injury at E11. **A) and B)** Sections from sham-operated embryos at E12. **C) and D)** Sections from injured embryos, 24 hours after injury at E11. There is no up-regulation of 3CB2 expression after injury. Scale bars: 500μm

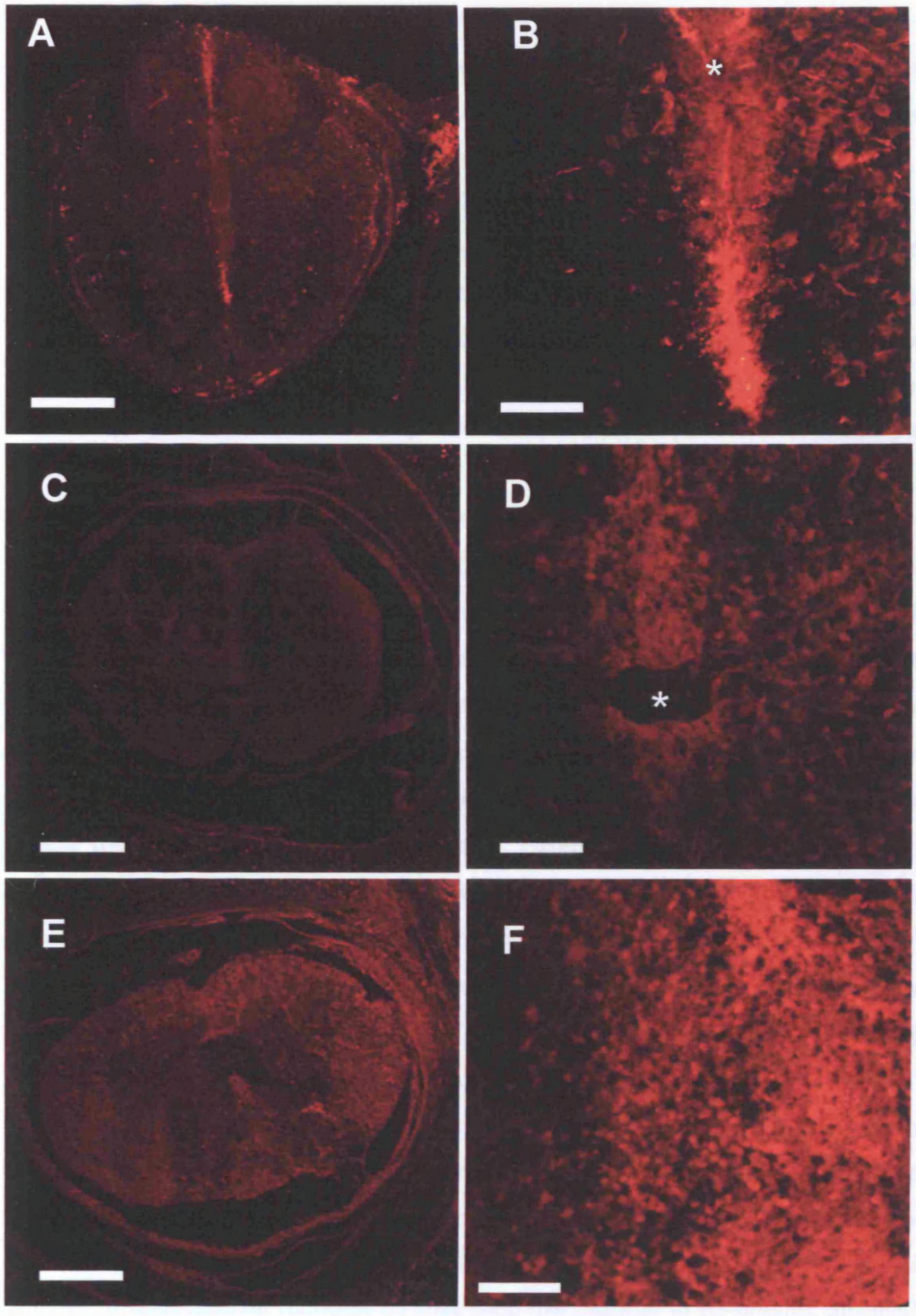


Fig. 7.8. Analysis of 3CB2 expression after injury at E15. Immunostaining for 3CB2 (red) in transverse sections of spinal cord, twenty four hours after injury at E15. **A) and B)** Section from a sham-operated embryo at E16. **C)-F)** Sections from an injured embryo, taken from two different levels, 24 hours after injury at E15. Scale bars A,C,E = 200 μm, B,D,F = 100 μm

for double labelling with BrdU and markers of a neuronal phenotype. According to this strategy, cells dividing at the time of, or subsequent to, an injury will be labelled with BrdU and, if they go on to become neurons, will begin to express neuronal markers. By assessing the spinal cord for cells which are double-labelled for both BrdU and the neuronal marker it should be possible to identify neurons that have been generated in response to the injury. Previously existing neurons or dividing cells that adopt a glial phenotype will be labelled with only one of the markers (Fig. 7.9). This strategy required the selection of multiple specific markers for neuronal differentiation and, therefore, several different commonly-used antibodies were considered for this purpose.

The first antibody tested for the purpose of assessing neurogenesis was anti-β3-tubulin, an early marker of post-mitotic neurons. β3-tubulin is expressed in the cell body and processes of neurons and has been used in a number of similar double labelling studies in conjunction with BrdU. In order to determine the usefulness of β3-tubulin for this purpose, embryos were collected during the period of neurogenesis, at E4, and stained for β3-tubulin and BrdU. As shown in Fig. 7.10.A, at E4, 24 hours after labelling with BrdU, β3-tubulin was expressed in the ventral and lateral regions of the neural tube, where the post-mitotic neurons are residing. Expression of β3-tubulin could also be observed in the axonal projections exiting the ventral and dorsal regions of the neural tube. Double labelling of cells in these regions with BrdU and β3-tubulin could be observed, suggesting that at this developmental stage cells begin to express β3-tubulin within 24 hours of dividing (Fig. 7.10.A'). At E12, 24 hours after BrdU labelling, β3-tubulin expression was widespread throughout the spinal cord (Fig. 7.10.B). This widespread expression, together with the non-nuclear localisation of β3-tubulin, means that it was virtually impossible to determine conclusively whether cells are double labelled with this antibody and BrdU. Consequently, it was decided that this neuronal marker would be unsuitable for double-labelling analysis. Analysis of the expression pattern of doublecortin (Chapter 6) suggested that, similar to β3-tubulin, this marker would be unsuitable for double-labelling owing to its widespread, non-nuclear expression.

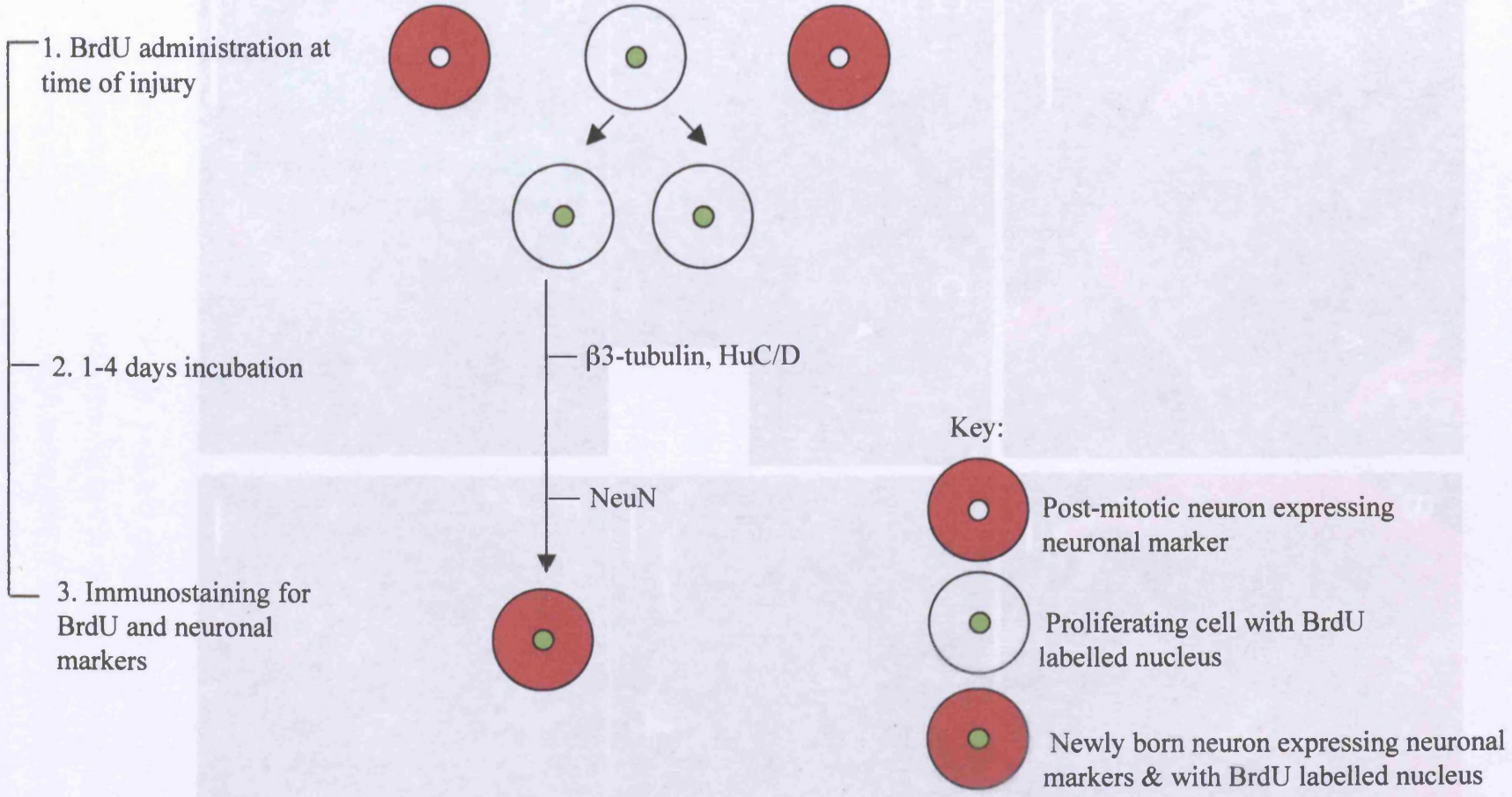
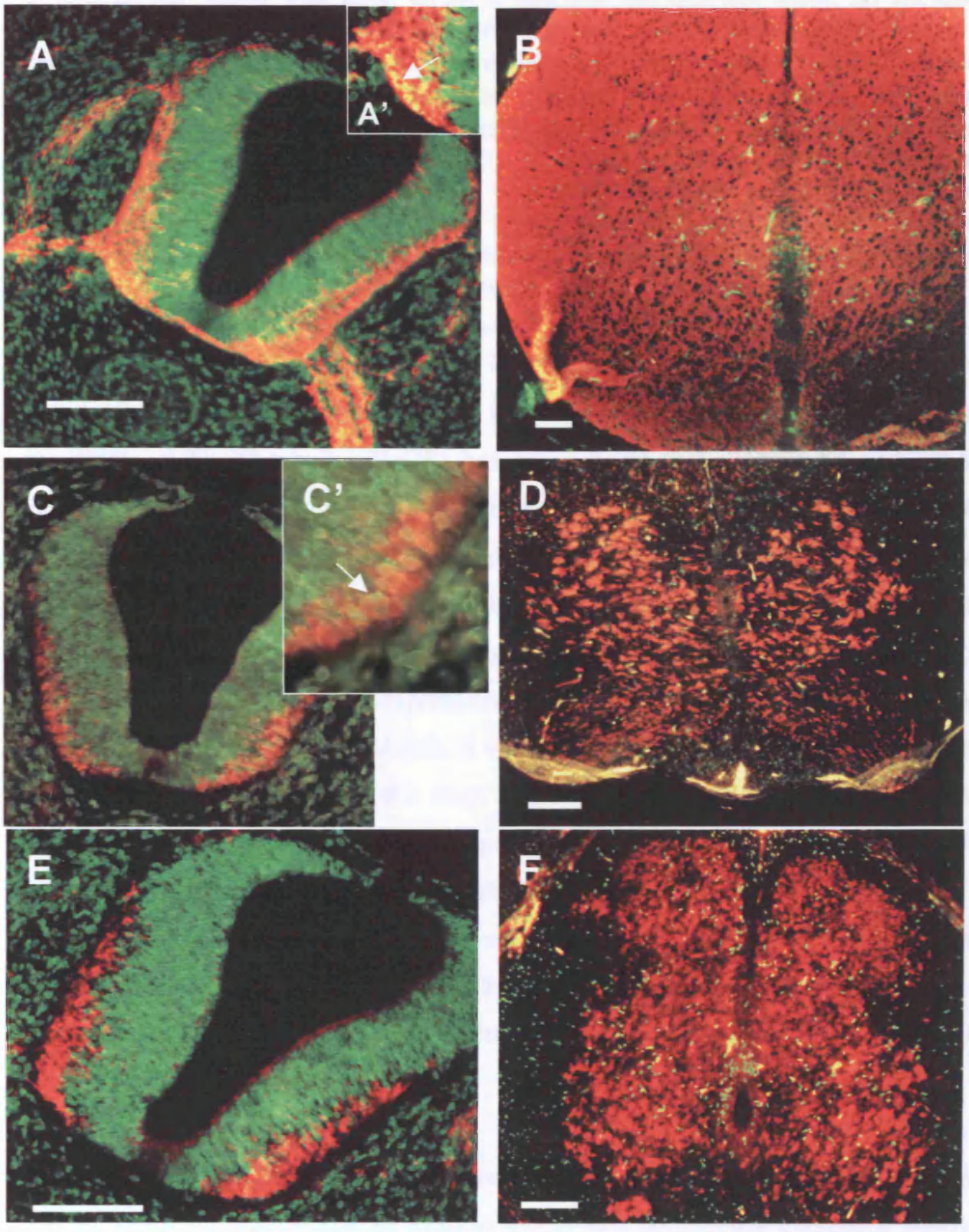


Fig. 7.9. Strategy for assessing neurogenesis in the chick spinal cord. Schematic diagram illustrating the use of double-labelling studies to detect neurogenesis. 1) cells dividing at the time of or subsequent to the injury are labelled with BrdU (green). 2) During the following incubation period of 1-4 days, cells which are becoming neuronal will begin expressing early (β3-tubulin, HuC/D), then late (NeuN) neuronal markers (red). 3) Immunostaining for BrdU and neuronal markers unequivocally identifies newly generated neurons (red and green).


