Investigation of endogenous stem cells and reactive astrocytes in post-traumatic syringomyelia

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Introduction: Around a quarter of patients with spinal cord injury develop post traumatic syringomyelia (PTS), causing progressive neurological deficits. Current surgical treatment is unsatisfactory. Endogenous stem cell therapy, aiming at replacing lost tissue and repairing damaged ones by endogenous progenitors, may offer hope. Investigation into the reaction of endogenous progenitors in PTS may extend our knowledge about stem cell biology and help to develop a new treatment option for PTS. Endogenous stem cells were found to differentiate into astrocytes. Reactive astrocytes and gliosis are shown to have an important role in spinal cord injury, such as protecting neurons, limiting inflammation and regulating local environment to suit progenitors. We hypothesize that reactive astrocytes may play an important protective and potential therapeutic roles in PTS. The aim of this thesis is to study proliferation, differentition and location of endogenous progenitors and their roles in PTS.

Materials and methods: Excitotoxic injury model of PTS was performed in adult Wistar rats. Proliferating cells were marked by either exogenous mitotic marker bromodeoxyuridine or endogenous mitotic marker Ki67. Immunofluorescence techniques targeting mitotic markers were used to trace the proliferating cells. Immunofluorescent double staining techniques were used to phenotype the proliferating cells.

Results: A large number of endogenous progenitors appear in PTS from 24 hours to at least 8 weeks post injury (PI). They proliferate much faster in PTS than in the control animals. Although less endogenous progenitors are observed after 4 weeks PI, their number is still much higher than that in the control animals. Immediately after injury, progenitors exist mainly in the white matter, but the majority of them shift their position closer to the lesion within 2 days. In the chronic stage, the majority of stem cells are located in and around the lesion site. Endogenous progenitors differentiate into astrocytes but not oligodendrocytes or neurons within 8 weeks. Astrocytes respond to injury by upgrading GFAP (1 day PI), becoming hypertrophic (7 days PI) and forming glial scar (2 weeks PI) in PTS. The development of a glial scar corresponds with the stage of cyst stability or reduction in size.

Conclusions: Endogenous progenitors exist in PTS and they respond to injury by proliferating and shifting their position towards the lesion. These studies are important in understanding the endogenous stem cell response to PTS and lay the groundwork for future studies examining stem cell therapy for the condition. Endogenous progenitors in the PTS model differentiate into astrocytes, which help to form the glial scar lining the syrinx. Reactive gliosis may play an important role to seclude the injury site from healthy tissue, prevent a cascading wave of uncontrolled tissue damage and restrict the syrinx enlargement.

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Investigation of Endogenous Stem Cells and Reactive Astrocytes in Post-traumatic Syringomyelia

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A thesis submitted to fulfil the requirements for the degree of Doctor of Philosophy

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Certificate of Originality

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree of diploma at UNSW or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

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Abbreviations

ABC	ATP-binding cassette
ACEC	Animal care and ethics committee
aFGF	Acidic fibroblast growth factor
ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CCg	Glycosylated form of cystatin
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSF	Cerebral spinal fluid
CT	Computed tomography
DAPI	4,6-diamidino-2-phenylindole
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESCs	Embryonic stem cells
EZ	Ependymal zone
FGF-2	Fibroblast growth factor 2
GABA	Gamma-aminobutyric acid
GCL	Granule cell layer
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM	Grey matter
GRPs	Glial restricted progenitors
Hcl	hydrochloride acid
IGF-1	Insulin like growth factor 1
IL-1α, -1β, -6	Interleukin-1α, -1β, -6
LA	Left anterior area
LP	Left posterior area
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
NCAM	Neural cell adhesion molecule
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NPCs	Neural progenitor cells
NSCs	Neural stem cells

NT-3	Neurophine-3
OB	Olfactory bulb
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PI	Post injury
PNA	Peanut agglutinin
PSA-NCAM	Polysialated glycoprotein neuronal cell adhesion molecule
PTS	Post-traumatic syringomyelia
PZ	Pial zone
QA	Quisqualic acid
RA	Right anterior area
REST	RE silencing transcription factor
RMS	Rostral migration stream
RNA	Ribonucleic acid
RP	Right posterior area
SCI	Spinal cord injury
SDF-1α	Stormal cell derived factor-1α
SE	Standard error
SEZ	Subependymal zone
SGZ	Subgranular zone
SPZ	Subpial zone
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
TGF	Transforming growth factor
TNFα	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WM	White matter

Chapter One Introduction

1.1 Post-traumatic syringomyelia

1.1.1 Definition

Fluid filled cavities within the spinal cord were described by Charles Estienne in 1546. The term "syringomyelia" was first used 281 years later by Ollivier d'Angers after the Greek, in which syrinx means pipe, tube or channel and myelos means marrow [1]. Although terms such as "hydromyelia", "syringohydromyelia" and "cystic myelopathy" were used in the past, nowadays syringomyelia is a well-accepted term used to describe all abnormal fluid-filled cavities with the spinal cord except small cavities that are secondary to trauma or necrosis and not enlarging [2]. The term "post-traumatic" is used to define syringomyelia occuring after spinal cord injury.

1.1.2 Epidemiology

Spinal cord injury or arachnoiditis was thought to be the aetiological factor in about a quarter of the patients presenting with syringomyelia [3]. Syringomyelia is found in 21-28% of spinal cord injury patients between 1 and 30 years after spinal cord injury [4-8]. Since spinal cord injury affected 261 people in Australia in the year 1999-2000, as many as 73 of them will be expected to develop post-traumatic syringomyelia [9]. Eighty percent of patients suffering from post-traumatic syringomyelia (PTS) are male, which matches the gender distribution in spinal cord injury [9, 10]. The chance of developing syringomyelia following quadriplegia was shown to be similar to that after paraplegia [7, 11].

1.1.3 Classification and Pathology

1.1.3.1 Classification

According to pathological findings, syringes can be classified into three main categories, namely: 1) communicating central canal syrinxes; 2) noncommunicating central canal syrinxes and 3) extracanalicular syrinxes [12].

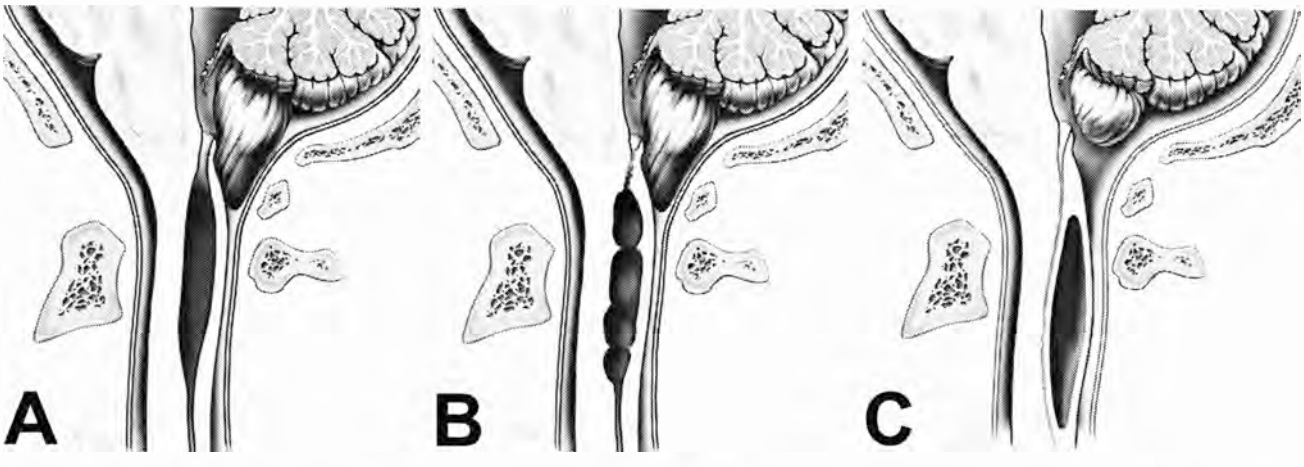

Figure 1-1. Classification of syringomyelia. A: communicating central canal syrinx; B: noncommunicating central canal syrinx; C: extracanalicular syrinx

Communicating central canal syringes are simple dilated central canals that communicate with the fourth ventricle. They are usually found in children and young adults and associated with conditions such as the Chiari II malformation or Dandy Walker cyst. The cysts are wholly or partially lined by ependyma, which is surrounded by varying amount of spongy glial tissue. Grossly enlarged cavities are sometimes lined almost exclusively by compressed glial tissue. Communicating central canal syringes are usually asymptomatic. This is thought to be because rupture of the dilated central canals into the surrounding tissue seldom occurs [12-14].

Noncommunicating central canal syringes are also dilated central canals. However, they do not communicate with the fourth ventricle and most of them are closed at both ends by central canal stenosis. Noncommunicating central canal syringes

are usually associated with Chiari I malformation, spinal arachnoiditis, cervical spinal stenosis or basilar impression. The cavities have extensive ependymal denudation, intracanalicular septations composed of spongy glial tissue and paracentral dissection. Paracentral dissections are lined by simple glial tissue and cause central chromatolysis and neuronophagia in the affected grey matter. In the white matter, demyelination, infiltration of macrophages and Wallerian degenation can be found. Compared with communicating central canal syringes, noncommunicating central canal syringes are more likely to cause segmental neurological deficits, due to paracentral dissection, which is seen in more than 40% of cases [12-15].

Extracanalicular syringes are cavities separate from the central canal and are associated with conditions such as spinal cord injury, infarction, haemorrhage and myelitis. Segmental neurological deficits correlate with the extent of spinal cord damage. PTS is an extracanalicular syrinx, which has distinct pathological features compared with other syrinxes [12-14].

1.1.3.2 Pathology of human PTS

PTS has the following macroscopic features. PTS usually occurs in the cervical and upper thoracic spinal cord. They are usually juxtaposed to the sites of spinal cord injury: rostral in 81%, caudal in 4% and in both directions in 15% of cases [4, 16]. In some reports, over half of the syrinxes extend 2-5 levels and 40% of them extended 6-10 levels, while only 3-17% of the syrinxes extended more than 10 levels. They were even found to extend into the brain stem in some studies [10, 12]. Most of the post-traumatic syrinxes form in the central and dorsolateral grey matter, while 9% occur in the white matter alone. They are irregular in shape and may be composed of single or multiple cysts [12, 17, 18].

Histological studies report the following features of PTS. Syrinx walls are formed by compressed glial tissue surrounded by gliosis, microglia and haemosiderin-containing macrophages, but not ependymal cells [12, 19, 20]. Wallerian degeneration, macrophage infiltration, demyelination and gliosis are found in the white matter. Central chromatolysis and neuronophagia, as well as gliosis, are observed in the grey mater [12, 19-21]. In addition, vascular changes occur around the syrinx, which include hyalinized and thickened vessels, oedema and haemorrhages [22]. Perivascular spaces may be enlarged and can communicate with the subarachnoid space at the dorsal nerve root entry zones or the ventromedian fissure [12, 19]. Furthermore, arachnoiditis is often associated with PTS. Because of the proliferation of collagen in the subarachnoid compartment, the dura is firmly affixed to the underlying spinal cord. The proliferated collagen forms a thick layer embedding the injured spinal cord and entrapping nerve roots and vessels. Neovascularisation and osseous metaplasia can also be found [21, 23].

1.1.3.3 Pathology of PTS in animal models

In addition to the pathological studies carried out in human patients, various animal models of PTS have been established. Traditionally, weight-drop injury models have been used. The re-producibility of syrinx is enhanced when the injury is combined with arachnoiditis produced with an injection of kaoline into the subarachnoid space [24]. The most reliable method of syrinx production is the excitotoxic model with or without kaolin-induced arachnoiditis [24-30]. Despite the different techniques, there are similar histological features in the animal PTS models, which mimic human PTS.

In the animal models, syringes usually form in the spinal cord within a week after the injury. Compared with human syrinxes that form usually months or years after spinal cord injury, experimental syrinxes form much more rapidly. The reason is not

clear. Some studies showed that syrinx formation in rats closely depended on the dose of excitotoxic acid, which is thought to cause initial formation of a cyst [25, 26]. Thus, a possible reason is that the amount of excitotoxic acid following the initial spinal cord injury in human beings is not high enough to cause the rapid formation of an initial cyst. A more likely explanation is that arachnoiditis takes longer to develop after human spinal cord injury and that CSF dynamics therefore change more slowly. The site and size of syrinxes vary in different studies, largely depending on the length of survival. The syrinx wall consists of gliosis, macrophages and inflammatory cells, but no ependymal cells. Neuronal loss is significant in the grey matter near the syrinx and bilateral neuronal damage has also been reported [26]. In the white matter, demyelination is common, which is thought be caused by damage to the oligodendrocytes [31, 32]. Reactive astrocytes can be found not only in the syrinx wall but also in the border zone between syrinx and normal tissue. Compared with human PTS, animal models have a more severe acute inflammatory reaction. Macrophages are present throughout the parenchyma, especially in the immediate vicinity of syrinx. Brodbelt and his colleagues reported that the inflammatory cell infiltration was highest in the first three weeks and reduced steadily thereafter [25].

1.1.3.4 Gliosis in post-traumatic syringomyelia

Pathological studies have illustrated that gliosis is one of the important histological features in PTS. Gliosis or glial scar is a response to injury in the brain or spinal cord, whenever the CNS is damaged. The glial scar is composed primarily of reactive astrocytes and the extracellular matrix molecules of proteoglycans which are produced by reactive astrocytes [33-35]. Some studies showed that there is widespread astrocytic reaction after CNS injury, which may extend for up to 1 cm from the injury

[36, 37]. Astrocytes around this area become hypertrophic, produce many fine processes and some undergo cell division. As a result, astrocytic tissue develops, consisting of tightly packed, hyperfilamentous astrocytes, with many of their processes tightly apposed to one another, limited extracellular spaces, and with many gap and tight junctions [33]. Glial scar or gliosis may have a double-edged nature in the damaged CNS. The reactive astrocytes and the gliosis are found to inhibit axonal regeneration, but they are also shown to be important for neuroprotection and self-repair [33-35, 38].

Glial scar has a negative role in injuried CNS. Various *in vivo* and *in vitro* studies show that gliosis can be inhibitory to axon regeneration [39-45]. Enlarged and entangled reactive astrocytes surround dystrophic endballs at the tips of non-regenerating fibres. Glial scar develop into a rubbery, tenacious, growth-blocking membrane. Silver and Miller summarised that reactive astrocytes and glial scars are responsible for failed regeneration through the formation of physical wall [34].

Little is known about gliosis in PTS. Gliosis may play some positive roles in the evolution of PTS, namely restricting inflammation, protecting neurons and limiting the cystic enlargement. Firstly, gliosis may help to restrict inflammation in PTS. Faulkner and his colleagues found that gliosis contributes significantly to restrict inflammation after SCI [46]. Other pathological studies indicated that although some inflammatory cells distribute throughout the spinal cord, most of them are mainly located around the syrinx, which is surrounded by gliosis [12, 25, 26]. Thus, a question is inevitably raised whether gliosis helps to restrict the infiltration of inflammatory cells in PTS. Secondly, gliosis possibly protects neurons in PTS. This possibility is supported by the evidence that considerable neuronal damage in PTS usually appears in the area around the syrinx, which is surrounded by gliosis [12, 25, 26]. This possibility is also supported by

Faulkner's study that gliosis protects neurons after SCI. Thirdly, gliosis may be helpful in limiting the enlargement of syrinx. Currently, it is commonly accepted that the enlargement of the syrinx is caused by the pressure of the fluid. Compared with normal spinal cord tissue, the gliosis has very strong structure consisting of tightly packed, hyperfilamentous astrocytes, with many of their processes tightly apposed to one another, limited extracellular spaces, and with many gap and tight junctions [33]. This particular structure is probably able to resist higher pressure than normal spinal cord tissue, which may limit the enlargement of syrinx.

The above possible roles of gliosis in PTS may lead to a new treatment of PTS by activating reactive gliosis. However, there is lack of knowledge about the details of gliosis formation in PTS limits the development of such new treatment, which requires further studies.

1.1.4 Aetiology

The aetiology of post-traumatic syringomyelia is unclear, although several possible mechanisms have been raised regarding the initial formation of a cavity and syrinx enlargement [1].

Initial formation of a cavity in the spinal cord parenchyma may have different mechanisms. Inflammatory responses in the spinal cord secondary to injury cause oedema and sometimes cyst formation [47, 48]. An intraparenchymal haematoma caused by spinal cord trauma may result in cyst formation [10]. Ischaemia after primary spinal cord injury and subsequent arachnoiditis might lead to the formation of a cyst from infarction [10, 12]. Excitatory amino acids released after spinal cord injury, which is regarded as secondary cord damage, may lead to glutamate receptor activation, calcium influx, neuronal cells death and subsequent cyst formation [49-51]. Other

potential mechanisms involved in secondary cord damage include proteolytic enzyme release, liberation of free fatty acids, free radical production, phospholipid degradation and abnormality of prostaglandin production [10, 15, 52, 53].

Syrinx enlargement must result from an imbalance between inflow and outflow of fluid. Very little is known about fluid outflow in syringomyelia. Suggested mechanisms of fluid inflow include: 1) passive inflow into a cavity that has been enlarged by dissection caused by respiratory or vascular pressure waves in the subarachnoid space; 2) increased CSF flow from the subarachnoid space caused by focal pressure changes resulting from arachnoiditis, canal stenosis or cord compression [5, 10, 54-61].

1.1.5 Clinical Features

Post-traumatic syringomyelia develops as soon as 3 months or as late as 34 years after spinal cord injury and symptoms progress gradually in most patients [4, 14, 62, 63]. Segmental pain, sensory loss and weakness are common symptoms. The pain may be dull, aching or burning and can be mild or severe, constant or intermittent. The pain is usually above the level of injury and can be exacerbated by coughing, sneezing and straining. Complete loss of sensation is more common than classic dissociated sensory loss in ascending segments. Progressive asymmetrical weakness tends to occur after the onset of sensory loss. It presents as a gradual loss of motor function above the level of the original injury. Other associated symptoms and signs include asymmetrical reduction in reflexes, hyperhydrosis, autonomic dysreflexia, Horner's syndrome, dysphagia and cardiorespiratory dysfunction [10, 62, 63]. Patients without symptoms who are diagnosed with MRI are likely to have smaller and shorter syrinxes than symptomatic ones [8].

1.1.6 Radiological Investigation

Before MRI was used in the investigation of post-traumatic syringomyelia, it was difficult to make the diagnosis. Because of the degradation of the spinal cord image by the effect of surrounding bone, plain CT is not reliable [11, 22]. Although it is better than plain CT, CT scan combined with contrast myelography failed to detect as many as 50% of the syrinxes [64, 65].

So far, MRI has proved to be the best diagnostic test for post-traumatic syringomyelia. It is non-invasive and more sensitive than CT combined with contrast myelography, which has the advantage of detecting most associated abnormalities [11, 22, 59, 66, 67]. However, MRI may not detect small syrinxes or those in patients with bone deformities and scoliosis [22, 68].

1.1.7 Natural History

Although the symptoms and signs of a few patients with post-traumatic syringomyelia progress rapidly, most have a slow progression [22, 63, 69, 70]. According to some reports, up to half of the patients remained stable without treatment during a period of more than ten years [70, 71]. Even spontaneous resolution, possibly due to decompression of the syrinx into subarachnoid space, has been reported [72, 73].

1.1.8 Treatment

Treatment of post-traumatic syringomyelia is generally unsatisfactory. Surgical treatment is usually reserved for patients with progressive neurological deterioration or pain. Corrections of deformity or compression, various shunting procedures, arachnolysis with or without duraplasty, cord transection and foetal spinal cord tissue

implantation have been reported. Shunting procedures and arachnolysis with or without duraplasty are the most widely used surgical options [10, 11, 74-76].

During a shunting procedure, the proximal end of the tube is inserted into the syrinx and the other end is put into the peritoneal, pleural or subarachnoid space. The main purpose of shunting is continued drainage of the syrinx fluid. According to some studies, 12-53% of the patients had their symptoms and signs improved following shunting, 10-56% were unchanged and 12-32% were worse [10, 11, 77].

Arachnolysis with duraplasty appears to be the preferable treatment to shunting in most cases, in which the dura is extensively opened and arachnoid scarring is resected with sharp dissection. The goals of this procedure are to restore free CSF passage in areas of arachnoid scarring and to untether and decompress the spinal cord. Klekamp and Samii reported that 31% of the patients treated with arachnolysis with duraplasty improved, 54% were unchanged and 15% were worse [11]. It was also pointed out that in patients with extensive arachnoid adhesions or calcification, the risks associated with arachnolysis and duraplasty are so high that shunting is preferable [11, 60, 74].

As other spinal cord injuries, the traditional treatment of PTS is unsatisfactory. Recently, more attention has been drawn to the area of stem cell therapy, largely due to advanced knowledge about stem cells. The stem cells may play an important role in the treatment of PTS by replacing the damaged cells, helping remyelination, filling the cavity. However, no stem cells therapy for PTS has been reported so far.

1.2 Adult endogenous neural stem cells

1.2.1 Definition

So far, no uniform definition has been given to "stem cell". However, it is widely accepted that "stem cell" is used to describe cells having the capacity to divide, self-renew and differentiate into cells characteristic of the particular tissue. When a stem cell divides asymmetrically, it gives rise to two daughter cells. The properties of one of the cells are the same as those of the original cell, which is regarded as self-renewal. The other daughter cell is more mature and ready to migrate and differentiate further. Self-renewal enables a stem cell population to be retained. Usually, stem cells are defined by the organ from which they are derived or by where they are observed in vivo [78].

According to the ability to form other cells and the state of the body's development, there are three types of stem cells, namely totipotent, pluripotent and multipotent. Totipotent stem cells are those that can form a full organism. A fertilized egg (zygote) is totipotent. Pluripotent stem cells are those that can give rise to every cell of the organism except the trophoblasts of the placenta. Embryonic stem cells are pluripotent. Multipotent stem cells are those that can only form specific cells in the body. Neural stem cells (NSCs) are multipotent. By childhood, most pluripotent stem cells become multipotent, while there are no totipotent stem cells and only a few hard-to-isolate pluripotent stem cells [79, 80].

"Neural stem cell" is used to describe the cells that: 1) are derived from the nervous system 2) have the capacity to self-renew, and 3) can give rise to both neurons and glial cells. NSCs are multipotent stem cells and they can further differentiate into neuronal restricted progenitors (NRPs) and glial restricted progenitors (GRPs). NRPs

and GRPs are intermediate precursors. They have a limited capacity to self-renew (commonly by symmetrical rather than asymmetrical division) and can give rise to neurons and glial cells (including astrocytes and oligodendrocytes) respectively. NRPs and GRPs are also known as neuroblasts and glialblasts [78, 79].

Apart from stem cells, other terms, such as progenitor or precursor cell, are also widely used. In the current text, "progenitor (cells)" and "precursor (cells)" are used to describe both multipotential stem cells and lineage restricted stem cells, such as NRPs and GRPs.

1.2.2 History

For the most of the 20th century, it was believed that the mammalian adult central nervous system (CNS) lacked the capacity of regeneration. However, as early as in the 1910s, scientists reported the presence of the mitotically active subependymal layers around the wall of the anterior lateral ventricle of the adult rat brain [81].

In the 1960s, thymidine-^{3}H autoradiography was introduced to label the deoxyribonucleic acid (DNA) of dividing cells. Altman and other scientists found that many areas in the adult rat brain retained postnatal neurogenesis [82-88]. During the 1970s and 1980s, there was more evidence reported sporadically that neurogenic areas existed in the postnatal mammalian brain [89, 90]. Moreover, Goldman and Nottebohm reported the production of new neurons from progenitors in the adult canary brain [91].

Despite the evidence, postembryonic mammalian neurogenesis was not widely accepted until the early 1990s. Scientists identified and isolated the progenitors from postnatal mammalian CNS, which differentiated into neurons and glial cells *in vivo* and *in vitro* [92-94]. In the last decade, stem cell research has become one of the most exciting areas in the field of neuroscience.

1.2.3 Methodology of stem cell research

1.2.3.1 *In vivo* study

NSCs can be detected *in vivo* with thymidine-^{3}H or BrdU labelling or through the use of retroviruses [82, 86, 95, 96]. Retroviruses infect only dividing cells and they can be passed on to all progeny of the infected cells. However, this procedure is generally inefficient and nonquantitative. Furthermore, retroviral expression is often down-regulated with terminal differentiation. Thymidine and BrdU can be used to label nucleotides and are quantitative. However, if the cells continue to divide, the label will be diluted. Thus, an *in vivo* study can provide information such as the position and the number of dividing cells at a particular time [79].

Fates of dividing cells *in vivo* are determined by staining with cell markers that discriminate between different cell types or different states of a cell type. Once a NSC divides asymmetrically, the more mature progenitor migrates. As the progenitor migrates, it matures further until it reaches a site where it stops and either becomes quiescent or differentiates fully into a functional cell [97, 98].

1.2.3.2 *In vitro* study

The standard method of isolating NSCs is to dissect out a region that has been demonstrated to be rich in dividing cells *in vivo*. The tissue obtained from the NSCs region is digested and then dissociated into single cells. These cells are exposed to mitogens, such as FGF-2 and EGF, in a defined or supplemented medium on a matrix as a substrate for binding [92, 99, 100]. Progenitor cells proliferate and form a cluster of cells called a neurosphere, which is a mixture of stem cells and lineage restricted progenitors. Neurospheres are induced to differentiate by exposure to other factors. Cells in the neurospheres can differentiate to neurons, oligodendrocytes and astrocytes.

Cellular fates are analysed by confirmation with different cell markers [79, 101]. Cells have been planted at low density and monitored to determine if a single cell can give rise to the three phenotypes [79, 102]. Cell properties can be further identified when cells are lineage tagged with a retrovirus *in vitro* by Southern analysis [103]. *In vivo* analysis is used to determine the fate potential of NSCs. After labelling NSCs, they can be grafted back to the CNS. The number, position and fates of grafted cells can then be examined [79].

1.2.3.3 NSC markers

Stem cells in general and NSCs in particular express a wide range of gene products rather than a small group of genes that specify their potency [104, 105]. Nestin is the most widely used marker for rodent neural progenitors. Some other markers, such as EGFR, D1x2, PNA/HAS, LeX/ssea-1 for murine as well as AC133 and Musashi for human, are also well accepted.

Nestin is an intermediate-filament neuroectodermal cell marker and it is expressed both in embryonic stem cells (ESCs) and adult NSCs *in vivo* and *in vitro* [100, 106-108]. Nestin expression is downregulated when NSCs differentiate and other members of the intermediate-filament family are expressed, primarily neurofilament in neurons and glial fibrillary acidic protein (GFAP) in astrocytes [106, 109, 110]. However, nestin is re-expressed after different types of cellular stress in the CNS, particularly in reactive astrocytes [111, 112]. Some scientists are also concerned that oligodendroglial progenitors may express nestin *in vivo* [113]. In addition, expression of nestin in the CNS turmours, differentiating myoblasts and endothelial cells was also reported [111, 114-117]. Today, the presence of nestin is considered necessary but not sufficient to define NSCs [118].

EGFR, the receptor for epidermal growth factor, is a transmembrane receptor strongly expressed on the cell surface of late ESCs and adult NSCs and in the C cells from subventricular zone (SVZ) of the mouse [100, 119]. However, it is also present in other cell types that use this pathway to growth, as found in some tumour cells and in other complex pathologies, such as AIDS [120]. Dlx2 is a homeobox-containing transcription factor implicated in the development of GABA-ergic neurons and oligodendrocytes that is also expressed in the SVZ [121]. Low binding and expression of peanut agglutinin (PNA) and heat stable (HSA, mCD24a) antigens, respectively, have been demonstrated by flow cytometry studies to give rise *in vitro* to the greatest frequency of neurosphere-forming cells [122]. Lewis X (LeX) is a complex carbohydrate highly expressed by the cell surface of embryonic multipotent stem cells [123]. AC133 is a five-transmembrane protein expressed in human haematopoietic stem cells. Antibodies against AC133 were used to detect a distinct subset of human foetal brain cells [124]. Musashi1 (Msi1) and Musashi2 (Msi2) are recently discovered RNA-binding proteins and are evolutionarily conserved across different species. Msi1 and Msi2 are cooperatively involved in the proliferation and maintenance of CNS stem cell populations [125].

1.2.4 Biological features of adult NSCs in the CNS

1.2.4.1 Adult NSCs in the brain

NSCs have been identified from the subventricular zone (SVZ) [126], hippocampus [127], olfactory bulb [128], striatum, septum, thalamus, hypothalamus [129], cerebral cortex [130], cerebellum [131] and ependymal layer [116]. However, most studies were focused on three areas, namely the SVZ, hippocampus and cortex.

1.2.4.1.1 The SVZ

The SVZ is a remnant of the enlarged perinatal periventricular germinative area. This area narrows to the most rostral part of the lateral ventricle and forms the SVZ during development [132]. The SVZ progenitors originate from the subependymal layer of the lateral ventricle [133]. The SVZ can give rise to both neurons and glial cells during development. Interestingly, neurogenesis persists in adulthood [94, 134-136].

In vivo, SVZ stem cells proliferate slowly and express GFAP (type B cells). Electron microscopy studies show that they have the ultrastructural characteristics of astrocytes which extend single cilia into the ventricle lumen through the ependymal barrier. During differentiation, they become rapidly dividing cells (type C cells) and generate neuroblasts (type A cells), which migrate in chain through the rostral migration stream (RMS) to the olfactory bulb (OB). Then, the neuroblasts or NRPs differentiate into neurons in the OB [132].

In vitro, the SVZ stem cells can be expanded in serum-free medium containing epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) and form neurospheres, which generate neurons and glial cells [92, 93, 137]. Progenitors were also isolated from RMS and OB. They self-renew *in vitro* with EGF and FGF-2 and retain their multipotentiality [128, 138].

1.2.4.1.2 The hippocampus

Hippocampus progenitors originate from the subgranular zone (SGZ) of the dentate gyrus (DG) [86, 139-141]. *In vivo*, like their counterparts in the SVZ, hippocampus stem cells also express GFAP and proliferate weakly (type B cells). They give rise to type D cells, which are small, dark transient cells and specific to the SGZ. Then they migrate into the granule cell layer (GCL) of the DG, where they differentiate

into granule neurons. These granule neurons extend axons to the CA3 region [86, 139-142]. Recently, a study highlighted the long-term persistence of these newly generated neurons, which were detected 11 months after BrdU labelling [143]. Interestingly, apart from in the GCL, neurogenesis also happens in the SGZ itself [130, 144, 145]. Furthermore, Kornack and Rakic reported the generation of glial cells in the DG of the Old World Monkey [146].

In vitro, the hippocampus progenitors from rodent and human have been isolated and studied. They were found to retain the potential of self-renewal and the ability to differentiate into neurons and glial cells [103, 147, 148]. However, Seaberg and van der Kooy reported that in contrast to SVZ derived neurospheres, which were passagable (i.e. self-renewing), the DG derived neurospheres did not generate secondary spheres following dissociation and replantation and were not multipotent. Thus, they believed that lineage restricted progenitors rather than NSCs were responsible for the neurogenesis in the adult DG [149].

1.2.4.1.3 The cortex

Few studies have identified NSCs in the mammalian cortex, compared to those in the SVZ and SGZ. *In vivo*, neurogenesis in the neocortex of adult macaques has been reported by Gould and colleagues [150]. These NSCs originate from the SVZ and migrate through the white matter to integrate into the cortex, where they differentiate into mature neurons. This ventricular-cortical migration may be the remnant of the tangential migration observed from the lateral and medial ganglionic eminences to the neocortex during development [151]. However, some investigators disagree with this demonstrating that BrdU positive cells detected in the neocortex of macaques were satellite glial cells closely apposed to the resident neurons [152, 153].

In vitro, NSCs have been isolated from the human cortex and amygdala [154]. Furthermore, GRPs were isolated from human subcortical white matter [155]. These progenitors appear to be multipotential cells retaining the capacity to give rise to neurons and glial cells *in vitro* and following transplantation.

1.2.4.2 Adult NSCs in the spinal cord

Compared to the brain, only a few *in vivo* studies have been performed in the adult spinal cord. The origin of stem cells in the spinal cord is still not clear. Alonso reported progenitors were located in the ependymal layer lining the central canal while Chiasson and other scientists found that NSCs were in the subependymal zone [156-158]. In addition, Horner and colleagues reported that numerous dividing cells were in the parenchyma of the adult rat spinal cord (mainly in the white matter) with a few proliferating cells in the ependymal layer [95]. Based on the above findings, two models were hypothesized to explain the origin of the progenitors in the adult spinal cord. In the first one, progenitors may reside throughout the spinal cord, including the ependymal layer and parenchyma. In the second, progenitors probably originate from the ependymal zone and their progeny migrate to the parenchyma [95]. No solid evidence of cell migration from the ependymal zone to the parenchyma has been found. Horner and colleagues' study also showed the differentiation from NG2 (chondroitin sulphate) positive glial progenitors to oligodendrocytes and astrocytes [95]. The NG2 positive population of dividing cells may represent a heterogeneous mix of progenitors and differentiated phenotypes [159]. There is increasing evidence that NG2 positive progenitors self-renew and are stem cell-like in character [159, 160].

In an *in vitro* experiment by Weiss and his colleagues, cells from various levels of the spinal cord were suspended in a medium enriched with EGF and FGF-2. The

lumbosacral segment provided the greatest number of NSCs [161]. In addition, NSCs were isolated from regions of the adult spinal cord separated from the ependymal and subependymal zone [113]. This provides further evidence that NSCs reside in not only ependymal and subependymal zones but also the spinal cord parenchyma. Although neurogenesis is absent *in vivo*, spinal cord progenitors display the self-renewal and multipotential features of the stem cells *in vitro* [161, 162].

When cultured spinal cord progenitors are transplanted back to the adult spinal cord, their differentiation is restricted to the glial lineage [163]. However, new neurons form if cultured spinal cord NSCs are grafted into neurogenic areas of the brain [164]. Furthermore, if undifferentiated stem cells derived from ESCs are transplanted into the spinal cord, they give rise to neurons [165]. Apart from that, genetically modified NSCs also differentiate into neurons after grafting into the adult spinal cord [166]. Recently, Fujiwara and colleagues reported that after being injected intravenously, NSCs migrated to the injured spinal cord and differentiated into both neurons and glial cells [167]. Therefore, there is evidence that spinal cord NSCs can differentiate into neurons and that the spinal cord is capable of supporting neurogenesis. However, there appears to be factor(s) in the adult spinal cord that limit or prevent neurogenesis *in situ*.

1.2.5 Reaction of endogenous NSCs to injury

1.2.5.1 In the brain

1.2.5.1.1 The SVZ

Several models have been utilized to study the reaction of SVZ progenitors to injury. It has been demonstrated SVZ progenitors are activated in response to different

insults, including seizure [168], ischaemia [169, 170], transection [171], demyelination [172] and in Huntington's disease [173].

The mobilization and recruitment of SVZ progenitors and their subsequent neurogenesis have been studied. In a rat epilepsy model, SVZ progenitors migrated more numerously in the RMS to give rise to neuroblasts. Some of the progenitors were ectopically recruited in the neighbouring injured regions [168]. In a stroke model induced by transient occlusion of the middle celebral artery, SVZ progenitors were selectively recruited and expressed neuroblast markers in the injured striatum but not in the contralateral intact side. Four weeks after the stroke, the number of mature neurons increased, which indicated the neuroblasts underwent differentiation [169]. However, in the above studies, most of the newly generated neurons survived less than 5 to 6 weeks and only a small amount (about 0.2%) of the striatal neurons were replaced [168, 169]. In a global ischaemia model which selectively induced the death of CA1 pyramidal neurons, SVZ progenitors were mobilized to the CA1 region of the hippocampus to differentiate and replace the pyramidal neurons. Importantly, the new neurons received synaptic input and established connections between the CA1 region and the subiculum. Functional recovery was also reported in this study [174]. Not only neurogenesis, but also gliogenesis, was found from SVZ progenitors in rodent models of inflammatory demyelination and encephalomyelitis [172, 175]. However, the fate and functionality of newly generated oligodendrocytes have not been examined.

The above studies revealed the abilities of SVZ NSCs to proliferate, migrate and differentiate multipotentially after injury. However, they did not display a massive replacement.

1.2.5.1.2 The hippocampus

Various animal models of neurological disease were used to investigate the reaction of NSCs in the hippocampus. Hippocampus progenitors reacted to seizure and ischaemia by proliferating [168, 170, 176]. Although SGZ cells normally migrate a short distance to integrate into the GCL, long distance migration has not been found in these pathological models.

The presence of lesion-induced neurogenesis and gliogenesis was studied by Liu and colleagues [170, 176, 177]. Using a transient global ischaemia model, Liu and colleagues reported most of the BrdU labelled cells were in the SGZ but these cells were negative for neuronal markers 2 weeks after ischaemia. However, from 4 to 6 weeks after the injury, traced cells progressively migrated to the GCL and over 60% of them expressed neuronal markers. In the same study, SGZ progenitors were also reported to migrate to the hilus to generate glial cells [177].

The above evidence shows that SGZ progenitors were activated to proliferate, migrate a short distance and give rise to neurons and glia. However, the long term persistence of newly generated neurons has not been confirmed.

1.2.5.1.3 The Cortex

Although the presence of neurogenesis in the intact mammalian cortex is still controversial, *in situ* cortical neurogenesis can occur in the pathological situation. Magavi and colleagues induced synchronous apoptotic degeneration of the corticothalamic neurons in the cortex of adult mice. Two weeks later, proliferation was found throughout the whole cortex. Around 2% of proliferating cells were neurons and they were located in the lesion site. Some of these neurons formed long distance corticothalamic connections and they survived at least 28 weeks [178].

1.2.5.2 In the spinal cord

Although Adrian and Walker found endogenous progenitors in the injured spinal cord over 40 years ago [82], current knowledge about the reaction of adult spinal cord NSCs to spinal cord injury (SCI) is still limited. Most studies have been focussed on nestin positive (nestin+) NSCs and NG2+ glial progenitors. Although there is no controversy about proliferation of progenitors in the SCI, it seems that they behave in different ways in response to various pathologies [179-181]. However, one common finding is that no neurogenesis in the adult spinal cord has been found *in vivo*.

Nestin+ progenitors have been found in the ependyma lining the central canal [179, 180, 182-184], throughout the parenchyma [180, 181] and in the subpial area [185] in different injury models. Firstly, ependymal cells were found to be nestin+ as early as 12 hours after the SCI. At the same time, ependymal cells were reported to proliferate and cause enlargement of the ependymal zone (EZ). The proliferation lasts up to 4 weeks after SCI and usually peaks at 1 to 2 weeks [179, 182, 183, 185]. Furthermore, Namiki and colleagues found these nestin+ cells from the EZ migrated to the subependymal zone (SEZ) 3 days after injury [182] and Mothe and Tator reported that they migrated into grey matter 2 weeks after injury [184]. In addition, these progenitors from the EZ were found to differentiate into glial cells [183, 184]. Secondly, nestin+ NSCs were found throughout the spinal cord parenchyma after SCI [180, 181]. Lang and colleagues reported that nestin+ progenitors appeared in the grey and white matter 1 day after SCI and they were found throughout the parenchyma 1 week after injury. The proliferation lasted 2 weeks and decreased thereafter [180]. They occupied around 12% of all the proliferating cells [181]. These parenchymal NSCs were isolated and found to have the capacity to differentiate into both neurons and glial cells in culture [180, 181]. Thirdly, nestin+ cells were found in the subpial zone in a contusion

model. Shibuya and colleagues reported that these nestin+ progenitors moved toward the grey matter from the subpial zone. It took them around 4 weeks to reach the border between white matter and grey matter and a very small number entered the grey matter [185]. Questions persist about the nature and origin of these nestin+ progenitors in the SCI. Do they arise from ependyma, parenchyma or SEZ? Are they the same cells from different stem cell pools or different progenitors? Do they have similar multipotential features? Further studies need to be done on these issues. Ideally, strategies can be envisioned to label each of these populations specifically and to determine their fate.

In recent years, NG2 antibodies against a chondroitin sulphate proteoglycan have been incereasingly used in stem cell studies. Although some scientists insist that NG2 is a marker of oligodendrocyte progenitors [159], it is widely accepted that NG2+ progenitors are glial progenitors because they can differentiate into both astrocytes and oligodendrocytes *in vivo* and *in vitro* [186-188]. However, NG2 is not exclusive to GRPs. It is also expressed by macrophages, microglia, vascular smooth muscle cells and endothelia cells [159, 186, 189].