A
A'
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C
C'
D
E
F

Fig. 7.10 (previous page). Comparison of different neuronal markers for assessing neurogenesis. Immunostaining of transverse sections of spinal cord at different developmental stages with three different neuronal markers and BrdU. **A)** E4 spinal cord stained for β3-tubulin (red) and BrdU (green). **A')** High magnification view of cells in the ventrolateral neural tube double-labelled with β3-tubulin and BrdU (arrow). **B)** E12 spinal cord stained for β3-tubulin (red) and BrdU (green), demonstrating the extensive nature of β3-tubulin immunostaining at this stage. **C)** E4 spinal cord stained for HuCD (red) and BrdU (green). **C')** High magnification view of cells in the ventrolateral neural tube double-labelled with HuCD and BrdU (arrow). **D)** E15 spinal cord stained for HuCD (red) and BrdU (green). **E)** E4 spinal cord stained for NeuN (red) and BrdU (green). No double-labelling can be observed at this stage. **F)** E12 spinal cord stained for NeuN (red) and BrdU (green). Scale bars = 0.5mm

Another antibody used to detect neurons is anti-HuC/D. This antibody detects a neuron specific family of RNA binding proteins, including HuC, HuD and Hel-N1 (Marusich *et al.* 1994). Like β3-tubulin, HuC/D has been shown to be expressed early during neuronal differentiation and has a non-nuclear expression pattern (Fornaro *et al.* 2003). However, since HuCD is not expressed in neuronal processes its expression was expected to be slightly less widespread than that of β3-tubulin, and it was hoped that this might provide a more suitable neuronal marker for double labelling analysis. As shown in Fig. 7.10.C, HuC/D expression at E4 was localised towards to ventral and lateral parts of the neural tube. Again, it was possible to observe some overlap between HuC/D and BrdU labelling at this stage (Fig. 7.10.C'). At E12, HuC/D is expressed throughout the grey matter of the spinal cord (Fig 7.10.D). Unlike β3-tubulin expression, it was possible to distinguish individual cells using this antibody and to determine more easily whether cells co-express BrdU, although the non-nuclear expression pattern still presented a challenge for double labelling. Therefore, it was decided that HuC/D would be an appropriate neuronal marker for future double-labelling studies.

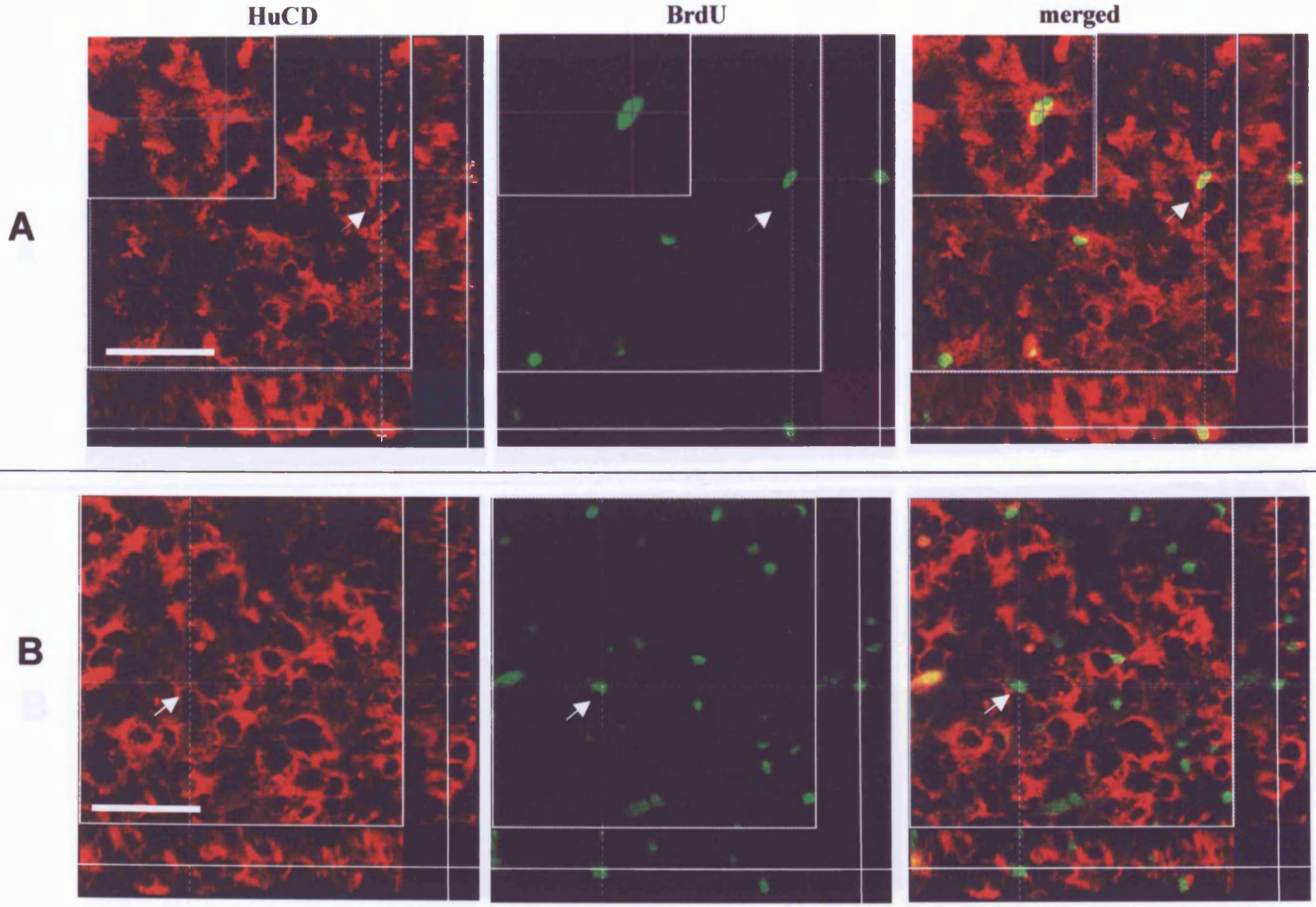
As described previously, the early neuronal markers β3-tubulin and HuC/D present complications for double-staining analysis due to their non-nuclear expression patterns. NeuN is a neuronal marker that has a nuclear expression pattern (Mullen *et al.* 1992). NeuN is expressed in post-mitotic neurons and is reported to be expressed relatively late during neuronal development. As shown in Fig. 7.10.E, at E4, cells in the ventral and lateral parts of the neural tube were shown to express NeuN. However, at this stage, after 24 hours labelling with BrdU, no cells double labelled for BrdU and NeuN could

be observed. This suggests that more than 24 hours are required for a newly born neuron to begin to express NeuN at this developmental stage. At E12, NeuN was expressed throughout the grey matter of the spinal cord (Fig, 7.10.F). Due to the nuclear localisation of the NeuN labelling it was possible to distinguish cells double-labelled for BrdU and NeuN relatively easily since the signals for both overlap. It was therefore decided that NeuN would be an appropriate marker for double-labelling studies, although it was expected that longer incubation periods would be required for newly born neurons to begin to express NeuN.

7.2.5. Analysis of neurogenesis in the chick spinal cord at 24 or 48 hours after injury at E11.

In order to determine whether neurogenesis occurs in the chick spinal cord following an injury at regenerating stages, transection injuries were performed at E11 and BrdU labelling was carried out at the time of injury. Immunostaining for HuC/D and BrdU was carried out on 30 μm sections of spinal cord, 300 μm from the injury site, 24 hours after injury and labelling with BrdU. Since the egg represents a closed system, BrdU is expected to remain available over the course of the 24 hour period and this therefore represents a cumulative labelling strategy. Confocal imaging was used to generate stacks of images in order to visualise cells three dimensionally. For each section up to two individual stacks in different regions of the spinal cord were constructed. These were further sub-divided into 4 higher magnification stacks using the 'separation' function in the confocal software. Once a stack of images had been generated these were exhaustively searched for potentially double labelled cells using the 'cross section' function, as illustrated in Fig. 7.11.

Fig. 7.11. (next page) **Analysis of neurogenesis in sections of sham operated spinal cord at E12 .** Confocal images of immunostaining for HuC/D (red) and BrdU (green) in 30 μm transverse sections of spinal cord 24 hours after BrdU labelling at E11. The central panel of each images shows a single confocal scan in the x-y plane taken from a stack of confocal images. Side panels to the right and bottom show cross sectional view in the x-z and y-z planes through the stack in the places indicated by the dashed white lines. **A)** Sham-operated spinal cord in which one double labelled cell was identified (arrows). Inset pictures show high magnification view of the double-labelled cell. **B)** Sham operated spinal cord in which no double labelled cells were found. The arrow indicates a potentially double-labelled cell which was eliminated from the analysis using this technique, Scale bars = 50 μm (same magnification in each)


HuCD
BrdU
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A
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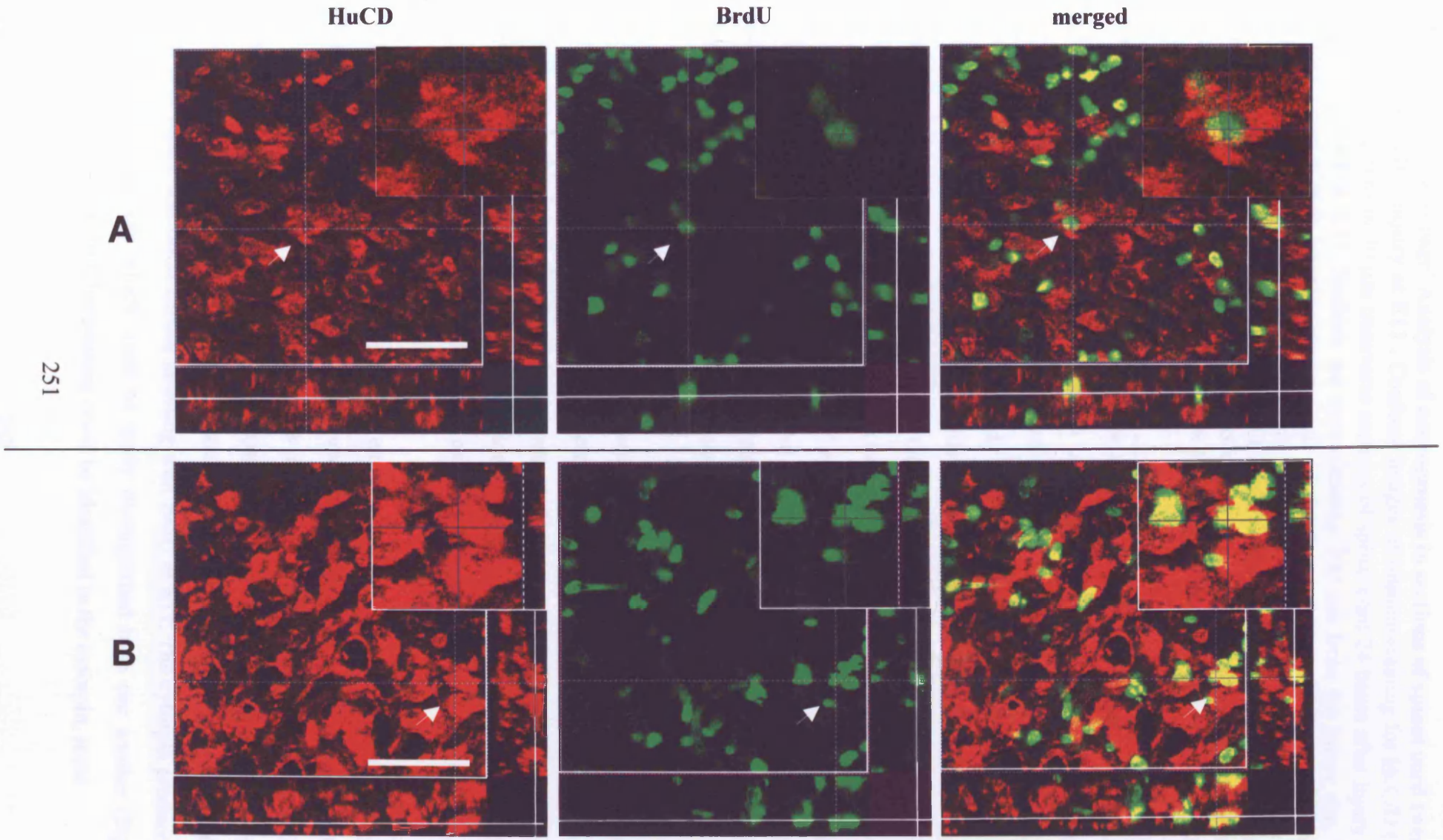

HuCD
BrdU
merged
A
B

Fig. 7.12. (previous page) **Analysis of neurogenesis in sections of spinal cord twenty-four hours after injury at E11 .** Confocal images of immunostaining for HuC/D (red) and BrdU (green) in 30 μm transverse sections of spinal cord 24 hours after injury and BrdU labelling at E11. Sections are approximately 300 μm from the injury site. The central panel of each images shows a single confocal scan in the x-y plane taken from a stack of confocal images. Side panels to the right and bottom show cross sectional view in the x-z and y-z planes through the stack in the places indicated by the dashed white lines. Double-labelled cells are indicated by the arrows. Inset pictures show a high magnification view of the double-labelled cells, Scale bars = 50 μm (same magnification in all pictures)

Since HuC/D does not label the nucleus and will therefore not overlap with BrdU labelling directly it was decided that, in order for a cell to be classified as double labelled there must be unequivocal evidence of a BrdU labelled nucleus (green) surrounded by a HuC/D labelled cell body (red) in all dimensions. As shown in Fig 7.11, and Fig 7.12, using this method double-labelled cells were identified in both sham operated and injured spinal cord samples. Only one double-labelled cell was found out of 6 sections taken from 3 different sham-operated embryos was identified. A total of 8 double labelled cells were found out of 7 sections taken from three different injured embryos were identified. This suggests that neurogenesis is up-regulated after injury, although the sample numbers of cells which could be identified even in these thick sections used were not compatible with statistical analysis.

Although the analysis of neurogenesis in sections with HuC/D and BrdU indicated that neurogenesis does occur after injury, there were still a number of difficulties in identifying double labelled cells with this method. The closely packed nature of neurons in the spinal cord means that it is often difficult to determine whether the BrdU labelled nucleus lies within a single HuC/D labelled cell or between two or more labelled cells.