NG2+ progenitors make up 70% of the proliferating cells in the intact adult spinal cord. They are located throughout the parenchyma and divide *in situ* rather than migrating from the ependyma [186]. Their reaction to damage includes cell proliferation, changes in cell morphology (shorting and thickening of processes) and increase in NG2 immunoreactivity [189-191]. In spinal cord injury models, although proliferation was observed throughout the spinal cord at the lesion levels, it is most obvious near and in the lesion [189, 191-193]. The reaction of NG2+ progenitors to SCI is quick but transient. It was reported that they start to proliferate as early as 24 hours after injury and peak at around 1 to 2 weeks. However, this response lasted no longer than 8 to 10

weeks [189, 191-193]. Although they were reported to migrate short distances from the periphery of a demyelinating lesion into the lesion, no evidence of long distance migration was found [192]. The differentiation of NG2+ progenitors into oligodendrocytes was observed in the demyelinating and contusion model 1 to 2 weeks after injury, but there was no evidence of functional remyelination [191, 192].

1.2.6 Regulation of adult NSCs

Regulation of adult NSCs is presumably controlled by intrinsic and extrinsic factors. Current knowledge about the intrinsic regulation of NSCs is very limited. However, there is evidence that NSCs do not behave uniformly in the CNS environment. Region-specific expression of transcription factors such as Sox2 occurs during embryogenesis [194]. In the adult spinal cord, homeodomain transcription factors, Pax6, Pax7 and Nkx2.2 are distinctly and differentially expressed, indicating the potential for multiple progenitor subtypes [195]. In addition, there is also evidence of different electrophysiological properties in embryonic and adult brain and spinal cord derived NSCs [196, 197].

Some studies provide solid evidence for the role of environmental or extrinsic factors in the fate of NSCs. For instance, adult hippocampus-derived progenitors gave rise to granule cell neurons only, when they were grafted back into hippocampus. However, when they were transplanted into RMS, they generated site-specific tyrosine-hydroxylase-positive interneurons in the olfactory bulb [198]. In another example, NSCs derived from non-neurogenic regions differentiated into glial cells when transplanted back into non-neurogenic areas. However, they gave rise to neurons when transplanted into hippocampus [199, 200]. These studies demonstrate that adult NSCs

from different regions may not be fate restricted by intrinsic programs but that extrinsic cues from the local environment may control their fate.

1.2.6.1 Cellular control

Anatomical studies have shown that the vasculature may influence neurogenesis. In the adult hippocampus, proliferating cell clusters are found in close proximity to the blood vessels, which implicates a possible role of the vasculature or blood derived factors in neurogenesis [201]. In addition, Zhu and colleagues reported that vascular endothelial growth factor promoted proliferation and neurogenesis in the mammalian forebrain, indicating an important relationship between these two processes [202].

Regional differences in the astrocyte population are also important for the neurogenic microenvironment. In an *in vitro* study, adult hippocampal astrocytes were reported to actively regulate neurogenesis by promoting the proliferation of NSCs and instructing them to adopt a neuronal fate. However, the effects of astrocytes are regional specific. Astrocytes from adult spinal cord do not have the same effects [203]. Furthermore, they are also likely to contribute to maturation and synapse formation of newly generated neurons in the hippocampus [204, 205]. Astrocytes in the SVZ have the same effect on proliferation and differentiation [206].

1.2.6.2 Molecular control

A large number of growth factors [100, 200, 202, 207-219], hormones [220-225] and neurotransmitters [221, 222, 226-229] have been implicated in the regulation of NSCs. However, the physiological mechanisms underlying these factors are unclear, the relation between different factors is not understood, and whether they act directly or indirectly to regulate NSCs has not been determined.

1.2.6.2.1 Regulation of proliferation

Growth factors play an important role in the regulation of proliferation. FGF-2 and EGF-receptor ligands are the primary mitogens used to control the proliferation of NSCs *in vivo* and *in vitro* [100, 207, 213-217]. Delivery of either FGF-2 or EGF to the adult rodent CNS by different routes has been demonstrated to increase proliferation in the SVZ [230-232]. Moreover, a null-mutation for the EGF receptor ligand TGFα caused a significant decrease of proliferation of the NSCs in the SVZ [218].

Vascular endothelial growth factor (VEGF) and brain derived neurotrophic factor (BDNF) are also important for the regulation of adult NSCs. They were reported to have a stimulatory effect after being intraventricularly infused or virally overexpressed [129, 202, 219, 233]. Platelet derived growth factor (PDGF) was also found to have stimulatory effects *in vitro* and *in vivo* [234-236].

NSC derived factors and inflammatory mediators are also essential for the regulation of proliferation. In both *in vitro* and *in vivo* studies, the glycosylated form of cystatin (CCg) is expressed in the NSCs from hippocampus and it is necessary as a cofactor for FGF-2 dependent proliferation [207]. Chemokines, such as CCL5, CX and CL1, were reported to promote proliferation and survival of NSCs *in vivo* and *in vitro* [237-240].

1.2.6.2.2 Regulation of differentiation

A wide range of factors play important roles in the regulation of differentiation of NSCs both *in vivo* and *in vitro*. Some factors may help to guide NSCs to differentiate into glial lineages, such as bone morphogenetic protein (BMP), Notch and interleukin (IL-6) [241-244]. Adult NSCs express members of the BMP family that instruct them to adopt a glial cell fate [241, 245-247]. In the SVZ, the BMP inhibitor noggin is secreted

by ependymal cells in the lateral ventricle and presumably serves to block the gliogenic effect of BMP [245]. However, noggin itself is probably not sufficient to induce neuronal differentiation of adult NSCs.

Some factors have been found to induce neuronal differentiation. Recently, Wnt-signalling was demonstrated as one candidate pathway that instructs NSCs to adopt a neuronal fate [248-251]. Wnt is expressed in adult hippocampus astrocytes, and blocking astrocyte derived Wnt causes a significant decrease in neuronal differentiation of NSCs. Wnt also is secreted by NSCs themselves. Apart from Wnt, Ciliary neurotrophic factor (CNTF) [252, 253], erythropoietin [223, 254], glial derived neurotrophic factor (GDNF) [255, 256], transforming growth factor (TGF) - β [257, 258] and interleukin-1β [242, 259, 260] have also been reported to help induce neuronal differentiation.

1.2.6.2.3 Regulation of migration

Some mechanisms and molecules that are necessary for long distance migration of newly generated neurons have been reported. The interaction of the migrating neurons with their environment through expression of the polysialated glycoprotein neuronal cell adhesion molecule (PSA-NCAM) is necessary for proper migration [261-263]. Members of the ephrin-B family [264], Slit [265], integrin family members [266] and astrocyte derived factors of unknown identity [267] have also been demonstrated to direct migration through the RMS.

1.2.7 Application of adult NSCs in SCI

There are at least two possible strategies involving neural progenitors to repair injured spinal cord. They are transplantation of exogenous NSCs and stimulation of endogenous NSCs.

1.2.7.1 Transplantation of exogenous adult NSCs

There have been attempts to transplant various cells, such as neurons, glial cells and Schwann cells, to repair damaged spinal cord. The main objectives of these transplantation experiments are: 1) growth facilitation: the transplant fills the lesion site and serves as a cellular bridge; 2) new neurons: the transplant can provide new neurons, which in turn provide new targets and sources of innervation and thus repair the damaged neural circuits; 3) factor secretion: the transplant can produce a variety of substances, such as neurotrophic factors, that may aid in the repair process [268]. Several characteristics of NSCs make them potentially suitable for repair after SCI. Firstly, they can serve as a renewable supply of transplantable cells by clonal expansion in culture. Secondly, they are of CNS origin and the cells generated from the grafts have neural characteristics. Thirdly, NSCs can be manipulated by genetic engineering methods to produce specific proteins, such as neurotrophins, neurotransmitters and enzymes [269].

The efficacy of transplantation largely depends on a grafting method that optimizes the survival of the transplanted cells and minimizes the graft-induced lesion. To optimize the grafting procedure, the following issues have been considered.

The time of transplantation. The optimal time for transplantation may not be immediately after injury. The levels of various inflammatory cytokines (TNFα, IL-1α, IL-1β and IL-6) in the injured spinal cord peak 6-12 hours after injury and remain elevated until the 4^{th} day. Although these inflammatory cytokines are known to have both neurotoxic and neurotrophic actions, they are believed to be neurotoxic within a week after injury, which causes the microenvironment to be unsuitable for survival of the grafted cells [270]. However, if too much time passes after the injury, glial scar

forms around the lesion site and inhibits axon regeneration. In addition, Caselle and colleagues reported that the formation of new vessels occurs most actively 1 to 2 weeks after SCI [271]. Thus, it is considered that 7 to 14 days after injury is the optimal time for transplantation [272, 273].

The routes of administration. Most transplantation studies involved intraparenchymal injection into the CNS, in which cells were grafted directly into or adjacent to the lesion [272, 274, 275]. More conveniently, NSCs can be delivered intravenously [276]. Fujiwara and colleagues reported that NSCs migrated to the lesion site, survived and differentiated in the contused spinal cord after being injected intravenously [167]. However, it seems that intrathecal lumber puncture and intraventricular routes allow more efficient delivery of the cells to the injured spinal cord [277, 278].

The vast majority of transplantation studies used embryonic or foetal neural progenitor cells (NPCs) [279-282]. Only a few studies of adult NPCs transplantations to repair damaged spinal cord have been reported. When grafted into injured adult spinal cord, adult rodent NSCs were found to integrate along axons surrounding the lesion and differentiate into astrocytes and oligodendrocytes [163]. In another report, when adult NPCs were transplanted with fibroblasts, they enhanced axonal regeneration [283]. In addition, Teng and colleagues reported functional recovery after implantation of a scaffold seeded with NPCs [284]. Adult human NSCs were also involved and they were reported to elicit extensive remyelination when grafted into the demyelinated rodent spinal cord [285].

Due to limited information from studies of adult NSCs transplanted in SCI, the behaviours of adult NSCs after transplantation, such as proliferation and migration, and

the mechanisms of functional recovery are still not clear. Moreover, no report of neurogenesis has been found after transplantation of adult NSCs into the adult spinal cord.

1.2.7.2 Stimulation of endogenous NPCs

Compared to transplantation of exogenous NPCs, the possible advantages of stimulation of endogenous NPCs to repair damaged spinal cord are as follows: 1) it avoids the ethical issue of embryonic and foetal cells; 2) it is usually less invasive; 3) it may avoid immunological reaction [286].

Delivery of various growth factors seems to be the most common way to stimulate NPCs. The following growth factors have been reported: EGF, FGF-2 [212, 287, 288], bFGF [289], aFGF [290], BDNF [291, 292], NGF, NT-3 [291], VEGF [293], GDNF [294], IGF-1 [292] and SDF-1 alpha [295]. They were administrated by intraventricular [212], intraparenchymal [290-293] or intrathecal [287-289, 294] injection. They were reported to not only enhance the proliferation, migration and gliogenesis of NPCs [212, 287, 288, 295] but also to protect the spinal cord from further damage [292-294]. In addition, these growth factors facilitated the regrowth of axons and remyelination [290, 291, 296]. Functional recovery was also reported after they were delivered into injured spinal cord [288, 290]. However, the underlying mechanism of functionary recovery is still not clear.

Not only growth factors, but also other molecules, were shown to stimulate endogenous NPCs. Proliferation of endogenous NPCs was demonstrated when the sodium channel blocker tetrodotoxin and the glycoprotein molecule sonic hedgehog were injected into the parenchyma [297, 298]. Imitola and colleagues reported that cognate receptor CXCR4 expressed by NSCs can regulate their proliferation and direct

their migration towards the injury site [295]. In addition, antibodies blocking interleukin (IL)-6 receptors were reported to not only inhibit differentiation of endogenous NSCs into astroglia *in vivo* and *in vitro*, but also to promote functionary recovery [299, 300]. Okano and colleagues assumed that the functionary recovery is probably due to blocking IL-6 and consequently inhibiting the formation of glial scars and promoting axonal regeneration [299, 301]. Notably, studies of ATP-binding cassette (ABC) transporters have emerged as a new field of investigation. ABC transporters (especially ABCA2, ABCA3, ABCB1 and ABCG2) are found to play an important role in proliferation and differentiation of NSCs [302-307].

Like adult NPCs transplantation studies in SCI, no neurogenesis has been reported from the stimulation of endogenous NPCs. Yamamoto and colleagues reported that lack of neuronal differentiation is related to upregulation of the Notch signal pathway [195]. The increased level of various cytokines within the microenviroment surrounding the area of injury may also cause a lack of trophic support for differentiation into neuronal lineage [111, 116, 273, 308].

Recently, more attention has been drawn to CBP/ p300-phosphorylated Smad complex. It was found that CBP/ p300-phosphorylated Smad complex can be bound in NSCs, which may decide the differentiation of NSCs. If the complex is bound with phosphorylated signal transducers and activator of transcription (STAT) 3, the NSCs differentiate into astroglia lineage cells. On the other hand, if the complex is bound with proneural-type bHLH factor, such as neurogenin 1 and 2, they differentiate into the neuronal lineage [301, 309, 310]. Apart from that, Peveny and Placzek reported that SOX gene may also play an important role in neural differentiation [311].

Once NSCs decide to differentiate into neuronal lineage, a cascade of hundreds of genes is regulated over time to lead the immature neuron into its mature phenotype. Many of these neural genes are controlled by RE1 silencing transcription factor (REST). REST acts as a repressor of neural genes in non-neural cells, while regulation of REST activates large networks of genes required for neural differentiation [312-314].

1.2.7.3 Possible roles of NSCs in the treatment of PTS

No study of NSCs in PTS has been done so far, neither endogenous nor exogenous. However, according to the important pathological features of PTS, such as form of syrinx in the spinal cord parenchyma, neuronal damage in and around the syrinx, loss of oligodendrocytes and demyelination in the surrounding white matter, and wide- spread inflammation [1, 12, 25, 26], NSCs may have the following possible therapeutic roles.

Firstly, NSCs may replace damaged neurons in PTS. There is evidence that endogenous NSCs differentiate into neurons and establish connections after trauma [168, 169, 174, 178]. Although neurogenesis has not been observed in the adult spinal cord, this remains a possibility, especially with a suitable microenvironment.

Secondly, NSCs may differentiate into oligodendrocytes to replace the lost ones and help remyelination. Some studies proved that after grafting into the injured adult spinal cord, NSCs have the ability to differentiate into oligodendrocytes, enhance axonal regeneration and elicit remyelination [163, 283, 284]. Thus, NSCs possibly have the similar therapeutic roles in PTS.

Thirdly, NSCs derived astrocytes may join the reactive astrocytes to form a glial scar, which possibly contributes to limit the inflammatory reaction and spread of aminotoxicity. Furthermore, the gliosis may help to prevent the enlargement of a syrinx.

Currently, it is believed that the enlargement of syrinx is caused by the pressure of the fluid in the syrinx [5, 10, 54-61]. Furthermore, histological studies showed that the syrinx wall is covered by gliosis [12, 25, 26]. A possibility is raised that the enhancement of gliosis, from either reactive astrocytes or NSCs derived astrocytes, of the syrinx wall will stop or slow the enlargement of a syrinx.

Fourthly, NSCs may help to fill the cavity in PTS. Teng and his colleagues reported that following implantation of the scaffold–NSCs unit into an adult rat hemisection model of SCI, NSCs led to functional improvement by filling the cavity with NSCs derived tissue, diminishing tissue loss and enhancing regeneration [284]. This study indicated that a cavity in the PTS may be filled as long as NSCs can be delivered into the cyst.

Last, but not least, NSCs may be manipulated to produce various substances, such as neurotrophin and growth factors, which consequently improve the microenvironment for neurogenesis and protect tissue from further damage in PTS. This is not uncommon in the studies of NSCs in other SCI models [268, 269].

1.3 **Summary**

The pathophysiology of post-traumatic syringomyelia remains unclear and its treatment is unsatisfactory. Future studies to understand the aetiology and improve treatment are needed. Gliosis is an important histological feature of PTS in human and animal models. Some of its characteristics, such as restricting inflammation, protecting neurons, make enhancing reactive astrocytes a possible new treatment to PTS. However, current knowledge about reactive astrocytes and gliosis in PTS is not sufficient. Here, a study is carried out to examine the responses of reactive astrocytes and the formation of

gliosis in PTS. This study will extend our knowledge about pathology in PTS model and may also contribute to establish a new treatment to PTS.

The rapid advancement in the field of NSCs research may also offer hope in the treatment of PTS. NSCs may have some therapeutic roles in PTS, such as replacing damaged neurons, assisting regeneration and remyelination, filling the cavities and so on. There are two possible strategies, transplantation of exogenous NSCs and stimulation of endogenous NSCs. Currently the vast majority of studies of exogenous NSCs in SCI are based on embryonic or foetal cells, which encounter ethical issues. Compared with grafting exogenous NSCs, stimulating endogenous cells bypasses the ethical issue of embryonic and foetal cells and avoids the possible immunological reaction from transplantation.

Although they have great potential, our knowledge about endogenous NSCs in adult spinal cord is very limited, in terms of their biological features and responses to injury. This makes it impossible to use endogenous NSCs to treat PTS. Some studies are carried out here to examine the distribution, migration, proliferation and differentiation of endogenous NSCs in adult PTS model. These studies extend our knowledge about biological features of adult endogenous NSCs and their responses to injury. These studies also make the first step to explore the possibility to treat PTS by adult endogenous NSCs.

Chapter Two General Methods

2.1 Animal care

2.1.1 Ethics

All experiments in this study were approved by the Animal Care and Ethics Committee of the University of New South Wales (ACEC reference numbers: 02/98 and 02/136).

2.1.2 General

Animals used in this study were handled in accordance with ACEC guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 6th Edition 1997. Rats were housed in standard cages of no more than four per cage. Bedding was changed twice a week, and food and water was provided *ad libitum*. Attempts were made to allow acclimatization to new surroundings (holding rooms and operating theatres) before any procedure, and to minimize the time postoperatively that animal spent alone in a cage (about 2 weeks).

2.1.3 Morbidity and mortality

Unilateral forelimb weakness occurred in all the animals developing a syrinx. It usually resolved within 2 weeks, but almost a quarter of the animals had weaknesses for longer than 2 weeks. Rats undergoing syrinx induction procedures had an average weight loss of 15-18% within the first week after surgery. In total, 1.6% (2 of 124) animals died during the operation or within 24 hours of the procedure. A further 1.6% (2 of 124) animals were euthanized early as they were considered to be suffering, indicated by weight loss greater than 20% of their initial body weight.

2.2 Syrinx induction procedure

2.2.1 Anaesthesia and operative care

Anaesthesia was induced with 4% isoflurane in oxygen in an induction chamber after weighing. Each animal was then placed prone in a nose cone and maintained with 2.5% isoflurane in 2 L/min of oxygen, increased if required to maintain an adequate level of anaesthesia as judged by the respiratory rate and response to painful stimulation (hindlimb web pinch). The animals lay on a warming blanket with a heat lamp overhead during surgery. Regular observations, including respiratory rate, core temperature, and withdrawal response, were taken at 0, 5, 10, 15 and every 15 minutes throughout the procedure. The operation site was shaved and prepared with povidine iodine. Local anaesthetic (0.15 mL of 0.5% bupivicaine) was infiltrated at the incision site, and 5 mL of sterile saline was given subcutaneously. All survivable procedures were performed in a sterile field with aseptic techniques. The surgical procedure was started only when a stable respiratory pattern and a consistent absence of pain responses were obtained.

At the end of the procedure 0.2 – 0.25 mL of temgesic (buprenorphine 0.3 mg/mL) and a further 5 mL of sterile saline were given subcutaneously. The animals were marked by an implantable microchip (Trovan). Inhalational anaesthesia was turned off whilst maintaining oxygen flow until the animal started to awaken. Once recovered the animals were transferred to the holding facility in the Prince of Wales Medical Research Institute.

A second dose of temgesic was usually given on the first postoperative day for animals undergoing a syrinx induction procedure. Post operative observations included

daily weight, assessment of mobility, and wound health for seven days, followed by weekly assessment thereafter.

2.2.2 Syrinx induction

This syrinx induction procedure was describeded by Brodbelt et al previously [25, 54]. Under general anaesthesia, the animal was placed prone with a pad under the chest to provide neck flexion. A 2 cm midline incision over the dorsal cervical spine was made extending just caudal to the palpable spine of T2. After a midline dissection demonstrating the spines and laminae, a C7 to T1 laminectomy was performed under a surgical microscope (Zeiss instruments, Germany).

The dura and arachnoid were opened longitudinally. A glass needle tipped (outside diameter 50 μm) 5 μL syringe (SGE International Pty Ltd., Austin, TX) held in a stereotactic micromanipulator was used to infiltrate four 0.5 μL injections of 24 mg/mL quisqualic acid (QA) and 1% Evans blue along the line of the right dorsal nerve rootlets between C7 and T1. Evans blue in the solution allowed any leakage of the quisqualic acid to be identified. Kaolin (10 μL of 250 mg/mL) was injected into the subarachnoid space to promote arachnoiditis using a 10 μL syringe attached to a needle blunted and bent to 70°. Muscle fascia was closed with 4/0 vicryl, and the skin with 3/0 silk suture.

Quisqualic acid (Tocris Cookson Ltd., Bristol, U.K.) was dissolved in four fifths 0.9% saline and one fifth 1% Evans blue. Evans blue (Sigma- Aldrich, St. Louis, MO) was made as 1% solution in 0.9% saline. Kaoloin (Sigma- Aldrich, St. Louis, MO) was dissolved in 0.9% saline.

2.3 **Labelling mitotic cells**

Bromodeoxyuridine (BrdU) is a widely used mitotic marker and it incorporates into DNA during S phase. After intraperitoneal injection, the bioavailability of BrdU lasts about 2 hours. Intraperitoneal injections of 10 mg/mL BrdU (Sigma- Aldrich, St. Louis, MO) were used to label proliferating cells systematically. There were two labelling methods used in this study (Figure 2-1.). Labelling method 1: one injection of BrdU (50 mg/kg body weight) was made 24 hours after syrinx induction in the study of reaction of endogenous progenitor cells in the early stage in PTS (Chapter 3). Labelling method 2: 12 single daily injections (same dose as method 1) were induced from the first postoperative day after surgery in the study of *in vivo* differentiation of endogenous progenitors in PTS (Chapter 5).

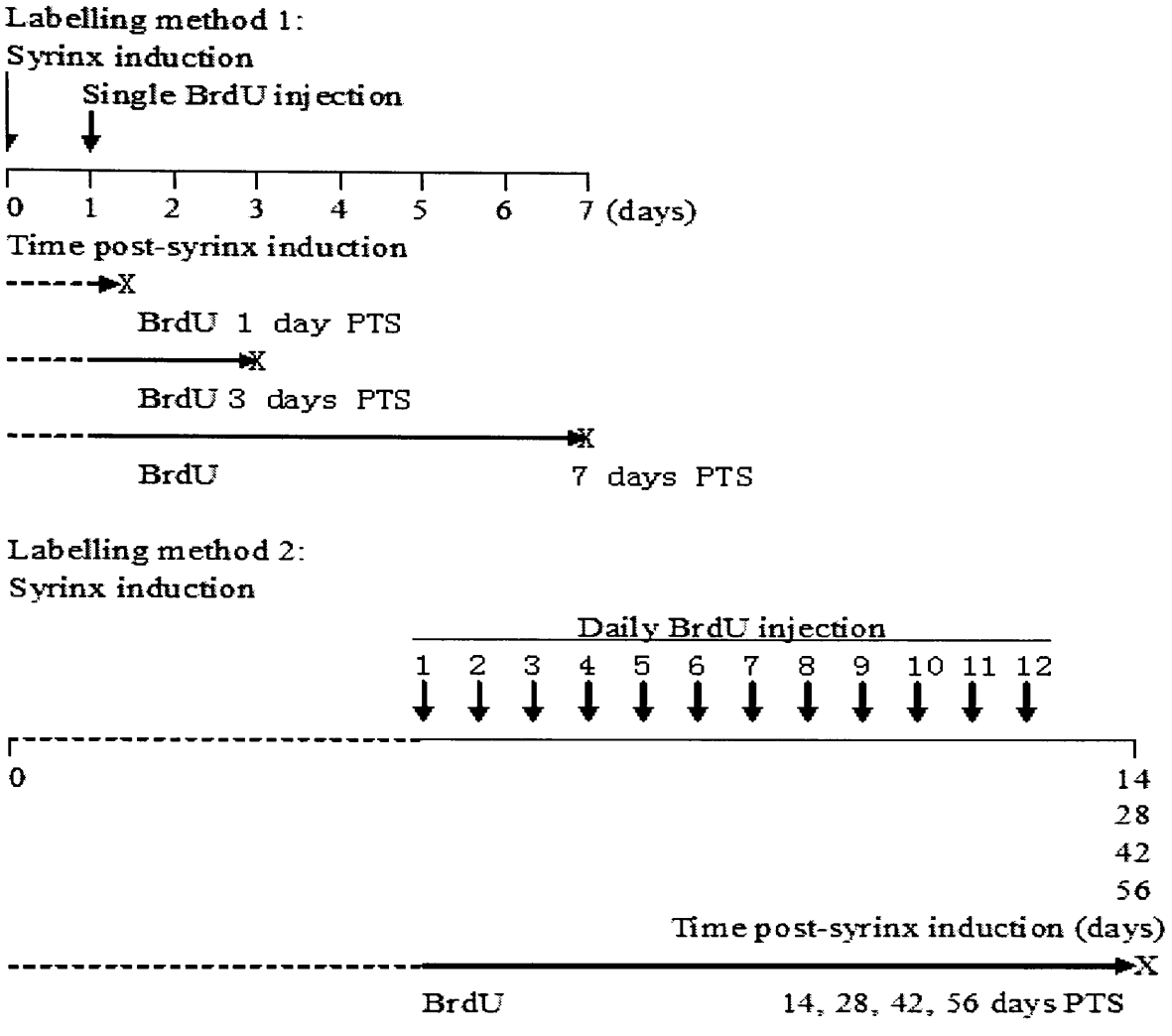

Figure 2-1. Time course of BrdU labelling, denoted by a solid line; time of sacrifice is indicated by "X". The sham-operated and syrinx animals were randomly divided into different time points following surgery. Rats received single BrdU injection with labelling method 1 and one BrdU injection per day for 12 days with labelling method 2.

2.4 Tissue processing

2.4.1 Perfusion fixation

Animals were anaesthetized with 4% isoflurane in oxygen through a nose cone and perfused by intracardiac injection of 5000 i.u. heparin in 1 mL of 0.9% saline followed by 100 mL phosphate buffered saline (PBS) and then 0.5 L of 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.45 over 20 minutes. The spinal cord and

brain stem were dissected out and post-fixed in 4% paraformaldehyde in 0.1 mol/L PBS overnight. Spinal cords were cut into segments from C2 to L5 according to the nerve roots and transferred to 32% sucrose overnight for cryopreservation.

2.4.2 Tissue embedding and sectioning

Spinal cord segments were embedded in cryomolds (Mile Inc., Elkhart, IN) in O.C.T. mounting medium (Sakura Finetek Co., Torrance, CA). Blocks were stored at -80℃ until sectioned coronally on a cryostat (Cryo-star HM 560, MICROM international GmbH, Germany) at 10 μm and stored on slides at -20℃.

2.5 Immunohistochemistry

2.5.1 Antibodies

The primary antibodies used in this study are shown in Table 2-1. The following secondary antibodies were chosen: ALEXA Fluor 594 goat anti-mouse, ALEXA Fluor 488 goat anti-rabbit and ALEXA Fluor 488 donkey anti-sheep (all in 1:800, Molecular Probes, USA).

Table 2-1 Primary antibodies

Primary antibodies	Antibody selectivity	Type	Dilution	Source
Anti-Ki67	Proliferating cells	Rabbit polyclonal	1:2000	Novocastra, UK
Anti-BrdU	Proliferating cells	Sheep polyclonal	1:200	Abcam, UK
Anti-nestin	Neural stem cells	Mouse monoclonal	1:4000	Chemicon, USA
Anti-NeuN (neuronal nuclei)	Neurons	Mouse monoclonal	1:200	Chemicon, USA
Anti-NCAM (neural cell adhesion molecule)	Neurons	Mouse monoclonal	1:1000	Chemicon, USA
Anti-β-tubulin (III)	Neurons	Mouse monoclonal	1:300	Chemicon, USA
Anti-NG2	Glial Progenitors	Mouse monoclonal	1:200	Chemicon, USA
Anti-MBP (myelin basic protein)	Oligodendroglia	Mouse monoclonal	1:50	Chemicon, USA
Anti-GFAP (glial fibrillary acidic protein)	Astrocytes	Mouse monoclonal	1:4000	Chemicon, USA
ED-1	Macrophages	Mouse monoclonal	1:400	Serotech, UK

2.5.2 Staining

2.5.2.1 Staining for BrdU

Sections were washed in PBS, pH 7.45 for 10 min twice to remove Tissue Tek; followed by 30 min in 2 mol/L hydrochlorice acid (HCl) at 37℃, 10 min in 0.1 mol/L Borate buffer, pH 8.5 twice and 10 min in PBS three times. Non-specific labelling was blocked by 10% normal horse serum in PBS for 20 min. Two primary antibodies (BrdU and another cell marker) were applied together overnight at 4℃. Sections were washed in PBS for 10 min three times, and then incubated with secondary antibodies for 2 hours in the dark. Following washing in PBS for 10 min three times, sections were mounted in mounting medium for fluorescence with 4,6-diamidino-2-phenylindole (DAPI, Vector

Laboratories, Burlingame, CA) and then coverslipped and sealed with nailpolish. Slides were kept in the dark at 4℃ until examined by microscopy.

2.5.2.2 Staining for Ki67

Sections were washed in PBS, pH 7.45 for 10 min twice to remove Tissue Tek, followed by 20 min in 100% dimethyl sulfoxide (DMSO) and 10 min in PBS. After blocking non-specific labelling with normal horse serum, two primary antibodies (Ki67 and another cell marker) were applied together overnight at 4℃. Sections were washed in PBS for 10 min three times, and then incubated with secondary antibodies for 3 hours in the dark. Following washing in PBS for 10 min three times, sections were mounted in mounting medium for fluorescence with DAPI and then coverslipped and sealed with nailpolish. Slides were kept in the dark at 4℃ until examined by microscopy.

2.5.3 Criteria for microscopic analysis of mitotic labelling

BrdU and Ki67 were used to label mitotically active cells in the study. BrdU is a thymidine analogue and is incorporated into DNA during DNA synthesis after a systemic intraperitoneal injection. BrdU is detectable in the nuclei of proliferating cells on further division. This is important when following proliferating cells for more than one cell cycle and determining long term differentiation. However, the level of BrdU will decrease due to dilution on further cell division. Ki67 antigen is a nuclear protein expressed in all active phases of the cell cycle (G1, S, G2 and mitosis) but not in resting cells (G0). Thus, Ki67 is ideal for marking all mitotically active cells.

The number of BrdU and Ki67 positive cells were assessed in spinal cord sections using a fluorescence microscope (Axioplan 2, Carl Zeiss, Germany) and imaging data acquired by a digital camera (AxioCam MRm, Carl Zeiss, Germany) using

Axiovision software (Carl Zeiss, Germany). Only uniformly labelled nuclei were considered as positive. An exception was when immunoreactivity was detected throughout the nucleus, but the intensity was higher at the periphery of the nucleus than the centre. This effect is probably due to decreased antibody penetration to the centre of the nucleus. The nuclear stain DAPI was used as a counterstain to identify all nuclei. DAPI stained double stranded DNA without staining of cytoplasm. BrdU or Ki67 stained nuclei were scored as positive only when co-location was present with DAPI staining, in terms of their positions and shapes. This was to prevent false positive staining for BrdU or Ki67.

All slides were viewed by two observers blinded to the nature of the specimen.

2.5.4 Criteria for microscopic analysis of phenotypes

Cells were considered positive for a particular phenotype when well defined BrdU or Ki67 labelled nuclei were associated with immunopositive (eg. GFAP, NG2, Nestin etc.) cell bodies. Positive nuclei were followed through the z-stack re-construction and only cells with well-circumscribed immunopositive cell bodies were classed as positive. At least 100 cells were selected randomly from each section and examined for phenotype.

2.5.5 Data analysis

Differences among different groups were evaluated by a one way ANOVA. Significance was accepted as a p value < 0.05.

Chapter Three Acute Responses of Endogenous Progenitors in an Adult Rat Model of Post-traumatic Syringomyelia

3.1 Introduction

A quarter of patients suffering from spinal cord injury develop PTS. Currently, the treatment of PTS is unsatisfactory [74, 77, 315, 316], which is partially due to poor understanding of the underlying pathophysiology. It is believed that syrinx formation consists of two stages: initial cyst formation and subsequent enlargement. Initial cyst formation is likely caused by inflammation, hematoma, infarction or excitatory amino acid overproduction after trauma [48-51]. A temporal profile of syrinx formation has unveiled that macrophages are the predominant inflammatory cell type in the syrinx wall at the acute stage of post-traumatic syringomyelia and that the formation of an initial cyst predisposes to syrinx formation [25, 26]. It was originally thought that trauma-induced central nervous system (CNS) inflammation is dominated by hematogenous macrophages recruited from blood and CSF [317, 318], recent data cannot exclude the possibility of resident macrophages occurring at the site of injury since systemic depletion of infiltrating macrophages failed to deplete the cells effectively [319, 320].

The diverse functions of progenitor cells have prompted considerable interest in their acute responses to post-traumatic syringomyelia.We hypothesize that endogenous progenitors exist in the spinal cord parenchyma and they react by proliferating and differentiating. A population of resident macrophages may differentiate from endogenous progenitor cells *in situ*. In this study, we aim to examine the existence of

endogenous progenitors in the early stages of the PTS model and their proliferation, location and phenotypes.

3.2 Materials and methods

3.2.1 Animal groups

Thirty-six male Wister rats (10 - 12 weeks old and 200 – 380 g body weight) were used in this study. There were 3 groups at 4 time points (Table 3-1). Tissues used for the time point at 14 days post-injury (PI) were harvested from the rats involved in the study of "*In Vivo* Differentiation of Adult Endogenous Progenitors in PTS" (Chapter 5). Animals in the intact group had no surgery and rats in the sham operated group had laminectomies only, while animals in the syrinx group had syrinx induction procedures (decribed in detail in General Methods). Twelve rats were sacrificed 1 hour post injection of BrdU (1 day PI), 3 days PI and 7 days PI respectively. A further 12 rats were sacrificed 1 day after daily intraperitoneal injections of BrdU for 12 days (14 days PI).

Table 3-1 Animal groups

	1 day PI	3 days PI	7 days PI	14 days PI
Intact	4	4	4	4*
Sham operated	4	4	4	4*
Syrinx	4	4	4	4*

PI: post injury; * Tissue harvested from rats used in Chapter 5: *In Vivo* Differentiation of Adult Endogenous Progenitors in PTS

3.2.2 Mitotic cell labelling

Labelling method 1: each animal was given a single intraperitoneal injection of 50 mg/kg BrdU (10 mg/mL) 24 hours after the syrinx induction procedure. Rats were sacrificed 1 hour, 2 days or 6 days after the injection.

Labelling method 2: to achieve a higher availability of BrdU, animals received daily intraperitoneal injections of BrdU (same dose as in method 1) for 12 days commencing 24 hours after surgery in the sham-operated and syrinx groups. Twelve successive daily injections of BrdU were given to the intact controls. Rats were sacrificed 1 day after the last injection.

3.2.3 Immunohistochemistry

Nine primary antibodies (Table 3-2) were used in this study. The secondary antibodies were: Alexia 594 (goat anti-mouse, 1:800) and Alexia Fluor 488 (donkey anti-sheep IgG, 1:800). Details of the above antibodies and tissue processing were described in Chapter Two. Spinal cord sections were mounted in fluorescence mounting medium with DAPI.

Table 3-2 Primary antibodies

Primary antibodies	Type	Concentration
Anti-BrdU	Sheep polyclonal	1:200
Anti-nestin	Mouse monoclonal	1:4000
Anti-NG2	Mouse monoclonal	1:200
Anti-β-tubulin (III)	Mouse monoclonal	1:300
Anti-NCAM	Mouse monoclonal	1:1000
Anti-NeuN	Mouse monoclonal	1:200
Anti-MBP	Mouse monoclonal	1:50
Anti-GFAP	Mouse monoclonal	1:4000
Anti-ED1	Mouse monoclonal	1:400

3.2.4 Quantification of BrdU+ cells

The number of BrdU positive cells taken from segment C8 was counted using a fluorescence microscope and digital camera. Only uniformly labelled nuclei, which co-located with DAPI, were considered as positive. BrdU nuclei were excluded from the

study if they exhibited punctate staining in part of the nucleus. Two sections from each animal were taken for cell counting.

In order to study the location of proliferating cells in spinal cord section, two templates were used in this study. The first template (Figure 3-1) divided the section into 6 parts: ependymal zone (EZ), subependymal zone (SEZ), grey matter (GM), white matter (WM), subpial zone (SPZ) and pial zone (PZ). The EZ was the area where ependymal cells lined the central canal. In this study, the SEZ was defined as 50 μm from the EZ. The SPZ was defined as 100 μm from the pial surface. In the second template (Figure 3-2), a cross centred at the central canal divided the coronal section into four parts: the right anterior area (RA), right posterior area (RP), left anterior area (LA) and left posterior area (LP). The lesion caused by the syrinx induction procedure was mainly located in the RP.

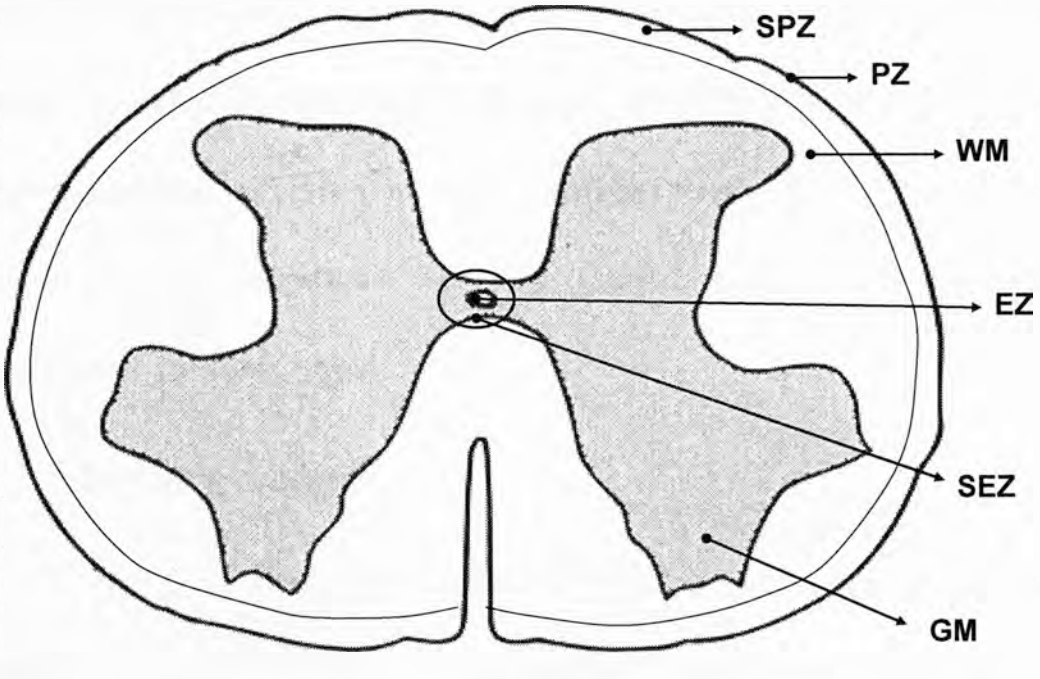

Figure 3-1. Template one: the spinal cord section is divided into 6 regions. EZ: ependymal zone; SEZ: subependymal zone (50 μm from EZ); GM: grey matter; WM: white matter; SPZ: subpial zone (100 μm from PZ); PZ: pial zone.