This means that it is possible to mistakenly exclude a double-labelled cell or to wrongly identify a cell as double labelled. To overcome this problem each cell would need to be visualised at a single cell level. For this reason, it was decided that analysis of neurogenesis on dissociated spinal cord preparation would be advantageous to confirm these results. Cytospin preparations of dissociated spinal cord cells were prepared 48 hours after injury and simultaneous labelling with BrdU at E11. The cytospin produced an even spread of cells which could be easily distinguished from one another (Fig. 7.13A). In order to confirm that neurons could be identified in the cytospin, some

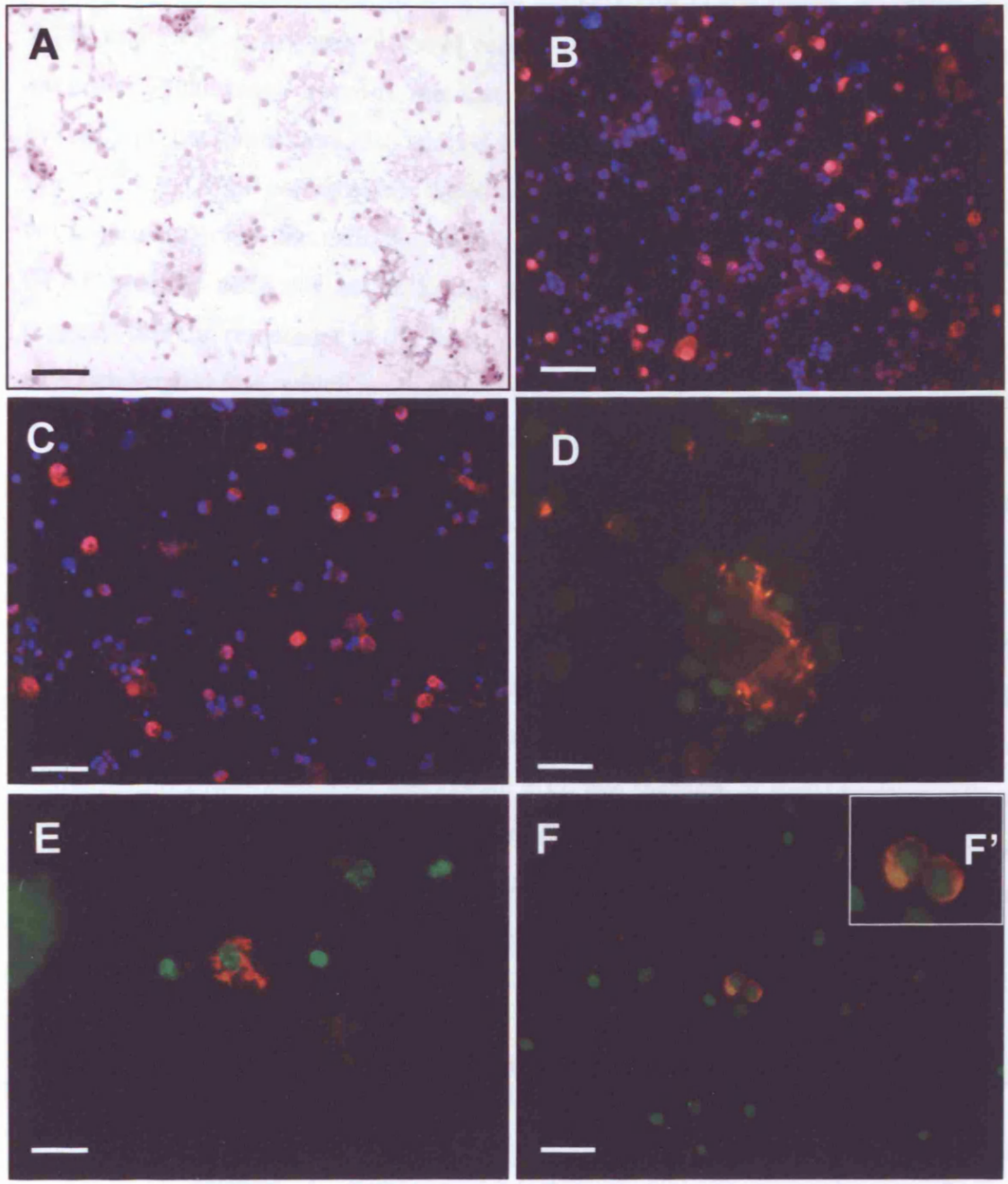


Fig. 7.13. Analysis of cell types present in cytospin preparations of spinal cord. A) H&E staining of a cytospin preparation. **B) and C)** Immunostaining for HuC/D (B) or NeuN (C) (red) in a cytospin taken from E13 spinal cord. Nuclei were counterstained with Hoechst (blue). **D), E) and F)** Immunostaining for GFAP (red) and BrdU (green) in cytospin preparations from E13 (D) and E15 (E and F) spinal cord, after continuous labelling with BrdU from E11. Only a low number of neurons or GFAP positive cells were found in a typical cytospin preparation. Scale bars A-C = 50μm D-F =16 μm

preparations were stained for the neuronal markers HuCD and NeuN. As shown in Fig. 7.13B and 7.13C, a low proportion of cells in the preparation were identified as neurons, suggesting that many neurons are lost during the preparation of the cytospin. The cytospin preparations were also stained for a marker of astrocytes, GFAP. As shown in Fig 7.13D-F, GFAP positive cells could be identified in the cytospin preparation. This is not surprising given that astrocyte development is ongoing at this stage; however again GFAP positive cells did not make up the majority of the cells in the sample. This suggests that the remainder of the BrdU positive cells in the spinal cord would adopt an oligodendroglial fate, which is consistent with the timing of the birth of these cells (Ono *et al.* 2001). Immunostaining for GFAP and BrdU revealed a number of double-labelled cells, indicating that, as expected, some cells undergoing division at this time point are fated to become astrocytes (Fig. 7.13D-F).

Immunostaining for HuC/D and BrdU in cytospin preparations demonstrated the existence of double-labelled cells in both sham operated and injured spinal cord samples in agreement with the findings in spinal cord sections (Fig. 7.14A-E). The numbers of double-labelled cells were very low (Table 7.1); less than 2% of BrdU positive cells co-expressed HuC/D. Although the percentage of double-labelled cells in the injured spinal cord was higher than that in the sham-operated spinal cord this did not prove statistically significant (p=0.432, Mann Whitney Test).

Table 7.1. Quantification of double labelling with BrdU and HuC/D in cytospin preparations 48 hours after injury at E11

	No. of samples	Total BrdU+ve cells counted	Total double-labelled cells	% total BrdU+ve cells which co-expressed HuCD	Average % double-labelled cells in group (±SEM)
Sham	9	2455	16	0.65	0.65 (± 0.195)
Injured	4	800	10	1.25	1.65 (± 0.89)

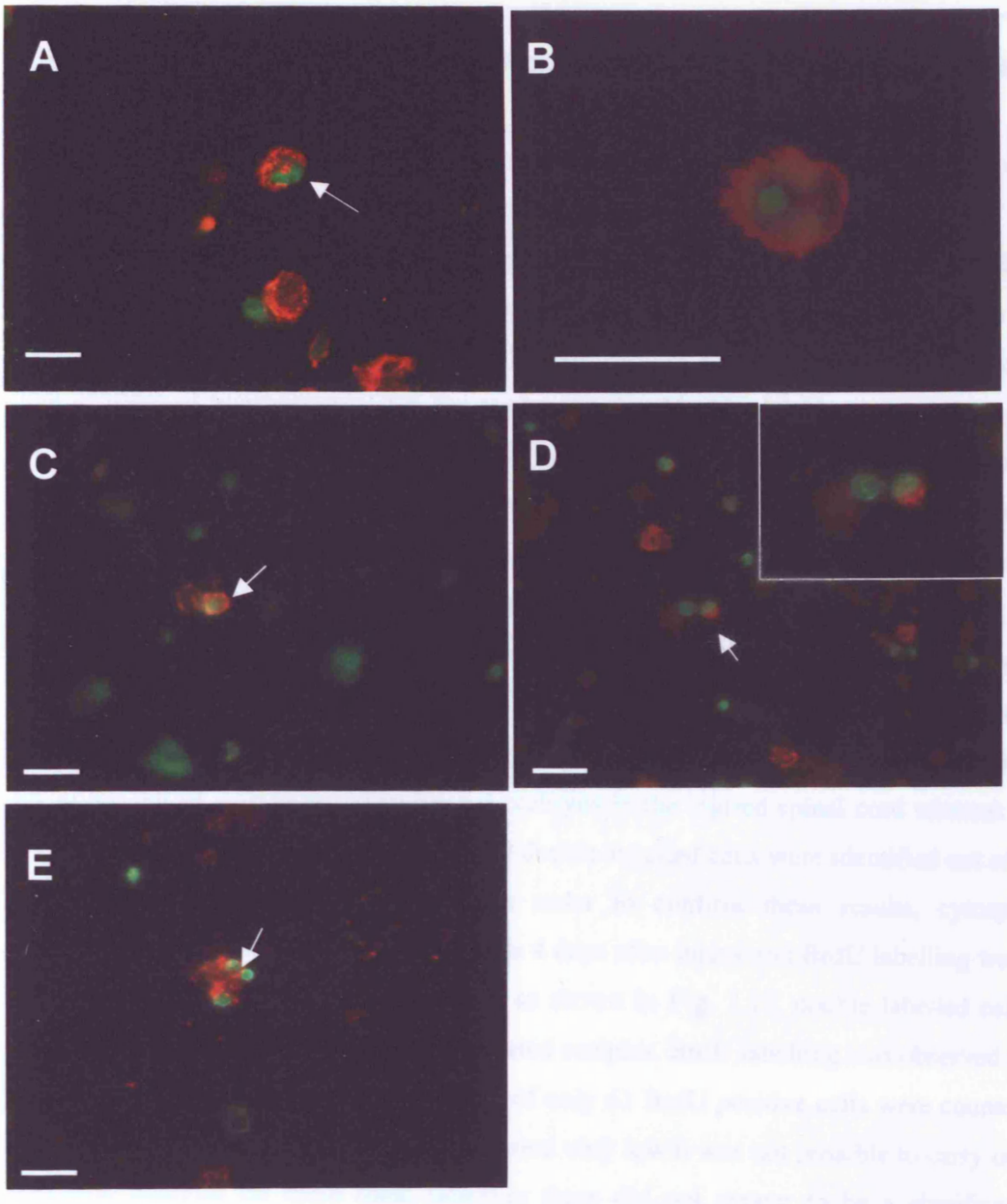
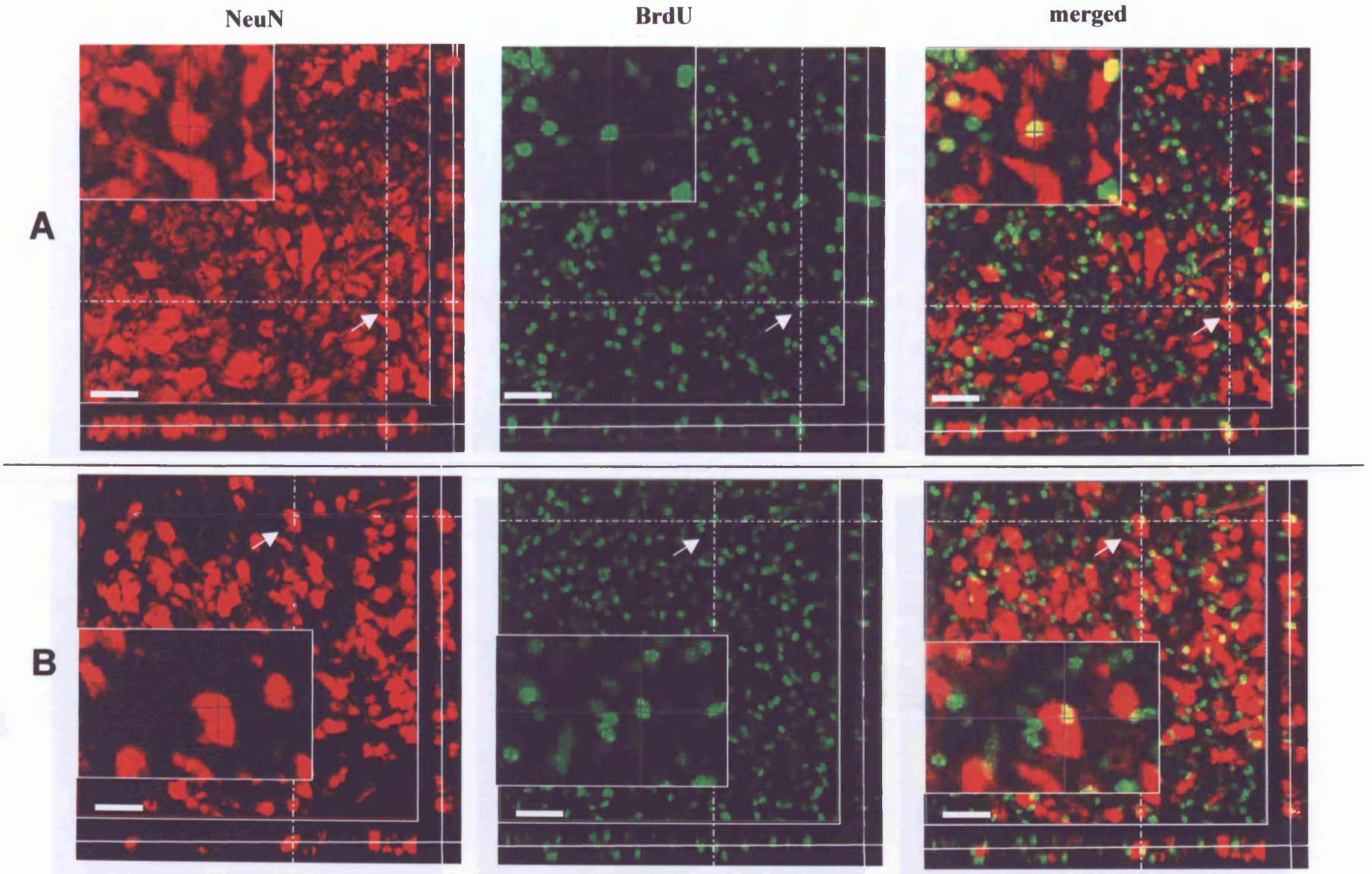


Fig. 7.14. Analysis of neurogenesis in spinal cord cytospin preparations 48 hours after injury at E11. A)-E) Immunostaining for HuC/D (red) and BrdU (green) in cytospin preparations taken from injured (A and B) or sham-operated (C-E) spinal cords 48 hours after injury at E11. Double-labelled cells were observed in both sham-operated and injured samples (arrows). Scale bars = 16 μm.

7.2.6. Analysis of neurogenesis in the chick spinal cord at 4 days after injury at E11

The results of the experiments described above had unexpectedly revealed the presence of apparent neurogenesis in sham-operated spinal cords. However despite the use of the cytospin preparation, there were still some difficulties in identifying double labelling with HuC/D and BrdU. The non-nuclear labelling of HuC/D made if difficult to be absolutely certain a cell was labelled with both markers. Consequently, it was decided that analysis of neurogenesis with the nuclear marker NeuN might improve confidence in these findings. Since NeuN is expressed later than HuC/D, in order to use NeuN as a marker for neurogenesis, spinal cords were collected 4 days after injury. BrdU was administered daily from E11 to result in cumulative labelling and prevent dilution of the BrdU signal in dividing cells. Confocal analysis was used to assess double labelling of BrdU and NeuN in 30μm spinal cord sections of injured and sham-operated embryos. As shown in Fig. 7.15 and Fig. 7.16, cells double labelled with NeuN and BrdU were found in both injured and sham-operated embryos. In total, 4 double-labelled cells were identified out of 7 sections taken from 4 embryos in the injured spinal cord whereas in the sham-operated spinal cord a total of 10 double-labelled cells were identified out of 9 sections examined from 6 embryos. In order to confirm these results, cytospin preparations of dissociated spinal cord cells 4 days after injury and BrdU labelling were also stained for BrdU and NeuN. Again, as shown in Fig. 7.17, double-labelled cells were found in both injured and sham-operated samples. BrdU labelling was observed to be lower in these samples and an average of only 61 BrdU positive cells were counted per sample. Because the sample numbers were very low it was not possible to carry out statistical analysis on these data; however there did not appear to be a significant difference in the percentage of double-labelled cells between the injured and sham-operated cords (Table 7.2). It was notable that the percentage of double-labelled cells

Fig. 7.15. (next page) **Analysis of neurogenesis in the sham-operated spinal cord 4 days after BrdU labelling at E11.** Confocal images of immunostaining for NeuN (red) and BrdU (green) in 30 μm transverse sections of spinal cord 4 days after BrdU labelling at E11. The central panel of each images shows a single confocal scan in the x-y plane taken from a stack of confocal images. Side panels to the right and bottom show cross sectional view in the x-z and y-z planes through the stack in the places indicated by the dashed white lines. Double labelled cells are indicated by arrows. Inset pictures show high magnification view of the double-labelled cells. Scale bars = 40 μm


NeuN
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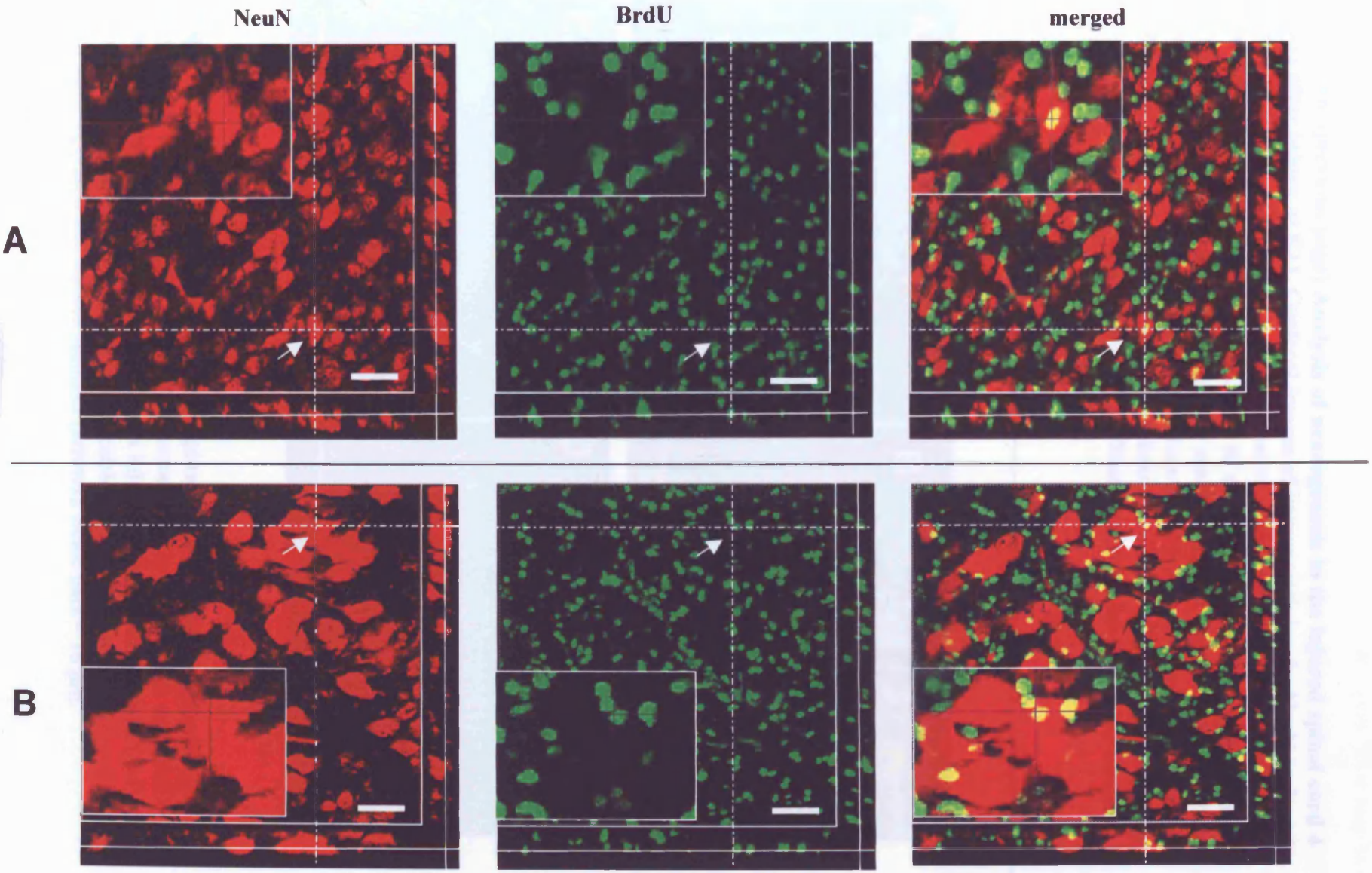
NeuN
BrdU
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Fig. 7.16. (previous page) **Analysis of neurogenesis in the injured spinal cord 4 days after injury at E11.** Confocal images of immunostaining for NeuN (red) and BrdU (green) in 30 μm transverse sections of spinal cord 4 days after injury and BrdU labelling at E11. The central panel of each images shows a single confocal scan in the x-y plane taken from a stack of confocal images. Side panels to the right and bottom show cross sectional view in the x-z and y-z planes through the stack in the places indicated by the dashed white lines. Double labelled cells are indicated by arrows. Inset pictures show high magnification view of the double-labelled cells. Scale bars = 40 μm

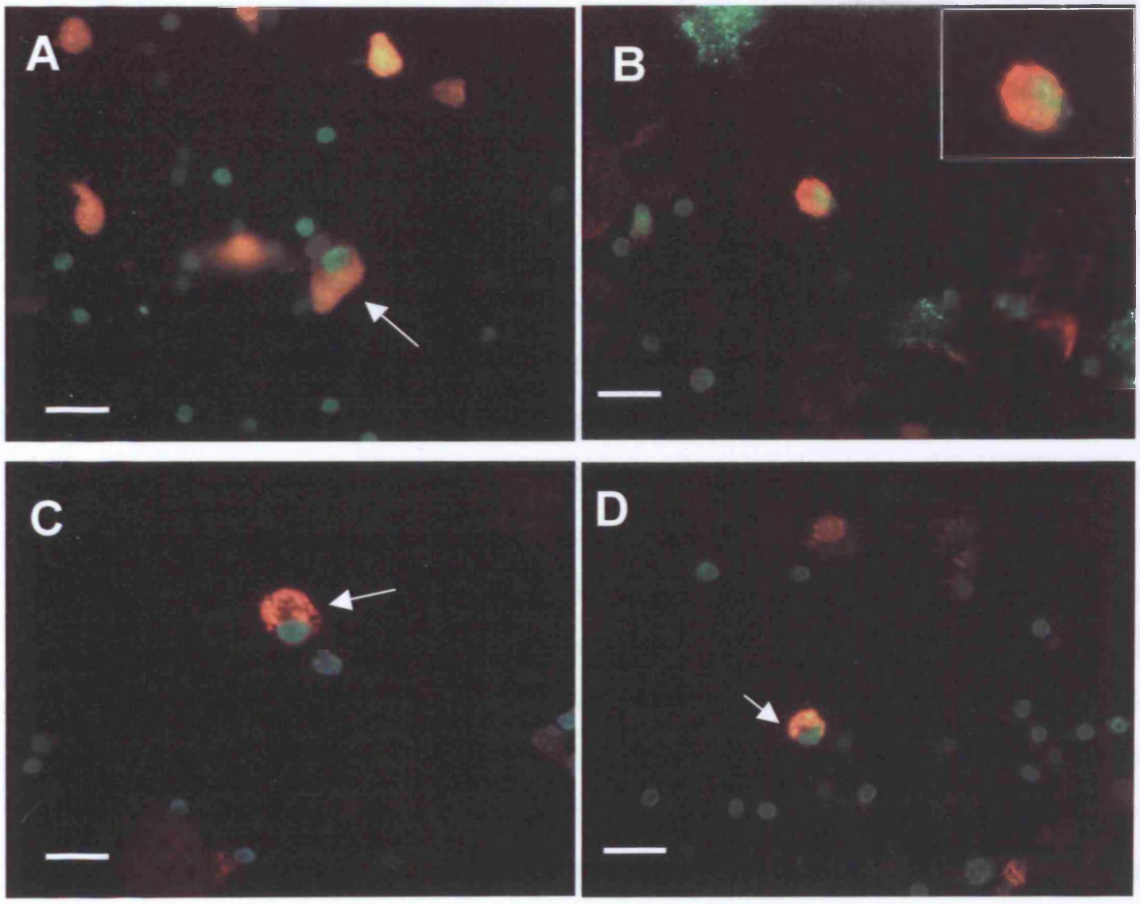


Fig. 7.17. Analysis of neurogenesis in spinal cord cytospin preparations 4 days after injury at E11. A)-D) Immunostaining for NeuN (red) and BrdU (green) in cytospin preparations taken from injured (A) or sham-operated (B-D) spinal cords 4 days after injury at E11. Double-labelled cells were observed in both sham-operated and injured samples (arrows). Scale bars = 16 μm.

much lower in the cytospin preparations assessed at 4 days after injury. This may have been a result of a greater loss of neurons in the preparation of a cytospin from these older embryos.

Table 7.2. Quantification of double-labelling with BrdU and NeuN in cytospin preparations 4 days after injury at E11

	No. of samples	Total BrdU+ve cells counted	Total double-labelled cells	% total BrdU+ve cells which co-expressed NeuN	Average % double-labelled cells in group (±SEM)
Sham	2	1833	2	0.109	0.11 (± 0.08)
Injured	2	1833	1	0.555	0.07 (± 0.05)

7.2.7. Analysis of proliferation after injury in the regenerating chick spinal cord

The results described above revealed unexpectedly that neurogenesis is not only observed in the injured spinal cord, but is also present in the normal spinal cord at E11. These results, however, did not confirm whether this process is up-regulated after injury at this stage. In order to investigate further how the spinal cord responds to injury, it was important to determine whether there is an increase in overall proliferation following an injury. Sections located approximately 300 μm caudal or rostral to the injury site were stained for BrdU. Two confocal images, one of each half of a transverse section of spinal cord, were used for image analysis. Using image analysis software, each section was divided into three regions; the ependymal region consisting of a pseudostratified layer of cells around the central canal, the grey matter and the white matter (Fig. 7.1.D). The number of BrdU positive cells was counted in each region and expressed relative to the total area of that region. Sections from a total of 4 injured and 6 sham-operated spinal cords were measured, with up to two sections (one caudal and one rostral) taken from each embryo. As shown in Fig. 7.18, there was a significant increase in the number of BrdU positive cells in the grey matter of the spinal cord 24 hours after injury, but no up-regulation of proliferation in the ependymal region or the white matter. This further supports the hypothesis that neurogenesis might increase after injury at E11.

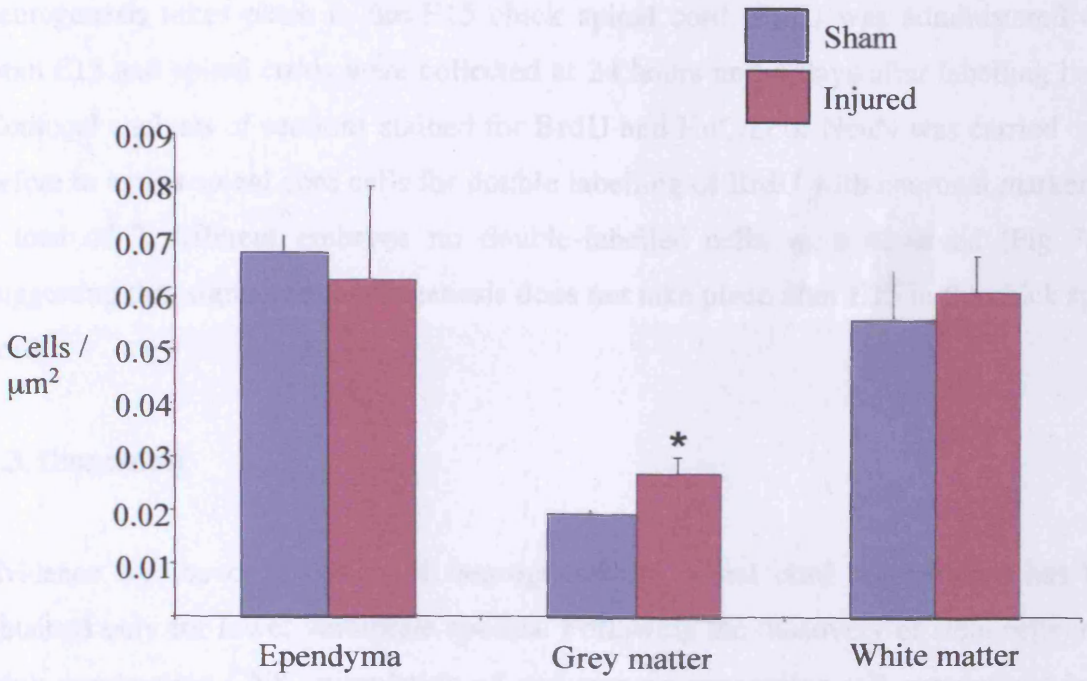


Fig. 7.18. Analysis of proliferation in the injured chick spinal cord by BrdU labelling. BrdU positive cells were counted in three spinal cord regions in transverse sections 300 µm from the injury site, 24 hours after injury and labelling with BrdU at E11. Data shows mean cell count/ μm^2 ± SEM in sham and injured embryos. There was a statistically significant increase in proliferation in the grey matter. * p= 0.033 for grey matter p=0.83 for ependyma and p=0.394 for white matter (Mann Whitney Test)

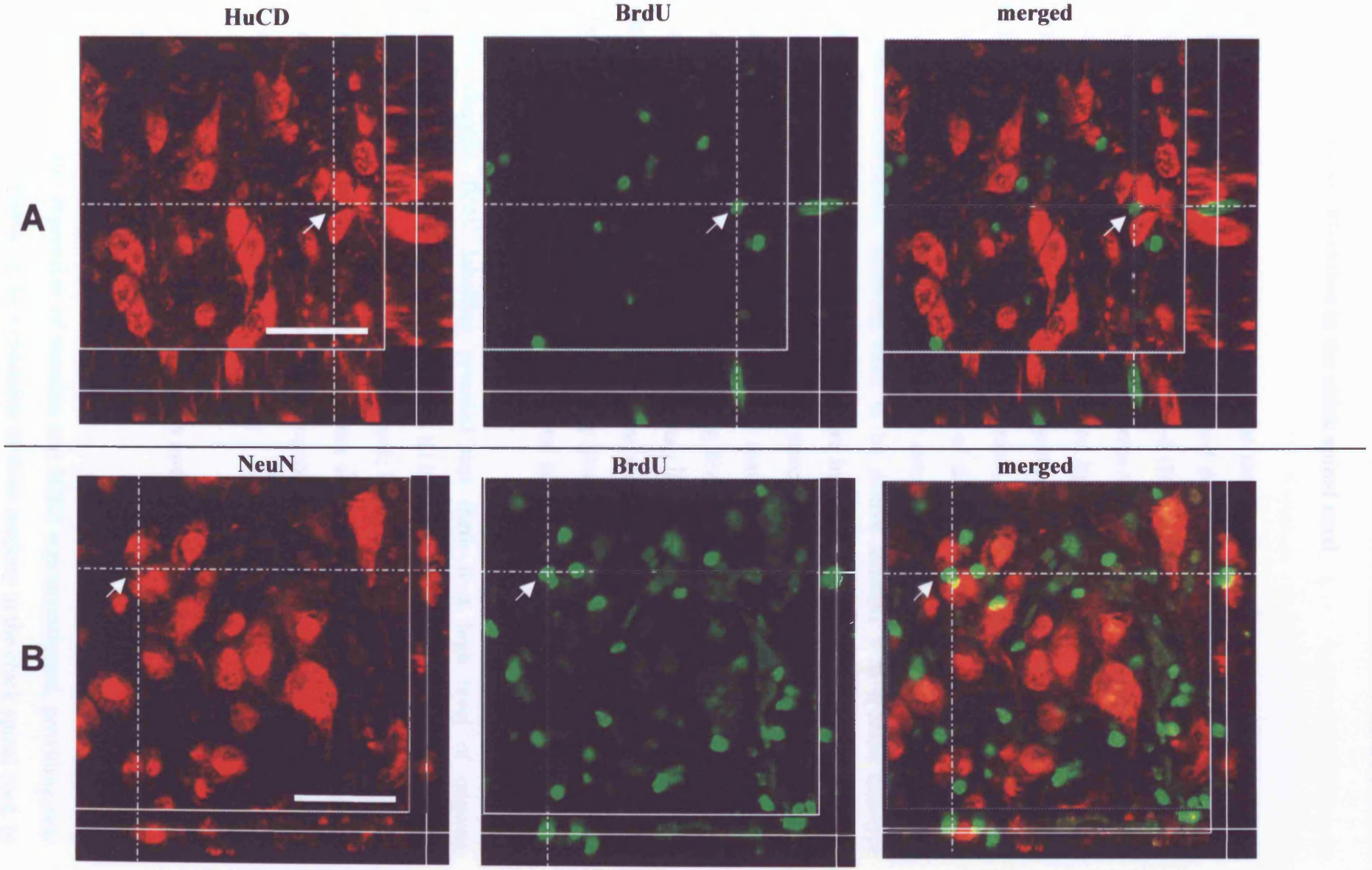
7.2.8. Analysis of neurogenesis in the E15 chick spinal cord

As described above, neurogenesis in the chick spinal cord may continue at low levels beyond the expected developmental stage, possibly contributing to the regenerative capacity of the E11 spinal cord. Since a subsequent reduction in this process might play a role in the loss of regenerative capacity, it was necessary to determine whether neurogenesis takes place in the E15 chick spinal cord. BrdU was administered daily from E15 and spinal cords were collected at 24 hours and 4 days after labelling began. Confocal analysis of sections stained for BrdU and HuC/D or NeuN was carried out as before to assess spinal cord cells for double labelling of BrdU with neuronal markers. In a total of 7 different embryos no double-labelled cells were observed (Fig 7.19), suggesting that significant neurogenesis does not take place after E15 in the chick spinal cord.