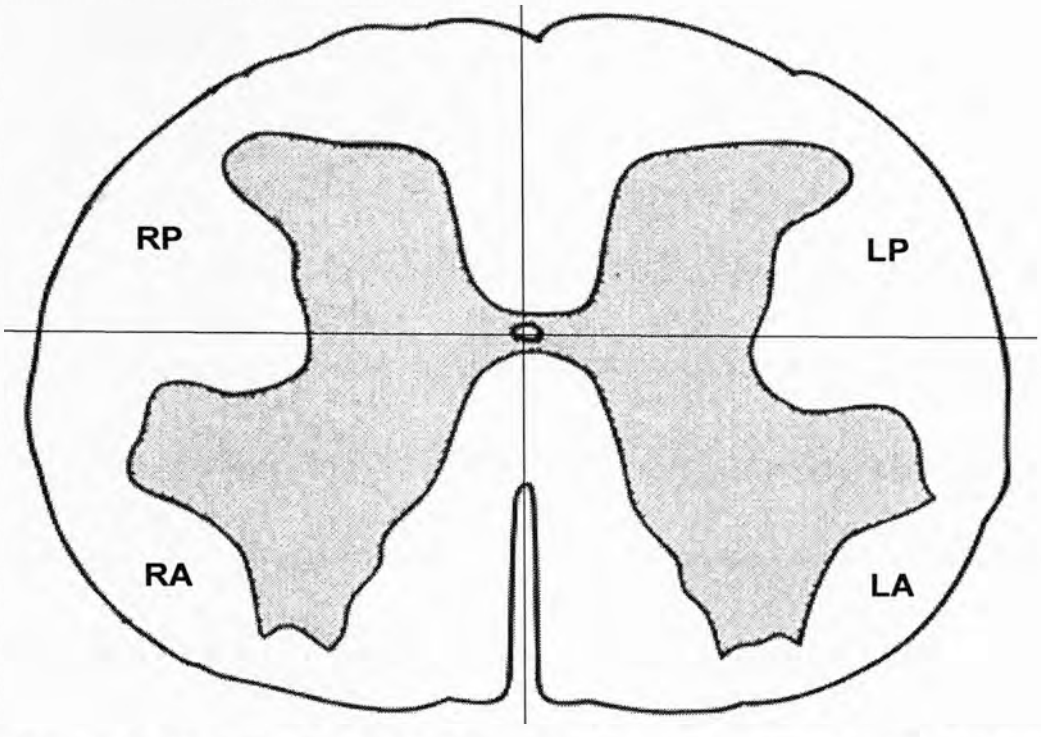

Figure 3-2. Template 2: the spinal cord section is divided into 4 regions. RA: right anterior area; RP: right posterior area; LA: left anterior area: LP: left posterior area.

3.2.5 Quantification of immunofluorescecent images

Cells were considered positive for a particular phenotype when well defined BrdU labelled nuclei were associated with immunopositive cell bodies. The cell nuclei were followed through the z-stack and only cells with well-circumscribed immunopositive cell bodies were considered as positive. One section from each animal was examined and at least 100 cells selected randomly from each section were examined for phenotypes.

3.2.6 Data analysis

Data are expressed as mean ± SE. Differences among groups were evaluated by one way ANOVA. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Syrinx model

No spinal cord cysts were found in the intact and sham-operated groups. In the syrinx group, no obvious cyst formed at 1 day after surgery. However, in all the syrinx animals, cysts were found in the right grey matter 3 and 7 days after surgery. The cysts were separate from the central canal. According to template two, cysts were mainly located in the RP, extending to RA in very few situations.

3.3.2 BrdU+ cells

BrdU+ cells were found in not only syrinx animals but also intact and sham-operated groups. Cells with BrdU staining matched those with DAPI+ cells in terms of their position and shape (Figure 3-3). At 1 day PI time point, the vast majority of BrdU positive cells were present as single cell profiles in all the animals groups. However, at the time points of 3 days PI and 7 days PI, many cells were found in clusters of two, which were present more commonly in syrinx animals than the other 2 groups (Figure 3-3). BrdU incorporation was detectable after multiple cell divisions.

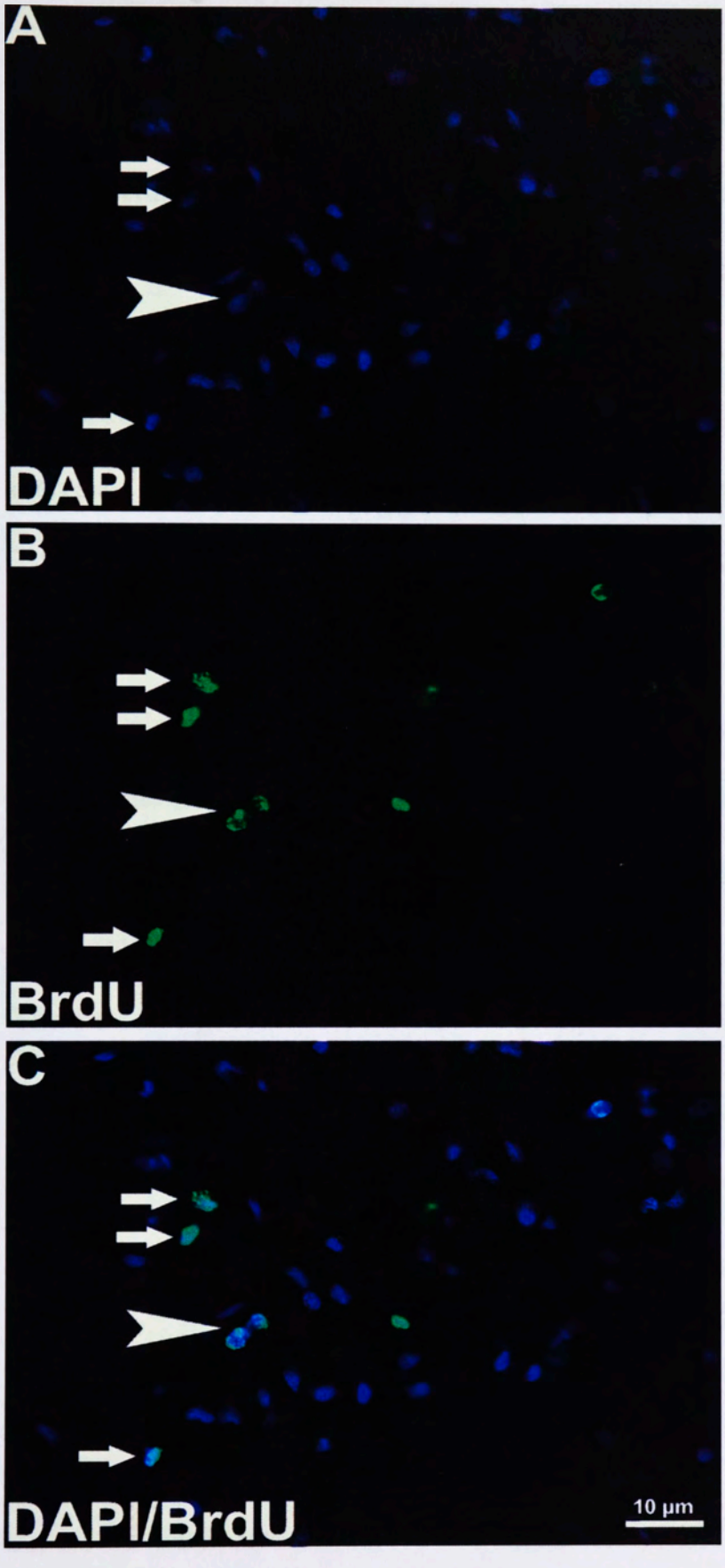

Figure 3-3. The immunofluorescent images were taken from the syrinx animals 3 days PI. A: DAPI single staining. B: BrdU single staining. C: DAPI and BrdU double staining. Arrows indicate BrdU+ cells matching with DAPI+ cells in terms of their position and shape. Arrow head shows a cell undergoing division.

3.3.3 Proliferatiion

Using labelling method 1, we found very few proliferating cells in the intact and sham-operated animals. There were around 4 BrdU+ cells per section at 1 day PI, 6 BrdU+ cells at 3 days PI and 8 BrdU+ cells at 7 days PI in the control groups. In the syrinx animals, however, many more proliferating cells were found. There were over 280 BrdU+ cells per section found in the syrinx animals at 1 day PI. This number increased to over 650 at 3 days PI but decreased to around 310 at 7 days PI (Figure 3-4, 3-5, 3-6 and 3-7). There was a significant difference in the number of dividing cells between syrinx and control groups ($n = 8$; $P < 0.001$).

Using labeling method 2, we were able to detect a higher cell division rate and could demonstrate that these dividing cells persist for 14 days post-syrinx induction. At this time point, the number of BrdU positive cells was 30 ± 4, 29 ± 5, and $3{,}392 \pm 275$ cells/section in the intact, sham-operated controls, and syrinx group, respectively. There was a significant difference in the number of dividing cells between syrinx and control groups ($n = 8$; $P < 0.001$).

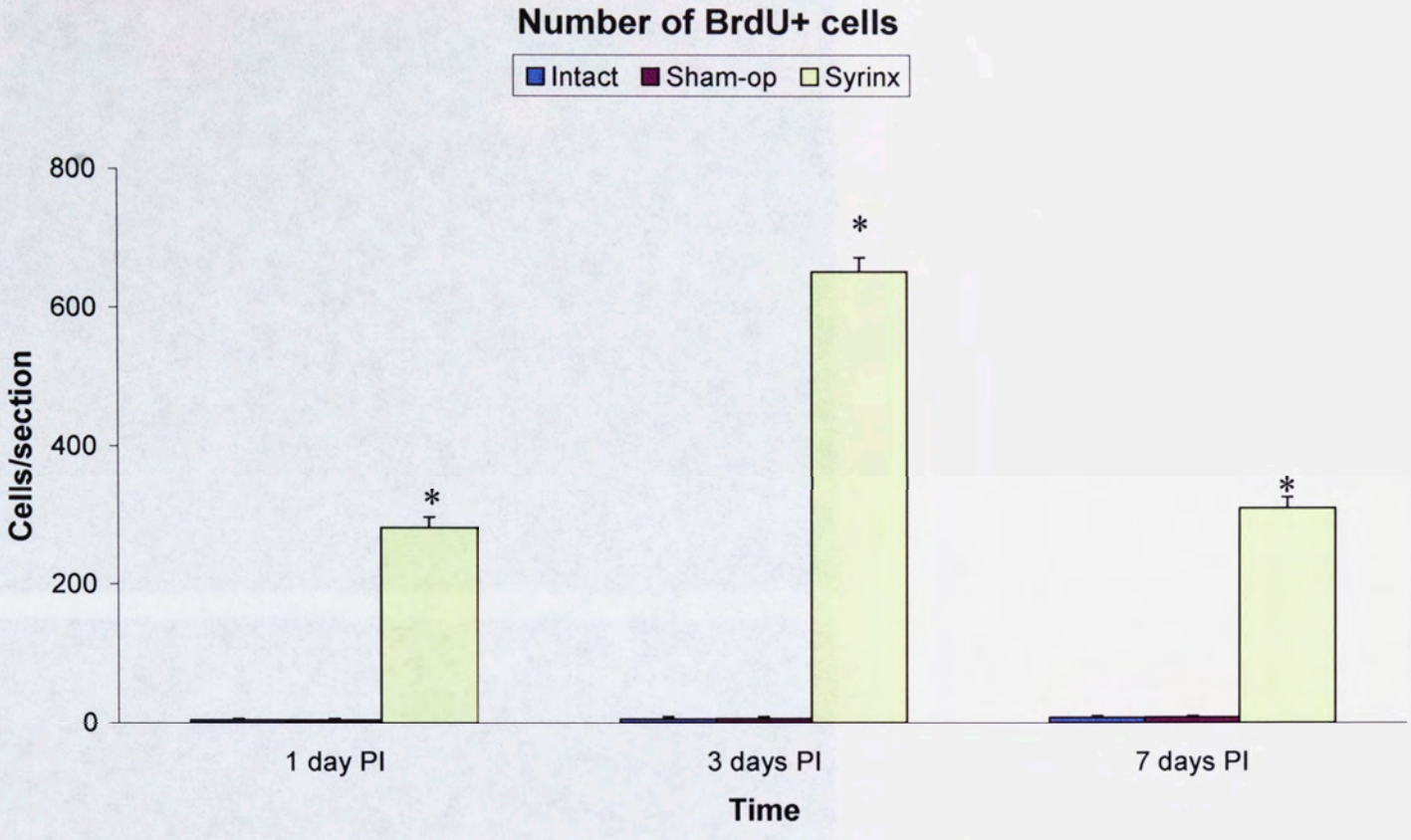

Figure 3-4. Number of BrdU+ cells in the spinal cord section (C8) in the intact, sham-operated and syrinx animals. Data presented as mean ± SE (n=8). *p < 0.01 vs intact and sham-operated animals. The numbers of BrdU+ cells in the syrinx animals are much higher than those in the intact and sham-operated animals.

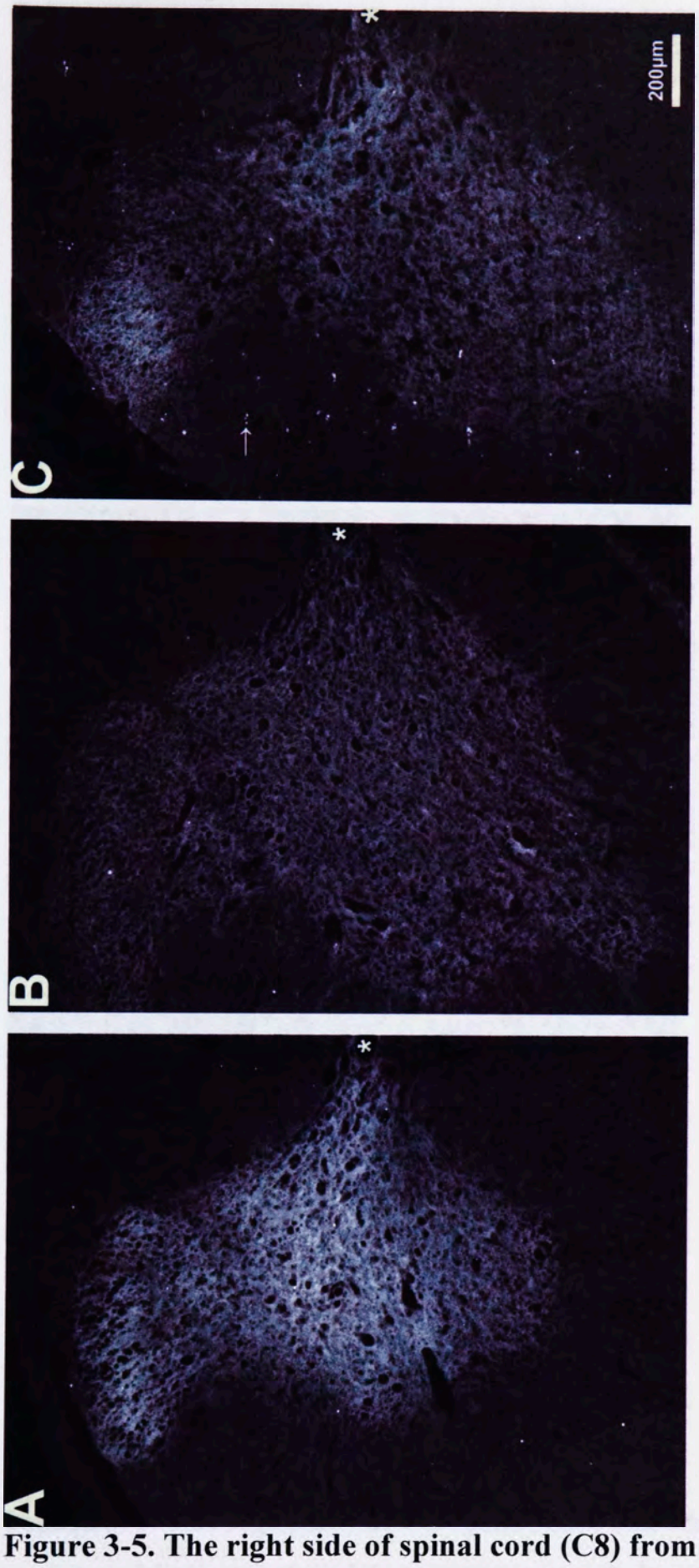

Figure 3-5. The right side of spinal cord (C8) from the intact, sham-operated and syrinx animals at 1 day PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. White dots (arrow) are BrdU+ cells. These BrdU+ cells are located mainly in the white matter.

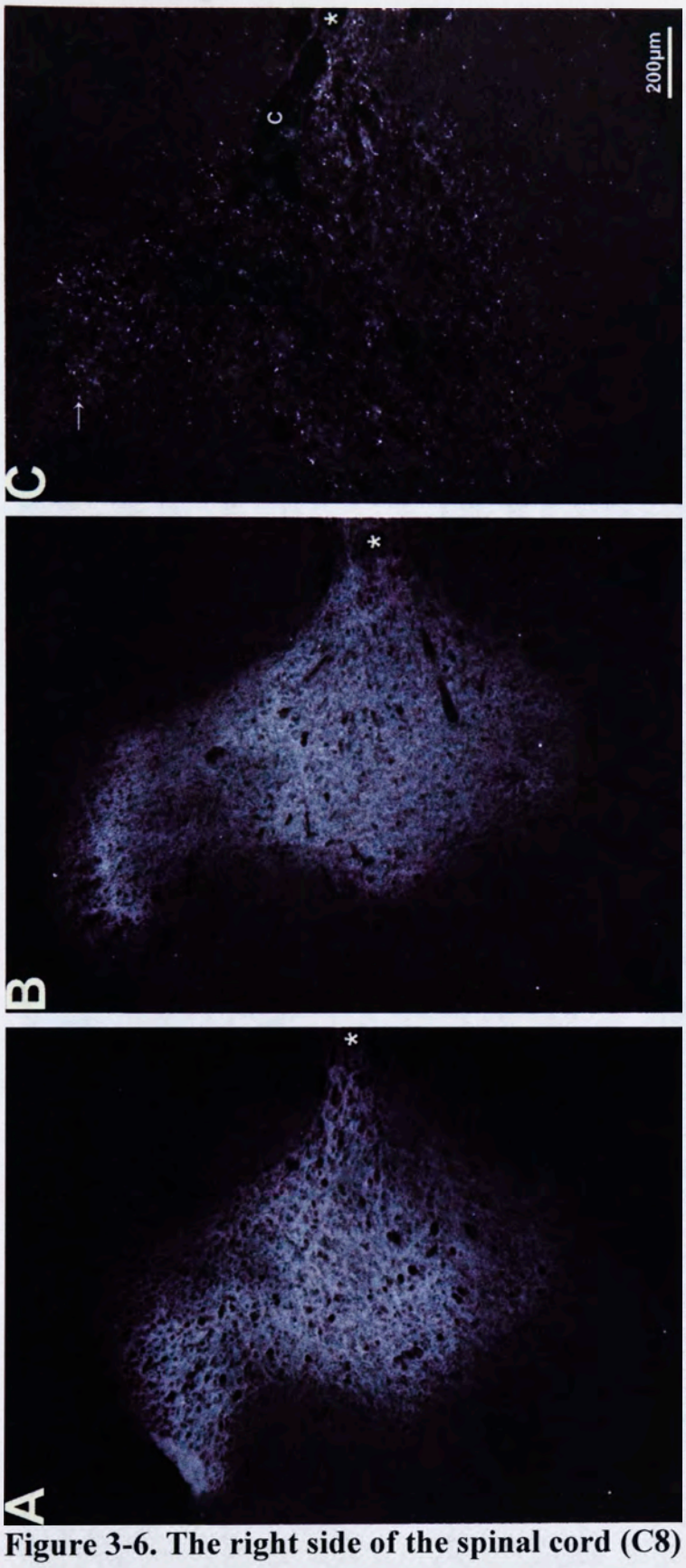

Figure 3-6. The right side of the spinal cord (C8) from intact, sham-operated and syrinx animals at 3 days PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. White dots (arrow) are BrdU+ cells. In the intact and sham-operated animals, BrdU+ cells are mainly in the white matter, while they are located throughout the grey and white matter in the syrinx animals. Syrinx animals at this time point have much more BrdU+ cells than the other 2

time points. "C" indicates cysts formation. Multiple small cysts form in the grey matter in the syrinx animals. Intact and sham-operated animals have no cyst.

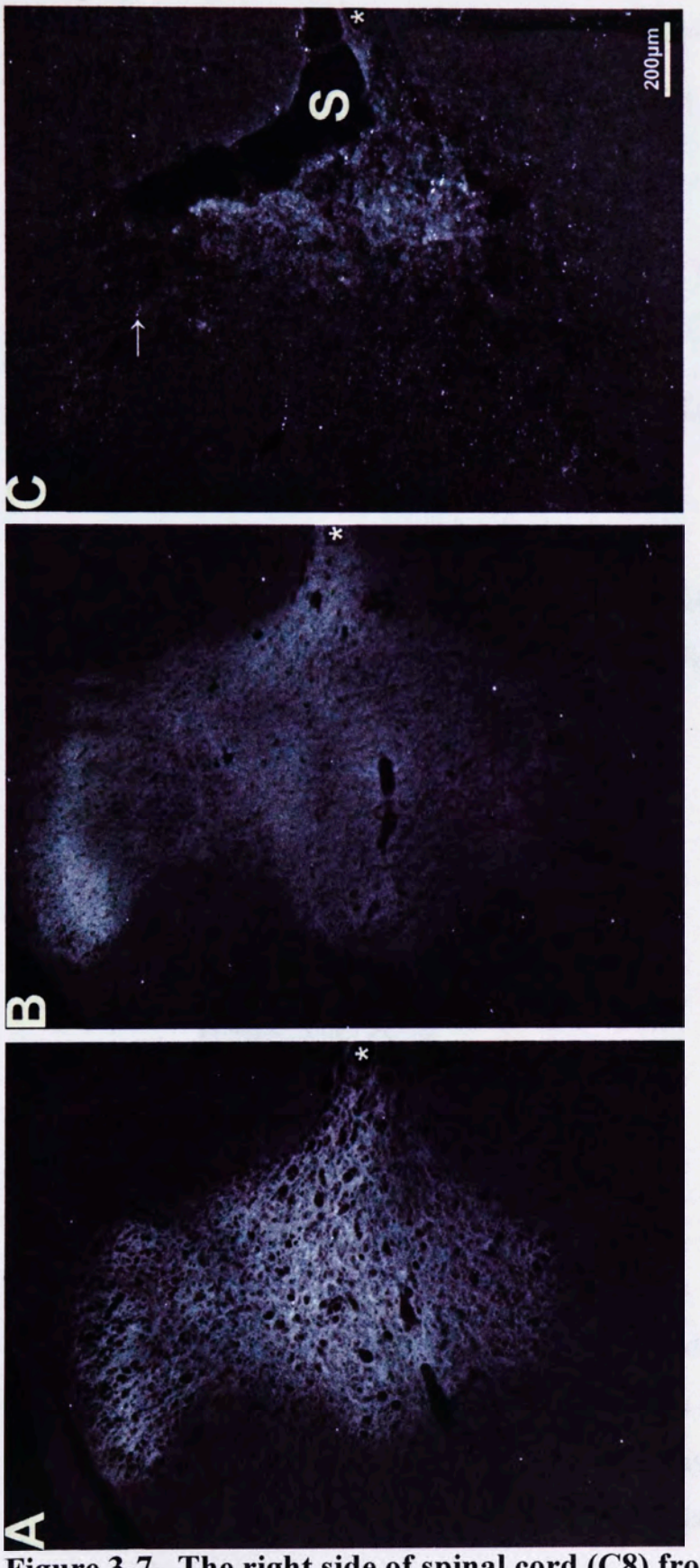

Figure 3-7. The right side of spinal cord (C8) from intact, sham-operated and syrinx animals at 7 days PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. White dots (arrow) are BrdU+ cells. In the intact

and sham-operated animals, as in the other two time points, BrdU+ cells are mainly in the white matter, while they are located throughout the grey and white matter in the syrinx animals. "S" indicates cysts formation in the grey matter in the syrinx animals. Intact and sham-operated animals have no cyst.

The number of BrdU+ cells in the intact and sham-operated animals increased with time. The number of BrdU+ cells doubled from 1 to 7 days (Figure 3-8), suggesting the doubling time in control groups is 6 days. However, the number of BrdU+ cells in the syrinx animals increased from 280 to over 650 cells per section and then dropped to 310 cells per section at 7 days PI. The doubling time of BrdU+ cells in the syrinx group was less than 2 days (Figure 3-9).

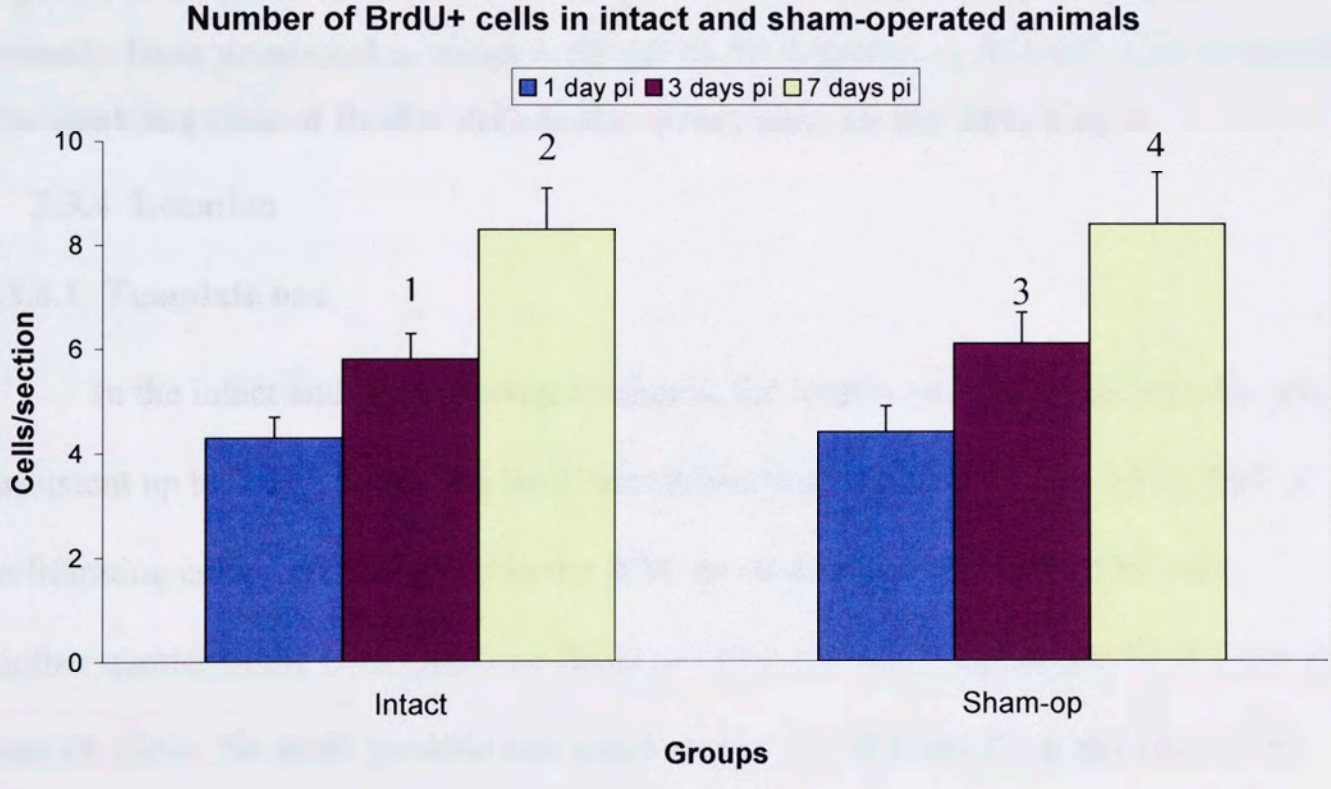

Figure 3-8. Number of BrdU+ cells in spinal cord sections (C8) from the intact and sham-operated animals. Data presented as mean ± SE (n=8). [1] $P < 0.05$ vs intact at 1 and 7 days PI; [2] $P < 0.05$ vs intact at 1 and 3 days PI; [3] $P < 0.05$ vs sham-op at 1 and 7 days PI; [4] $P < 0.05$ vs sham-op at 1 and 3 days PI. The doubling time of BrdU+ cells in the intact and sham-operated animals is about 7 days.

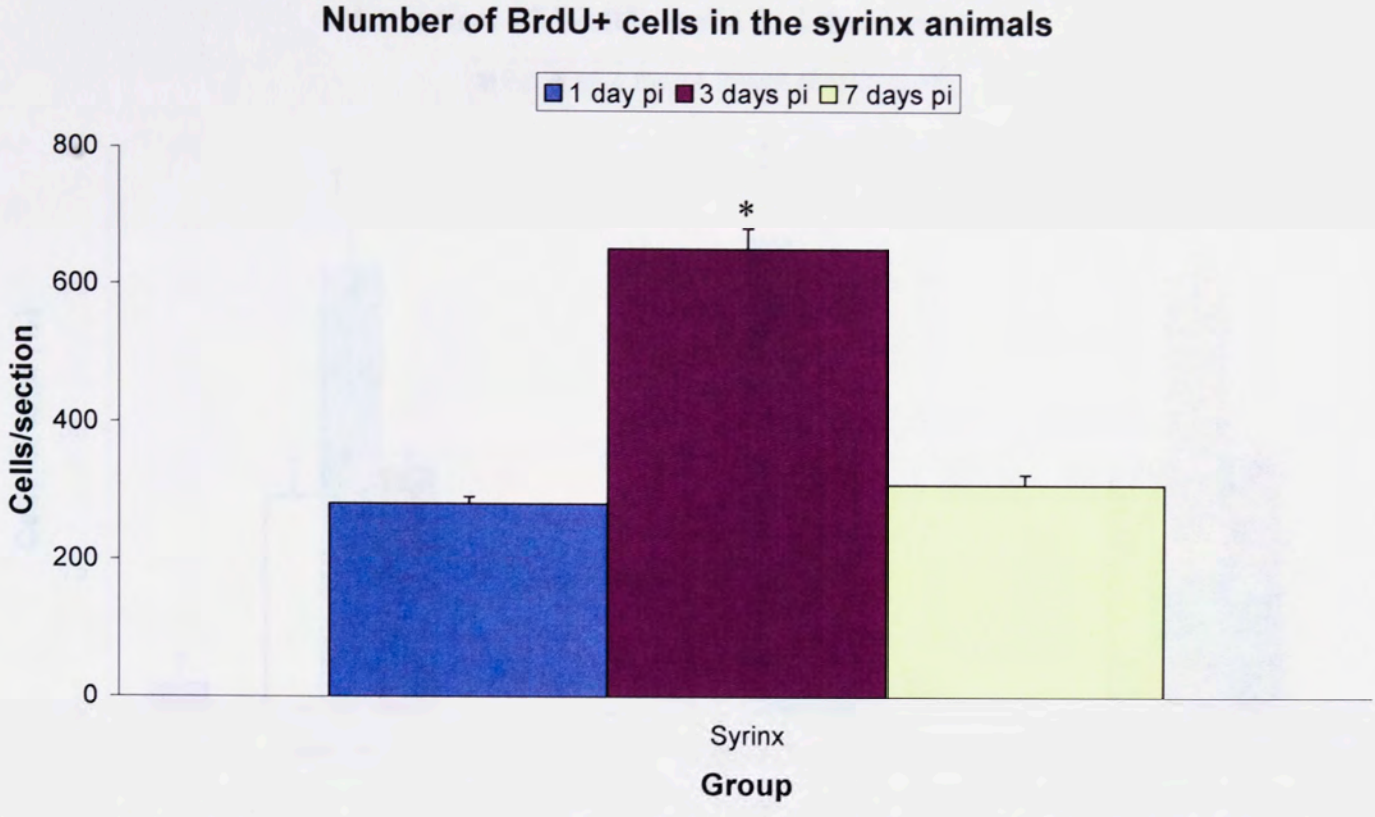

Figure 3-9. Number of BrdU+ cells in spinal cord sections (C8) in the syrinx animals. Data presented as mean ± SE (n=8). *p < 0.01 vs syrinx at 1 and 7 days PI. The doubling time of BrdU+ cells in the syrinx animals is within 2 days.

3.3.4 Location

3.3.4.1 Template one

In the intact and sham-operated animals, the location of BrdU-labeled cells was consistent up to 7 days following BrdU administration (Figure 3-10 and 3-11). Half of proliferating cells were identified in the WM, around one quarter in the SPZ and another quarter in the GM. Only one BrdU positive cell was found in the EZ at 1 day PI from 48 slides. No BrdU positive cell was found in the SEZ and PZ at any time point.

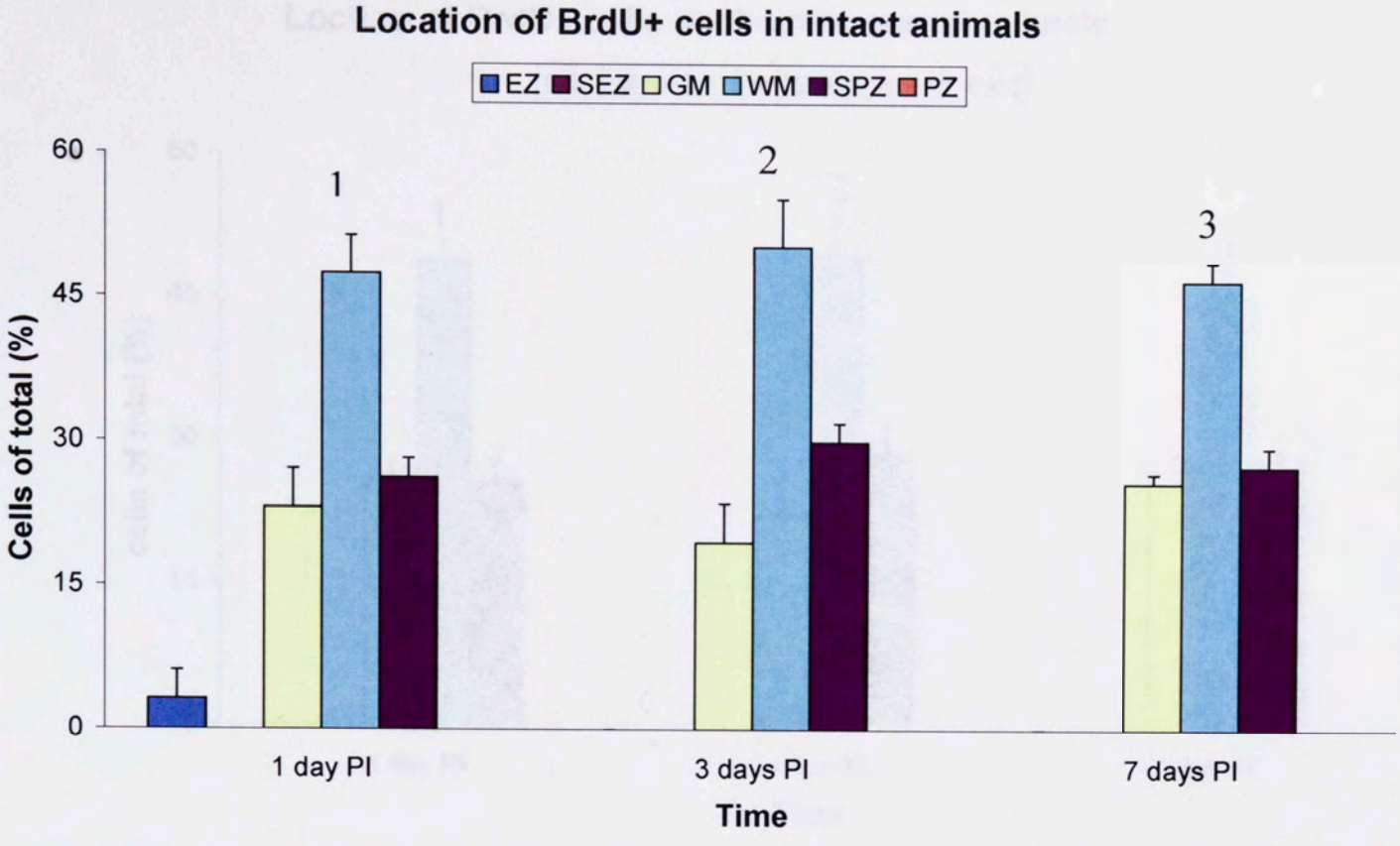

Figure 3-10. Percentages of BrdU+ cells (of total) in different regions of the spinal cord sections (C8) in the intact animals. Data presented as mean ± SE (n=8). EZ: ependymal zone; SEZ: subependymal zone; GM: gray matter; WM: white matter; SPZ: subpial zone; PZ: pial zone. [1] $P < 0.01$ vs EZ, SEZ and PZ at 1 day PI; [1] $P < 0.05$ vs GM at 1 day PI; [2] $P < 0.01$ vs EZ, SEZ and PZ at 3 day PI; [2] $P < 0.05$ vs GM at 3 days PI; [3] $P < 0.01$ vs EZ, SEZ and PZ at 7 days PI. Almost all the BrdU+ cells are in the GM, WM and SPZ in the intact animals.

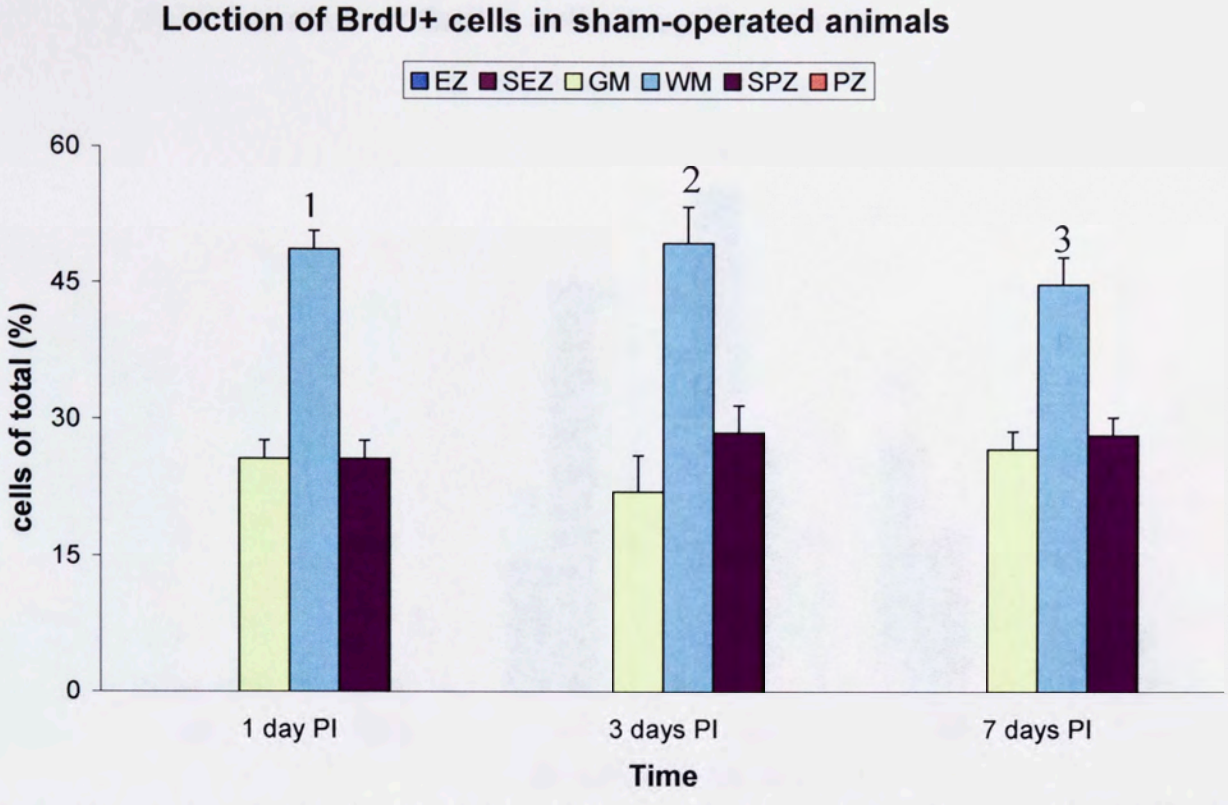

Figure 3-11. Percentages of BrdU+ cells (of total) in different regions of the spinal cord (C8) in the sham-operated animals. Data presented as mean ± SE (n=8). EZ: ependymal zone; SEZ: subependymal zone; GM: gray matter; WM: white matter; SPZ: subpial zone; PZ: pial zone. [1] $P < 0.01$ vs EZ, SEZ and PZ at 1 day PI; [1] $P < 0.05$ vs GM and SPZ at 1 day PI; [2] $P < 0.01$ vs EZ, SEZ and PZ at 3 days PI; [2] $P < 0.05$ vs GM at 3 days PI; [3] $P < 0.01$ vs EZ, SEZ and PZ at 7 days PI; [3] $P < 0.05$ vs GM at 7 days PI. All the BrdU+ cells are in the GM, WM and SPZ.