7.3. Discussion

Evidence for the contribution of neurogenesis to spinal cord regeneration has been obtained only for lower vertebrate species. Following the discovery of stem cells in the adult mammalian CNS, stimulation of endogenous progenitor cell populations is one option that is being considered as a therapeutic strategy for spinal cord injury. For this reason, it was of interest to determine the extent to which the loss of these capabilities might contribute to reduced regenerative capacity in the embryonic chick. In Chapter 6, an increase in the expression and phosphorylation of doublecortin was observed at E11, which might reflect increased levels of neurogenesis. In order to test this hypothesis, this study aimed to investigate neurogenesis in the injured chick spinal cord.

Fig. 7.19. (next page) **Analysis of neurogenesis in the E15 chick spinal cord. A)** Confocal images of immunostaining for HuC/D (red) and BrdU (green) in a 30 μm transverse section of spinal cord 24 hours after BrdU labelling at E15. **B)** Confocal images of immunostaining for NeuN (red) and BrdU (green) in a 30 μm transverse section of spinal cord 4 days after BrdU labelling at E15 The central panel of each image shows a single confocal scan in the x-y plane taken from a stack of confocal images. Side panels to the right and bottom show cross sectional view in the x-z and y-z planes through the stack in the places indicated by the dashed white lines. The arrows indicate potentially double-labelled cells which were eliminated from the analysis using this technique, Scale bars = 50 μm (same magnification in all pictures)


HuCD
BrdU
merged
A
NeuN
BrdU
merged
B

7.3.1. Assessing proliferation in the chick spinal cord

A number of different techniques exist for the detection of cell proliferation. These include antibodies against proteins expressed during specific phases of the cell cycle, such as PCNA and phosphorylated histone-3 (H3), DNA incorporation of BrdU or ^{3}H-thymidine and retroviral labelling with proteins such as green fluorescent protein (GFP). In this study, cell proliferation was detected by labelling DNA in dividing cells with BrdU. This method labels all cells that synthesise DNA during the period in which BrdU is available in the egg. Once incorporated, BrdU will remain present in the DNA of the cell throughout subsequent rounds of division and, ultimately, differentiation. Unlike ^{3}H-thymidine, which is detected by autoradiography, BrdU can be detected by immunohistochemistry, allowing cells to be double labelled with specific cell-type markers (Gratzner 1982). These factors were important for this study since they allow the detection of cells which have divided, incorporating BrdU and differentiated into neurons at a later time point. In contrast, markers such as PCNA and H3 are only expressed for a relatively limited time during division, which would hinder such double-labelling analysis. The use of BrdU rather than ^{3}H-thymidine also avoids the need to use radioactive isotopes and is a more efficient method of labelling cells than retroviral labelling. As the results of the current study show, it was possible using this technique to label proliferating cells at all developmental stages tested.

In this chapter, BrdU labelling revealed that there is a high level of ongoing proliferation in the chick spinal cord at both E11 and E15. Cells which were positive for BrdU were detected throughout the spinal cord; between E11 and E15 there was a trend towards increased labelling in the white matter and reduced labelling in the grey matter, which fits with the transition between neurogenesis and astrocyte and oligodendrocyte development.

7.3.2. Timing of the expression of transitin and 3CB2 in the developing chick spinal cord

In this chapter, the expression of transitin and 3CB2 was investigated, providing new information on the timing of the expression of these markers in the chick spinal cord. In a number of species, nestin has been used as a marker of neural precursor or stem cells,

although it has also been shown that nestin is expressed by reactive astrocytes (Dahlstrand *et al.* 1995; Lendahl *et al.* 1990; Reynolds and Weiss 1992; Weiss *et al.* 1996). At present, no chick homologue of nestin has been identified; however a member of the same family of intermediate filament proteins, transitin, has been shown to share 45% homology to nestin in the α-helical core domain, and to share a similar developmental expression pattern (Cole and Lee 1997; Lee and Cole 2000; Napier *et al.* 1999; Yuan *et al.* 1997). The results of the present study confirmed and extended these previously reported patterns of transitin expression in the developing chick spinal cord and showed that, by E11, most expression has become restricted to the dorsal midline with only low levels of expression persisting in radial fibres to relatively late developmental stages (at least E16). Similarly, staining with the radial glial marker 3CB2, showed that the pattern of radial glial expression in the chick spinal cord matches that observed in other species (McMahon and McDermott 2002). Again expression became restricted mainly to the dorsal midline at late stages, and only very low-level expression persisted in radial fibres. Both transitin and 3CB2 have been reported to be expressed in radial glia, and radial glia have been suggested to act as neural precursors during development. This expression therefore indicates that radial glial cells, which might potentially give rise to new neurons are mostly down-regulated by E11, although the persistence of low-level staining might be associated with radial glial that would go on to transform into astrocytes.

7.3.3. Changes in the expression of neural precursors and radial glia in the chick spinal cord after injury

Initial experiments showed that there are high levels of ongoing proliferation in the spinal cord at E11. Under normal circumstances at this stage, it was presumed that most of these cells would become astrocytes or oligodendrocytes as the developmental period of neurogenesis has been reported to be over by E9 (Fujita 1964). It was therefore important to determine whether, in response to injury, any of the dividing cells might adopt a phenotypic fate which is untypical for this stage of development and become neuronal, in order to replace neurons lost in the injury. This was suggested by the results of Chapter 6, in which the early neuronal marker doublecortin was shown to be up-regulated after injury.

In this chapter it was shown that transitin is also up-regulated after injury at E11. This is in agreement with a number of studies which have demonstrated an up-regulation of nestin expression after spinal cord injury (Namiki and Tator 1999; Shibuya *et al.* 2002). It has been suggested that the up-regulation of nestin may represent an increase in the number of neural precursor cells and may subsequently contribute to the production of glial cells and the formation of a glial scar (Frisen *et al.* 1995). However, a glial scar has not been observed in the developing chick spinal cord and, at the stages being examined, the spinal cord is too immature for this up-regulation to represent reactive astrocytes. Alternatively, transitin expression might represent an up-regulation or activation of radial glial or other multipotential progenitor cells that could help to replenish lost neurons after injury.

The up-regulation of transitin observed after injury at E11 might therefore represent an up-regulation in the formation of radial glial cells or the conversion of another cell type to a radial glia phenotype. A marker of radial glial, 3CB2, has been demonstrated previously to be up-regulated after spinal cord injury in the rat (Shibuya *et al.* 2003) . However, no up-regulation of 3CB2 expression was observed after injury at E11 or E15. The discrepancy between these results and those reported previously might be explained by experimental differences; in the current study, 3CB2 expression was measured at twenty-four hours after injury wheras in the rat spinal cord 3CB2 was first up-regulated at 1 week after injury. It is therefore possible that such an up-regulation might have been missed in the present study. The up-regulation of transitin but not 3CB2 observed in the current study might also be a reflection of heterogeneity of the radial glial cell population. It has been shown previously that different subtypes of radial glia may be destined towards different fates (Hartfuss *et al.* 2001; Kriegstein and Gotz 2003). On the other hand, up-regulation of transitin expression may have occurred in radial glia, already present in the spinal cord, although the staining patterns observed in the current study would suggest that any such population of cells would be very small. This was partly addressed by double-labelling for BrdU and transitin, which indicated that some, but not all, of the transitin expression might be found in cells which have recently divided, although it was difficult to assess the correlation between the filamentous staining of transitin and the nuclear BrdU staining.

7.3.4. Assessing neurogenesis in the chick spinal cord

While the up-regulation of transitin and doublecortin at E11 may be indicative of an increase in the numbers or activity of neural stem cells it remained unclear to what extent this might lead to the generation of mature neurons in the injured spinal cord. It was therefore necessary to develop a technique to visualise the new-born neurons directly. This issue was addressed by double staining for both BrdU incorporation, beginning at the time of injury, and neuronal phenotypic markers, HuC/D and NeuN. These markers were chosen from several available because it was hoped that their localisation in the cell body (HuC/D) and the nucleus (NeuN) would simplify the identification of double-labelled cells. In contrast, the early neuronal markers β3-tubulin and doublecortin were rejected from this study as their extensive labelling of axonal tracts in addition to the cell body severely hampered the ability to determine conclusively whether a particular cell was double labelled.

In the present study double labelling with BrdU and HuC/D or NeuN was demonstrated by two different methods: three-dimensional confocal analysis of spinal cord sections and cytospin preparation of dissociated spinal cord cells. These two different approaches to investigating double labelling were employed in order to reduce the possibility of falsely identifying cells as double labelled. Confocal analysis of thick sections provided the advantage of allowing the analysis of large numbers of cells and the visualisation of each potentially double-labelled cell from three dimensions, as well as providing positional information about the localisation of any double-labelled cells. The cytospin preparation provides better single-cell resolution of double-labelled cells but removes all positional information and may alter the morphology of the cells being studied. Both methods are time-consuming; however it was felt that this type of in-depth analysis was required in order to be fully confident of the results obtained. Many double-labelling studies in the past have relied on analysis of sections by fluorescent microscopy alone. However the experience gained during the current work suggests that it is easy to obtain false positive identification of double-labelling by this method as it is difficult to distinguish a single cell labelled with both markers from two cells closely positioned to one another, each labelled with only one marker.

7.3.5. Ongoing neurogenesis in the E11 chick spinal cord

One of the most interesting findings of these investigations was that neurogenesis in the chick spinal cord appears to still be ongoing at E11, albeit at a relatively low level. This is further supported by the findings of the previous chapter, in which expression of doublecortin, which has been proposed to be a marker of neurogenesis, was shown to be relatively high at this stage. It has been believed previously that all neurogenesis in the spinal cord ends several days before this time point. However, in reality, there have been very few studies examining the question of the timing of the end of the neurogenic period in the chick. Many more studies have tended to focus more on the initiation of neurogenesis. One study which has attempted to comprehensively investigate this issue used ^{3}H-thymidine to label cells undergoing division at specific time points during development (Fujita 1964). As with BrdU labelling in the chick, this results in a cumulative labelling and all cells dividing after the administration are labelled, while those which have already undergone their terminal differentiation remain unlabelled. Using this technique, it was shown that the last day at which cells which subsequently became neuronal could be labelled was at E9 in the cervical spinal cord and E8 in the lumbar spinal cord (Fujita 1964). There may be a number of reasons for the conflicting findings of the current study and this previous report. For example, the methods of identifying neurons were different, with the older study relying on the distribution of cells at various time points during development, rather than labelling with neuronal markers as in the current experiments. Importantly, as shown in the present study, the numbers of double-labelled cells that are found represent a fairly low proportion of the total number of cells in the spinal cord. In the study by Fujita, very low numbers of embryos were studied (for example, only two embryos were labelled at E11 and examined after hatching) and relatively thin microtome sections were examined. Use of the confocal microscope which could visualise all cells in a 30μm section, has allowed the examination of many more cells in the current study, making it more likely that these relatively rare double-labelled cells would be identified. Double labelling was confirmed using four different experimental protocols and a total of 33 embryos (including both sham-operated and injured samples): confocal analysis of BrdU and neuronal markers at 24 hours and 4 days after injury, and cytospin preparations taken at 48 hours and 4 days after injury. This variety of different approaches all produced the

same results, adding to confidence in the conclusion that neurogenesis takes place in the E11 chick spinal cord.

Having stated that the number of cells undergoing neurogenesis at this stage is relatively low it is important to consider whether this level of ongoing neurogenesis is of biological relevance, and in particular whether it may make a contribution to the regenerative capacity of the embryonic chick spinal cord at E11. From the numbers of double-labelled cells counted in this study it is suggested that around 2 double-labelled cells might be identified in one 30μm section of spinal cord, giving a total of 66 double-labelled cells per millimetre length of spinal cord. It is possible that the true number would be higher than this, since the stringent criteria used to identify double-labelled cells in this study are likely to underestimate the extent of neurogenesis. It remains to be determined whether this proportion of newly born neuronal cells does make an important contribution to the repair process after spinal cord injury. However the existence of ongoing neurogenesis does suggest that the E11 chick spinal cord is less mature than previously thought and that the regenerative process might involve the replacement of lost cells from endogenous progenitors in addition to axonal regeneration.

This study also raises the issue of when neurogenesis does end in the chick spinal cord. Due to the cumulative labelling strategy it is not possible to know at what time point after labelling began the double-labelled cells were actually born. As shown in these results, no double-labelled cells were observed in the chick spinal cord after labelling at E15, suggesting that by this stage, neurogenesis is complete. A more comprehensive study, involving BrdU labelling at successive stages between E11 and E15 could provide an answer to this question.

7.3.6. Is neurogenesis up-regulated after injury at E11?

As outlined above, the results of this investigation revealed double-labelled cells, indicating neurogenesis, present in the injured spinal cord and also, unexpectedly, in sham-operated spinal cords. The original assumption was that no double-labelled cells would be found in the sham-operated spinal cords and so any double labelling in the

injured spinal cord would therefore represent an up-regulation of neurogenesis after injury. Therefore, the primary purpose of the study was not to quantify the number of double-labelled cells. Consequently, the number of samples that there was time to collect and analyse was insufficient for a thorough quantitative analysis of neurogenesis. This was made more difficult by the low levels of neurogenesis observed – an average of only 2 cells per section were double labelled. However, in light of the identification of new-born cells in the normal post-E11 spinal cord, attempts were made to determine whether this process is up-regulated in the injured spinal cord. In support of the suggestion that neurogenesis might increase after injury, a significant increase in proliferation in the grey matter was observed at 24 hours after injury, which could be indicative of an increase in the birth of neuronal precursors, although it is not possible to know whether this increase might, in fact, indicate an increase in the proliferation of glial progenitors. Furthermore, as shown in Chapter 6, the microtubule protein, doublecortin, which is expressed in early post-mitotic neurons, was shown to be up-regulated after injury at E11, as was transitin, a putative marker of neural precursors. In spinal cord sections, the number of cells double-labelled with BrdU and HuC/D at 24 hours after injury was greater in the injured samples examined than in the sham-operated spinal cords. This might therefore represent an up-regulation of neurogenesis in the injured spinal cord. However, when examining the frequency of double labelling between BrdU and NeuN at 4 days after injury the situation was reversed, with larger numbers of double-labelled cells being identified in the sham-operated samples. This might represent an increase in cell death in the injured spinal cord. Unfortunately, the numbers obtained were insufficient for statistical comparison.

The cytospin preparation represented an opportunity to carry out cell counting in order to investigate this further. The number of double-labelled cells was compared to the total number of BrdU labelled cells for two reasons. Firstly, due to the fact that nuclear dyes do not appear to work correctly after BrdU administration, it was difficult to obtain a measure of total cell numbers and secondly, this method was also expected to take into account any possible variation in BrdU up-take between embryos. Using both HuC/D and NeuN as neuronal markers, it was found that there was no significant difference in the frequency with which double-labelled cells were found in the injured spinal cord in comparison to the sham-operated spinal cords. Again, the sample numbers were relatively low and there was a large degree of variability within each experimental

group, suggesting that a small increase in neurogenesis would not be detected by this method.

Overall, these results do not provide sufficient evidence of an up-regulation of neurogenesis after injury at E11 in the chick spinal cord. However, it would be of great interest to carry out a more quantifiable comparison of double labelling in the injured and sham-operated spinal cord. This would require much larger sample numbers, which is no trivial given the low survival rates, particularly at 4 days after injury for analysis with NeuN, and variability in the up-take of BrdU which means that around half of the embryos do not become labelled.

7.3.7. Conclusions

In this chapter, the potential contribution of proliferation and neurogenesis to the regenerative capacity of the E11 chick spinal cord has been considered. A number of difficulties and precautions necessary when using BrdU labelling in conjunction with neuronal markers have been identified. The findings of these investigations have demonstrated for the first time that, in the E11 chick spinal cord, proliferation and neurogenesis are ongoing processes, whereas neurogenesis appears to be complete by E15. The regeneration-competent chick spinal cord is therefore less mature than has previously been expected which may contribute to its favourable response to injury. There appears to be a correlation between the end of neurogenesis, which was demonstrated to occur somewhere between E11 and E15, and the loss of regenerative capacity. An increase in proliferation in the spinal cord grey matter, together with an increase in transitin and doublecortin expression, may indicate an up-regulation of neurogenesis after injury at E11, although further work will be required to examine this quantitatively.

Chapter 8. Final Discussion

8.1. Background

The chick spinal cord undergoes a dramatic loss of regenerative capacity at a key transitional stage of embryonic development, around E13. This model system therefore provides an opportunity to examine both the processes involved in spinal cord regeneration and those that are involved in preventing such regeneration, in the same species. The embryonic chick represents a system that shares features both with highly regeneration-competent lower vertebrates and with mammalian species. Understanding the factors which govern changes in regenerative capacity in the embryonic chick may provide insights into why the adult mammalian spinal cord is incapable of regeneration after injury, and may therefore suggest strategies to improve the outlook for spinal cord injury patients.

The majority of previous studies, both in the chick and in other regeneration competent and incompetent species have tended to focus on promoting axonal re-growth as a central issue. These studies have shown that strategies which down regulate or block inhibitory signalling from a range of myelin and glial-scar derived molecules may improve axonal re-generation. Indeed, in the chick, delaying myelination of the spinal cord has been shown to extend the regeneration-competent period (Keirstead *et al.* 1992; Keirstead *et al.* 1997; Keirstead *et al.* 1995). Axonal regeneration could also be enhanced by strategies which boost the intrinsic capacity of CNS neurons to regenerate and also by the use of factors which directly drive and encourage axonal extension. However, although the re-growth of axonal tracts is clearly an essential component of any functional recovery after disruption of such tracts, it is likely that a multitude of different factors contribute to the determination of regenerative capacity. Future therapeutic strategies will almost certainly have to deal with more than one aspect of the response to injury in the non-regenerating spinal cord in order to achieve success.

It is apparent that the success or failure of regeneration will be influenced by the degree of cell loss – that is, the number of viable cells which remain in the spinal cord in order to re-grow their axons or receive and respond to restored synaptic connections. It is important to consider that spinal cord injury results in the disruption, and often complete

destruction of the cells which make up of local neuronal circuits, in addition to the long axonal tracts which once passed through the lesion site. Restoring function to these local circuits cannot therefore be achieved by axonal re-growth alone. Furthermore, cell loss contributes to the failure of axon regeneration through the generation of cavities within the cord that act as a barrier to growth. Given that some degree of cell loss is inevitable after an injury, two factors may affect this parameter: protective mechanisms that minimise the extent of cell death, and cellular replacement mechanisms which may compensate for those cells lost. The extent to which these mechanisms contribute to the success of regeneration in the embryonic chick pre-E13 has not been fully determined and was therefore the focus of this thesis. The overall objective of the project was to increase our knowledge about whether a decrease in the number of viable cells in the spinal cord, as a result of increased cell death or deficient recruitment and proliferation of replacement cells, contributes to the decreased regenerative capacity observed after E13 in the chick spinal cord, and to identify underlying mechanisms which contribute to this change.

8.2. Factors contributing to cell death in the non-regenerating chick spinal cord

8.2.1. Reduced haemorrhage after injury correlates with reduced apoptosis and tissue damage

Which factors govern the extent of cell loss after spinal cord injury? Secondary injury mechanisms play a well-established role in expanding the damage which occurs in the mammalian spinal cord after injury. In mammals, a progression of vascular and biochemical events and cellular responses, including haemorrhage, ischemia and inflammation, ultimately results in axonal damage, apoptotic cell death and the formation of fluid-filled cavities within the cord (Profyris *et al.* 2004; Hausmann 2003). Many of these responses occur identically in the E15 chick spinal cord after injury, suggesting that this model provides an accurate representation of a non-regenerating system similar to the adult mammal. Previous studies in our laboratory, together with results presented in this thesis have demonstrated that there is a dramatic change in the response of the spinal cord to trauma between E11 and E15, in terms of the degree of tissue damage, haemorrhage and apoptotic cell death occurring after injury (O'Neill 2002; Whalley *et al.* 2006). The correlation between the extent of haemorrhage within

the cord and the subsequent apoptotic response was particularly notable, suggesting that a causal relationship might exist between these two factors. The extent of haemorrhage is related to the vascularisation of the spinal cord, which increases dramatically between E11 and E15 (O'Neill 2002; Whalley *et al.* 2006). These observations have been further supported by additional previous work which demonstrated that artificially enhancing the haemorrhagic response to injury at E11 by diazepam administration resulted in a massively expanded apoptotic response (O'Neill 2002; Whalley *et al.* 2006). In this thesis the results of the converse experimental approach, the pharmacological reduction of haemorrhage by administration of a haemostatic compound, have been presented. The results of this study support the previous findings, providing further evidence for a causal relationship between the presence of blood in the spinal cord after injury, apoptosis and cavitation. It is not possible to determine from these experiments whether increased haemorrhage and apoptosis is the sole or even the main factor underlying loss of regeneration in the chick spinal cord. More long-term studies, investigating whether treatment with desmopressin results in improved functional outcome, would be required to answer this question. It may well be the case that the presence of inhibitory factors preventing axonal re-growth, or the requirement for a supply of replacement cells would additionally have to be addressed to restore function completely. Nevertheless, a reduction in apoptosis and subsequent cavitation certainly reduces the degree of axonal reconstitution that would be required and would help to provide a more axon-friendly environment for such re-growth. Neuroprotective mechanisms or the absence of apoptotic stimuli might therefore make a significant contribution to the successful regeneration of the E11 spinal cord.

The combination of these results with previous work suggests that haemorrhage may be a key secondary injury mechanism which results in apoptotic cell death and that strategies for reducing haemorrhage could provide neuroprotection in non-regenerating systems such as the post-E13 chick or the adult mammal. Haemorrhage is one of the earliest events to occur after injury and it could therefore prove difficult to find ways to reduce or block haemorrhage within a realistic therapeutic timeframe. Nevertheless, since haemorrhage continues to expand over a period of hours after the initial insult there might still be room for a reduction in the overall impact of this event. The delay between the onset of haemorrhage and the first signs of apoptosis (around 8 hours in the chick) does, however, indicate that understanding the downstream factors which link

these two phenomena might suggest ways in which to minimise the impact of haemorrhage. Therefore, the main focus of this half of the project was to investigate further the underlying mechanisms that might link haemorrhage to apoptosis.

8.2.2. Caspase-dependent mechanisms are involved in apoptosis after spinal cord injury in the E15 chick.

A preliminary study presented in this thesis, together with a subsequent study by Steeves and colleagues, indicate that caspase-inhibition can reduce apoptosis following injury in the chick spinal cord, suggesting that caspase-dependent mechanisms are at least partially involved in this response and that inhibition of caspase activity might reduce the secondary injury response (McBride *et al.* 2003). However, caspase activation represents a relatively late step in the progress of apoptosis and direct inhibition of caspases might act too far down these pathways to save cells from damage. It might be of greater use for therapeutic purposes to identify factors acting upstream of caspases, which are responsible for triggering the apoptotic response. Given the link between haemorrhage and apoptosis at least some of these factors are likely to be either blood-borne, or activated or induced by factors carried in the blood. Although a wide range of different factors and cell types could fit this description, the serine protease family of coagulation factors can be considered among the candidates for this role.

8.2.3. Thrombin is unlikely to be involved in the apoptotic response to spinal cord injury in the chick

The serine protease thrombin has been linked by a number of different *in vivo* and *in vitro* studies to affect cell survival and cell toxicity (Donovan *et al.* 1997; Junge *et al.* 2003; Lee *et al.* 1997; Masada *et al.* 2000; Nishino *et al.* 1993; Pike *et al.* 1996; Smirnova *et al.* 1998; Smith-Swintosky *et al.* 1995; Striggow *et al.* 2000; Turgeon *et al.* 1999; Vaughan *et al.* 1995; Xue and Del Bigio 2001). Thrombin is endogenously expressed in the central nervous system, as are its receptor, PAR-1 and cognate serpins, such as PN-1, suggesting that thrombin has a function in the normal spinal cord (Choi *et al.* 1990; Deschepper *et al.* 1991; Dihanich *et al.* 1991; Weinstein *et al.* 1995). In chick spinal motorneuron cultures, thrombin activation of the PAR-1 receptor has been demonstrated to cause apoptotic cell death by caspase-dependent mechanisms

(Smirnova *et al.* 1998; Turgeon *et al.* 1999; Turgeon *et al.* 1998). Furthermore, increased thrombin expression and activity has been demonstrated in the injured rat spinal cord (Citron *et al.* 2000b). Thrombin was therefore an obvious candidate for initial investigations into serine protease function in the injured chick spinal cord.

There are several possible mechanisms by which thrombin could be linked to apoptosis in response to haemorrhage, and thereby linked to the developmental loss of regeneration: the precursor prothrombin, present in the spinal cord, might be activated by blood-borne factors entering the spinal cord after injury; or alternatively thrombin, entering the nervous tissue at high quantities, might trigger apoptosis, either directly or via activation of another factor. In these cases, the presence of larger quantities of blood, and hence larger amounts of thrombin or thrombin-activators, would explain the differential response of the E15 spinal cord compared to the E11 spinal cord, in which haemorrhage is reduced. It is also worth considering however, that the response of spinal cord cells to thrombin might be regulated developmentally, with the E11 spinal cord being more resistant to thrombin-induced apoptosis. Indeed a change in the susceptibility of spinal cord cell to apoptosis with development was suggested in the current work by the increased sensitivity to low serum conditions in E15 spinal cord slice cultures compared to E11. Finally, although unrelated to haemorrhage it is also possible that endogenous prothrombin gene expression might be induced in the spinal cord in response to injury, as was observed in the rat spinal cord, presumably to a greater extent in the E15 spinal cord than the E11 spinal cord. These possibilities were therefore all considered in this study. The results of this study indicated firstly that prothrombin expression in the normal spinal cord is similar at E11 and E15. The results also show that neither the expression nor activity of thrombin is increased after injury in the non-regenerating chick spinal cord. While these experiments were not exhaustive, with only two time-points tested and limitations associated with sample sizes, these results suggest that thrombin is not significantly activated or induced endogenously in response to injury, in contrast to the studies in the adult rat (Citron *et al.* 2000b). Whether this is due to species- or developmental age-related differences, or a difference in the techniques used is unknown. Furthermore, experiments conducted in organotypic cultures suggest that exogenous thrombin does not readily cause apoptosis in the chick spinal cord and that there is no developmental regulation of the response to thrombin with developmental age, in contrast to the change in response to low serum conditions.

It was however, not possible to rule out the possibility that a second blood-borne factor is required in addition to thrombin to trigger apoptosis.

Interestingly, several of the results pointed towards the potential involvement of an alternative serine protease in the response to injury at E15. A large increase in overall protease activity, as indicated by non-specific cleavage of a chromogenic substrate, suggests that up-regulation of serine proteases other than thrombin may occur. In further support of these findings, the relatively non-specific serine protease inhibitor PPACK provided slight, though non-significant, protection against apoptosis in the slice culture system. A number of other serine proteases, including the plasminogen activators tPA and urokinase, have also been linked to nervous system damage (Abe *et al.* 2003; del Zoppo 1998; Figueroa *et al.* 1998; Flavin *et al.* 2000; Kataoka *et al.* 2000; Masos and Miskin 1997; Melchor *et al.* 2003). Of the plasminogen activators only urokinase is expressed in the chick CNS. Although the results of this study suggest that endogenous urokinase expression is not regulated by injury, it would be of great interest to investigate changes in the activity of this protease after injury.

This study has also highlighted the difficulties associate with investigating changes in individual genes or proteins in a complex scenario such as spinal cord injury. With a multitude of possible factors to choose from, these results have demonstrated that even with reasonable grounds for the choice, it may not be possible to select the most interesting and important factor. With the availability of multi-gene and multi-protein analysis techniques now expanding to include species such as the chick, a good approach for the future would be to examine a number of different factors at one time, allowing a more exhaustive analysis.