In the syrinx animals, a higher proportion of BrdU positive cells initially appeared in the white matter and subpial zone. In contrast, most cells were in the grey matter 2 days later (Figure 3-12). A 38% decrease in the percentage of BrdU-labeled cells represented a 13.5% reduction in the number of dividing cells in the white matter and subpial zone during the same period. A 30% rise in the percentage of BrdU positive cells exhibited a 132% increase in the population of progenitors in the grey matter during the same period. The location pattern of dividing cells remained relatively similar between 3 and 7 days post-syrinx induction.

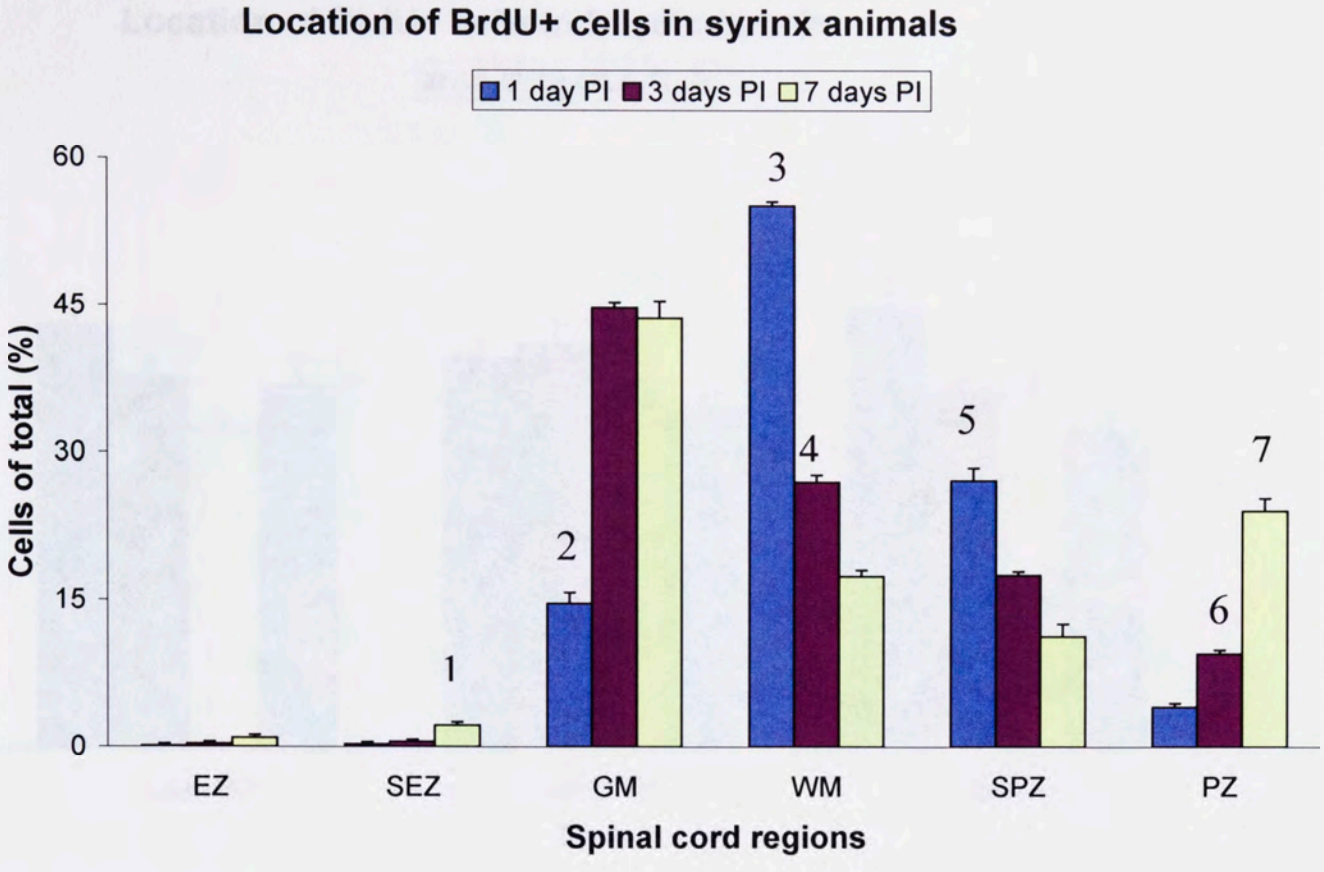

Figure 3-12. Percentages of BrdU+ cells (of total) in different regions of the spinal cord (C8) in the syrinx animals. Data presented as mean ± SE (n=8). EZ: ependymal zone; SEZ: subependymal zone; GM: gray matter; WM: white matter; SPZ: subpial zone; PZ: pial zone. [1] P < 0.01 vs SEZ at 1and 3 days PI; [2] P < 0.01 vs GM at 3 and 7 days PI; [3]P < 0.01 vs WM at 3and 7 days PI; [4] P < 0.01 vs WM at 1 and 7 days PI; [5] P < 0.01 vs SPZ at 7 days PI; [6] P < 0.01 vs PZ at 1 and 7 days PI; [7] P < 0.01 vs PZ at 1 and 3 days PI. The largest BrdU+ cell population was in the WM at 1 day PI, while it appeared in the GM at 3 and 7 days PI.

3.3.4.2 Template two

In the intact and sham-operated animals, BrdU+ cells were distributed equally in the four quadrants. This pattern remained consistent for 7 days following BrdU injection (Figure 3-13 and 3-14).

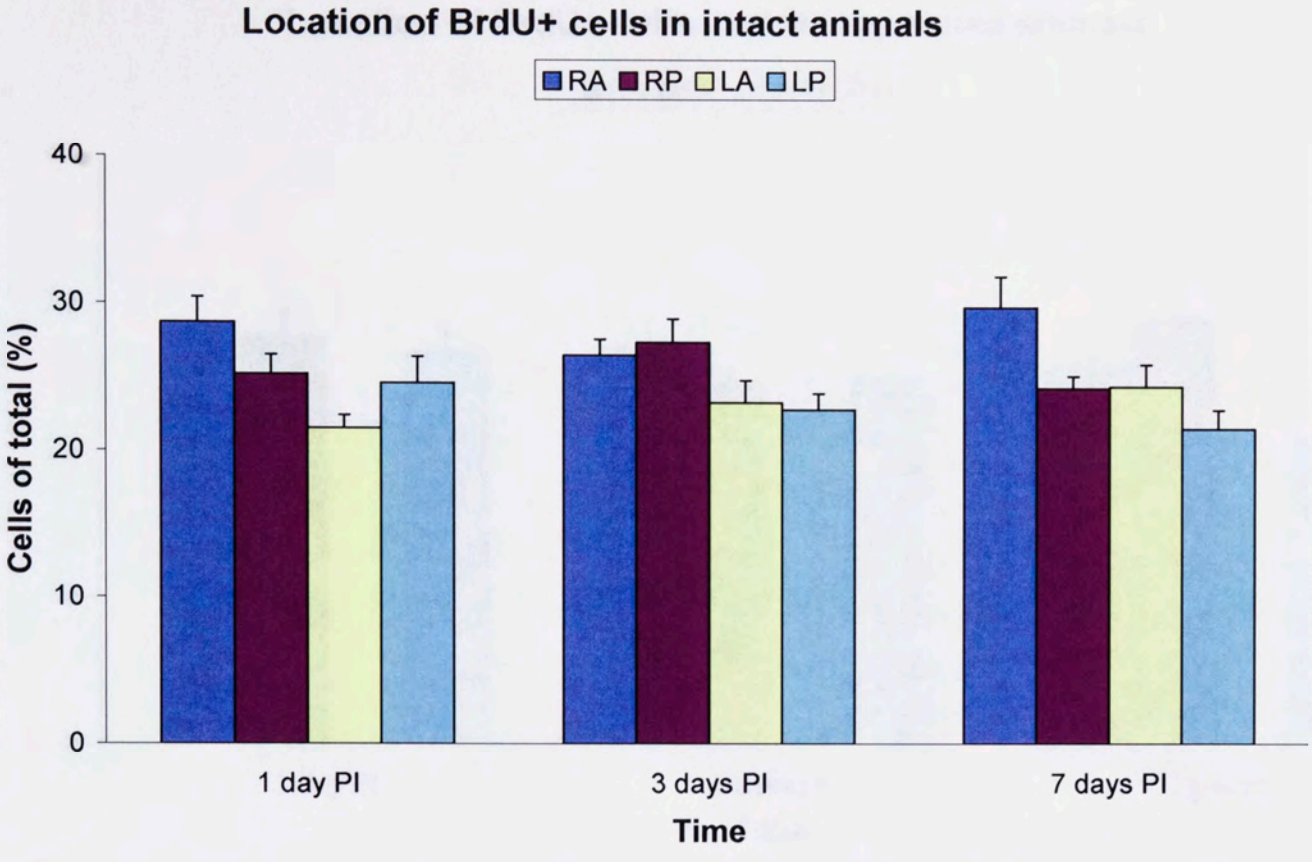

Figure 3-13. Percentages of BrdU+ cells (of total) in different regions of the spinal cord (C8) in the intact animals. Data presented as mean ± SE (n=8). RA: right anterior area; RP: right posterior area; LA: left anterior area; LP: left posterior area. The BrdU+ cells are located equally in the 4 regions.

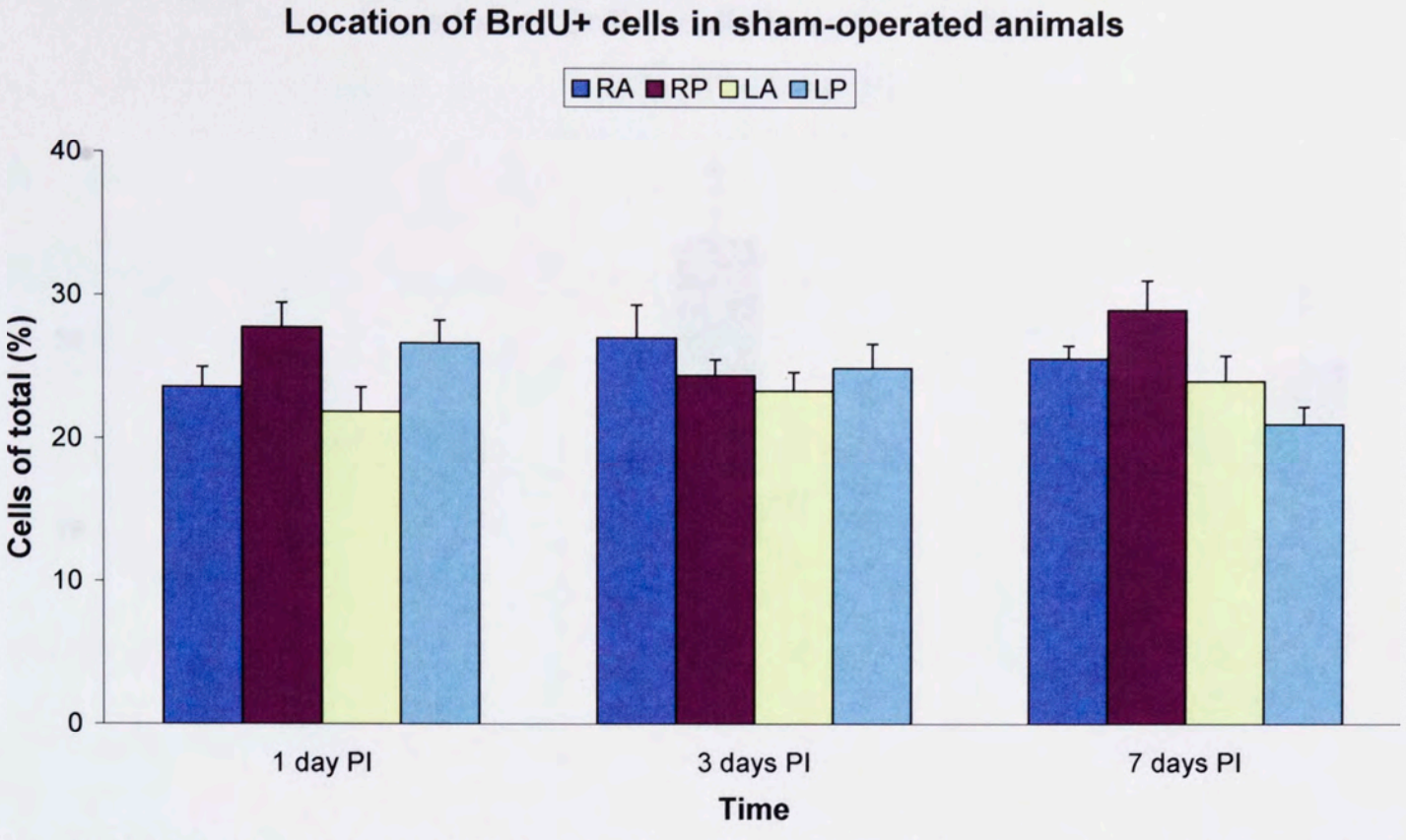

Figure 3-14. Percentages of BrdU+ cells (of total) in different regions of the spinal cord (C8) in the sham-operated animals. Data presented as mean ± SE (n=8). RA: right anterior area; RP: right posterior area; LA: left anterior area; LP: left posterior area. The BrdU+ cells are located equally in the 4 regions.

In the syrinx animals, there was 5% more dividing cells in the right posterior sector than any of other 3 sectors at 1 day post-injury (Figure 3-15). Two days later, a 9% rise in the percentage of BrdU positive cells represented a 204% increase in the population of progenitors in the right posterior sector. Seven days post-injury, the pattern of the location of dividing cells was similar to that at 1 day post-injury.

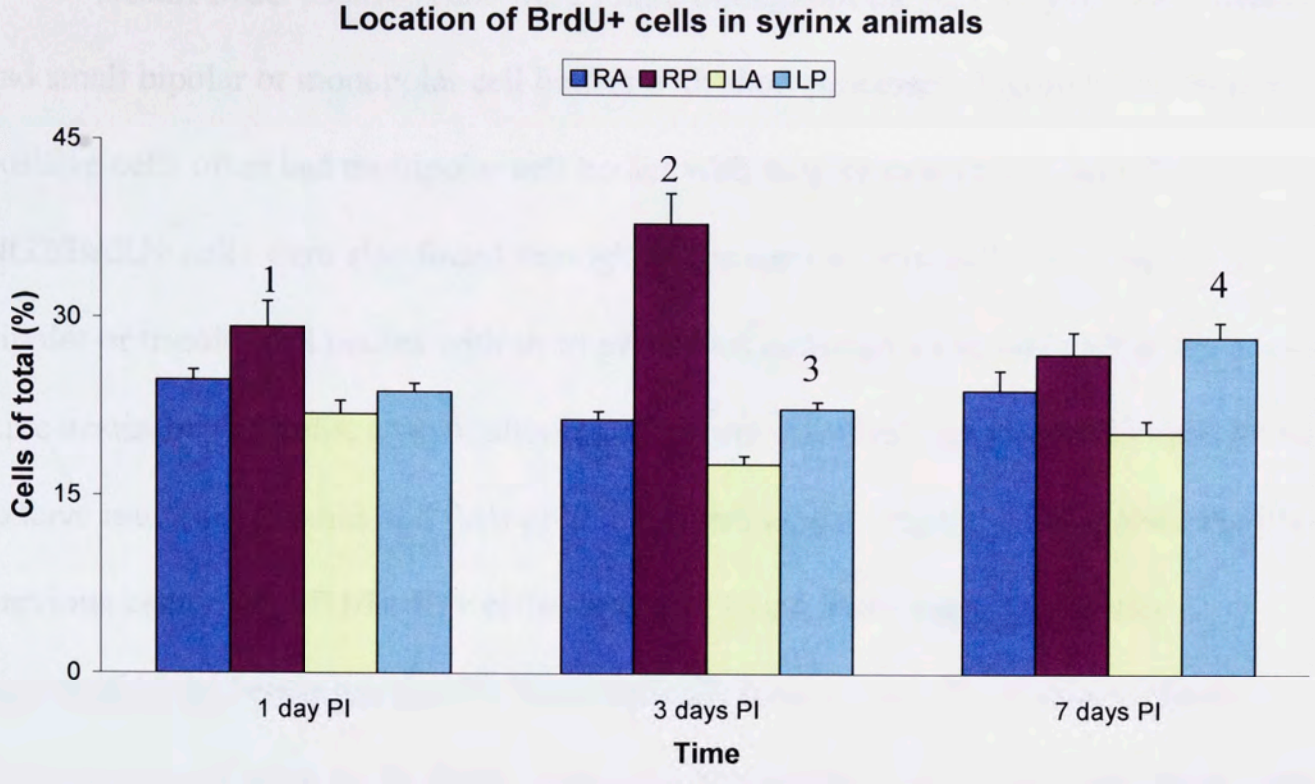

Figure 3-15. Percentages of BrdU+ cells (of total) in different regions of the spinal cord (C8) in the syrinx animals. Data presented as mean ± SE (n=8). RA: right anterior area; RP: right posterior area; LA: left anterior area; LP: left posterior area. [1] $P < 0.01$ vs LA and LP at 1 day PI; [2] $P < 0.05$ vs RA, LA and LP at 3 days PI, vs RP at 7 days PI; [2] $P < 0.01$ vs RP at 1 day PI; [3] $P < 0.05$ vs LA at 3 days PI; [4] $P < 0.05$ vs LA at 7 days PI. RA has slightly more BrdU+ cells than other regions at 1 day PI, while the difference was significant at 3 days PI. There was no significant difference in the percentages of BrdU+ cells between RA and other regions at 7 days PI. The proportion of BrdU+ cells in RA increased from 1 to 3 days PI, but decreased after that.

3.3.5 Phenotypes

Neither syrinx animals nor intact and sham-operated groups had BrdU+ cells expressing NCAM, β-tubulin, NeuN (neuronal markers) or MBP (oligodendrocyte marker). On the other hand, expression of nestin (NSCs marker), NG2 (glial progenitor marker), GFAP (astrocyte marker) and ED1 (macrophage marker) were found in the BrdU+ cells in all three animal groups.

Nestin/BrdU positive cells were found throughout the parenchyma. They usually had small bipolar or monopolar cell bodies with short processes (Figure 3-16). Nestin positive cells often had multipolar cell bodies with long processes at 7 days PI. NG2/BrdU+ cells were also found throughout the section. Typically, they had small bipolar or tripolar cell bodies with short processes, although some had multipolar bodies. Like nestin/BrdU+ cells, a week after injury, more NG2/BrdU positive cells were found to have multipolar bodies and their processes were longer (Figure 3-17). Unlike the two previous cell types, ED1/BrdU+ cells were only found in the right grey matter surrounding the lesion one day PI. They typically had big round cell bodies with no or few processes (Figure 3-18). BrdU+ cells also co-expressed GFAP and they usually had a small cell body with multiple long thin processes. However, some GFAP/BrdU+ cells had few short but thick processes, a typical morphological feature of reactive astrocytes (Figure 3-19).

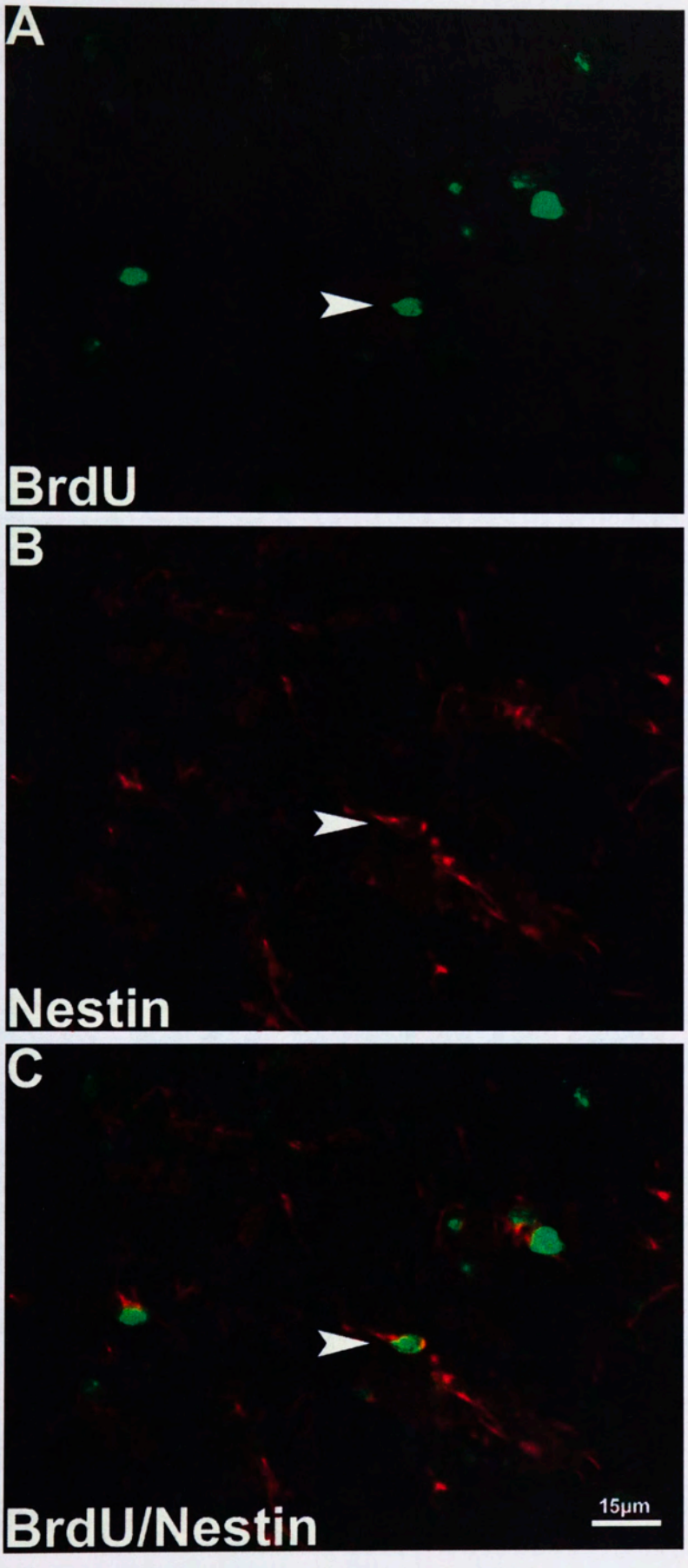

Figure 3-16. The immunofluorescent images were taken from spinal cord section (C8) in a syrinx animal at 3 days PI. A: BrdU single staining; B: nestin single staining; C: double staining with BrdU and nestin. Arrow head shows a BrdU/nestin+ cell with a small monopolar cell body with a short process.

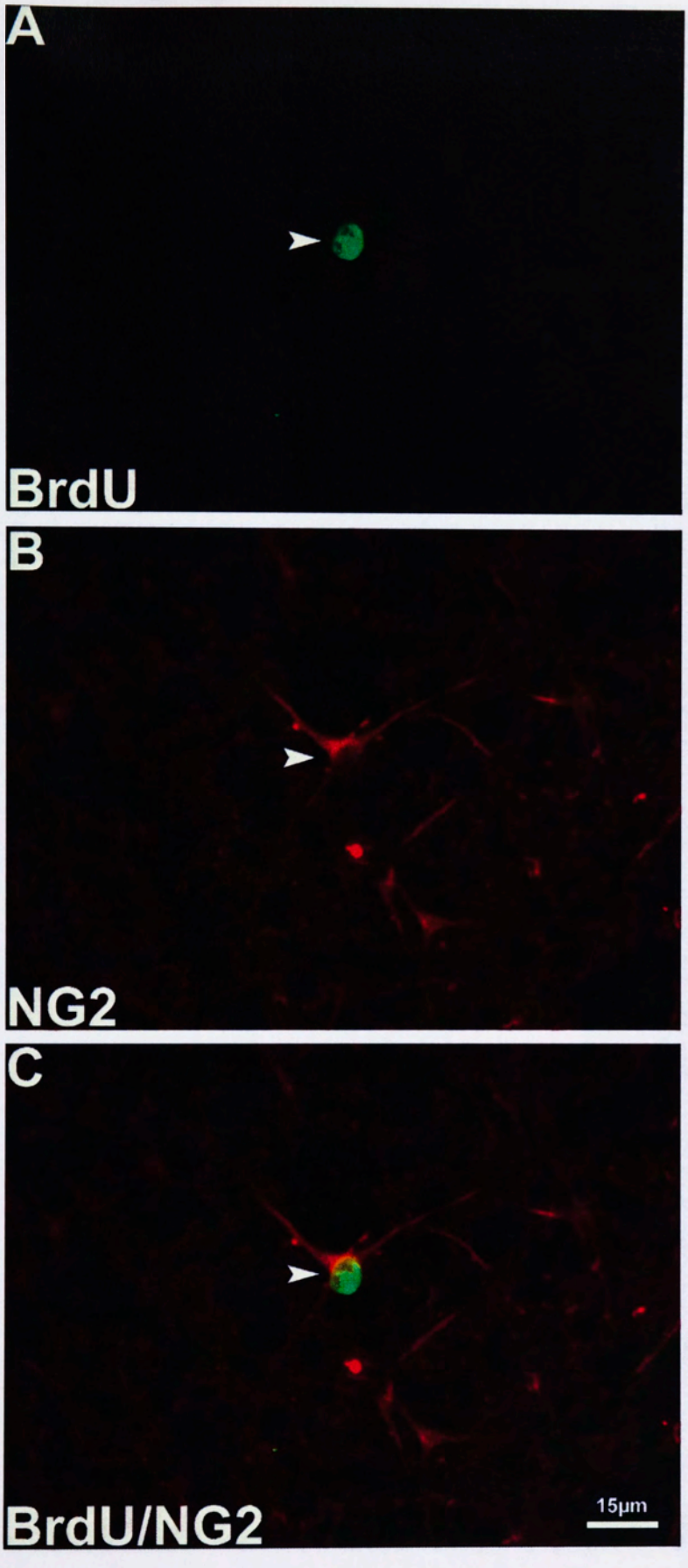

Figure 3-17. The immunofluorescent images were taken from spinal cord section (C8) in a syrinx animal at 3 days PI. A: BrdU single staining; B: NG2 single staining; C: double staining with BrdU and NG2. Arrow head shows a BrdU/NG2+ cell with a small tripolar cell body with long processes.

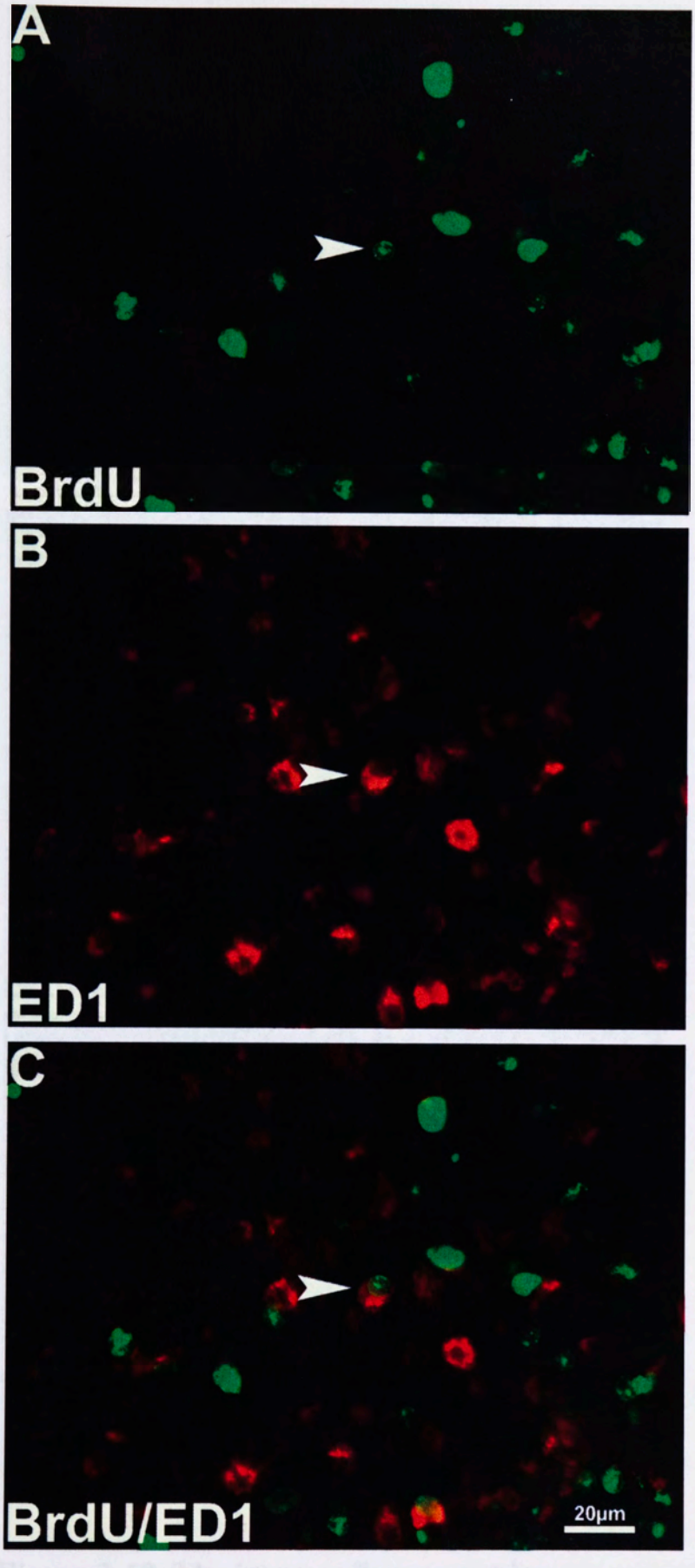

Figure 3-18. The immunofluorescent images were taken from spinal cord section (C8) in a syrinx animal at 3 days PI. A: BrdU single staining; B: ED1 single staining; C: double staining with BrdU and ED1. Arrow head shows a BrdU/ED1+ cell with a round cell body without process.

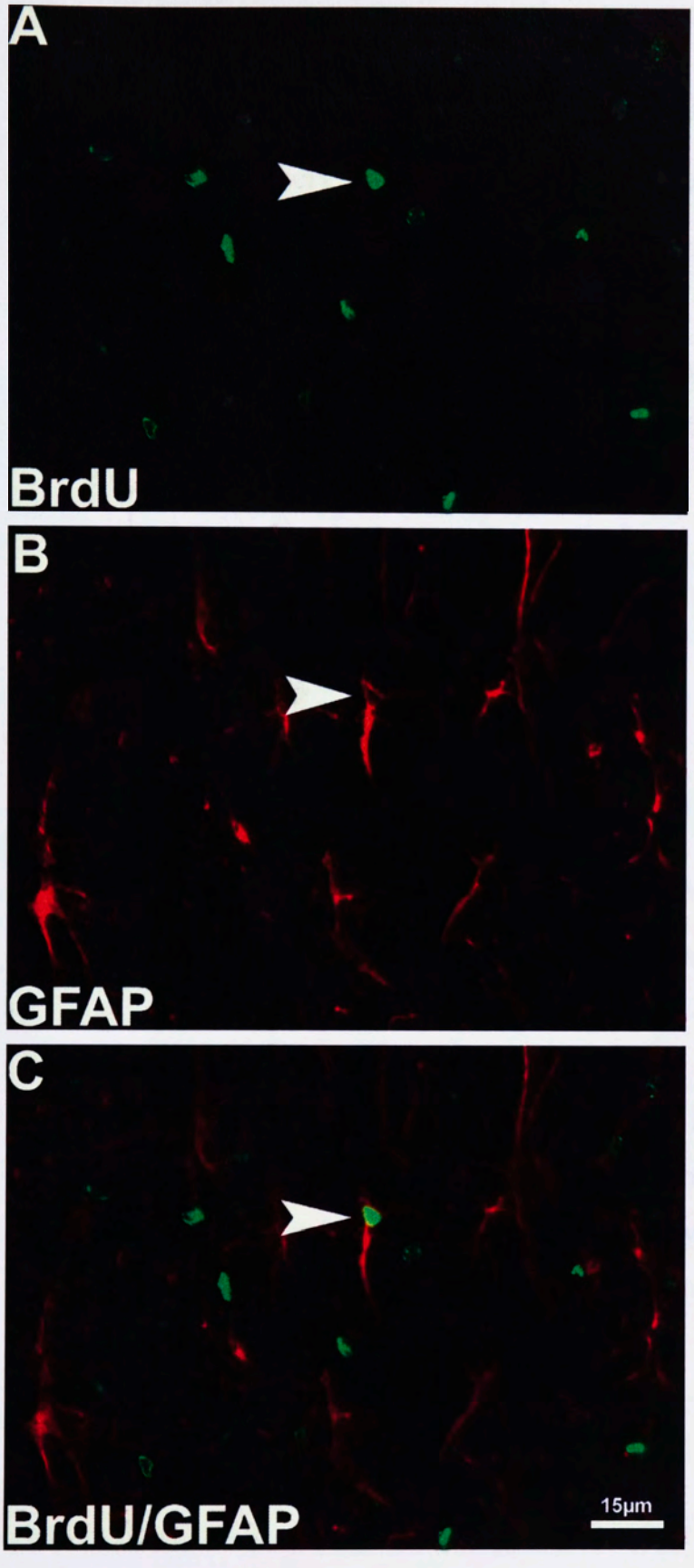

Figure 3-19. The immunofluorescent images were taken from spinal cord section (C8) in a syrinx animal at 3 days PI. A: BrdU single staining; B: GFAP single staining; C: double staining with BrdU and GFAP. Arrow head shows a BrdU/GFAP+ cell with a very small cell body and few processes. Its processes are shorter and thicker than those of typical astrocytes.

In the intact and sham-operated animals, around two thirds of the BrdU+ cells were NG2 positive, while about 6% of them were nestin and GFAP positive respectively. Only very few (around 1%) proliferating cells expressed ED1. These percentages did not change significantly with time (Figure 3-20 and 3-21).

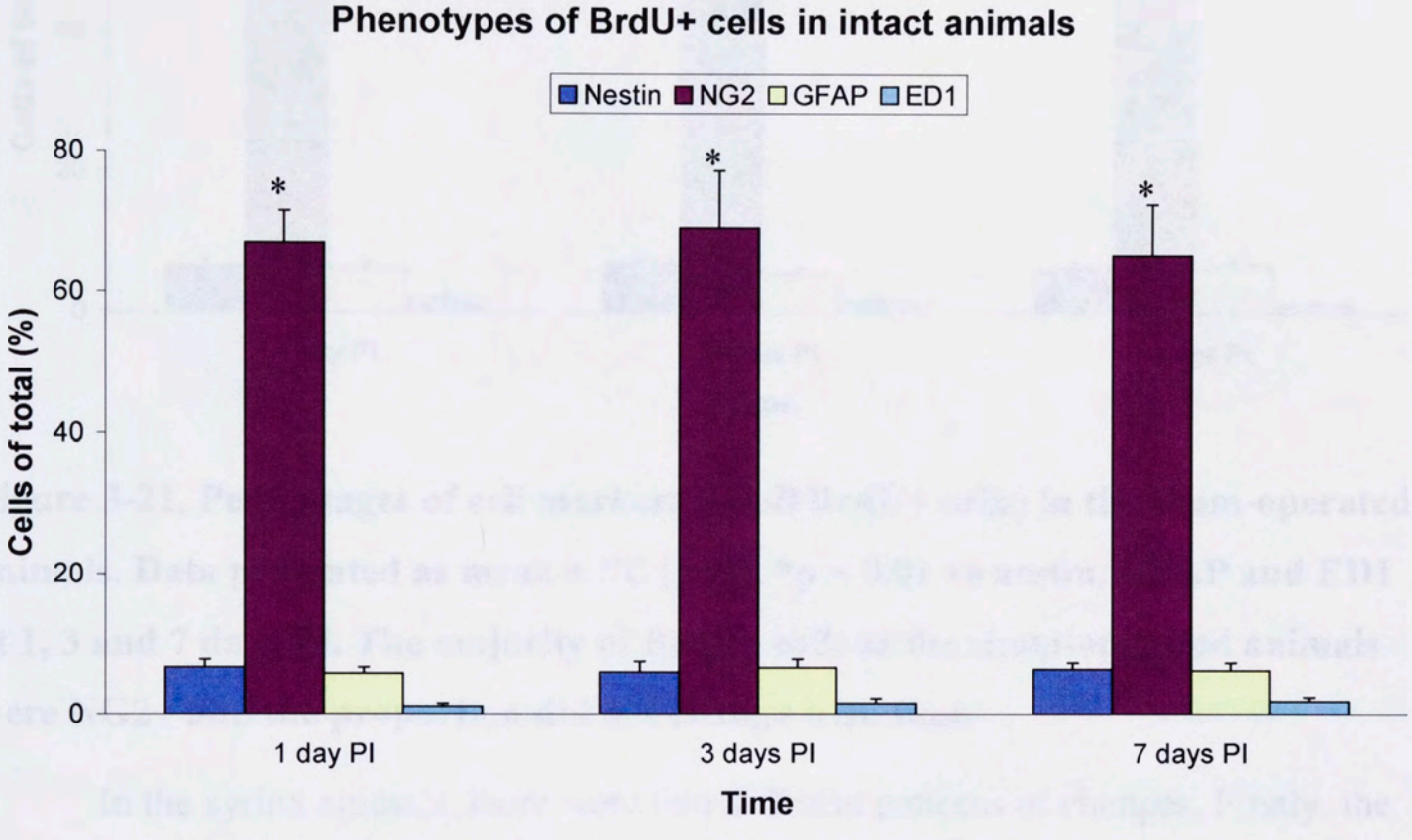

Figure 3-20. Percentages of cell markers (of all BrdU+ cells) in the intact animals. Data presented as mean ± SE (n=4). *p < 0.01 vs nestin, GFAP and ED1 at 1, 3 and 7 days PI. The majority of BrdU+ cells in the intact animals were NG2+ and the proportion did not change with time.

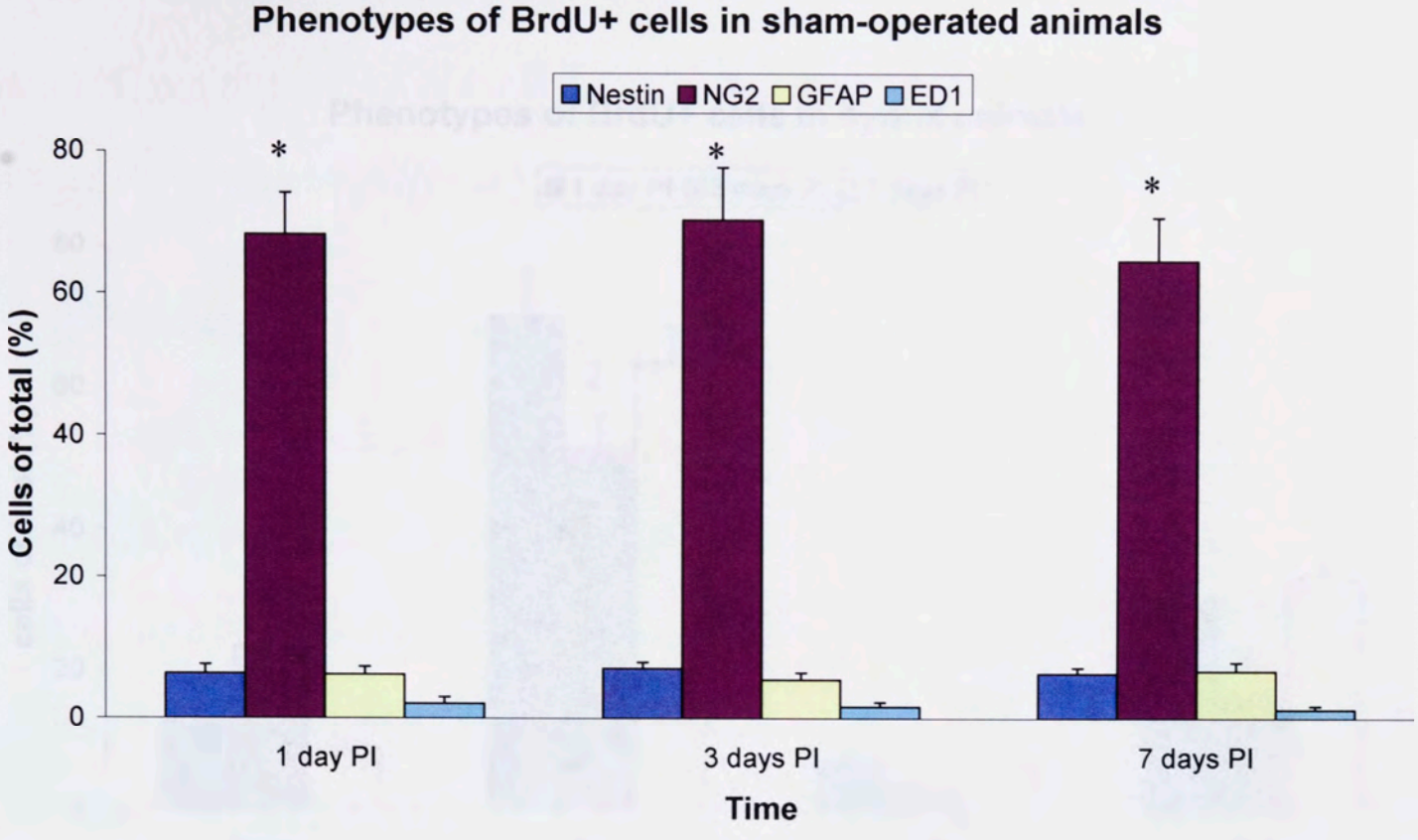

Figure 3-21. Percentages of cell markers (of all BrdU+ cells) in the sham-operated animals. Data presented as mean ± SE (n=4). *p < 0.01 vs nestin, GFAP and ED1 at 1, 3 and 7 days PI. The majority of BrdU+ cells in the sham-operated animals were NG2+ and the proportion did not change with time.

In the syrinx animals, there were two different patterns of changes. Firstly, the percentage of Nestin/BrdU+ cells doubled in the first 3 days then dropped to less than 9% at 1 week after injury. Secondly, the proportion of NG2/BrdU, GFAP/BrdU and ED1/BrdU positive cells decreased by various levels from 1 day PI to 3 days PI, then they increased at 7 days PI. Overall, NG2/BrdU+ cells were the dominant cell type at any time point, while GFAP/BrdU+ cells were the least common (Figure 3-22).

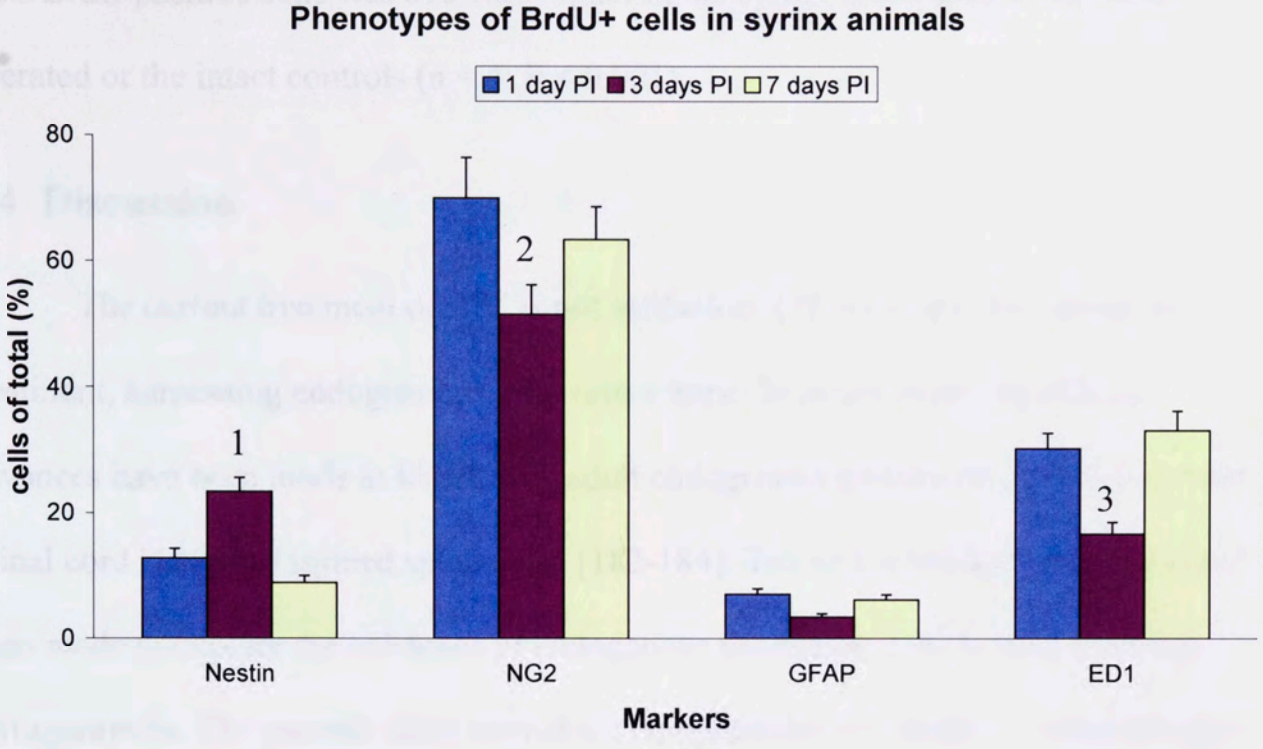

Figure 3-22. Percentages of cell markers (of all BrdU+ cells) in the syrinx animals. Data presented as mean ± SE (n=4). [1] P < 0.05 vs nestin at 1 and 7 days PI; [2] P < 0.05 vs NG2 at 1 day PI; [3] P < 0.05 vs ED1 at 1 and 7 days PI. The percentage of Nestin/BrdU+ cells increased from 1 day PI to 3 days PI and then decreased at 7 days PI. A reverse change was found in the proportions of NG2/BrdU+ and ED1/BrdU+ cells.

Using labeling method two at 14 days post-syrinx induction, we detected 7% nestin/BrdU positive cells in both the intact and sham-operated controls, and 22% in syrinx rats. The number of nestin/BrdU positive cells was 364-fold greater in syrinx rats than in the intact and sham-operated controls ($n = 4$; $P < 0.001$). The percentage of NG2/BrdU co-expression cells was similar in all groups, being 70%, 68%, and 65% in the intact, sham-operated, and syrinx animals, respectively. The number of NG2/BrdU positive cells was 104-fold more in syrinx rats than in the intact and sham-operated controls ($n = 4$; $p < 0.001$). A 6-7% GFAP/BrdU co-expression was observed in all groups. There was a 1-2% co-localization of ED1 and BrdU positive cells in the sham-

operated or the intact controls, but 26% was seen in syrinx animals. The number of ED1/BrdU positive cells was 895-fold higher in the syrinx group than in the sham-operated or the intact controls ($n = 4$; $P < 0.001$).

3.4 **Discussion**

The current treatment of PTS is not satifactory [1]. As a possible option of treatment, harnessing endogenous NSCs raises hope. In recent years, significant advances have been made in identifying adult endogenous progenitor cells in the intact spinal cord [186] and injured spinal cord [182-184]. To our knowledge, little effort has been made to explore the existence of endogenous progenitor cells in post-traumatic syringomyelia. The present study provides a comprehensive evaluation of proliferation, distribution patterns, and the degree of differentiation of mitotically active cells at the acute stage of post-traumatic syringomyelia.

It is worth mentioning here that a rat model of PTS was developed from the previous studies in our group [25, 54]. It mimics the pathological changes of PTS in human beings. In this study, 100% of animals in the syrinx group developed syrinxes in the spinal cord, while no rats had a cyst in the intact and sham-operated groups. It provides further evidence that our PTS model is reproducible and reliable.

The mitotic cells were labelled by BrdU in this study. BrdU only marked the cells that divided within a few hours after the injection. Thus the labelling method one can "lock" certain mitotic cells (BrdU+) and follow them in terms of their number, location and phenotype. The number of dividing cells labelled by this method is a relative indication since the number of BrdU positive cells could be affected by the bioavailability of BrdU that is gradually diluted as BrdU-incorporating cells continue to

divide. To clear this obvious concern, labelling method two, in which BrdU is available in abundance, was used as a reference.

3.4.1 Proliferation of NPCs in the PTS model

Proliferation of NPCs has been reported in injured and intact spinal cord [180, 181, 186, 321]. Immediately following the spinal cord injury there is a release of excitatory amino acids, including glutamate, at the site of injury, which triggers cellular damage [50, 322, 323]. In this rat model of post-traumatic syringomyelia, microinjection of quisqualic acid that is an agonist of ionotropic and group I metabotropic glutamate receptors, into the spinal cord grey matter can induce selective inflammation and neuronal death [25-27, 324]. Here, we show that such stimulation is sufficient for quiescent cells to divide in an adult model of post-traumatic syringomyelia. The majority of these cells incorporate BrdU and coincidentlly express NG2 (Figure 3-22), indicating that they are progenitors.

The *in vivo* division rate of the adult spinal cord progenitors is thought to be low [82] and Horner and colleagues reported that the doubling time of progenitors in adult rat spinal cord is 5 to 7 days [186]. Recent studies show such a low mitosis of the adult spinal cord progenitors can be increased after injury [179, 183]. The present work is in agreement with these reports supporting the concept that adult endogenous progenitors are responsive to injury with a capacity of proliferation. Using labelling method 1, we observed the number of proliferating cells increased 76 times in the first 3 days after trauma excluding inflammatory cells and astrocytes (Figures 3-4 and 3-22). Our results estimate that the doubling time is 2 days rather than 6 days, which is the cell-doubling time required for the normal spinal cord progenitors, suggesting a shorter cell cycle length during the formation of an initial cyst.

As mentioned above, the number of dividing cells labelled by labelling method one is affected by the bioavailability of BrdU that is gradually diluted as BrdU-incorporating cells continue to divide. To clear this obvious concern, labelling method two, in which BrdU is available in abundance, was used as a reference. Comparison of the results from these two methods reveals that the magnitude of the number of progenitors increased using labelling method one is similar to the magnitude using labelling method two.

3.4.2 Location of NPCs in the PTS model

The origin of NPCs in the adult spinal cord is not clear. Two models were hypothesized to explain the origin of the progenitors in the intact adult spinal cord. In the first one, progenitors may reside throughout the spinal cord, including the ependymal layer and parenchyma. In the second model, progenitors probably originate from the ependymal zone and their progeny migrate to the parenchyma [186]. In the injured adult spinal cord, apart from the ependymal zone [183, 184, 325] and parenchyma [180, 181, 321], the subpial zone was also regarded as a stem cell pool [185].

Our temporal profile of progenitor location reveals that the majority of proliferating cells appeared in the white matter and subpial zone initially prior to a shift of cell division in the grey matter (Figure 3-12). The shifting percentage of progenitors in the white matter between 1 and 3 days post-syrinx induction does not reflect in the number of BrdU positive cells in the sector, suggesting no significant migration of endogenous progenitors from the white matter to the grey matter. A likely explanaition for this observation is a greater activity of cell division in the grey matter on and after 3 days post-trauma. This explanaition is indirectly supported by the observation using the

second template, which shows a higher proportion of progenitors at 3 days post-syrinx induction in the right posterior where syrinxes are located (Figure 3-15).

One reason for the discrepancy of the original location of progenitors between previous reports and ours is possibly due to the different injury models used. If a compression injury model was used [182, 183, 185], the mechanical external forces might cause more severe tissue damage in the white matter than that in the grey matter. If it is the case, progenitor cells tend to show stronger responses near the white matter. It is also possible that mechanical external forces used by others [182, 183, 185], induced responses of progenitors that are different from that excitatory amino acid as used by us. Another explanation is that the responses of progenitors were observed at different stages post-traumatic injury. If one observed progenitor expression from the primary injury of tissue destruction and necrosis, and the other saw its responses from the secondary injury that produces subsequent enlargement of syrinx, associated variation between the two is obvious. In the current study, time- and area-dependent alterations of immunoreactivities of progenitor markers were analyzed for a period of 14 days post-trauma that represents the acute stage of post-traumatic syringomyelia development. Strikingly, there is an emerging population of progenitors from the pial zone (Figure 3-12), suggesting that there is a secondary stem cell pool to meet the increasing demand for progenitor cells.

3.4.3 Phenotypes of NPCs in the PTS model

Various markers have been identified for neural progenitors including neural multipotential cells, and nestin is one such marker [108]. Nestin is an intermediate filament protein found in stem cells in the neuroepithelial layer lining the central canal during development of the spinal cord, which becomes progressively reduced in the

ependymal layer as development proceeds [115]. Subsequently, nestin immunoreactivity disappears by day 6 postnatally in the rat spinal cord and is not detectable in the ependyma in normal adult rats [326]. However, nestin is re-expressed in ependymal cells after spinal cord injury [185]. In our experimental condition, nestin expression was detected at a low level in the intact and sham-operated adult spinal cord but the level tripled by 3 days post-syrinx induction (Figures 3-20, 3-21 and 3-22). Re-expression of immature intermediate filament nestin originally offered a ray of hope that multipotential progenitors can differentiate into both neurons and glial cells post-trauma. Our further examination shows that there was no expression of neuronal markers including beta-tubulin III, NCAM, NeuN or oligodendrocyte marker such as MBP in the spinal cord of normal, sham-operated, or syrinx rats. This is possibly due to the inflammatory microenvironment that inhibits differentiation from progenitors, in which there is the lack of specific neurotrophic factors to influence the differentiative potential of the progenitor cells. A recent study of transplantation of neural progenitor cells to repair CNS injury has provided evidence that the microenvironment is unsuitable for graft survival at the acute stage of injury [270]. An intriguing and important question raised by our study concerns the control of differentiation of progenitors post-trauma. This may be due to the lack of availability of growth factors or of receptors. An alternative possibility is that growth inhibitors may be responsible for the relative quiescent differentiation of progenitors *in vivo*. Although this may lead to a slow renewal of neurons, it is obvious that progenitors have a markedly proliferative potential.

At the acute stage post-trauma, it was originally thought that trauma-induced CNS inflammation is dominated by hematogenous macrophages recruited from blood and CSF [317, 318]. Our results suggest that ED1/BrdU labelled macrophages (Figure

3-18) may be an active population of macrophages is the progeny of *in situ* proliferating cells. The morphology of the former is basically ameboid that is distinct from the latter qualified as ramified. Recent reports [186] and our results (Figures 3-12 and 3-15) indicate that progenitors do not migrate, suggesting that local factors induce progenitors to differentiate into tissue type macrophages *in situ*. The kinetics of ^{3}H-thymidine labeling supports the view that tissue macrophages are maintained by a self-renewal process that is independent of bone marrow-derived cells [327, 328]. The ED1/BrdU labelled macrophages were also identified in the intact spinal cord (Figure 3-20) where blood-spine barrier is complete. Therefore, circulating macrophages do not cross vessel walls and thus do not penetrate into the spinal cord parenchyma [329, 330]. *In vitro* tissue culture has confirmed the differentiation of progenitors into tissue type macrophages [331, 332]. We propose that the fast differentiation of macrophage progenitors *in situ* is ideal for the initiation of primary immune responses in the lesion site. These tissue macrophages and the heterogeneous macrophages collectively contribute to immune surveillance at the acute stage of post-traumatic syringomyelia.

More importantly, macrophages may also contribute to the proliferation and neuronal differentiation of endogenous neural progenitors. Cytokine and chemokine expressed by macrophages and other inflammatory cell were reported to promote survival and proliferation of adult NSCs *in vivo* [238-240]. Furthermore, both *in vivo* and *in vitro* studies showed that macrophages combined with other inflammatory cells were found to promote neuronal differentiation of NSCs by expressing erythropoietin [223, 254, 333] and transforming growth factor-β (TGF-β) [257, 258, 334, 335]. This evidence, combined with our results suggests that macrophages may contribute to the regulation of NSCs in the early stage of PTS. It also implies a possible solution to

improve the microenviroment for endogenous NSCs by enhancing the expression of cytokines, chemokines, erythropoietin and TGF-β by macrophages.

3.5 **Conclusion**

The present study demonstrates proliferation, distribution, and differentiation of endogenous progenitor cells in an adult rat model at the acute stage of post-traumatic syringomyelia. These progenitors proliferate rapidly, and are mainly located in the grey matter where the syrinx is located. The main finding of this study is that an active population of macrophages is the result of *in situ* differention. Together with the heterogeneous macrophages, they contribute to immune surveillance and NSC regulation at the acute stage of post-traumatic syringomyelia.

Chapter Four Reaction of Endogenous Progenitors in the Chronic Stage of Experimental PTS

4.1 Introduction

In the previous chapter, the reaction of endogenous progenitors in the acute stage of experimental PTS was studied. However, PTS and its symptoms can appear a long period after the initial injury [4, 14, 62, 63], which makes it important to understand the reaction of the endogenous progenitors in the chronic stage of the PTS model.

BrdU was used to label the newly divided cells in the previous chapter and it enabled us to chase the further division of mitotic cells and their movement in a short period. However, BrdU is not suitable for the study of endogenous neural progenitors in the chronic stage, because the amount of BrdU will be too low to be detected due to the dilution after many divisions during a longer period. In this study, proliferating cells were marked by antibody against Ki67. Ki67 antigen is a nuclear protein expressed in all active phases of the cell cycle (G1, S, G2 and mitosis) but not in resting cells (G0) [336]. Thus Ki67 is ideal for marking all the mitotically active cells. Here, Ki67 staining makes it possible to study the proliferating cells at different time points after injury.

In the previous chapter, progenitors were found mainly in the white matter. Similar templates were used in this study to examine the position of proliferating cells, which possibly provide the comparison in the position of proliferating cells between acute and chronic stages.

Here, it is hypothesized that proliferating cells exist in the spinal cord at the chronic stage of PTS and keep mitotically active for a long term. Progenitors may appear around the syrinx and play a protective role in PTS. In this study, we aim to examine the existence of endogenous progenitors in the chronic stage of the PTS model and their reaction to injury, such as proliferation, distribution and phenotypes.

4.2 Materials and methods

4.2.1 Animal groups

Thirty-six male Wistar rats (10-12 weeks old and 200 – 380 g body weight) were used in this study. They were divided into three groups at three time points (Table 4-1). Animals in the intact group had no surgery and rats in the sham operated group had laminectomies only, while animals in the syrinx group had syrinx induction procedures. Sham-operated and syrinx animals were sacrificed 1, 2 or 8 weeks after the surgery.

Table 4-1 Animal groups

	1 week	2 weeks	4 weeks	8 weeks
Intact	4			
Sham-operated	4	4	4	4
Syrinx	4	4	4	4

4.2.2 Immunohistochemistry

Primary antibodies used in this study are shown in Table 4-2. The secondary antibodies were: ALEXIA Fluor 594 (goat anti mouse, 1:800) and ALEXIA Fluor 488 (goat anti-rabbit, 1:800). Details of the above antibodies are provided in General Methods.

Table 4-2 Primary antibodies

Primary antibodies	Type	Concentration
Anti-Ki67	Rabbit polyclonal	1:2000
Anti-nestin	Mouse monoclonal	1:4000
Anti-NG2	Mouse monoclonal	1:200
Anti-β-tubulin (III)	Mouse monoclonal	1:300
Anti-NCAM	Mouse monoclonal	1:1000
Anti-NeuN	Mouse monoclonal	1:200
Anti-MBP	Mouse monoclonal	1:50
Anti-GFAP	Mouse monoclonal	1:4000
Anti-ED1	Mouse monoclonal	1:400

4.2.3 Quantification of proliferating cells

In this study, proliferating cells were marked by antibody against Ki67. Ki67 positive cells at segment C8 were counted using a fluorescence microscope and digital camera. Two templates, which were similar to those used in the previous chapter, were used in this study to examine the position of proliferating cells. The first template divided the section into 2 parts, namely the grey matter (GM) and white matter (WM). In the second template (Figure 3-2), a cross centred at the central canal divided the coronal section into 4 parts, namely the right anterior area (RA), right posterior area (RP), left anterior area (LA) and left posterior area (LP). The lesion caused by syrinx induction procedure was mainly located in the RP.

4.2.4 Data analysis

Results were presented as mean ± SE. Differences among different groups were evaluated by one way ANOVA. Linear regressions were calculated by using the statistical computer package, Number Cruncher Statistical Systems [337]. $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Syrinx formation

Syrinx formation was observed as a cyst or a loose mesh of glial fibers. Syrinx size was expressed as a proportion of cross-section area (cyst size/cross-section x 100%). Syrinx size and area of spinal cord section were measured using AxionVersion 4.2 software (Zeiss, Germany). Animals in the experimental group developed a syrinx by 7 days after receiving injections of intraparenchymal quisqualic acid and subarachnoid kaolin. The average cyst size at 2 days was 2.15% of the cross-sectional area at the C8 level. Cyst size increased to 3.34% of the cross-section area at 14 days, and progressively enlarged up to 56 days (Figure 4-1). There was a positive correlation between cyst size and time ($r = 0.98$, $p < 0.01$). No control animal developed a syrinx.

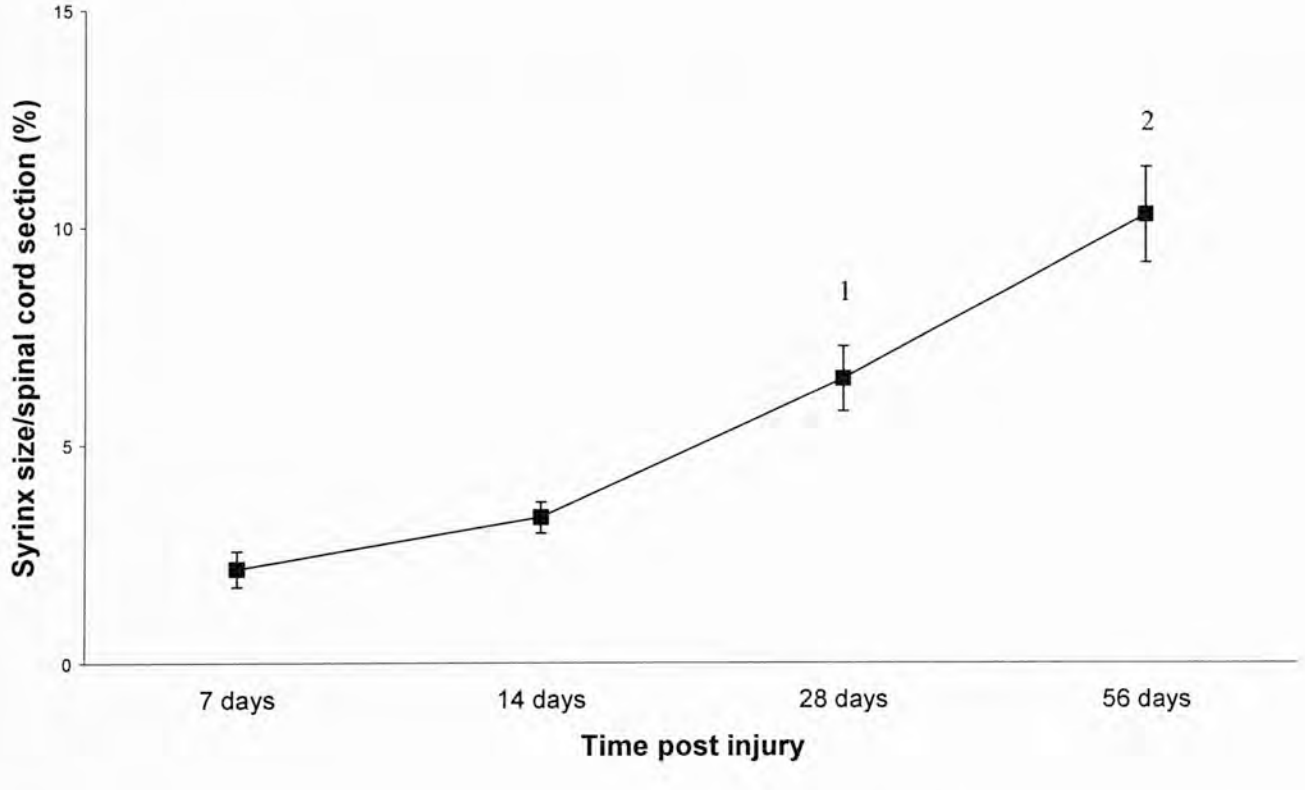

Figure 4-1. Correlation between syrinx size (at the level of C8) and time. Syrinx size expressed as % of cord cross sectional area and presented as mean ± SE (n=8). 1p < 0.05 vs 7, 14 and 56 days; 2p < 0.05 vs 7, 14 and 28 days.

4.3.2 Proliferating cells

Proliferating cells were Ki67 immunopositive. These cells were observed in syrinx animals with a few in the intact and sham-operated controls. Clusters of two Ki67 positive cells were identified in syrinx animals as evidence of dividing cells. The images of Ki67 positive cells are shown in Figures 4-2, 4-4, 4-5, 4-6 and 4-7. In syrinx animals, Ki67 positive cells were about 1,000 per section in the first 2 weeks post-syrinx induction, which was 34 times more than that in the intact and sham-operated controls (Figure 4-3). Two weeks later, this number had decreased by 60% and sustained up to 56 days post-trauma. In the intact and sham-operated controls, the number of Ki67 positive cells was consistent for a 56 day follow-up period. Because of tissue loss in syrinx animals, cell density is also estimated (Figure 4-8). Ki67 positive cells were 171 + 8 and 152 + 13/mm^2 at 7 and 14 days post-injury respectively, which decreased by 60% at 28 days post-injury and maintained at the same level up to 56 days post-syrinx induction.

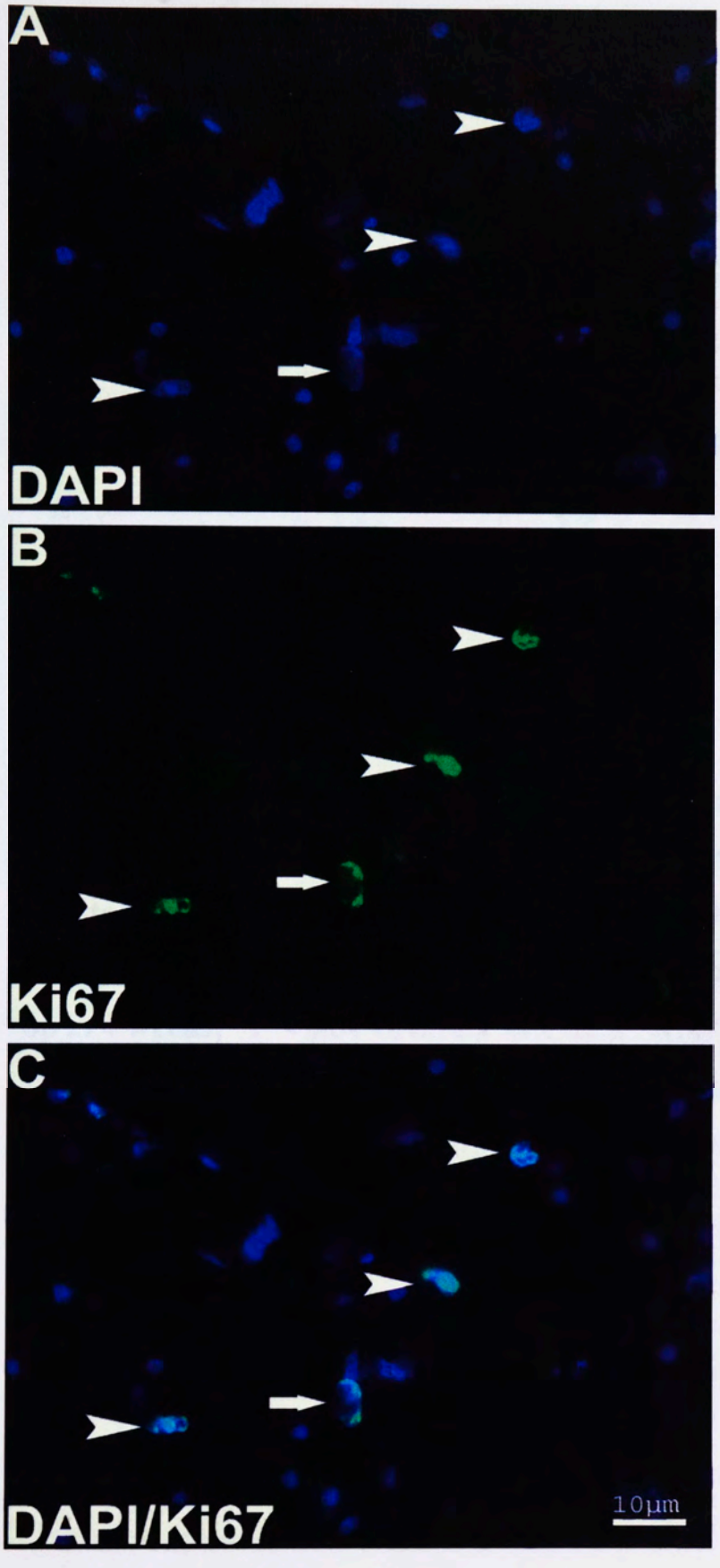

Figure 4-2. The immunofluorescent images were taken from the syrinx animals 1 week PI. A: DAPI single staining; B: BrdU single staining; C: DAPI and BrdU double staining. The arrow heads indicate that Ki67+ cells match with DAPI+ cells in terms of their position and shape. The arrow shows a proliferating cell undergoing division.

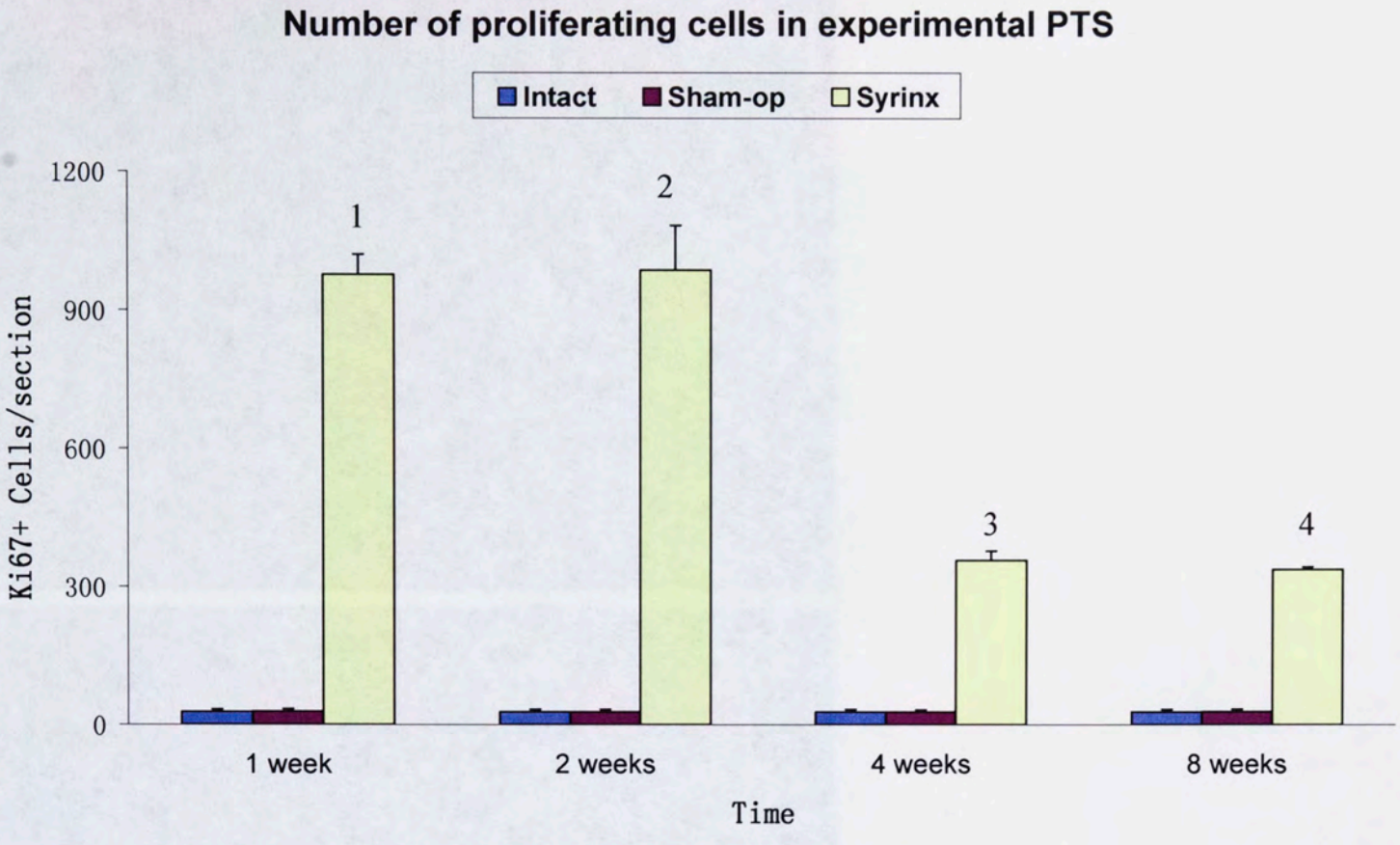

Figure 4-3. Number of Ki67+ cells in the spinal cord section (C8) in the intact, sham-operated and syrinx animals. Data presented as mean ± SE (n=8). [1] $P < 0.01$ vs intact and sham-op at 1 week, vs syrinx at 4 and 8 weeks; [2] $P < 0.01$ vs intact and sham-op at 2 weeks, vs syrinx at 4 and 8 weeks; [3] $P < 0.01$ vs intact and sham-op at 4 weeks; [4] $P < 0.01$ vs intact and sham-op at 8 weeks. More Ki67+ cells are found in the syrinx animals than the intact or sham-operated animals. The numbers of proliferating cells in syrinx animals are higher at 1 and 2 weeks PI than 4 and 8 weeks PI.

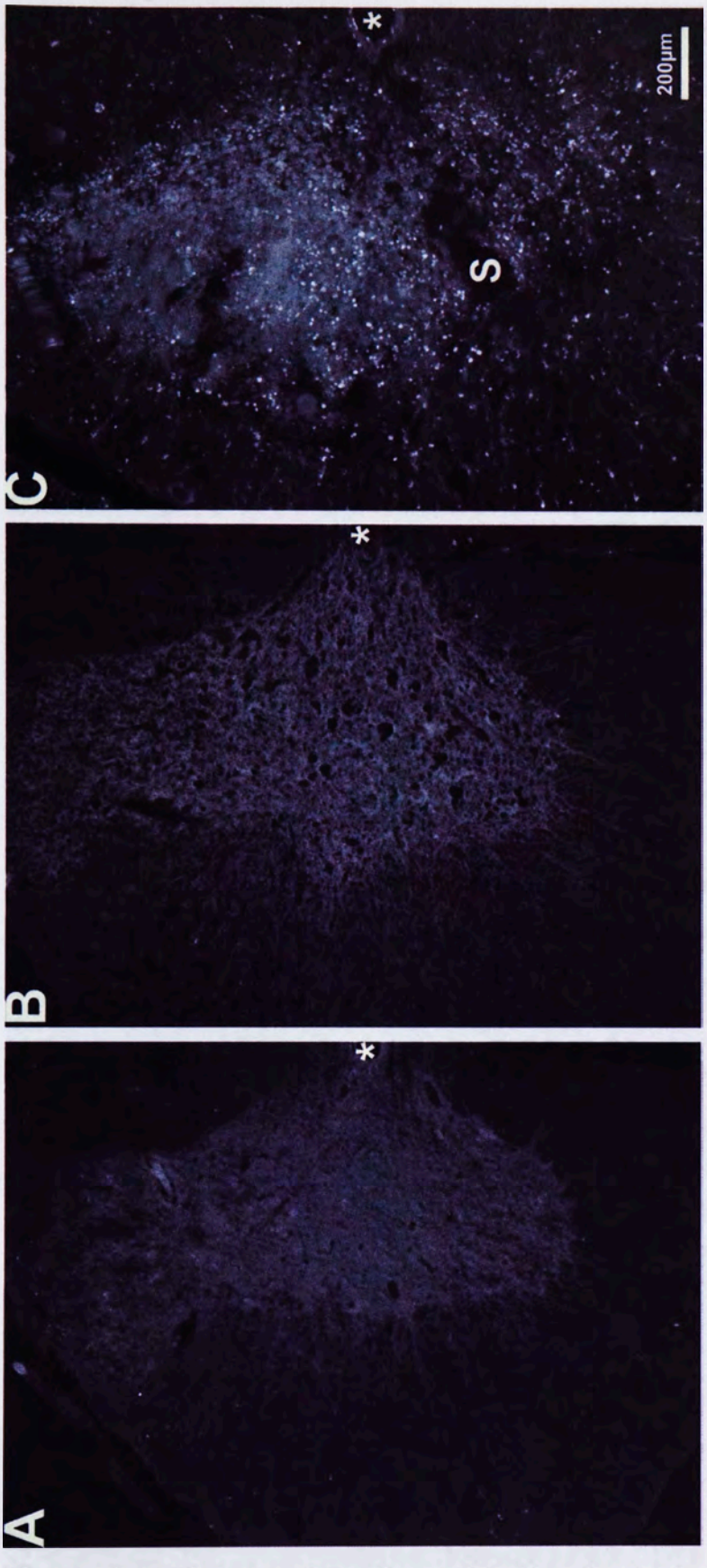

Figure 4-4. Pictures show the right side of spinal cord (C8) from the intact, sham-operated and syrinx animals at 1 week PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. White dots are Ki67+ cells. These Ki67+ cells are located throughout the parenchyma in the syrinx animals, while very few were in the intact and sham-operated animals. "S" indicates cyst in the right grey matter.

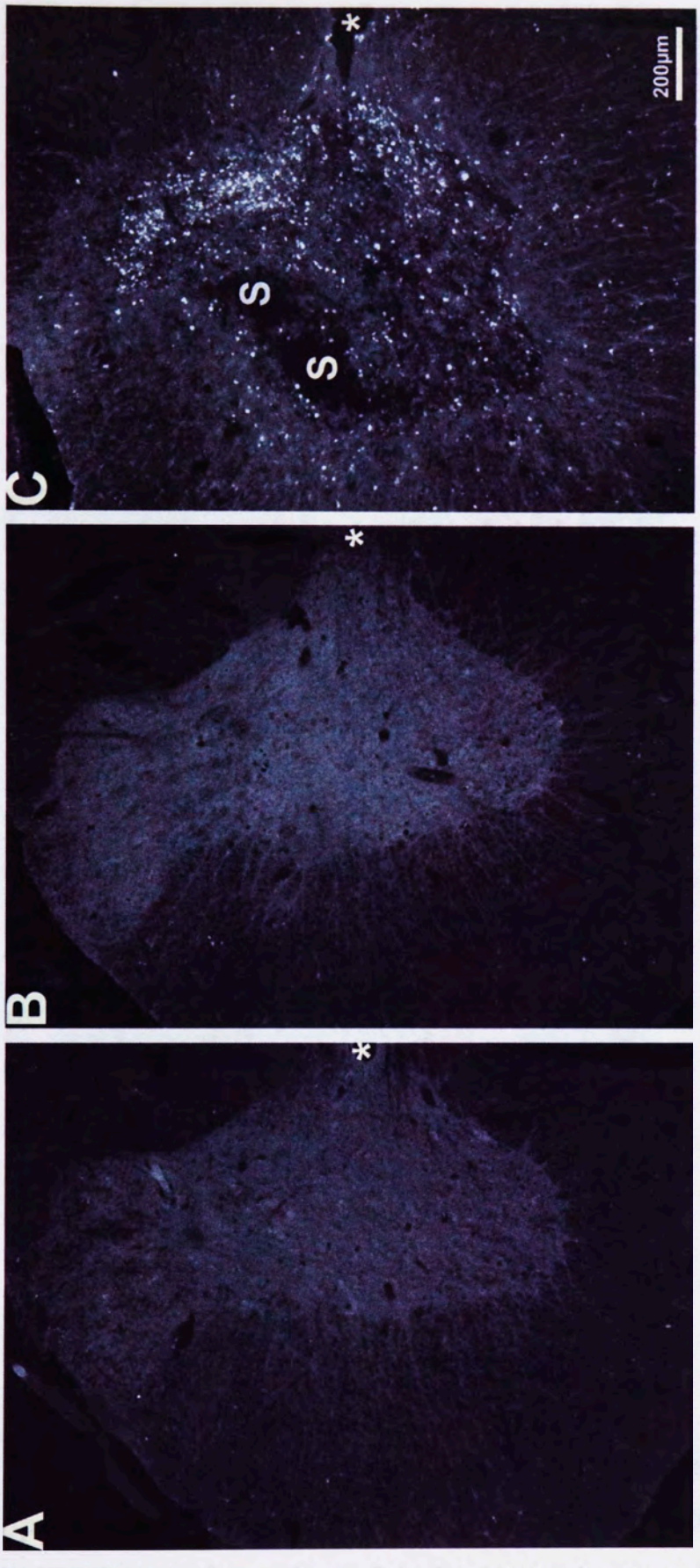

Figure 4-5. Pictures show the right side of spinal cord (C8) from the intact, sham-operated and syrinx animals at 2 weeks PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. Ki67+ cells are located throughout the parenchyma in the syrinx animal, while very few of them were in the intact and sham-operated animals. "S" indicates cyst in the right grey matter.