8.3. The identification of neurogenesis in the regenerating chick spinal cord

While the first part of this study concentrated on the apoptotic response as a potential similarity between the post-E13 spinal cord and other non-regenerating systems such as the adult mammal, in the second half of the study the focus moved to the investigation of responses in the E11 spinal cord which are potentially similar to those of other regeneration-competent systems. In lower vertebrates, proliferation and neurogenesis have been demonstrated to occur after spinal cord injury and may make an important

contribution to the regenerative response (Zhang *et al.* 2000; Benraiss *et al.* 1999; Anderson *et al.* 1994). In contrast, in the adult mammal, although the presence of endogenous stem cells within the spinal cord has been demonstrated, there is no evidence that these cells are effectively recruited in response to injury (Mothe and Tator 2005; Namiki and Tator 1999; Takahashi *et al.* 2003; Yamamoto *et al.* 2001). The lack of the ability to recruit replacement cells for those lost during trauma may therefore be a feature of non-regenerating systems. However, the contribution of such mechanisms to the regenerative ability of the chick spinal cord had not previously been investigated, with the majority of studies in this model focusing on axonal regeneration.

8.3.1. Doublecortin is regulated in response to injury in the E11 chick spinal cord

Studies into the activity and identity of precursor cells and neurogenesis are made difficult due to the lack of available markers to distinguish one cell type from another. This is further complicated in the chick, by the absence of some of the classical neural precursor markers such as nestin. One molecule which has risen to prominence over recent years is doublecortin, a microtubule associated protein linked to cortical migration (Bai *et al.* 2003; des Portes *et al.* 1998; Francis *et al.* 1999; Gleeson *et al.* 1999). A number of studies have used doublecortin as a marker of neurogenesis, exploiting the fact that this protein, unlike many neuron-specific proteins, is expressed only transiently during neuronal development (Couillard-Despres *et al.* 2005; Nacher *et al.* 2001; Rao M.S. and Shetty A.K. 2004). While this approach has been demonstrated successfully in the adult rodent brain, the expression and regulation of doublecortin in the chick spinal cord was unknown. I therefore set out to determine firstly whether doublecortin could be used as a neurogenic marker. The expression of doublecortin in the developing chick spinal cord was investigated, revealing high levels of expression throughout development (Fig 8.1). Although these results demonstrated that doublecortin would not be a useful marker of neurogenesis, because of the persistence of expression throughout development, the investigation of doublecortin expression did indicate some interesting differences between the E11 and the E15 spinal cord and point to a potential role for doublecortin after injury (Fig 8.1). Doublecortin protein was both present at higher levels and more highly phosphorylated at E11 in comparison to E15. Furthermore, in response to injury at E11 but not E15, an increase in doublecortin mRNA and protein levels occurred together with even greater levels of phosphorylation.

This suggests that phosphorylated doublecortin may be a hallmark of less mature neural development, a notion which is supported by the expression of this form of the protein in neuroblastoma cells. The return to a more phosphorylated state after injury might therefore be indicative of a recapitulation of developmental processes such as neurogenesis. Phosphorylation of doublecortin has been demonstrated to regulate its binding to microtubules, and hence phosphorylation may reduce microtubule stability (Schaar *et al.* 2004; Tanaka *et al.* 2004). Such a mechanism might be involved in a range of different cellular functions, including but not restricted to neurogenesis, many of which might be expected to contribute to the response to injury. For example, increased doublecortin levels might reflect an increase in axonal growth activity, which might be triggered by the injury. Alternatively, some *in vitro* studies have indicated that doublecortin might be neuroprotective against certain forms of toxic stimuli and it can be hypothesised that the increase in doublecortin expression acts to protect the E11 spinal cord from cell death-inducing factors. It is therefore of interest to determine which, if any, of these hypotheses are true.

8.3.2. Expression of transitin and 3CB2 in the developing chick spinal cord

In addition to doublecortin, several other potential neural markers were investigated in this thesis, providing new information on their expression patterns during normal spinal cord development in the chick. Although expression of the nestin homologue, transitin has previously been investigated in the chick brain, information on its expression in the spinal cord, particularly at late stages was sparse. Similarly, the radial glial antigen 3CB2 has been extensively investigated at early developmental stages in the chick spinal cord and in the adult chick brain. This thesis therefore adds to what was previously known about the timing of the down-regulation of these markers at later stages of spinal cord development (Fig 8.1). Both transitin and 3CB2 were demonstrated to be expressed throughout development, becoming restricted to the midline of the spinal cord after the end of neurogenesis. Only low level expression was observed in radial fibres at all developmental stages tested, suggesting that by these stages most radial glia have been down-regulated. However, the presence of a small population of radial glia that persist in the spinal cord provided the possibility, supported in part by the findings with doublecortin, that these cells could be activated or up-regulated to participate in neuronal replacement after injury.

	Developmental expression pattern	E11 spinal cord	Response to injury (at 24 hours)
Doublecortin	•Expressed throughout development •Levels reduced with development •Phosphorylation reduced with development •Becomes restricted mainly to dorsal horns from around E13	 Low levels throughout grey matter	•mRNA levels ⇑ •Protein levels ⇑ •Phosphorylation ⇑
Transitin	•Expressed in radial processes spanning width of tube •Around E11, expression becomes mainly restricted to dorsal midline	 Dorsal midline and some radial fibres	•Expression in radial fibres and scattered cells ⇑
3CB2	•Expressed in radial processes spanning width of tube •Around E11, expression becomes mainly restricted to dorsal and ventral midline	 Dorsal and ventral midline and some radial fibres	•No change

Fig 8.1. Expression of various markers of neural cells in the developing and injured spinal cord, as demonstrated in this thesis

8.3.3. Neurogenesis is ongoing in the chick spinal cord at E11

Does the increase in phosphorylated doublecortin represent a recapitulation of neurogenesis after injury? The results of the present study show that, as in most species tested, there is an increase in cell proliferation in the E11 spinal cord after injury. This occurred exclusively in the grey matter, which could point to an up-regulation in the proliferative activity of neural precursors. This concept was further supported by the increased expression of transitin (Fig 8.1), a chick protein which has been suggested to be similar to mammalian nestin, and is expressed in neural precursor and radial glia (Lee and Cole 2000; McCabe *et al.* 1992; Cole and Lee 1997). However both proliferation and nestin up-regulation have been shown to occur in both regeneration competent and incompetent species and are unlikely, therefore, to be important for regeneration (Mothe and Tator 2005; Namiki and Tator 1999; Takahashi *et al.* 2003; Yamamoto *et al.* 2001; Benraiss *et al.* 1999). It was therefore essential to determine whether this proliferation is followed by the production of new neurons, which is more likely to be a determining characteristic of regeneration-competency.

In the current study a thorough double-labelling approach was taken to identify cells which began proliferating at or after the time of injury and then subsequently differentiated to form neurons. Unexpectedly, this analysis revealed the presence of cells undergoing neurogenesis at stages much later than was expected in the normal chick spinal cord, confirmed by two separate approaches. This finding is supported by the persistent expression of doublecortin which was also noted in the chick spinal cord at these stages. In order to provide an indication of the end of the neurogenic period, identical analysis was carried out in the E15 spinal cord and no double-labelled cells were found, revealing that neurogenesis is complete at some point between E11 and E15. This is in contrast to previous estimates of the end of neurogenesis occurring by E9 at the latest (Fujita 1964). Therefore, although neurogenesis was also observed after injury at E11, it is not possible to know from these results whether this process is up-regulated in response to injury. In the future, more quantitative experiments will be required to determine whether this is the case. It seems likely, however, that the existence of ongoing neurogenesis in the spinal cord could in itself contribute to the regenerative response. These results suggest that the spinal cord is fairly immature at this time-point, which may make it simpler to recapitulate developmental processes

required for regeneration. Additionally, a spinal cord which is still in the process of generating neurons may be more permissive for neuronal development and plasticity and axonal growth. In support of this, the end of neurogenesis, between E11 and E15, corresponds well with the loss of regenerative capacity. Table 8.1 summarises the evidence which this study has provided that neurogenesis might play a role in determining the regenerative capacity of the chick spinal cord.

Table 8.1 Summary of the evidence for a contribution of neurogenesis to regenerative capacity in the embryonic chick spinal cord

	E11	**E15**
Ongoing neurogenesis	Yes	No
Up-regulation of transitin expression	Yes	No
Up-regulation of doublecortin expression	Yes	No
Up-regulation of proliferation in grey matter	Yes	-
Up-regulation of neurogenesis	Unknown	Unknown

8.4. Conclusions

This thesis aimed to provide some preliminary answers to the question of why the E11 chick spinal cord is able to regenerate, in contrast both to the E15 chick spinal cord and to the adult mammal. As outlined in the introduction to this thesis it is possible to hypothetically devise a set of conditions which must be met for spinal cord regeneration, meaning functional recovery, to occur (Fig 1.6). These can be broadly divided into 1) a requirement for sufficient numbers of viable cells to be present within the spinal cord, and 2) the ability of these cells to re-grow and thus restore functional axonal tracts and synaptic contacts. In this thesis I have investigated principally which factors may contribute to the first of these requirements. The project has dealt with two different, but related aspects of the early response to injury – mechanisms which result in cell death and those which result in cell replacement. Figure 8.2 summarises the new information which has been gained by this study, together with aspects which remain to be addressed and new questions which these findings have raised.

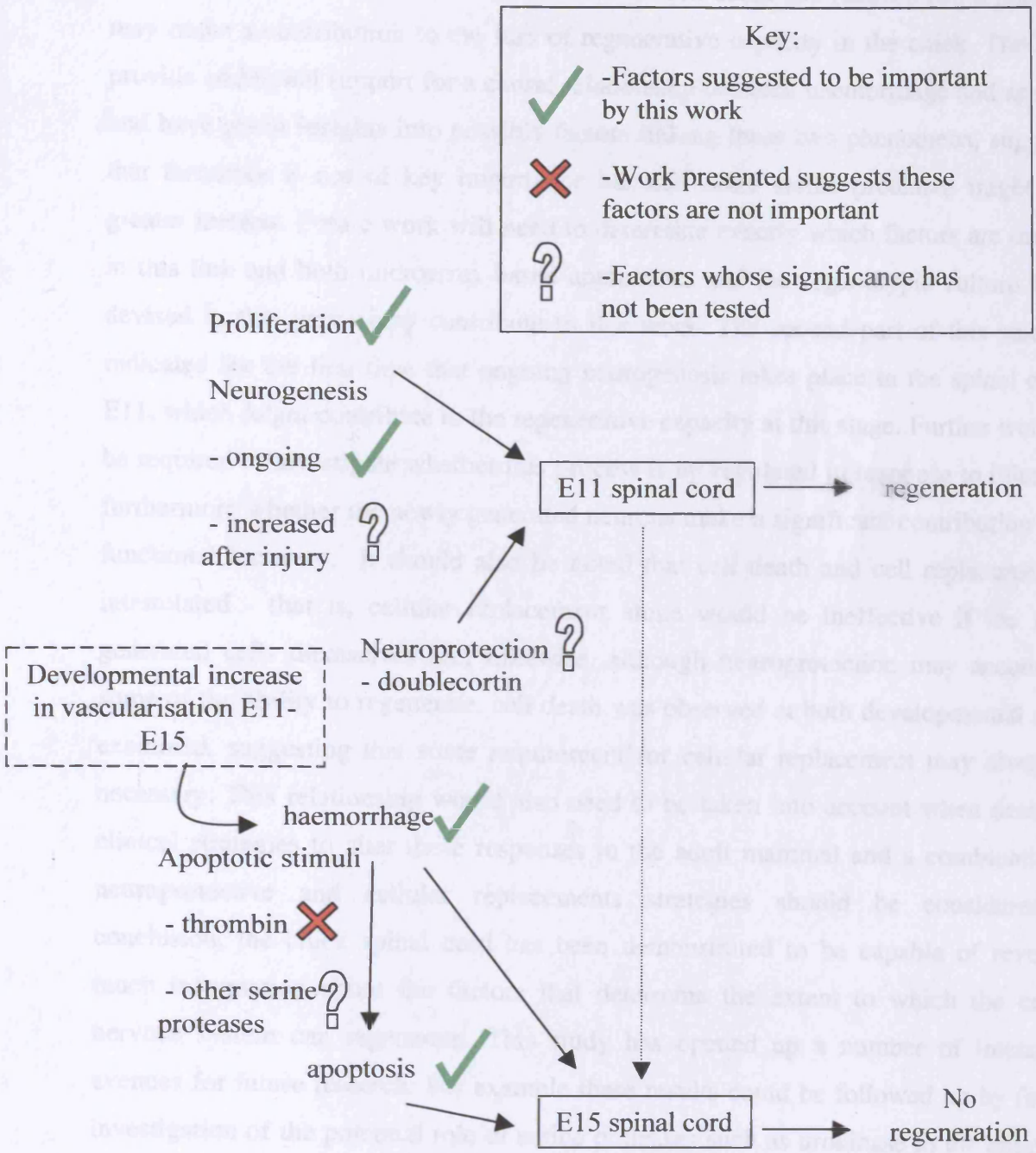
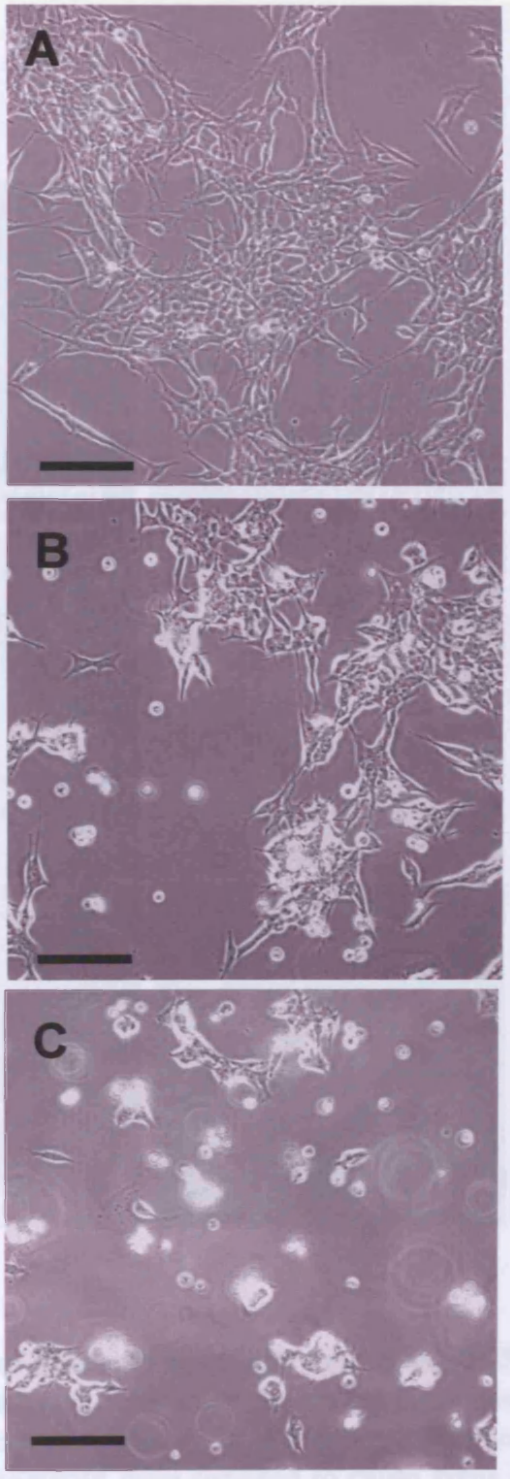
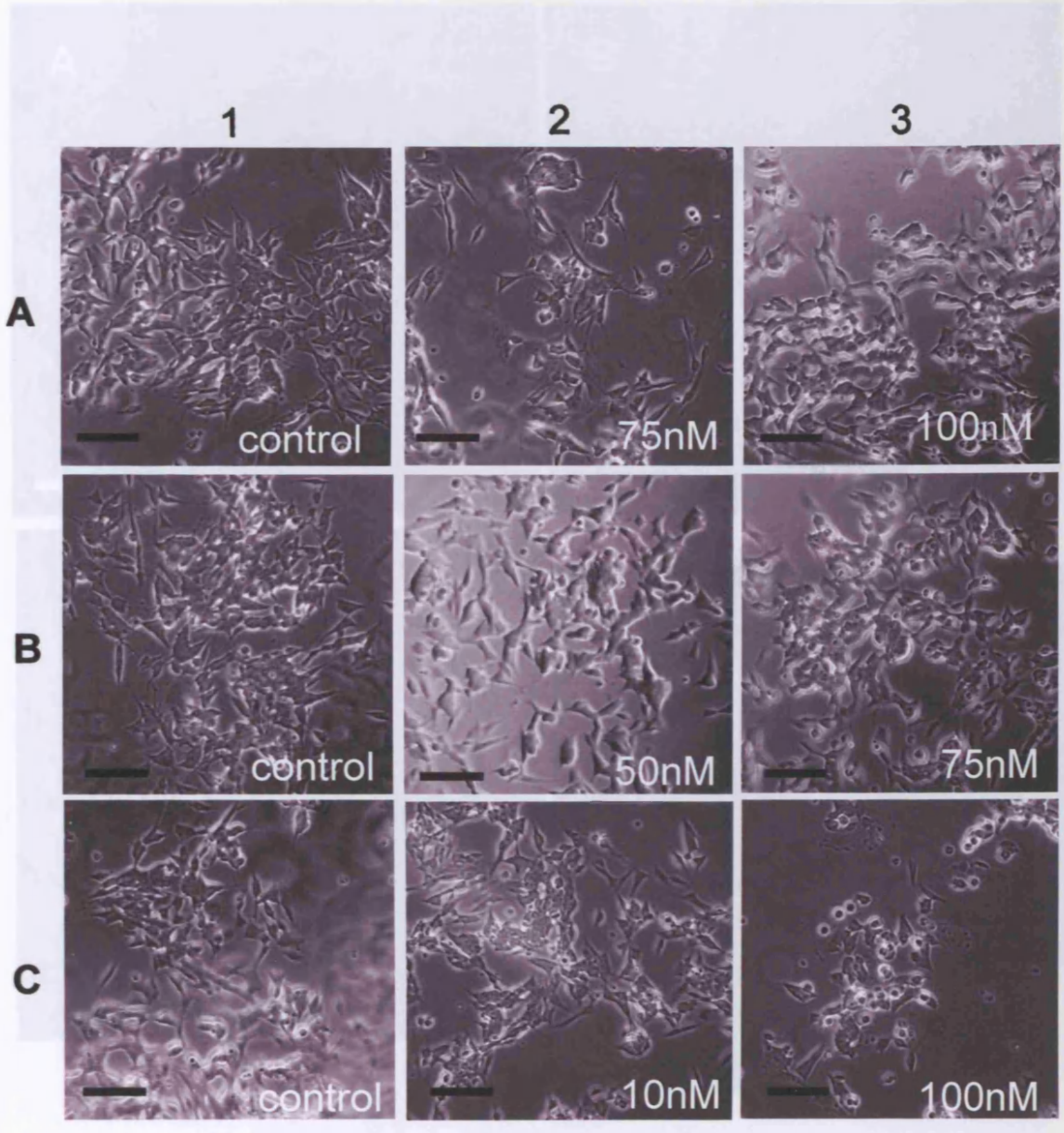


Fig. 8.2. Updated model of the early response to injury in the chick spinal cord. Figure illustrates the contribution of the experiments reported in this thesis to our understanding of the loss of regenerative capacity in the chick spinal cord between E11 and E15.

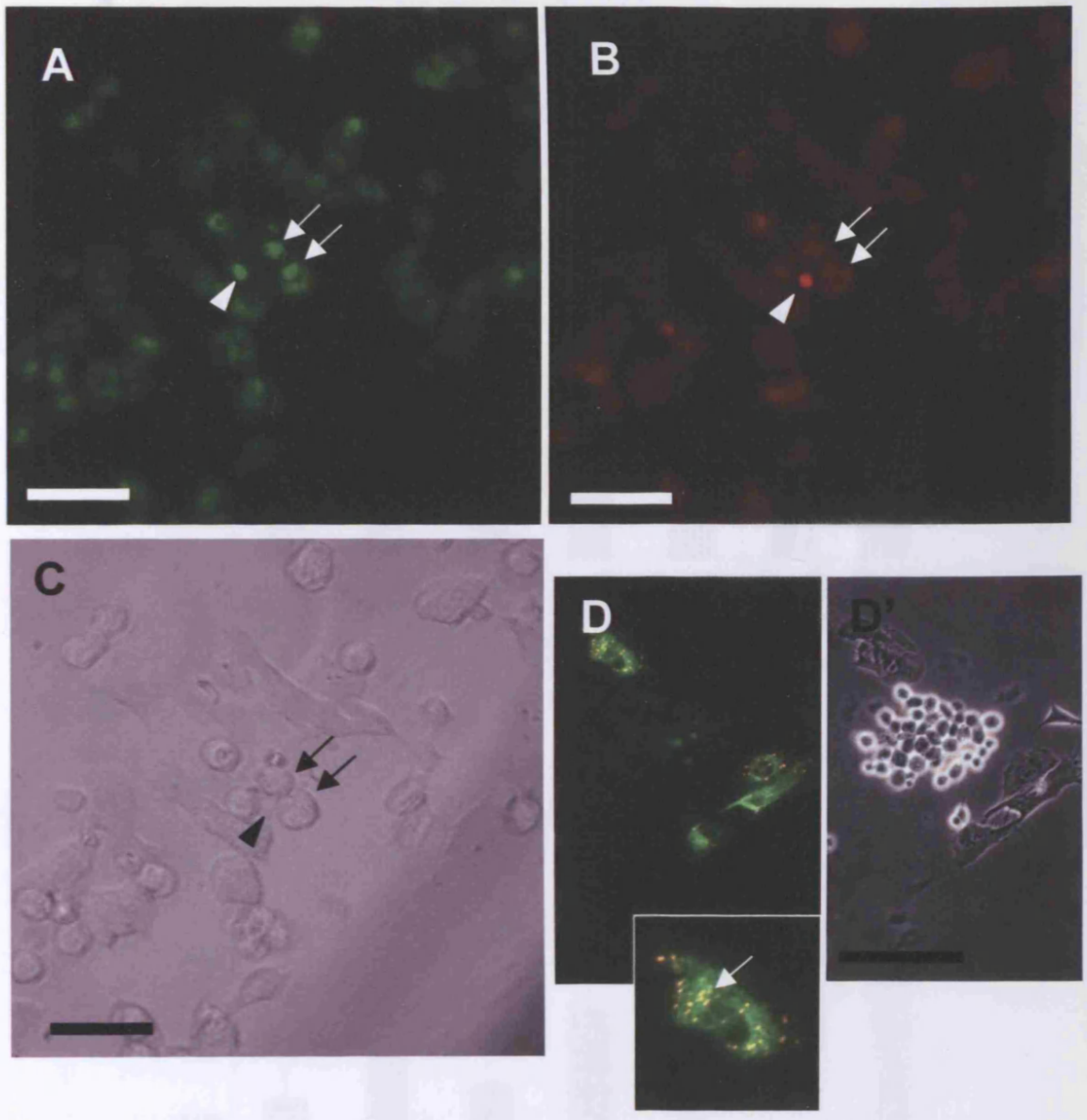
Overall, this study suggests that both increased cell death and reduced cell replacement may make a contribution to the loss of regenerative capacity in the chick. The results provide additional support for a causal relationship between haemorrhage and apoptosis and have given insights into possible factors linking these two phenomena, suggesting that thrombin is not of key importance but that other serine proteases might be of greater interest. Future work will need to determine exactly which factors are involved in this link and both microarray-based approaches and the organotypic culture system devised in this thesis may contribute to this work. The second part of this study has indicated for the first time that ongoing neurogenesis takes place in the spinal cord at E11, which might contribute to the regenerative capacity at this stage. Further work will be required to investigate whether this process is up-regulated in response to injury and furthermore whether the newly generated neurons make a significant contribution to the functional recovery. It should also be noted that cell death and cell replacement are interrelated - that is, cellular replacement alone would be ineffective if the newly generated cells themselves die. Likewise, although neuroprotection may account for some of the ability to regenerate, cell death was observed at both developmental stages examined, suggesting that some requirement for cellular replacement may always be necessary. This relationship would also need to be taken into account when designing clinical strategies to alter these responses in the adult mammal and a combination of neuroprotective and cellular replacements strategies should be considered. In conclusion, the chick spinal cord has been demonstrated to be capable of revealing much information about the factors that determine the extent to which the central nervous system can regenerate. This study has opened up a number of interesting avenues for future research. For example these results could be followed up by further investigation of the potential role of serine proteases such as urokinase in the apoptotic response to injury at E15. Further exploration of the potential functional role of doublecortin up-regulation and phosphorylation in the regenerative response on the E11 spinal cord could also be carried out. In particular, it would be of interest to investigate the potentially neuroprotective function of this protein.



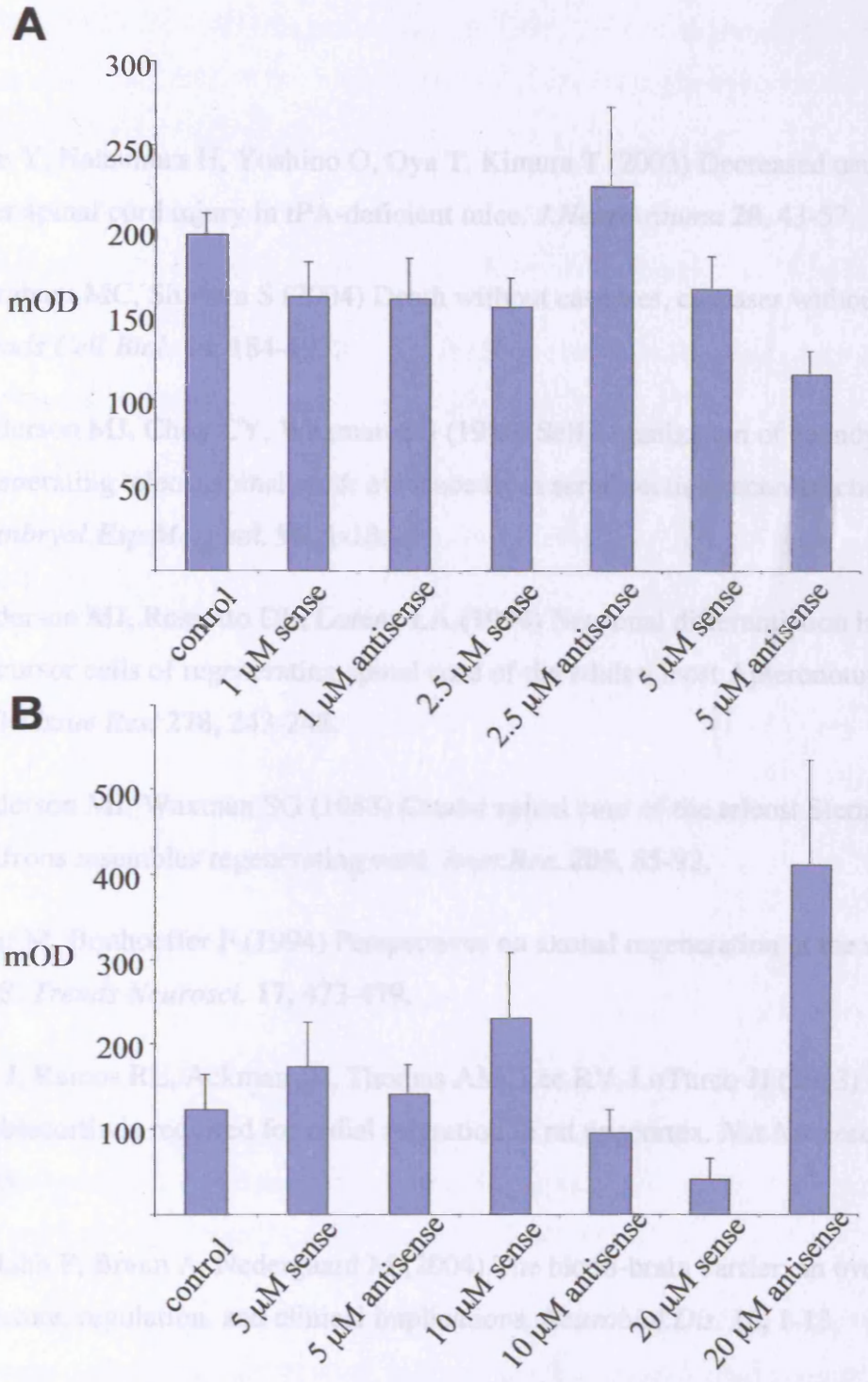
A1. The effect of roscovitine on SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were grown on plastic and incubated with the Cdk5 inhibitor, roscovitine. Images show typical phase-contrast images of the cells after 24 hours incubation. **A)** control culture, incubated with an equivalent amount of DMSO **B)** 10 μM roscovitine, **C)** 20 μM roscovitine. Treatment with the Cdk5 inhibitor resulted in signs of cell death such as cell rounding and detachment. Scale bars = 100 μm.



A2. The effect of different apoptotic agents on SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were grown on glass coverslips and incubated with various concentrations of thrombin, rotenone or nocodazole to determine the threshold dose required to cause signs of cell death . Images show typical phase-contrast images of the cells after 6 hours incubation. For each treatment the pictures from left to right show 1) control 2) sub-threshold dose and 3) above threshold dose. A) thrombin **B)** rotenone and **C)** nocodazole Scale bars = 100 μm



A3. Identification of apoptotic cells with ethidium bromide, acridine orange and JC-1. A)-B) Apoptotic cells identified by their condensed chromatin after staining with acridine orange (A), which stains chromatin in all cells and ethidium bromide (B), which stains chromatin in dead cells. Arrows indicate two cells, still living, which show evidence of condensed chromatin. Arrowhead shows one cell which is dead and shows evidence of apoptosis. **C)** Phase-contrast image of the cells shown in A and B. **D)** JC-1 staining of SH-SY5Y cells treated with roscovitine. Cells with normal mitochondrial membrane potential accumulate JC-1 in the mitochondria in aggregates which produce red fluorescence (arrow in high magnification view). Cells with loss of mitochondrial membrane potential do not accumulate JC-1 and exhibit only green fluorescence in the cytoplasm. **D')** Phase contrast image of cells shown in D. Scale bars = 50 μm



Appendix Fig. 4. Treatment of SH-SY5Y cells with doublecortin antisense oligodeoxynucleotides. SH-SY5Y human neuroblastoma cells were grown in a 96 well plate and incubated with various concentrations of low dose (A) or high dose (B) sense or antisense oligodeoxynucleotides against doublecortin for three days. Graphs show results of an ELISA using anti-doublecortin antibody to measure doublecortin protein levels. No consistent knockdown of doublecortin with antisense treatment could be observed.

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