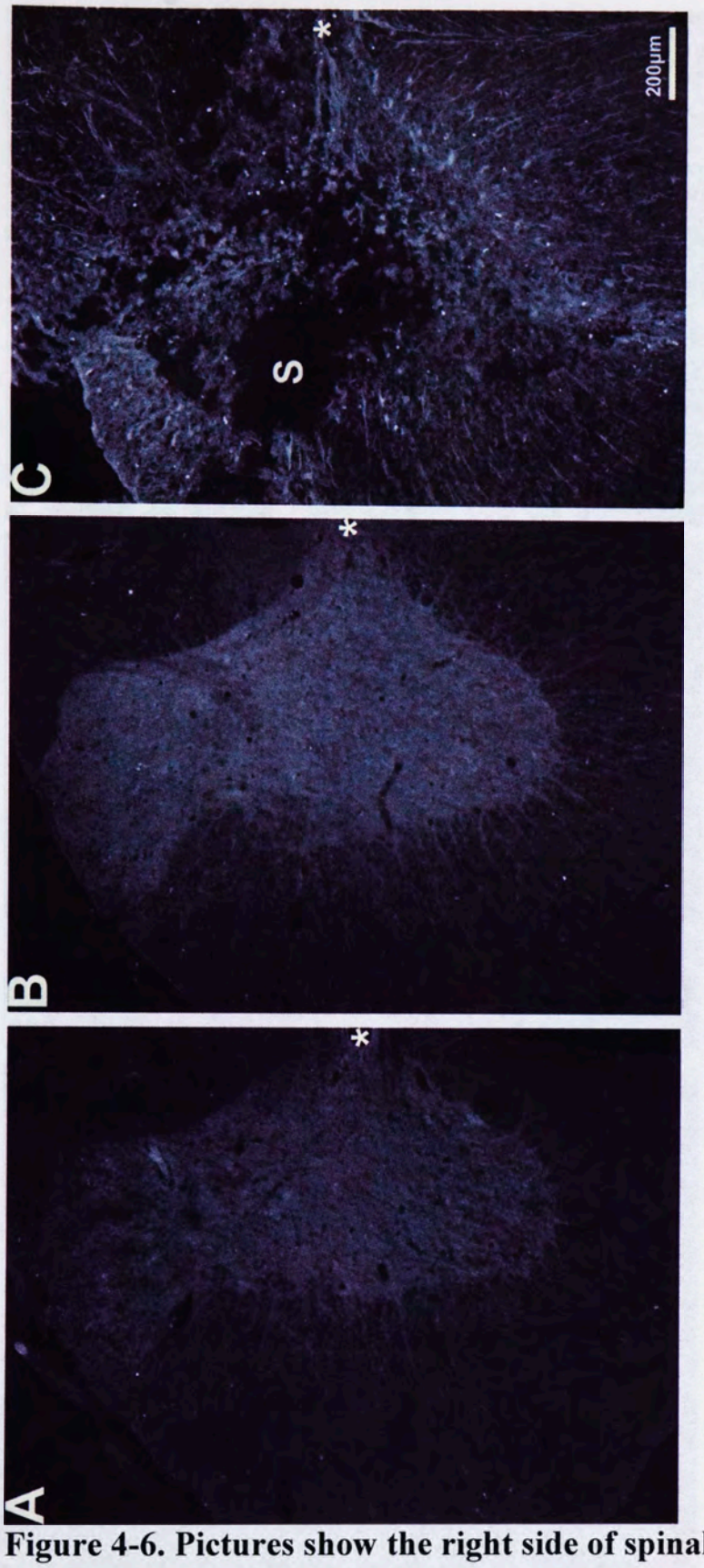

Figure 4-6. Pictures show the right side of spinal cord (C8) from the intact, sham-operated and syrinx animals at 4 weeks PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. Ki67+ cells are located throughout the parenchyma in the syrinx animal, while very few were in the intact and sham-operated animals. Fewer Ki67+ cells were found in the syrinx animals compared to those at 1 and 2 weeks post injury. "S" indicates cyst in the right grey matter. The size of the cyst is larger than those at 1 and 2 weeks post injury.

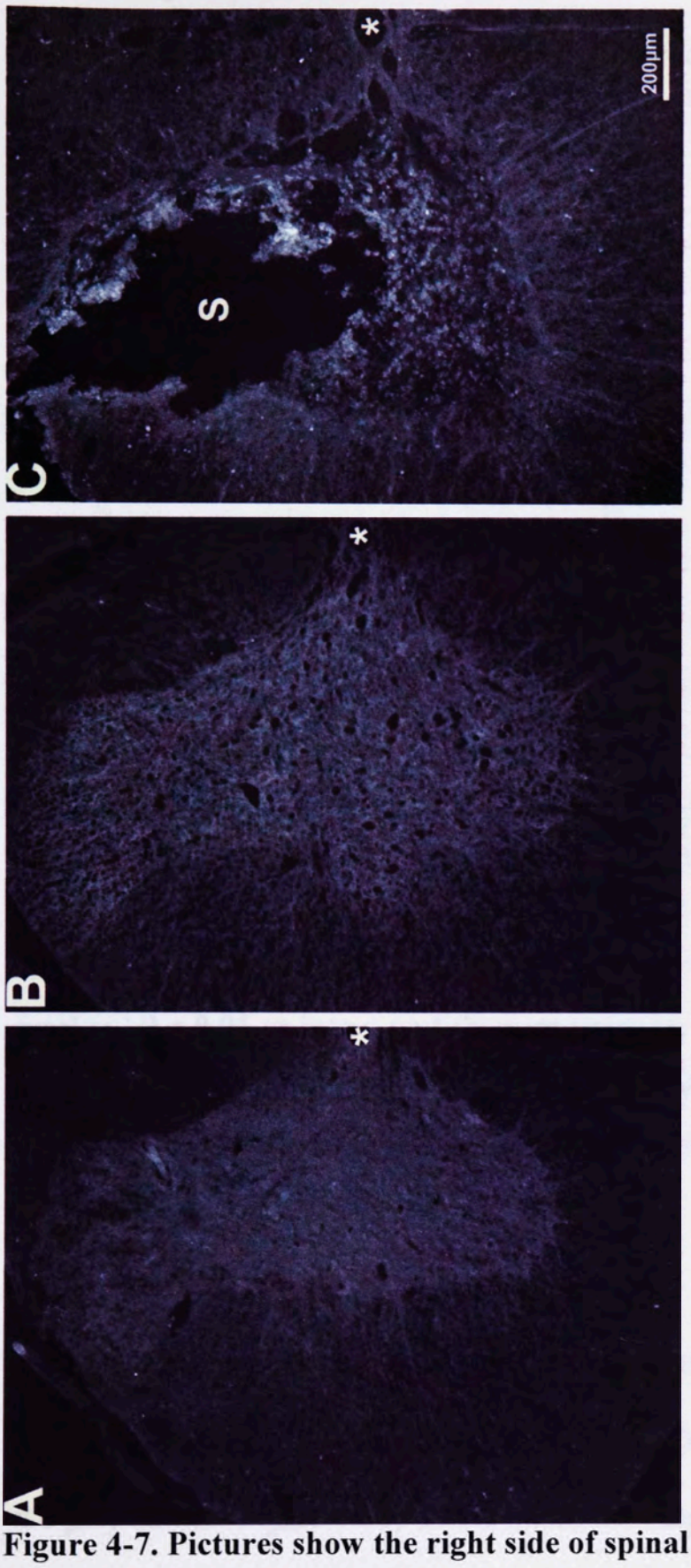

Figure 4-7. Pictures show the right side of spinal cord (C8) from the intact, sham-operated and syrinx animals at 4 weeks PI. A: intact animal; B: sham-operated animal; C: syrinx animal. * indicates central canal. The majority of Ki67+ cells are located in the grey matter in the syrinx animal. Fewer Ki67+ cells were found in the syrinx animals compared to those at 1 and 2 weeks post injury. "S" indicates cyst in the right grey matter.

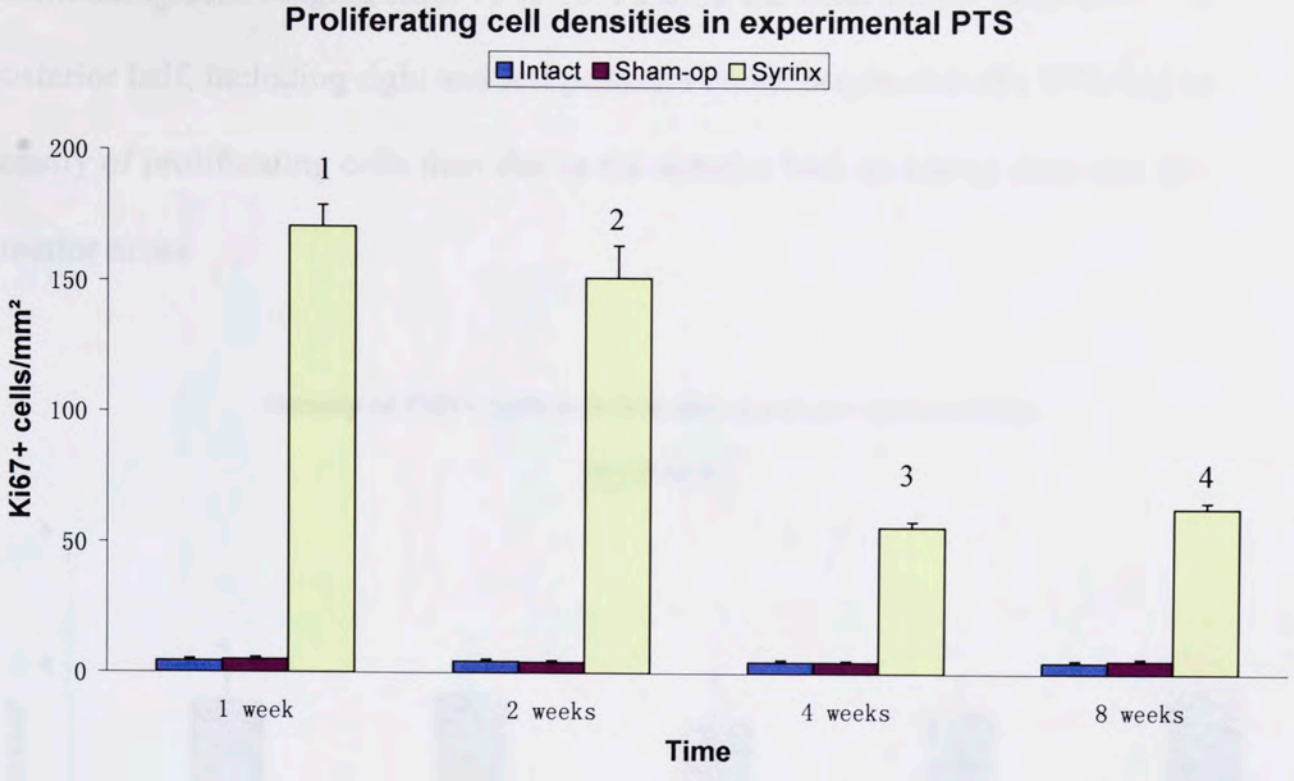

Figure 4-8. Density of Ki67+ cells in the spinal cord section (C8) in the intact, sham-operated and syrinx animals. Data presented as mean ± SE (n=8). [1] $P < 0.01$ vs intact and sham at 1 week, vs syrinx at 4 and 8 weeks; [2] $P < 0.01$ vs intact and sham at 2 weeks, vs syrinx at 4 and 8 weeks; [3] $P < 0.01$ vs intact and sham at 4 weeks; [4] $P < 0.01$ vs intact and sham at 8 weeks. More Ki67+ cells are found in the syrinx animals than the intact or sham-operated animals. The densities of proliferating cells in syrinx animals are higher at 1 and 2 weeks PI than 4 and 8 weeks PI.

4.3.3 Location

In the intact and sham-operated controls, proliferating cells were identified in the white matter (Figure 4-9). In syrinx animals, 79-87% of Ki67 positive cells were located in the grey matter (Figure 4-10). In the intact and sham-operated controls, Ki67 positive cells were 5/mm^2, equally distributed in each area at all time points (Figure 4-11). In syrinx animals, the right posterior area had the highest percentage of Ki67 positive cells, which was 36%, 38%, 34%, and 30% at 7, 14, 28, and 56 days post-syrinx induction, respectively (Figure 4-12). The left anterior area showed the lowest percentage of

proliferating cells ranging from 15 to 18% during the same follow-up period. The posterior half, including right and left posterior areas, demonstrated a 69% higher density of proliferating cells than that in the anterior half, including right and left anterior areas.

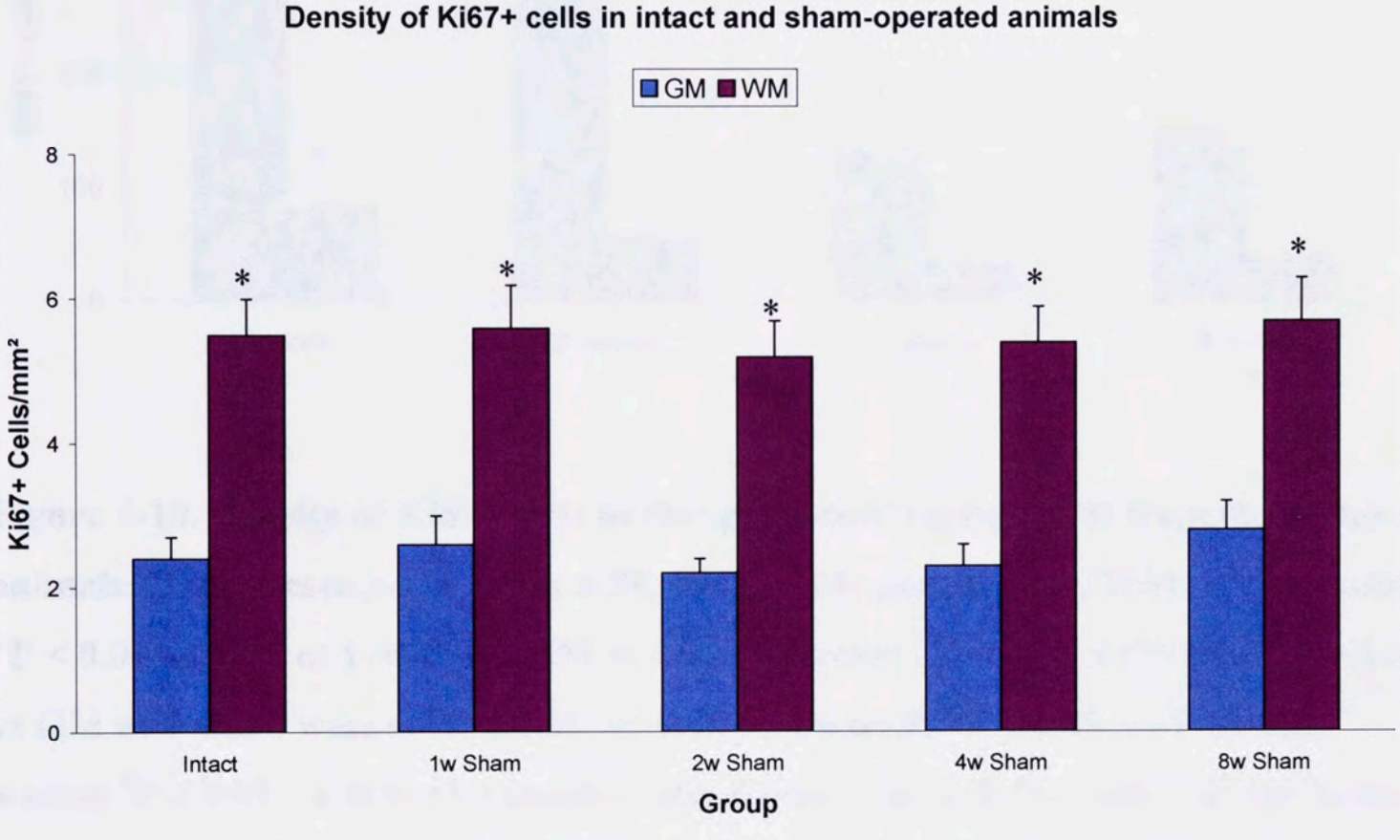

Figure 4-9. Density of Ki67+ cells in the spinal cord section (C8) from the intact and sham-operated animals. Data presented as mean ± SE (n=8); w: week. GM: grey matter, WM: white matter. *p < 0.05 vs GM in each group at every time point. The vast majority of Ki67+ cells are in the white matter in the intact and sham-operated animals.

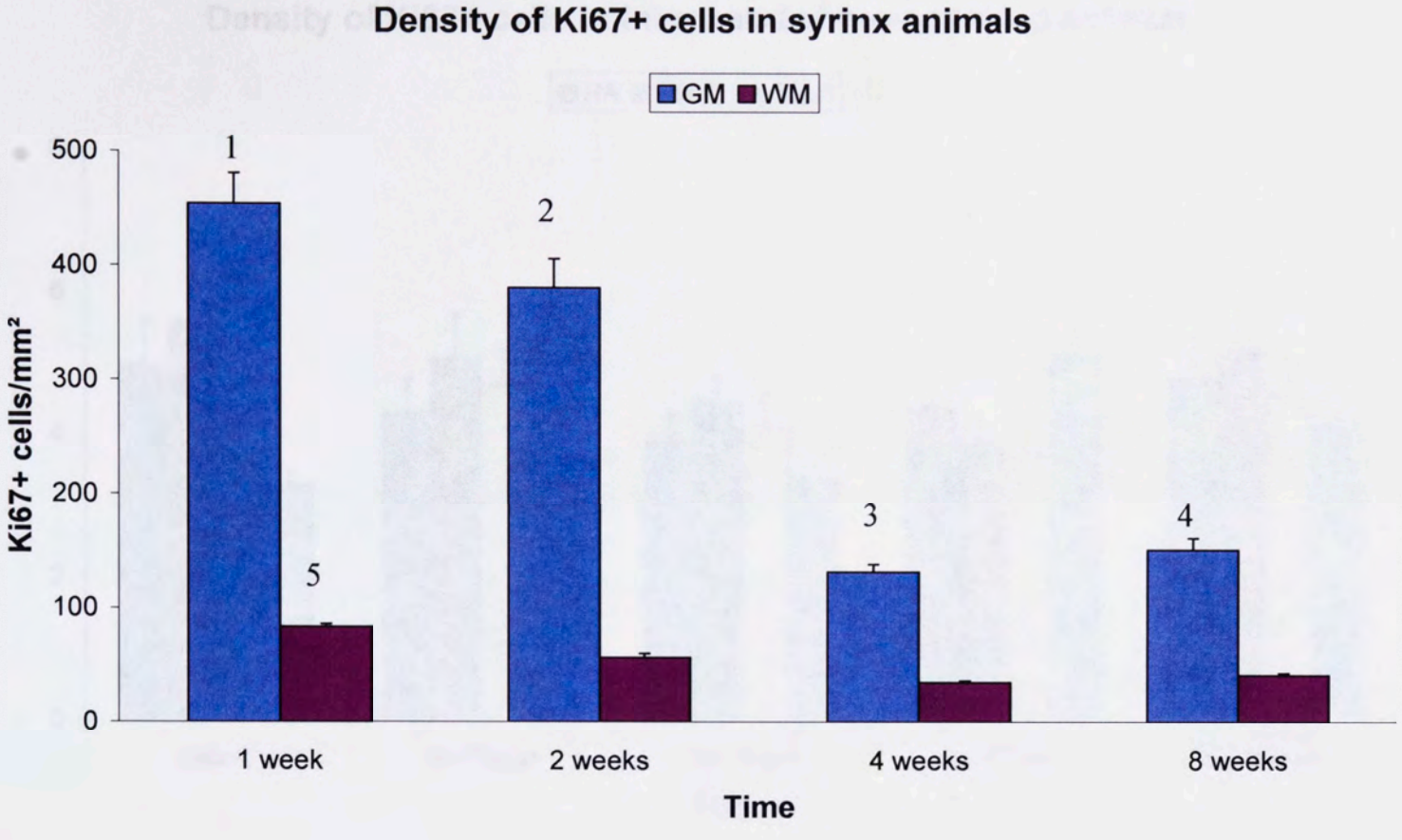

Figure 4-10. Density of Ki67+ cells in the spinal cord section (C8) from the syrinx animals. Data presented as mean ± SE (n=8). GM: grey matter, WM: white matter. [1] $P < 0.01$ vs WM at 1 week, vs GM at 4 and 8 weeks; [2] $P < 0.01$ vs WM at 2 weeks, vs GM at 4 and 8 weeks; [3] $P < 0.05$ vs WM at 4 weeks; [4] $P < 0.05$ vs WM at 8 weeks; [5] $P < 0.05$ vs WM at 4 and 8 weeks. Compared with the results in the intact and sham-operated animals, there is a higher density of Ki67+ cells in the GM than the WM in the syrinx animals. The density of proliferating cells in the GM decreased after 4 weeks PI.

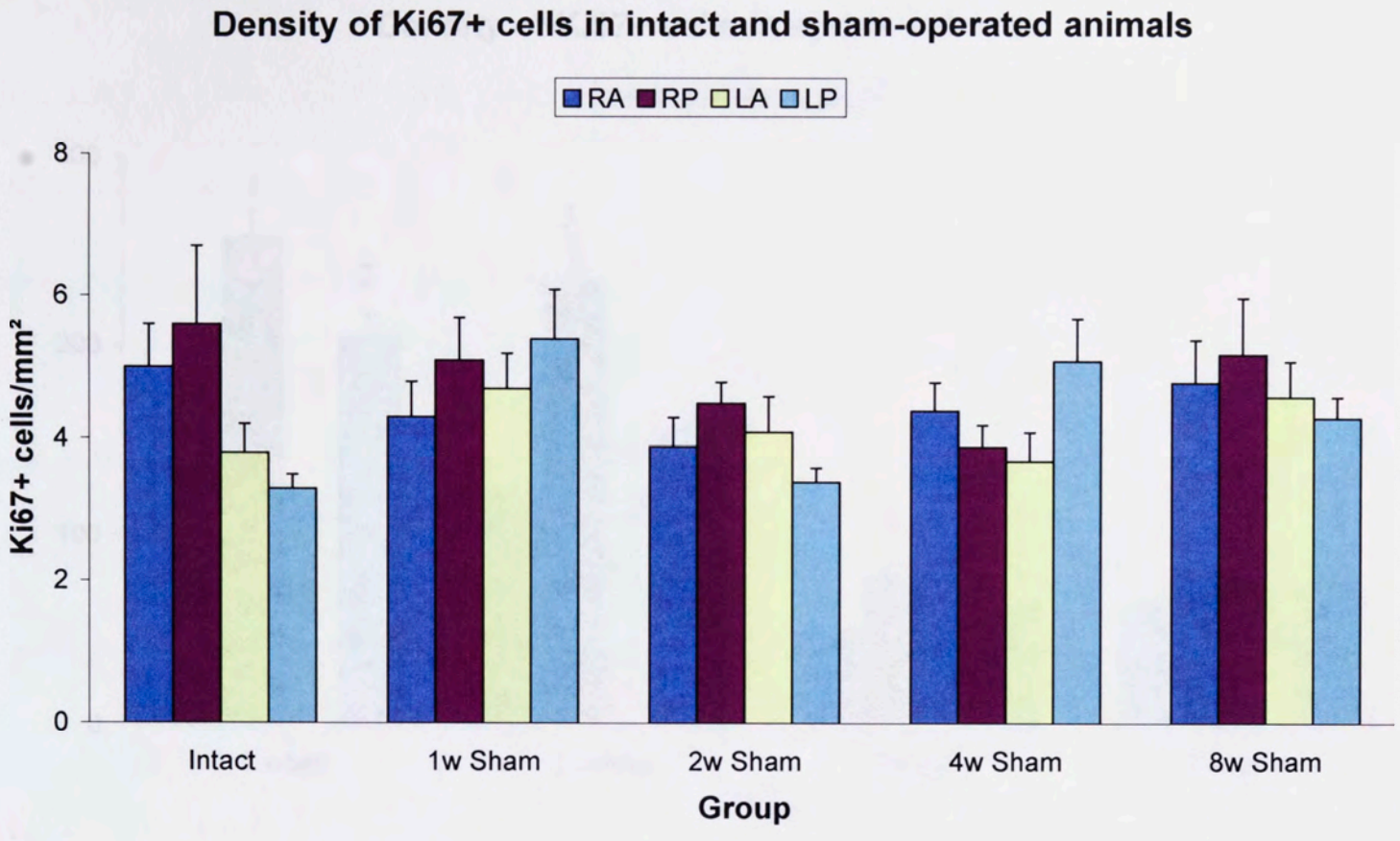

Figure 4-11. Density of Ki67+ cells in the spinal cord section (C8) from the intact and sham-operated animals. Data presented as mean ± SE (n=8); w: week. RA: right anterior area; RP: right posterior area; LA: left anterior area; LP: left posterior area. There is no difference in the density of Ki67+ cells among quadrants in the intact and sham-operated animals.

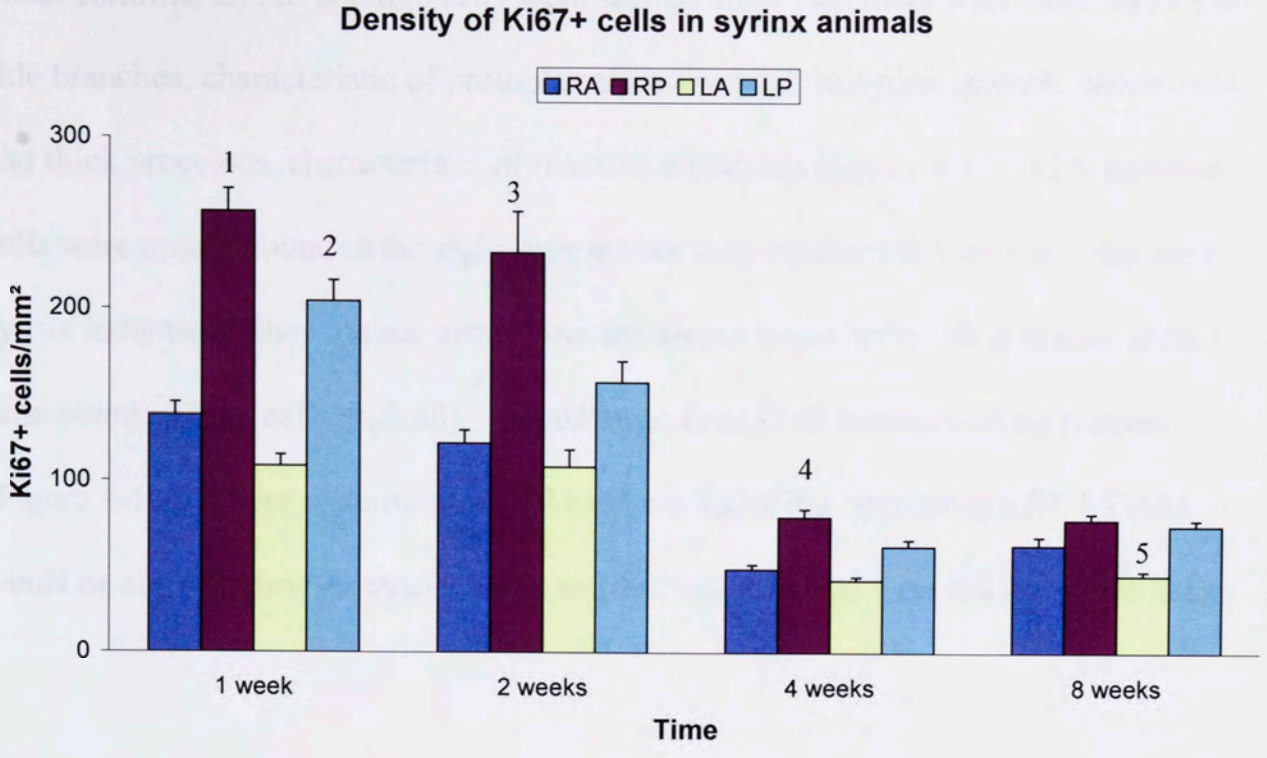

Figure 4-12. Density of Ki67+ cells in the spinal cord section (C8) from the syrinx animals. Data presented as mean ± SE (n=8). RA: right anterior area; RP: right posterior area; LA: left anterior area; LP: left posterior area. [1] $P < 0.01$ vs RA and LA at 1 week, [1] $P < 0.05$ vs RP at 4 and 8 weeks; [2] $P < 0.01$ vs LA at 1 week, vs LP at 4 and 8 weeks; [3] $P < 0.01$ vs RA and LA at 2 weeks, [3] $P < 0.05$ vs RP at 4 and 8 weeks; [4] $P < 0.05$ vs LA and RA at 4 weeks; [5] $P < 0.05$ vs RP and LP at 8 weeks. In syrinx animals, more Ki67+ cells were found in the RP in the first 4 weeks PI, but the difference between RP and other quadrants almost disappeared at 8 weeks PI. The density of proliferating cells in the RP decreased significantly after 4 weeks PI.

4.3.4 Phenotypes

Phenotypes of proliferating cells were unveiled by co-expression of Ki67 with progenitor markers (nestin and NG2), astrocyte marker (GFAP), or macrophage marker (ED1). Typical morphological features of nestin positive cells were seen as small bipolar or monopolar cell bodies with short processes (Figure 4-13). NG2 positive cells were noted as small bipolar or tripolar cell bodies with short processes (Figure 4-14). In the white matter of the intact controls, typical morphological features of GFAP positive cells appeared as small cell bodies with long slender processes. In the grey matter of the

intact controls, GFAP positive cells were seen as short processes with more thorn-like side branches, characteristic of protoplasmic astrocytes. In syrinx animals, these cells had thick processes, characteristic of reactive astrocytes (Figure 4-15). ED1 positive cells were mostly found in the right grey matter surrounding the lesion at 1 day post-syrinx induction. They moved away from the lesion towards the white matter at later time points. These cells typically showed large round cell bodies with no process (Figure 4-16). There were no neuronal markers including beta-tubulin III, NCAM, NeuN or oligodendrocyte marker such as MBP co-localized with Ki67 positive cells.

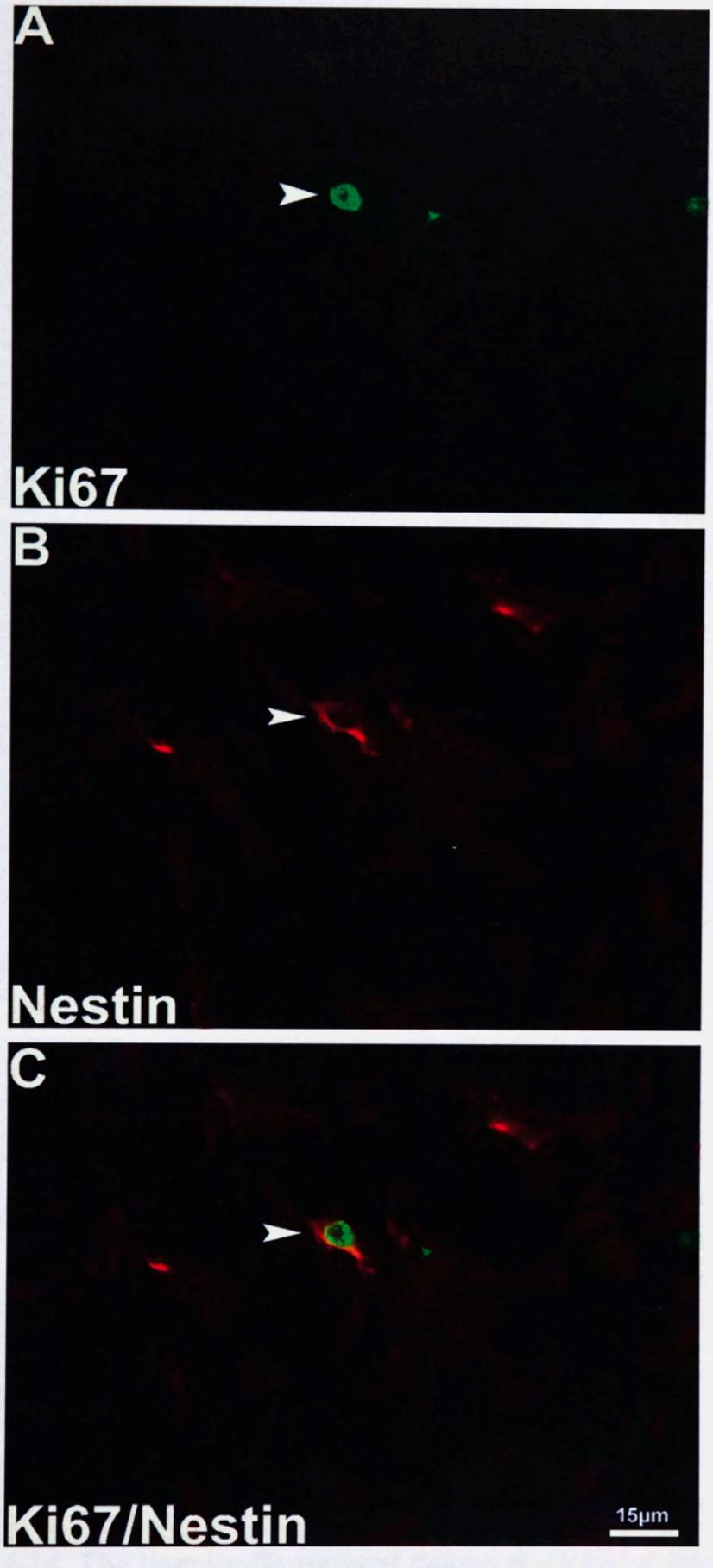

Figure 4-13. The immunofluorescent images were taken from a syrinx animal 1 week PI. A: Ki67 single staining; B: nestin single staining; C: double staining with Ki67 and nestin. Arrow head shows a Ki67/nestin+ cell. It has a small bipolar cell body with a short process.

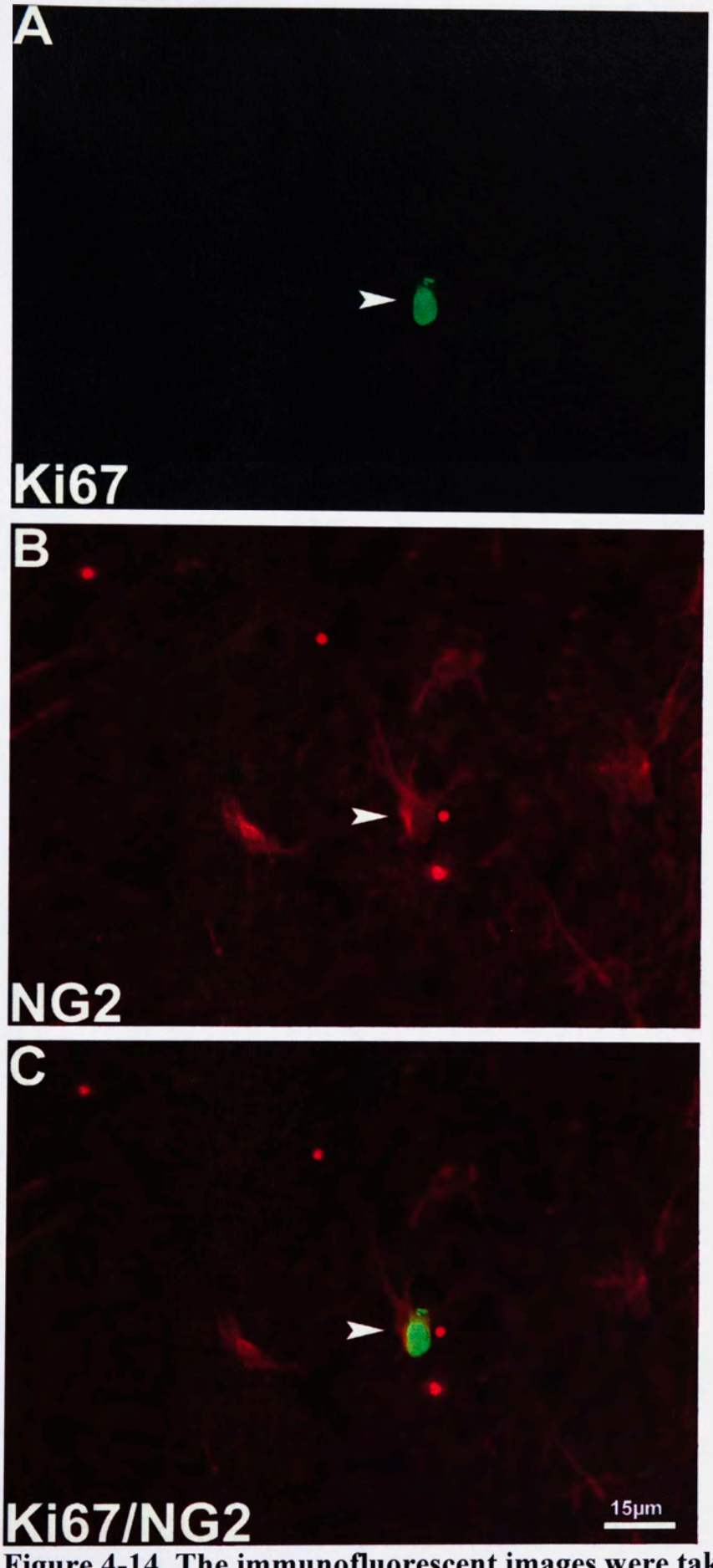

Figure 4-14. The immunofluorescent images were taken from a syrinx animal 1 week PI. A: Ki67 single staining; B: NG2 single staining; C: double staining with Ki67 and NG2. Arrow head shows a BrdU/NG2+ cell. It has a small multipolar cell body with few processes.

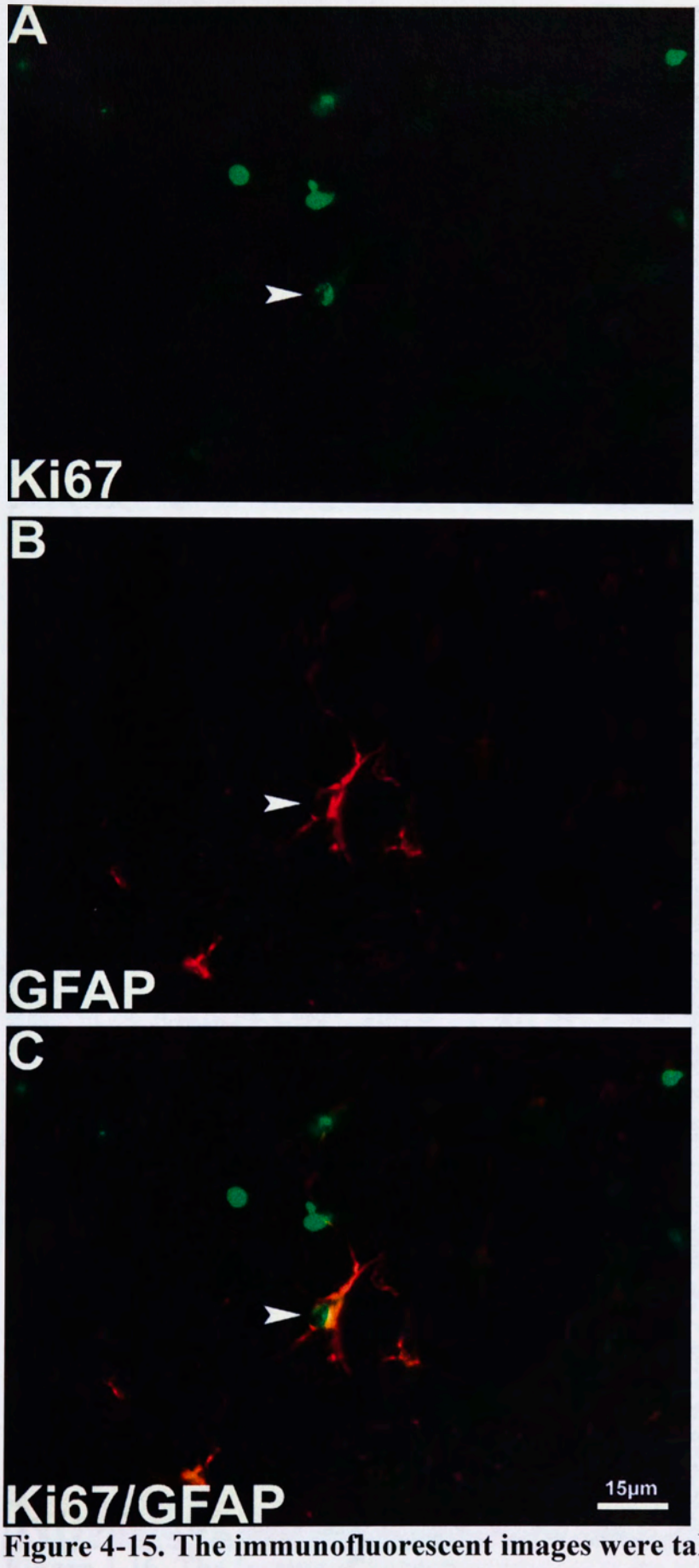

Figure 4-15. The immunofluorescent images were taken from a syrinx animal 4 weeks PI. A: Ki67 single staining; B: GFAP single staining; C: double staining with Ki67 and GFAP. Arrow head shows a Ki67/GFAP+ cell. It has a very small cell body with a few processes, including a typical long process. Most of its processes are shorter than those of typical astrocytes.

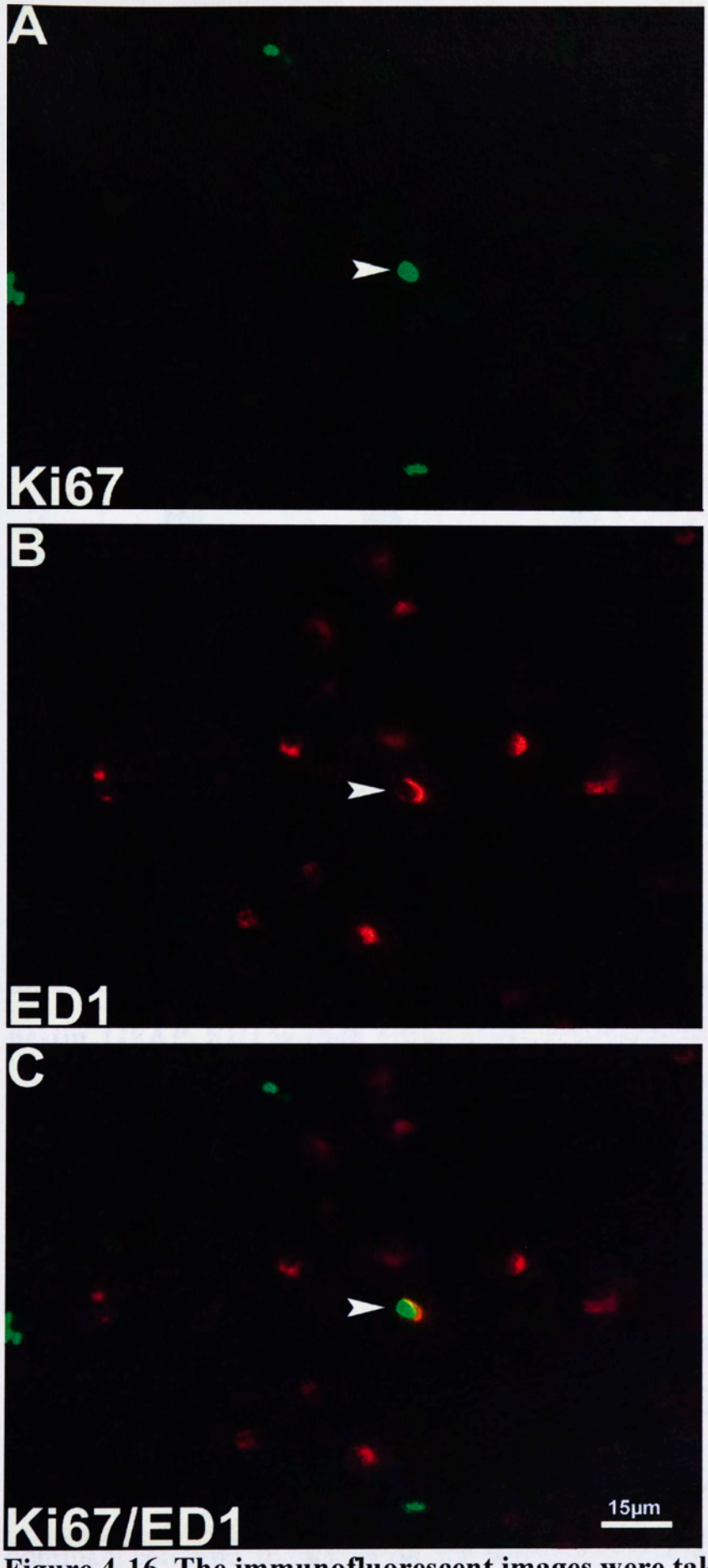

Figure 4-16. The immunofluorescent images were taken from a syrinx animal at 1 week PI. A: Ki67 single staining; B: ED1 single staining; C: double staining with Ki67 and ED1. Arrow head shows a Ki67/ED1+ cell. It has a round cell body without process.

In the intact and sham-operated animals, the vast majority of the proliferating cells were NG2+ and a minority were double stained with nestin and GFAP. Very few Ki67/ED1+ cells were found in the intact and sham-operated groups (Figure 4-17).

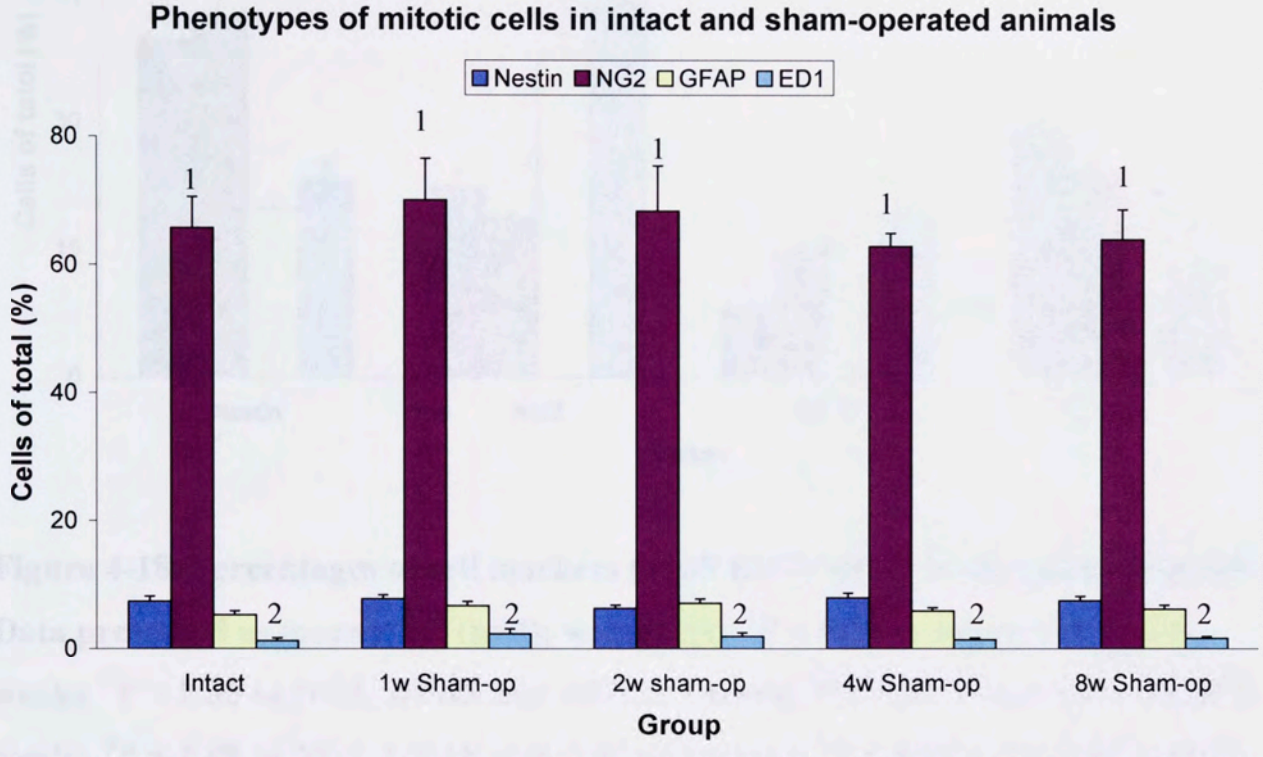

Figure 4-17. Percentages of cell markers (of all Ki67+ cells) in the intact and sham-operated animals. Data presented as mean ± SE (n=4); w: week(s). $^{1}P < 0.01$ vs nestin, GFAP, ED1 in each group at every time point; $^{2}P < 0.05$ vs nestin and GFAP in each group at every time point. The majority of mitotic cells in the intact and sham-operated animals were Ki67/NG2+ cells. Very few Ki67/ED1+ cells were found in the intact and sham-operated animals.

In the syrinx animals (Figure 4-18), Ki67/nestin+ cells were the most dominant phenotype (40% of all the Ki67+ cells) in the first 2 weeks after injury. They appeared throughout the parenchyma. However, from 4 weeks after injury, Ki67/NG2 cells (around 40%), which also were found throughout the parenchyma, had become the most dominant cell type. In this period, the percentage of Ki67/nestin+ cells decreased to around 20%. At 8 weeks post injury, the percentage of Ki67/ED1+ cells decreased steadily from almost 30% to just above 10%, while the proportion of Ki67/GFAP+ cells increased from 8% to almost 20%.

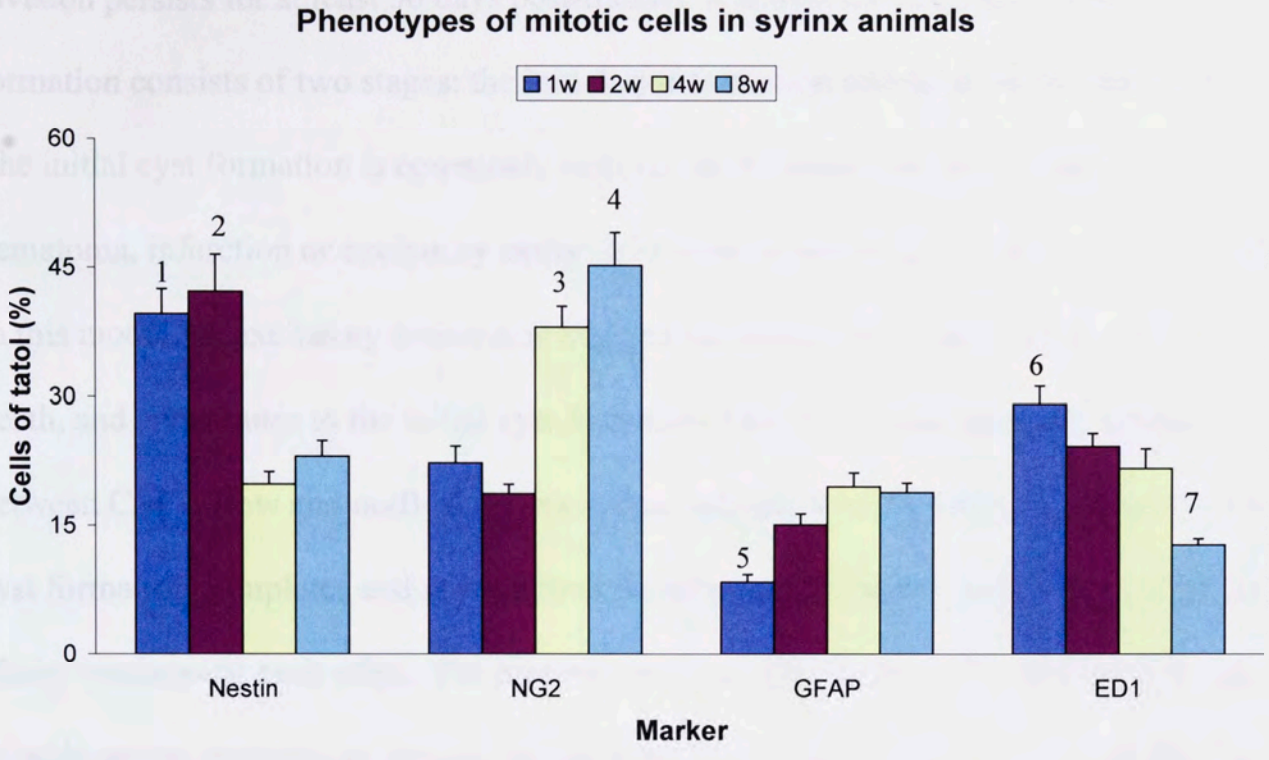

Figure 4-18. Percentages of cell markers (of all Ki67+ cells) in the syrinx animals. Data presented as mean ± SE (n=4); w: week(s). $^{1}P < 0.01$ vs nestin at 4 and 8 weeks, $^{1}P < 0.05$ vs NG2, GFAP and ED1 at 1 week; $^{2}P < 0.01$ vs nestin at 4 and 8 weeks, $^{2}P < 0.05$ vs NG2, GFAP and ED1 at 2 weeks; $^{3}P < 0.01$ vs NG2 at 1 and 2 weeks, $^{3}P < 0.05$ vs nestin, GFAP and ED1 at 4 weeks; ; $^{4}P < 0.01$ vs NG2 at 1 and 2 weeks, $^{4}P < 0.05$ vs nestin, GFAP and ED1 at 8 weeks; $^{5}P < 0.05$ vs GFAP at 4 and 8 weeks; $^{6}P < 0.05$ vs ED1 at 4 and 8 weeks; $^{7}P < 0.05$ vs ED1 at 1, 2 and 4 weeks. The Ki67/nestin+ was the most dominant phenotype in the first 2 weeks after injury, but Ki67/NG2+ cells took its place after 4 weeks PI. After 4 weeks PI, the proportion of Ki67/GFAP+ cells increased, while the percentage of Ki67/ED1+ cells decreased significantly.

4.4 Discussion

4.4.1 Proliferating cells

Ki67 antigen is a nuclear protein expressed in all proliferating cells during late G1, S, G2, and M phases of a cell cycle [336]. It is generally used as a marker to assess cellular proliferating activity. Our data demonstrate that post-traumatic syrinx formation is sufficient to stimulate predominantly quiescent cells to divide in adult rats. This cell

division persists for at least 56 days post-trauma. It is hypothesized that syrinx formation consists of two stages: the initial cyst formation and subsequent enlargement. The initial cyst formation is commonly believed to be caused by inflammation, hematoma, infarction or excitatory amino acid overproduction after trauma [48-51, 338]. In this model, an excitatory amino acid triggers inflammation, cellular injury, neuronal death, and contributes to the initial cyst formation [49-51]. A subsequent imbalance between CSF inflow and outflow causes syrinx enlargement [54-58]. It is unclear when cyst formation completes and at what time its enlargement begins. These two stages are likely overlapping each other. The present study provides evidence that the proliferation of endogenous progenitors persists during these two stages (Figure 4-8). It indicates that the progenitors are able to survive the complicated environmental changes in the spinal cord in PTS. Studies such as this one form the foundation for understanding stem cell response to syrinx formation and are necessary before attempting to use stem cell therapy for the condition.

4.4.2 Location of progenitors

The origin of NPCs in the adult spinal cord is not clear. Horner and colleagues hypothesized that progenitors in the intact adult spinal cord either exist throughout the spinal cord or originate from the ependymal zone and their progeny migrate to the parenchyma [186]. In the injured adult spinal cord, the ependymal zone [183, 184, 325] and parenchyma [180, 181, 321] were regarded as the location of the stem cell pool. The progenitors proliferate in the stem cell pool and then migrate to the lesion. In this study, we found that endogenous progenitors appear mainly in the grey matter of spinal cord from 7 to 56 days post injury in the PTS model (Figure 4-10). As discussed in the

previous chapter, the difference in the location of progenitors between our results and those from other studies may be due to different spinal cord injury models.

Interestingly, even in the same injury model, progenitors appear in different locations at different time points. The majority of proliferating cells exist in the white matter at 1 day PI (Figure 3-12), while they appear mainly in the grey matter after 7 days (Figure 4-10). At least two thirds of these proliferating cells are progenitors, either nestin+ NSCs or NG2+ glial progenitors (Figure 4-18). There are two possible explanations for this phenomenon.

Firstly, this may be the result of a shift of the stem cell pool in the spinal cord in the PTS model. Progenitor cells normally exist in the white matter of the spinal cord, which is supported by the location of proliferating cells in the control animals (Figure 3-10, 3-11 and 4-9). In the very early stage of PTS, the progenitors divide in the stem cell pool (in the white matter). From 7 days PI, as part of the reaction of endogenous progenitors to injury, the majority of the progenitor pool moves closer to the lesion (in the grey matter). The closer distance between stem cell pool and lesion may be important to the regulation of progenitors. For example, the microenvironment in the lesion may directly affect the progenitors in the stem cell pool. The preogenitors are endouraged to differentiate into macrophages to further enhance proliferation and survival of endogenous progenitors (details described in Chapter 3, "Acute response of endogenous progenitors in an adult rat model of PTS"). The microenviroment of a lesion may also directly affect progenitors in the pool to encourage glial differentiation, which play an important role in preventing a cascading wave of uncontrolled tissue damage and limiting syrinx enlargement (details described in Chapter 6, "Reactive astrocyte and gliosis in experimental PTS").

The cause of the shift of the stem cell pool is uncertain. We assume that imflammatory cells play important roles. Inflammatory cells were reported to support proliferation and survival of progenitors by producing inflammatory mediators (eg. chemokines) [237-240], growth factors (eg. BDNF and GDNF) [255, 256, 339-342] and other stem cell regulators (eg. Sonic hedgehog and Tenascin C) [211, 266, 343-347]. In the previous study of progenitors in acute stage of PTS, we observed a severe inflammatory reaction in the grey matter of the spinal cord within a week after syrinx induction, which is also supported by other studies [1, 12, 25, 26]. It is possible that imflammatory cells trigger the shift of the stem cell pool for further proliferation.

Secondly, the change of location of progenitors may result from the majority of progenitors moving closer to the lesion then dividing. However, this would be contrary to the current understanding of stem cell biology that progenitors dividing before migrating [78, 79, 348]. Further studies are required to clarify this issue. However, the difficulty of tracing mitotically silent cells *in vivo* makes it impossible at this stage.

4.4.3 Proliferation of progenitors

Progenitors can give rise to neurons and glial cells throughout adulthood in the brain, including the subventricular zone [132], hippocampus [86, 130, 139, 140, 142-145], and cortex [150]. However, the *in vivo* proliferation rate of progenitors has not been determined for the adult brain. The division rate of the adult spinal cord progenitors is thought to be low [82]. Recent studies have shown such an active population of progenitors to persist for 1 month in the adult rat spinal cord [186]. We demonstrated that mitotic activity persists for at least 56 days with a similar proliferation rate in the intact and sham-operated animals. Mitosis of adult spinal cord progenitors can be stimulated after injury [111, 179, 183]. In the present experiments,

the counts of proliferating cells have increased by 20 times in the first 2 weeks after trauma, excluding the effects of inflammatory cells and astrocytes. A difference in progenitor density between syrinx and controls decreased by 10 fold 4 weeks post trauma. It suggests that stimuli to the proliferation of progenitors are altered between the primary injury and the chronic stage. There is strong evidence to suggest that this discrepancy in mitotic activities between a chronic stage and the primary stage of post-traumatic syringomyelia is possibly associated with two factors. One factor is that the inflammatory reaction is decreased at the chronic stage that leads to a low level of inflammatory cytokines to stimulate mitotic activity of cells. Another factor is that micro-vessels had been destroyed as syrinx formed. This reduces the level of blood derived factors resulting in fewer stimuli to the proliferation of progenitors at a chronic stage than those at the primary stage of post-traumatic syringomyelia.

4.4.4 The optimal time to stimulating progenitors for repair

Previous studies of grafting NPCs to repair damaged CNS demonstrate that time is vital in transplantation therapy. The inflammatory reaction peaks within one week after injury, which causes the microenvironment to be unsuitable for the survival of the grafted cells [270]. However, if too much time passes after the injury, glial scars form around the lesion site and inhibit axon regeneration. In addition, Caselle and colleagues reported that the formation of new vessels occurs most actively 1 to 2 weeks after SCI [271]. Thus, it is considered that 7 to 14 days after injury is the optimal time for transplantation [272, 273]. No study about the optimal time to stimulate endogenous progenitors in SCI has been reported. The timing of stem cell therapy for PTS may be completely different than for SCI without syringomyelia.

The following facts support that the first 4 weeks after injury is the optimal time to start stimulating the endogenous progenitors. Firstly, the inflammatory reaction in the PTS peaks within 2 week after injury and decreases obviously after 2 weeks PI (Figure 4-18). As described above, the inflammatory cells produce different stem cells regulators to make the microenvironment suitable for the survival and proliferation of the progenitors. Secondly, the proliferation of endogenous progenitors is at a high level within 2 weeks post injury and it decreases between 2 and 4 weeks post injury (Figure 4-3). The progenitors are the basis of therapy of harnessing endogenous progenitors. Thirdly, phenotype study showed that majority of progenitors were multipotential progenitors within 2 weeks after injury, while NG2+ glial progenitors become more dominant after 4 weeks post injury (Figure 4-18). In theory, nestin+ multipotential progenitors can differentiate into both neurons and glial cells, but glial progenitors can only give rise to glial cells. Thus, nestin+ multipotential progenitors have much more potential.

Whether activating endogenous progenitors at an early time after SCI would influence syrinx formation is a matter of speculation. Strategies should be developed to improve the microenvironment for the endogenous progenitors to proliferate and differentiate and to inhibit the negative environmental signals. Further advances in understanding the biology of adult endogenous progenitors and the pathophysiology of PTS will facilitate their future clinical application.

4.5 **Conclusion**

Our results demonstrate proliferation, distribution, and phenotype of endogenous progenitor cells in an adult rat model of post-traumatic syringomyelia. These progenitors persist proliferating up to 8 weeks after injury. They are shifting their

location from white matter to grey matter after 7 days PI. These studies are important in understanding the endogenous stem cell response to PTS and lay the groundwork for future studies examining stem cell therapy for the condition.

Chapter Five *In Vivo* Differentiation of Adult Endogenous Progenitors in PTS

5.1 Introduction

In the intact adult rat spinal cord, endogenous progenitors give rise to astrocytes and oligodendrocytes but not neurons *in vivo* [186]. In various SCI models, progenitors harvested from the spinal cord parenchyma are able to give rise to both neurons and glial cells *in vitro* [180, 181]. Some *in vivo* studies of SCI showed endogenous progenitors were able to give rise to oligodendrocytes [191, 192, 321], while other studies did not replicate their results [181, 183, 184].

In the previous chapters, it was demonstrated that endogenous progenitors reacted to PTS by proliferating and shifting their location from white matter to the grey matter around the lesion. Regeneration of neurons from endogenous progenitors, which will be a crucial part in a strategy to treat PTS by using endogenous progenitors, was not observed. In the study of progenitors in the acute stage of PTS (Chapter 3), the endogenous progenitors had been traced for one week. The results of differentiation of endogenous progenitors were not conclusive, probably due to insufficient time for progenitors to display their differentiation potential within a week. In the study of progenitors in the chronic stage of PTS (Chapter 4), although endogenous progenitors were observed up to 8 weeks after injury, differentiation was not possible to be examined by Ki67 staining. Thus, it is important to study the differentiation of endogenous progenitors in a long period after injury. Multiple BrdU injections immediately after injury enable newly proliferated cells to be traced for a longer period, making it possible to examine the differentiation of endogenous progenitors in the

chronic stage in PTS. Here, we hypothesize that the endogenous progenitors keep proliferating during an 8 week period and most of them will differentiation into glial cells. In this study, we aim to examine proliferation and phenotypic fate of endogenous progenitors.

5.2 Materials and methods

5.2.1 Animal groups

Forty-eight male Wistar rats (10-12 weeks old and 200-380 g body weight) were used in this study. They were divided into three groups at four time points (Table 5-1). Animals in the intact group, which had no surgery, served as normal controls. Rats in the sham operated group had laminectomies only. Animals in the syrinx group had syrinx induction procedures. Animals were sacrificed 2, 4, 6 or 8 weeks after the surgery.

Table 5-1 Animal groups

	2 week PI	4 weeks PI	6 weeks PI	8 weeks PI
Intact	4	4	4	4
Sham-operated	4	4	4	4
Syrinx	4	4	4	4

PI: post injury

5.2.2 Labelling the mitotic cells

Daily intraperitoneal injections of BrdU (50 mg/kg) were administrated from day 1 to day 12 after surgery in the sham-operated and syrinx animals. In order to examine the differentiation of endogenous progenitors in normal rats, 12 successive daily injections of BrdU were also done in the intact animals. Four animals from each group at each time point were sacrificed 1 day, 2 weeks, 4 weeks and 6 weeks after the last injection.

5.2.3 Immunohistochemistry

The primary and secondary antibodies used in this study are summarised in Table 5-2.

Table 5-2 List of antibodies

Antibodies	Type	Concentration
Primary antibodies		
Anti-BrdU	Sheep polyclonal	1:200
Anti-nestin	Mouse monoclonal	1:4000
Anti-NG2	Mouse monoclonal	1:200
Anti-β-tubulin (III)	Mouse monoclonal	1:300
Anti-NCAM	Mouse monoclonal	1:1000
Anti-NeuN	Mouse monoclonal	1:200
Anti-MBP	Mouse monoclonal	1:50
Anti-GFAP	Mouse monoclonal	1:4000
Anti-ED1	Mouse monoclonal	1:400
Secondary antibodies		
ALEXIA Fluor 594	Goat anti mouse	1:800
ALEXIA Fluor 488	Donkey anti sheep	1:800

5.2.4 Microscopic and statistical analysis

BrdU positive cells per section were counted using a microscope and imaging data acquired by a digital camera. BrdU uniformly-labelled nuclei were regarded as positive when they matched with DAPI staining. A particular phenotype of proliferating cells was identified when well defined BrdU labelled nuclei were also associated with immunopositively labelled cell bodies. The only exception was anti-NeuN, which also stains nuclei. Serial Z-projections were stacked, and only cells with well-circumscribed specifically stained cell bodies were considered as an immunopositive phenotype. Data were presented as mean ± SE. Differences among groups were evaluated by one way ANOVA. $P < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Syrinx model

No cysts were found in the intact and sham-operated animals. Cysts were found in the right grey matter at the level of C8 from 2 to 8 weeks after surgery in the syrinx animals. These cysts were not connected to the central canal and their size increased gradually with time. Tissue loss was significant, especially 4-8 weeks post injury (Figures 4-4, 4-5 and 4-6). Multiple cyst formation was a common finding.

5.3.2 Proliferating cells

BrdU+ cells were found in syrinx animals, intact, and sham-operated groups. Cells with BrdU staining matched with those DAPI+ cells in terms of their position and shape (Figure 3-3). In the intact and sham-operated groups, BrdU+ cells were found throughout the 8 week period. In the syrinx animals, BrdU staining was weak at 6 weeks post injury and even weaker at 8 weeks post injury.

5.3.3 Density of the BrdU+ cells

In the intact group, 5.7 ± 0.8 BrdU+ cells/mm^2 were found at 2 weeks after the first injection. The density of BrdU+ cells increased slightly to 6.1 ± 1.1 BrdU+ cells/mm^2 2 weeks later and then decreased to 3.4 ± 0.5 in the next 4 weeks (Figure 5-1). Similar densities were found in the sham-operated animals (Figure 5-1). Slightly higher density (6.7 ± 1.3 BrdU+ cell per mm^2) was found at 4 weeks post surgery, but it did not reach statistical significance. Much higher densities of BrdU+ cells were found in the syrinx group (Figure 5-2). There were 654.9 ± 53.1 BrdU+ cells/mm^2 at 2 weeks post injury. But the density decreased sharply by almost 50% 2 weeks later. After that, the densities of proliferating cells decreased further to 254.7 ± 30.2 BrdU+ cells/mm^2 at 6 weeks post injury and to less than a hundred by 8 weeks post injury.

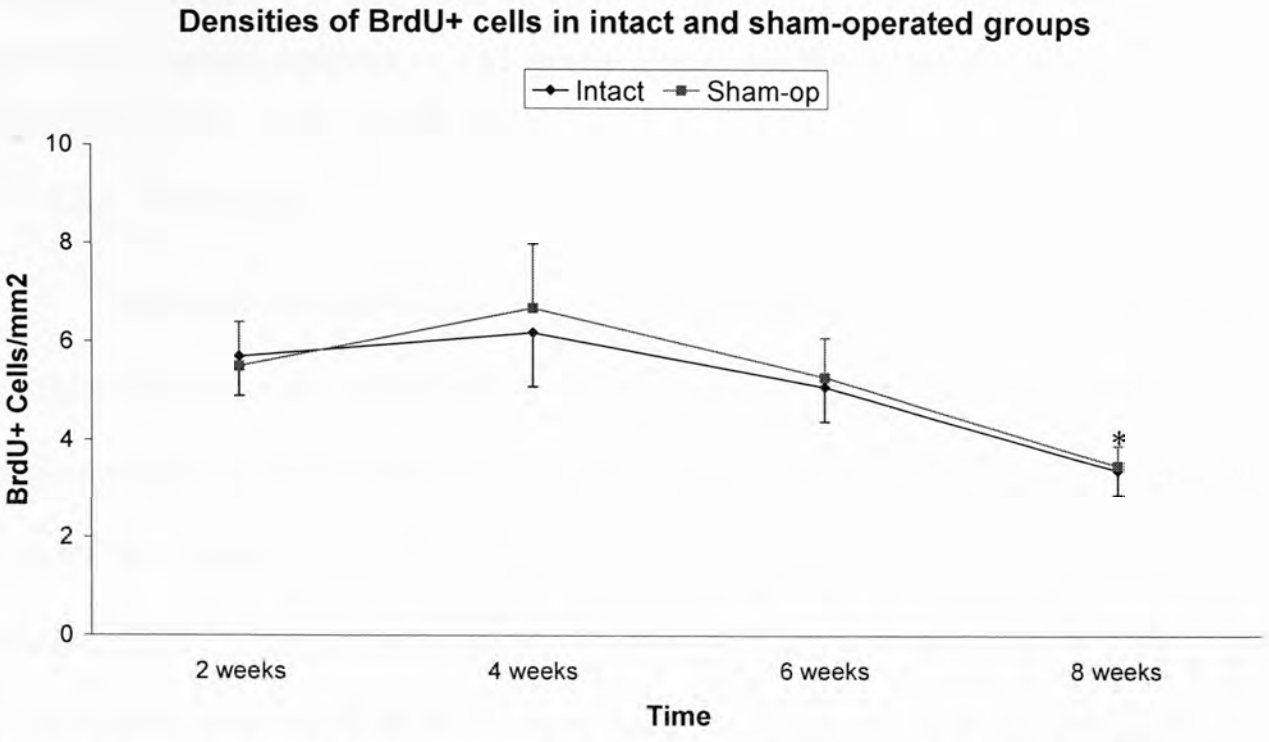

Figure 5-1. Densities of BrdU+ cells in spinal cord sections (C8) from the intact and sham-operated groups. Data presented as mean ± SE (n=8). *P < 0.05 vs intact and sham-op at 4 weeks. There is no significant difference in the density of BrdU+ cells between intact and sham-operated animals. Their densities decreased steadily with time.

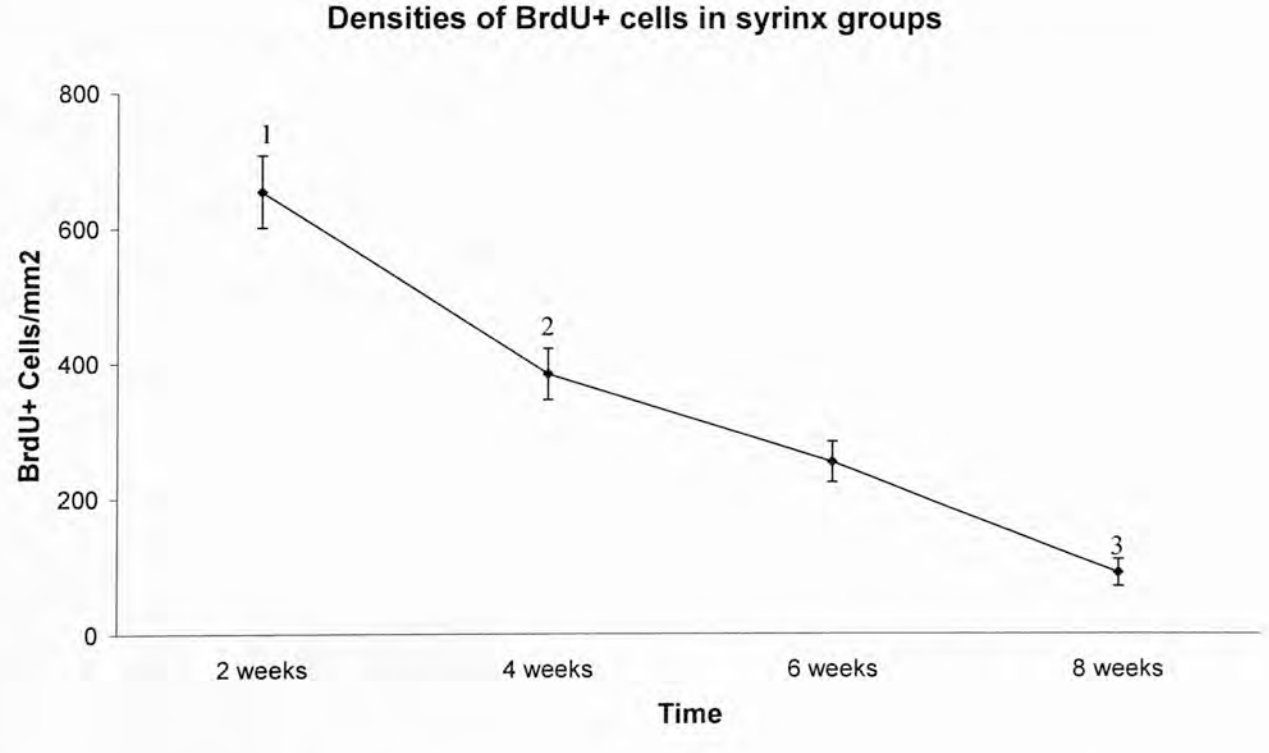

Figure 5-2. Densities of BrdU+ cells in spinal cord sections (C8) from the syrinx groups. Data presented as mean ± SE (n=8). [1]P < 0.05 vs 4, 6 and 8 weeks; [2]P < 0.05

vs 2 and 8 weeks; $^{3}P < 0.05$ vs 2, 4 and 6 weeks. Compared with intact and sham-operated animals, animals in PTS group have a much sharper decrease in the density of BrdU+ cells over 8 weeks.

5.3.4 Phenotypes

Phenotypes of proliferating cells were unveiled by co-expression of BrdU with markers for progenitor (nestin and NG2), astrocyte (GFAP), macrophage (ED1) or oligodendroglia (MBP). The morphological features of these cells have been described previously (Figures 3-16, 3-17, 3-18 and 3-19). There were no neuronal markers (beta-tubulin III, NCAM, and NeuN) co-localized with BrdU positive cells. MBP were expressed in the intact and sham-operated controls but not in the syrinx animals.

The percentage of different phenotypes of proliferating cells is summarized in Figures 5-3, 5-4 and 5-5. In the intact and sham-operated animals, BrdU/NG2 positive cells were the dominant phenotype throughout the 8 weeks period, although there was a small increase in the percentages of BrdU/GFAP and BrdU/MBP positive cells (Figures 5-3 and 5-4). In the syrinx animals, BrdU/NG2 positive cells were the dominant phenotype from 2 to 6 weeks PI, which were replaced by BrdU/GFAP positive cells at 8 weeks PI (Figure 5-5). The proportion of BrdU/ED1 positive cells decreased from more than 25% at 2 weeks PI to just over 10% at 4 weeks PI. It then dropped further to around 1% at 8 weeks PI.

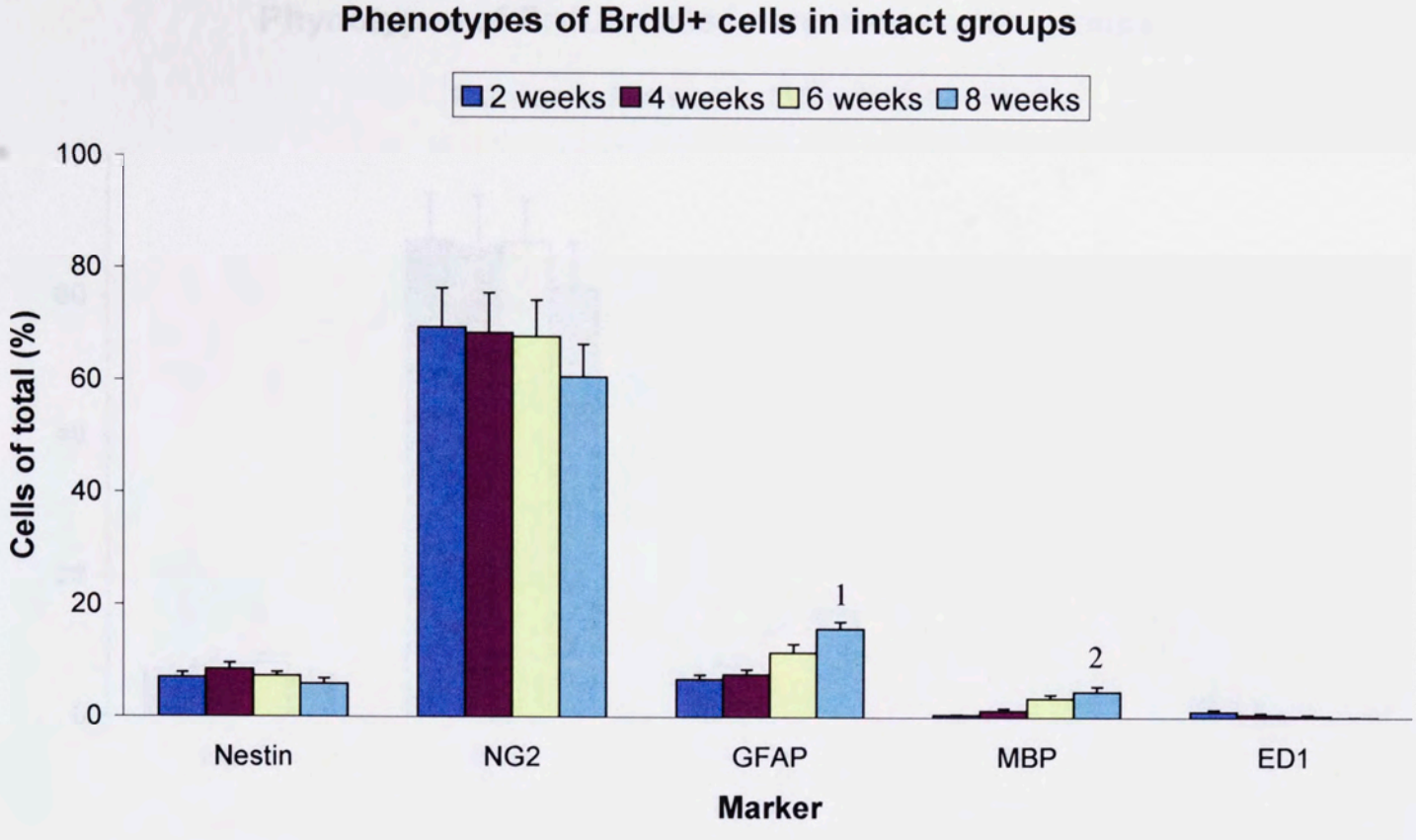

Figure 5-3. Percentages of cell markers (of all BrdU+ cells) in the intact animals. Data presented as mean ± SE (n=4). $^{1}P < 0.05$ vs GFAP at 2 weeks; $^{2}P < 0.05$ vs MBP at 2 weeks. The percentages of progenitor cells (Nestin/BrdU+ and NG2/BrdU+) do not change significantly, while the proportions of glial cells (GFAP/BrdU+ and MBP/BrdU+) increase with time. Very few macrophages appear in the intact animals.

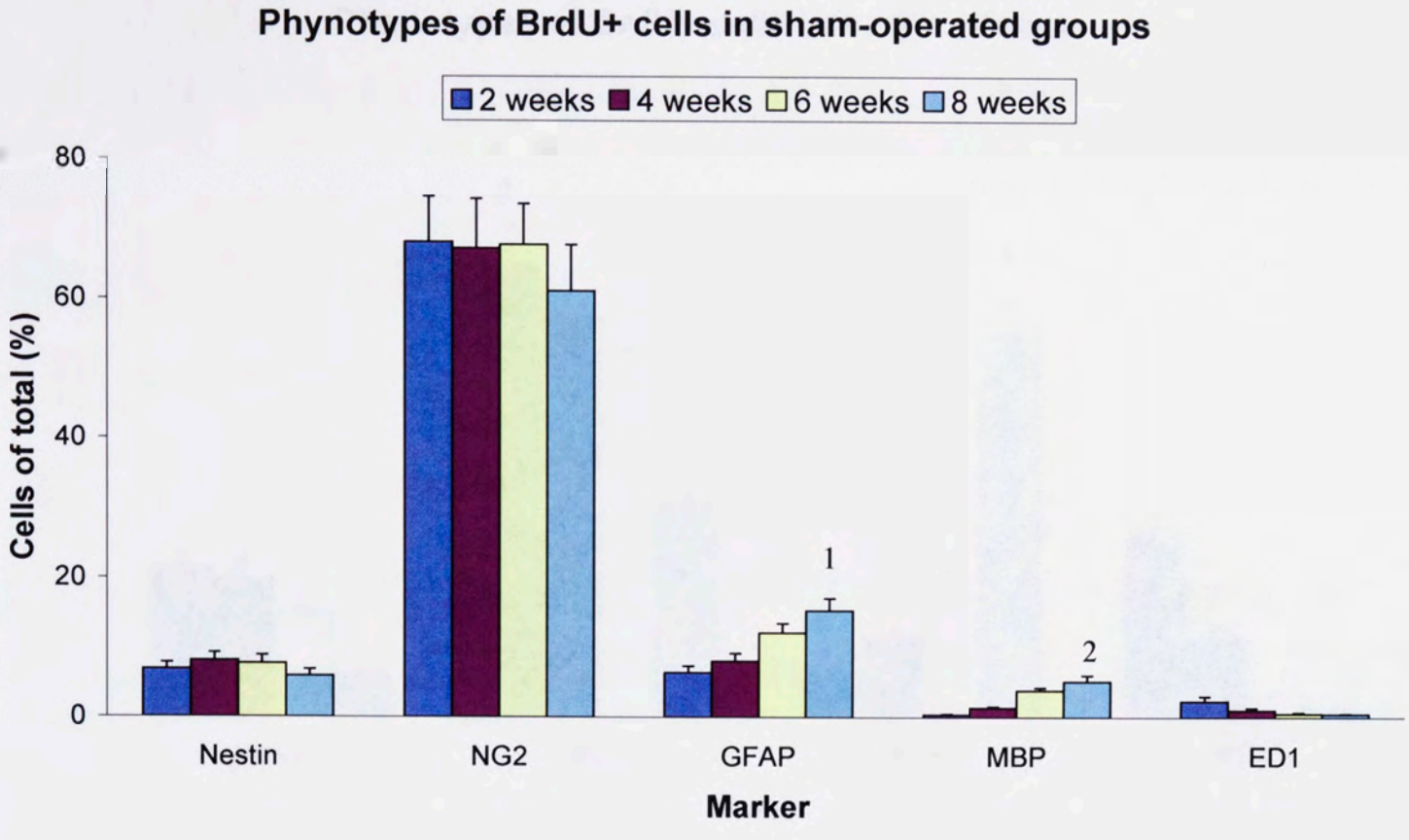

Figure 5-4. Percentages of cell markers (of all BrdU+ cells) in the sham-operated groups. Data presented as mean ± SE (n=4). $^{1}P < 0.05$ vs GFAP at 2 weeks; $^{2}P < 0.05$ vs MBP at 2 weeks. The percentages of progenitor cells (Nestin/BrdU+ and NG2/BrdU+) do not change significantly, while the proportions of glial cells (GFAP/BrdU+ and MBP/BrdU+) increase with time. The results are similar to those in the intact groups.

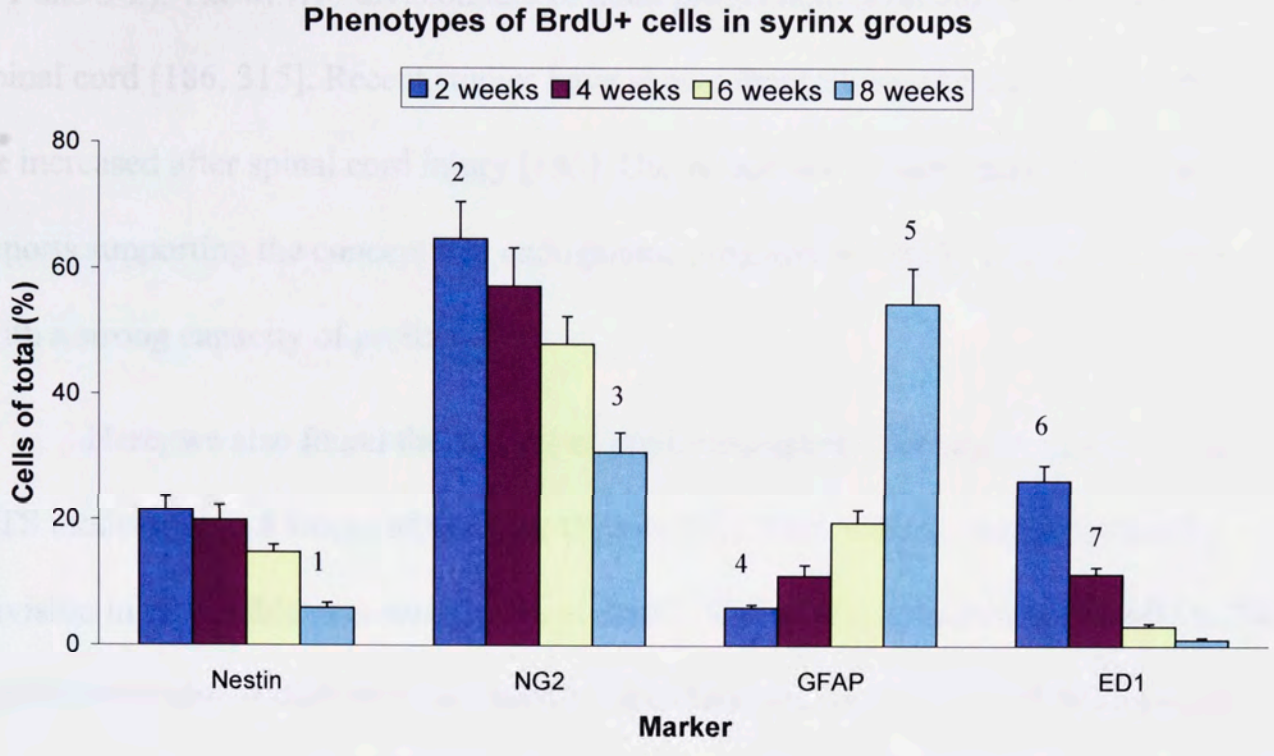

Figure 5-5. Percentages of cell markers (of all BrdU+ cells) in the syrinx groups. Data presented as mean ± SE (n=4). [1]P < 0.05 vs Nestin at 2 and 4 weeks; [2]P < 0.05 vs NG2 at 6 and 8 weeks; [3]P < 0.05 vs NG2 at 2, 4 and 6 weeks; [4]P < 0.05 vs GFAP at 6 and 8 weeks; [5]P < 0.05 vs GFAP at 2, 4 and 6 weeks; [6]P < 0.05 vs ED1 at 4, 6 and 8 weeks; [7]P < 0.05 vs ED1 at 6 and 8 weeks. The percentages of progenitors (Nestin/BrdU+ and NG2/BrdU+) decrease significantly with time in the PTS animals, while the proportion of astrocytes (GFAP/BrdU+) increases considerably. Over a quarter of BrdU+ cells are macrophages (ED1/BrdU+) at 2 weeks post injury and this percentage drops significantly with time.

5.4 Discussion

5.4.1 Proliferation of progenitors

Proliferation of endogenous progenitors in the intact and injured spinal cord has been reported by numerous researchers [179, 180, 182, 183, 186, 189, 191, 192, 349]. Here, we found that the majority of proliferating cells in the PTS model incorporate BrdU and co-express NG2 or Nestin, indicating that they are progenitors. The density of proliferating cells is much higher in the syrinx animals than the control animals (Figures

5-1 and 5-2). The *in vivo* division rate of adult progenitors is thought to be low in the spinal cord [186, 315]. Recent studies have shown that mitosis of adult progenitors can be increased after spinal cord injury [183]. Our results are in agreement with these reports supporting the concept that endogenous progenitors rapidly respond to injury with a strong capacity of proliferation.

Here, we also found the density of proliferating cells decreased sharply in the PTS model within 8 weeks after injury (Figure 5-2). This is likely due to rapid cell division in PTS, which causes dilution of BrdU. When the concentration of BrdU in the nuclei is too low, it will not be detected by the fluorescence microscope. In this study, the BrdU+ cell density in the syrinx animals decreased by around 86% within 8 weeks, while in the control animals, the proliferating cell density dropped 40% during the same period (Figure 5-1). In the previous study (Acute response of endogenous progenitors in the PTS model), we demonstrated that NPCs proliferate much faster in PTS than in control animals. This can explain why the proliferating cell density in the control animals decreased much slower. In this study, we again provide evidence that the proliferating cells divide faster in the PTS model than in normal animals.

5.4.2 Differentiation of progenitors

In various injury models, the differentiation of progenitors seems very different in different studies. Some researchers found the NPCs gave rise to both astrocytes and oligodendrocytes in contusion and demyelination models [191, 192, 321], but others believed that endogenous progenitors could only differentiate into astrocytes *in vivo* in different SCI models, including transection, hemitransection and various degrees of contusion [180, 181, 183, 184]. The reasons for these differences is unknown. Here, we

report that preogenitors give rise astrocytes, but not oligodendrocytes, in the adult rat model of PTS.

The astrocytes differentiated from progenitors may play important roles in the PTS model, including regulation of the microenvironment for progenitors to survive, proliferate and differentiate [203, 312]. Astrocytes induce and release vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), and neurotrophins, promoting proliferation of progenitors [129, 202, 219, 233, 350-352] and neurogenesis [352-354]. Astrocytes also produce some inflammatory mediators, which contribute to the regulation of micrenviroment. For example, chemokines (eg. CCL5 and CX_3CL1) are vital to the proliferation of progenitors [238-240] and interleukins (eg. IL-1β and IL-6) are important to the differentiation of progenitors [242, 243, 259, 260]. Apart from regulation of microenvironment, astrocyte may play some other roles in the PTS model, especially their association with the pathophysiology of PTS. For example, astrocytes and glial scar were observed to form the lining of syrinx cavities [12, 19, 20]. They may contribute to preventing further enlargement of syrinx by resisting higher pressure in the syrinx. Further studies are described in detail in the next chapeter, "Reactive astrocytes and gliosis in experimental PTS".

At present, our knowledge about the facts controlling the differentiation of progenitors *in vivo* is limited. It remains a puzzle why some studies reported that NPCs gave rise to oligodendrocytes while other studies did not, although these studies were performed using similar contusion models. In the current study, NPCs in the animals with laminectomy (sham-operated groups) differentiated into oligodendrocytes but those with injection of kaolin and excitatory amino acid did not. We assume that over production of excitatory aminoacid or subarachnoiditis or both may play important roles

in affecting the differentiation of NPCs. Demyelination is an important pathological feature of PTS and contributes to developping neurological dysfunction in patients with PTS [12, 19-21, 31, 32]. Oligondendrocytes differentiated from transplanted progenitors have been shown to repair demyelinated lesion in CNS [355, 356]. Recent studies showed that thyroid hormone induced the differentiation of oligodendrocytes *in vitro* and enhanced remyelination *in vivo* [357-360]. Administration of thyroid hormone to induce differentiation of oligodendrocyte from endogenous progenitors is a promising direction for future studies in our PTS model, which may help with improving neurological dysfunction. Other methods, such as activating basic helix-loop-helix protein [361], may also be explored in our PTS model to enhance the differentiation of oligodendrocytes.

5.5 **Conclusions**

Long term follow up study showed that the endogenous progenitors persist proliferating up to 8 weeks post injury in the PTS model. Endogenous progenitors differentiated into astrocytes, which may have an important role in regulating the microenvironment. No differentiation into oligodendrocytes was found in the PTS model, which may be due to over-production of excitatory amino acid. Further study is required to explore possible methods to enhance the differentiation of oligodendrocytes. The role of demyelination in PTS also needs to be examined.

Chapter Six Reactive Astrocytes and Gliosis in Experimental PTS

6.1 Introduction

Glial scar or reactive gliosis is a response to injury in the brain or spinal cord, whenever the CNS is damaged. The glial scar is composed primarily of reactive astrocytes and the extracellular matrix molecules of proteoglycans which are produced by reactive astrocytes [33-35]. Glial scars may have a double-edged nature in the damaged CNS. Reactive astrocytes and gliosis are found to be important for neuroprotection and self-repair. Following CNS injury, astrocyte active is often amongest the earliest responses that may limit cellular degeneration and restore the composition of the external environment as well as repair the blood-brain barrier. They produce and release growth factors, including NGF, BDNF, GDNF, FGF-2, TGFβ, VEGF, PDGF for synaptogenesis and neurogenesis [33, 35, 38, 362]. On the other hand, proteoglycans are shown to inhibit axonal regeneration [34, 363-365].

It has been reported that syrinx cavities are lined by glial tissue [12, 19, 20]. In a model of posttraumatic syringomyelia, cyst formation and subsequent enlargement occurred in a time dependent manner up to 42 days post-injury, then slowed or ceased by 84 days [25]. We hypothesize that this observation is likely due, in part, to an endogenous astrocytic response to injury or reactive gliosis that strengthens the cyst wall. In this study, we aim to examine the endogenous astrocyte response to injury and whether there is a temporal correlation between cavity development and gliosis.

6.2 Materials and methods

6.2.1 Animal groups

Animals were divided into intact (n = 24), and sham-operated (n = 24) controls as well as syrinx groups (n = 24) for the study. Animals in each group were equally subdivided into 6 time points: 1, 7, 14, 28, 42, and 56 days post-syrinx induction. Animals in the intact group had no surgery, while animals in the syrinx groups had syrinx induction procedures. Tissues used in this study were harvested from the rats involved in the other studies in the thesis. Briefly, tissues at 1 and 7 days PI in this study were from rats used in "Acute Response of Endogenous Progenitors in an Adult Rat Modle of PTS" and tissues at 2, 4, 6 and 8 weeks PI were from rats used in "*In Vivo* differentiation of Adult Endogenous Progenitors in PTS".

6.2.2 Labelling mitotic cells

Labeling method 1 (Figure 2-1): animals received an intraperitoneal injection of BrdU (10 mg/mL) at a dose of 50 mg/kg body weight 24 hours after the syrinx induction procedure. Rats were sacrificed 1 hour or 6 days after the injection.

Labeling method 2 (Figure 2-1): to achieve a higher availability of BrdU, animals received daily intraperitoneal injections of BrdU (same dose as in method 1) for 12 days following surgery in the sham-operated and syrinx groups. Twelve successive daily injections of BrdU were given to the intact controls. Rats were sacrificed 2, 16, 30, or 44 days after the last injection.

6.2.3 Immunohistochemistry

Two primary antibodies were used in this study. They were Anti-GFAP (mouse monoclonal, 1:4000) and Anti-BrdU (sheep polyclonal, 1:200). The secondary

antibodies were: ALEXIA Fluor 594 (goat anti-mouse, 1:800) and ALEXIA Fluor 488 (donkey anti-sheep IgG, 1:800).

6.2.4 Microscopic and statistical analysis

The number of BrdU positive cells per section was counted using a fluorescence microscope and digital camera using AxioVision image analysis software (AxioVision 4.2, Carl Zeiss, Germany). The size of syrinx and area of spinal cord section were also measured by using AxionVision software.

Data are expressed as mean ± SE. Differences among groups were evaluated by one way ANOVA. Linear regressions were calculated by using the statistical computer package, Number Cruncher Statistical Systems [337]. A value of $P < 0.05$ was considered statistically significant.

6.3 Results

6.3.1 Syrinx formation and temporal profile

Syrinx formation was observed as a cyst or a loose mesh of glial fibers. The temporal profile of syrinx formation and enlargement is shown in Figure 6-1. All animals in the experimental group developed a syrinx by 7 days. The average syrinx size at day 7 was 2.15 + 0.41% of the cross-sectional area at the C8 level (Figure 6-2). Syrinxes progressively enlarged up to 42 days. There was a positive correlation between cyst size and time up to 42 days ($r = 0.96$, $p < 0.01$). At 56 days, cyst size was 70% that at 42 days. No control animal developed a syrinx.

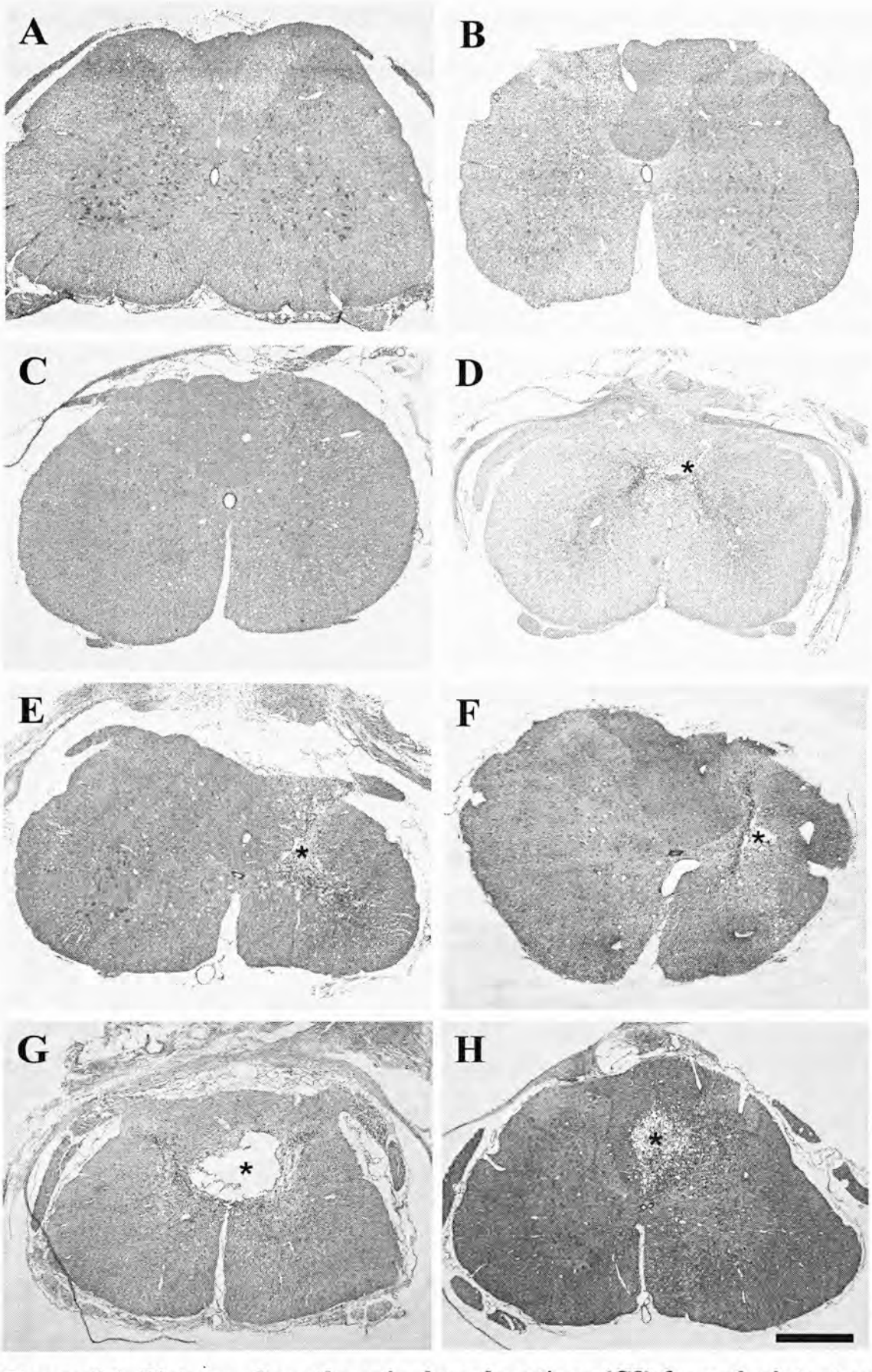

Figure 6-1. Pictures show the spinal cord sections (C8) from the intact animals, sham-operated animlas and syrinx animals at different time points. A: Spinal cord section from an intact animal. B: Spinal cord section from sham-operated animal 42 days PI. C: Spinal cord section from syrinx animal 1 day PI. No obvious syrinx is found at this time point. D: Spinal cord section from syrinx animal at 7 days PI.

A small syrinx can be identified (*). E: Spinal cord section from a syrinx animal 14 days PI. F: Spinal cord section from a syrinx animal 28 days PI. G: Spinal cord section from a syrinx animal 42 days PI. H: Spinal cord section from a syrinx animal 56 days PI. Scale bar = 500μm.

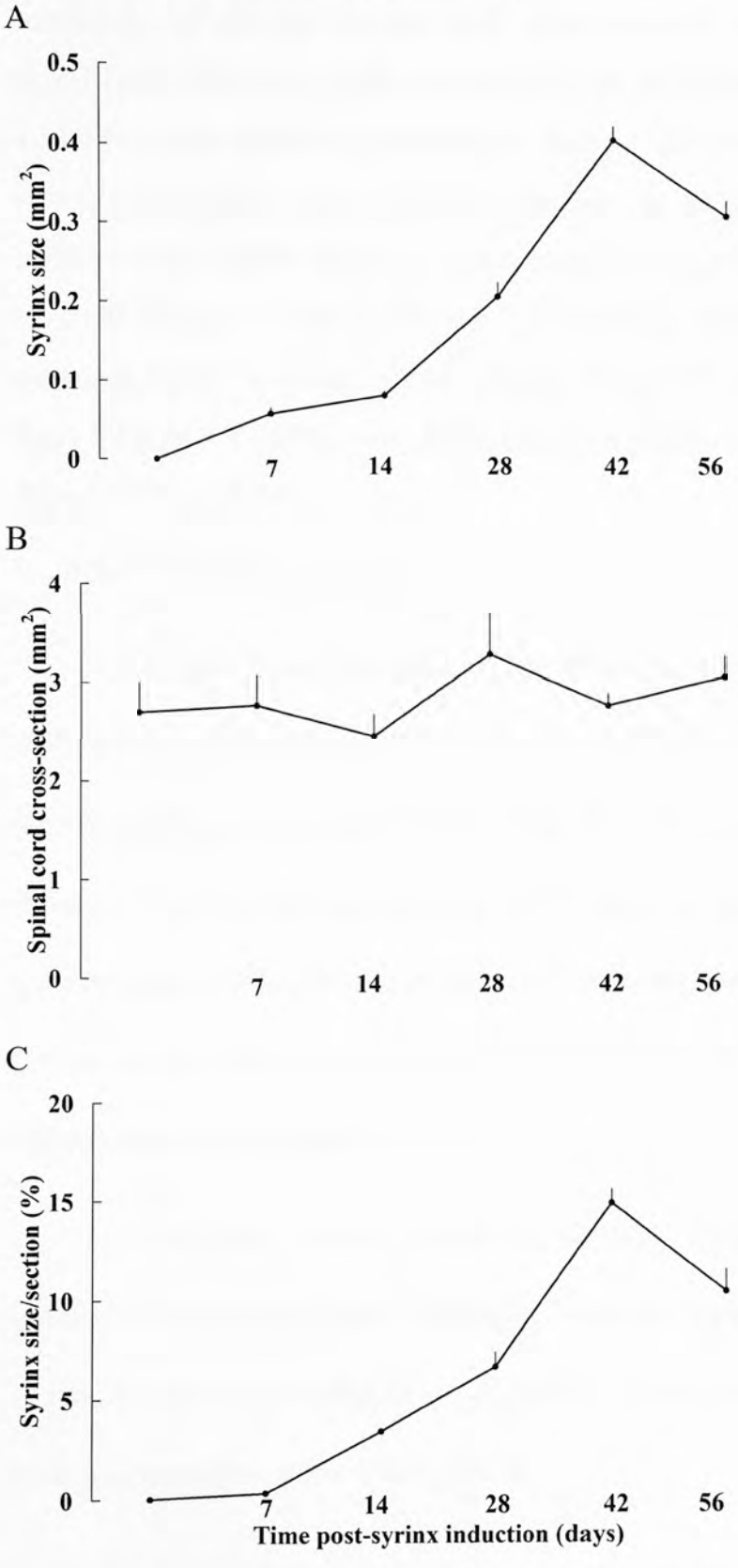

Figure 6-2. Correlation between syrinx size and time from 7 to 56 days post-syrinx induction. A: Syrinxes progressively enlarged up to 42 days. There was a positive

correlation between cyst size and time, up to 42 days ($r = 0.98$, $p < 0.01$), up to 56 days ($r = 0.88$, $p < 0.03$). There was a significant increase in cyst size between 28 and 42 days PTS ($p = 0.002$), and a significant reduction in cyst size between 42 and 56 days PTS ($p = 0.006$). B: Cross-sectional areas of spinal cords remained constant, and did not change with time over a periods of 56 days ($r = 0.46$), and no significant difference between time points. The number of animals was 4 per group, and 44 in total including the intact, sham-operated, and syrinx groups. C: Syrinx size is expressed as the % of the total cord cross sectional area. There was a positive correlation between cyst size and time, up to 42 days ($r = 0.96$, $p < 0.01$), up to 56 days ($r = 0.85$, $p < 0.05$). There was a significant increase in cyst size between 7 and 14 days PTS ($p < 0.05$), 14 and 28 days PTS ($p < 0.02$), 28 and 42 days PTS ($p < 0.0001$), and a significant reduction in cyst size between 42 and 56 days PTS ($p < 0.01$).

6.3.2 Reactive astrocytes

In intact control animals, astrocytes demonstrated the typical morphological features of small cell bodies with long slender processes (Figure 6-3). They were located in the pial zone and white matter, their long fine processes extending towards the grey matter. In the grey matter, GFAP-immunopositive cells showed short processes with more thorn-like side branches, a characteristic of protoplasmic astrocytes (Figure 6-4 A). In the white matter, most GFAP-positive cells morphologically resembled fibrous astrocytes (Figure 6-4 B).

In the syrinx group, the number of GFAP-positive cells increased significantly within 24 hours post-syrinx induction. These cells had thick processes, a characteristic of reactive astrocytes (Figure 6-4 C and D). Most of them were observed in the pial zone and throughout the white matter.

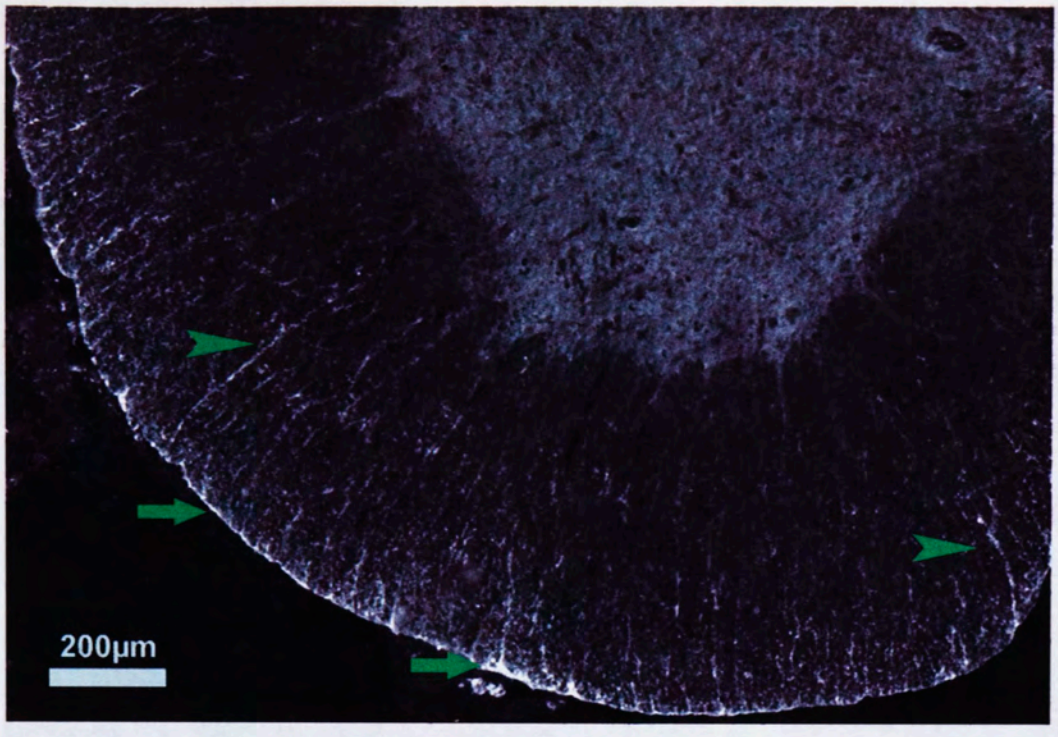

Figure 6-3. GFAP staining in the spinal cord section (C8) from an intact animal. Astrocytes have predominantly small cell bodies with long slender processes. They were located mainly in the pial zone (arrows), with long processes extending from the pial zone towards the grey matter (arrowheads). Scale bar = 200µm.

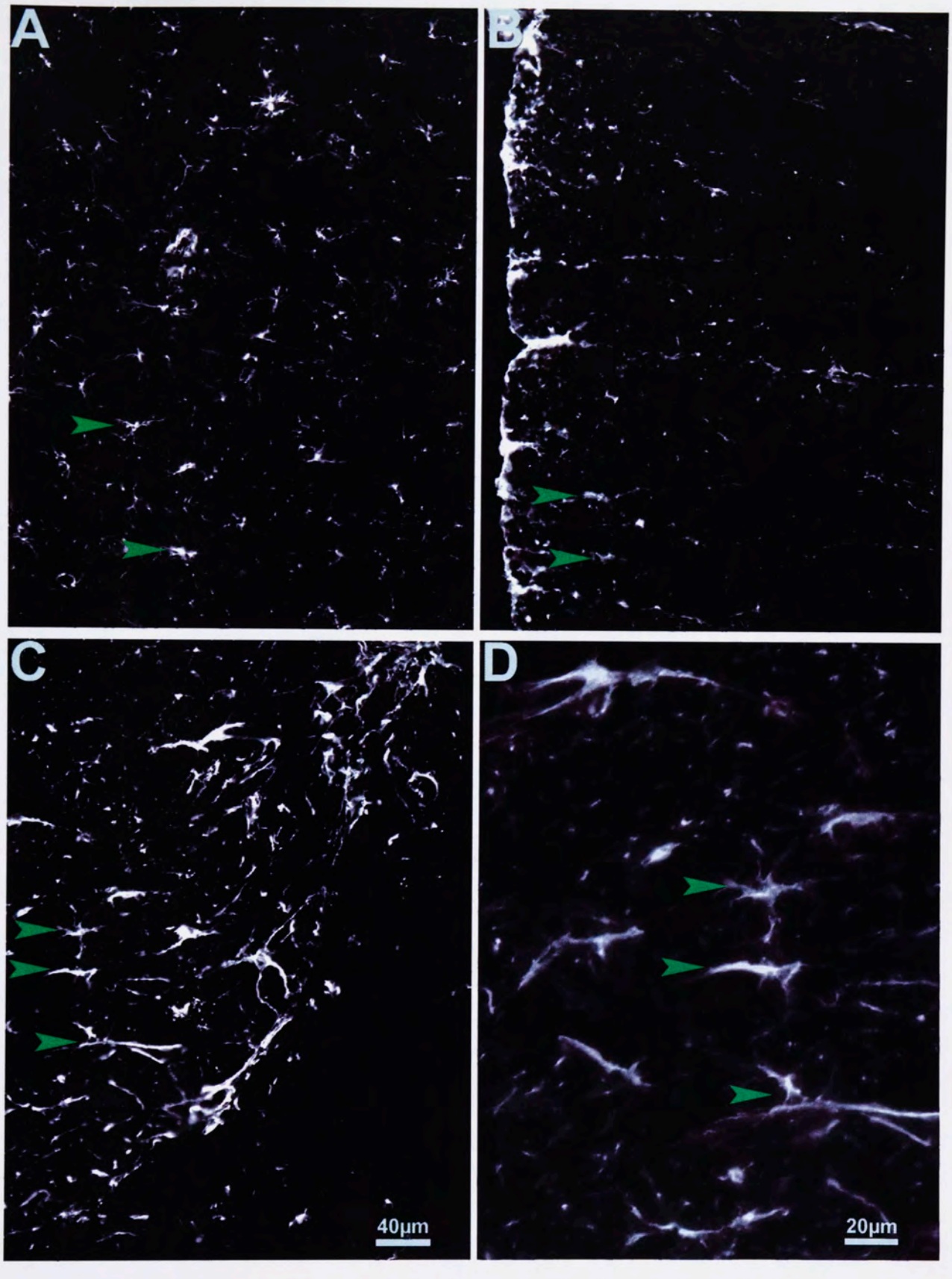

Figure 6-4. GFAP staining in the spinal cord section (C8) from the intact animlas and syrinx animals. A: GFAP immunoreactive cells in the grey matter in intact animals, showing morphological characteristics of protoplasmic astrocytes (arrowheads). B: Intact animals demonstrated fibrous astrocytes along the pial zone and in the surrounding tissue of the white matter (arrowheads). C and D: Syrinx animals showed reactive astrocytes with small cell bodies with short and

thick processes (arrowheads). Scale bar (A, B and C) = 40μm. Scale bar (D) = 20μm.

More BrdU-expressing proliferating cells were observed in the syrinx group with counts of BrdU-positive cells being 92 ± 5, 108 ± 6, and 655 ± 53 cells/mm^2 at 1, 7, and 14 days, respectively, before being reduced by half at 28 days, 255 ± 30, and 97 ± 19 cells/mm^2 at 42 and 56 days, respectively. Density of BrdU positive cells in syrinx animals is shown in Figure 6-5 A and B.

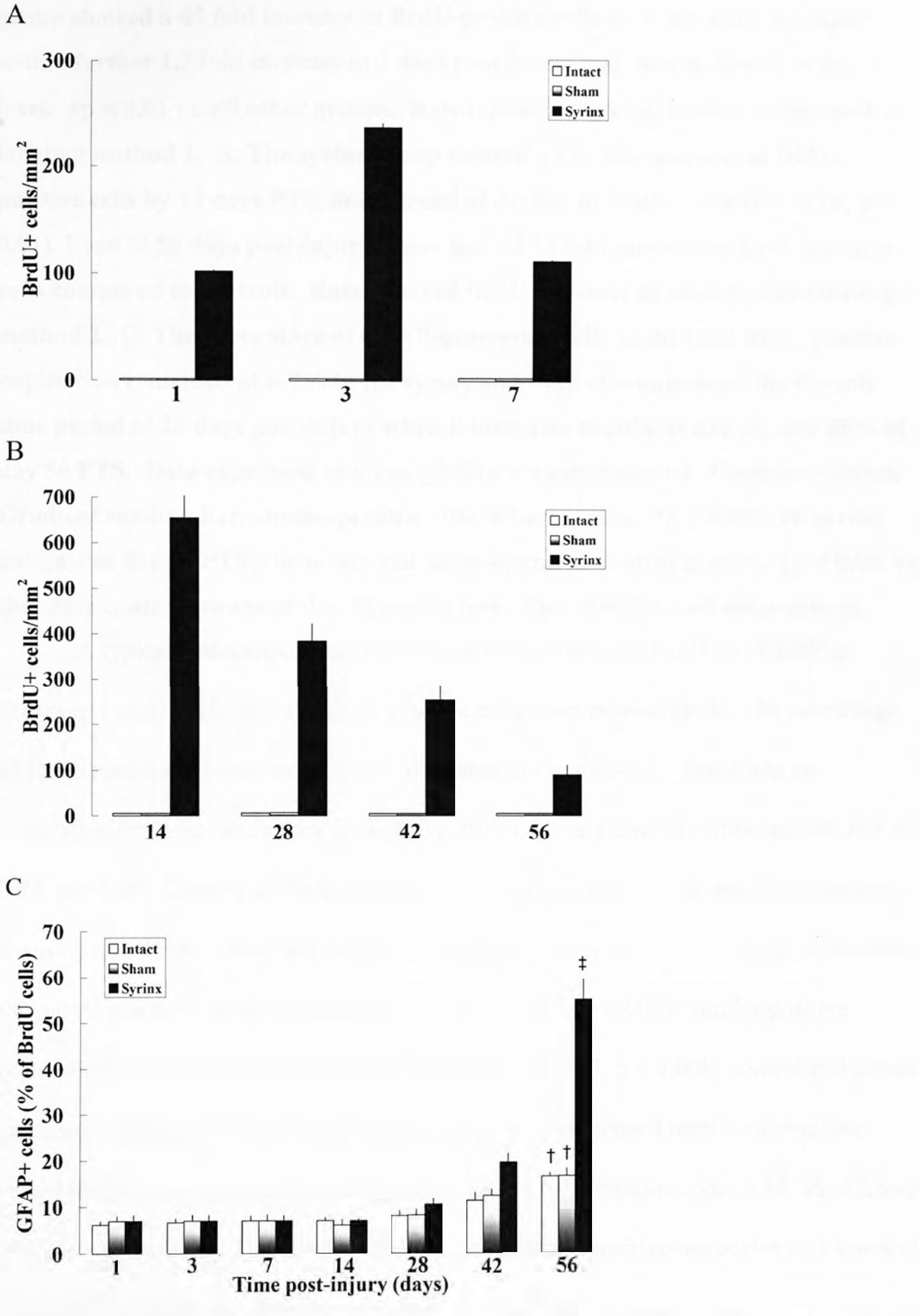

Figure 6-5. Quantification of BrdU-positive cells in the control and syrinx groups at periods from 1 -56 days post-injury; and the number of GFAP expressing cells as a percentage of the total BrdU+ cells at the same time periods. A: The syrinx

group showed a 65 fold increase in BrdU-positive cells by 1 day after the injury with a further 1.3 fold increase at 3 days post-lesion and then declining to day 1 level. *p < 0.01 vs. all other groups. Rats received BrdU injection as described in labeling method 1. B: **The syrinx group showed a 114 fold increase in BrdU-positive cells by 14 days PTS, and a trend of decline in BrdU+ cells ($r = -0.96$, $p < 0.01$). Even at 56 days post-injury, there was an 18 fold increase in BrdU-positive cells compared to controls. Rats received BrdU injection as described in labeling method 2.** C: **The percentage of GFAP-expressing cells in the total BrdU-positive population remained at 6-7% in the syrinx and control groups until the chronic time period of 28 days post-injury when it increases to 20% at day 42, and 55% at day 56 PTS. Data expressed as mean ± SE (n = 4 experiments). Open bar: intact. Gradient shading bar: sham-operated. Black bar: syrinx. *p < 0.005, vs. syrinx animals at day 28 PTS, the intact and sham-operated control groups. †p < 0.05, vs. the same control groups at day 42 post-injury. ‡p < 0.005, vs. all other groups.**

A typical endogenous reactive astrocyte co-expressing BrdU and GFAP is shown in Figure 3-19. Not all GFAP positive cells co-expressed BrdU. The percentage of BrdU positive reactive astrocytes is indicated in Figure 6-5 C. There was no correlation between the density of BrdU positive cells and time in syrinx animals ($r = -0.74$, $p = 0.09$). Density of BrdU positive cells was consistent in the intact control and sham-operated rats. There was a positive correlation between the percentage of reactive astrocytes and time in the intact control rats ($r = 0.94$, $p = 0.006$). Similar positive correlations were observed in the sham-operated ($r = 0.93$, $p = 0.008$) control and syrinx groups ($r = 0.86$, $p = 0.03$). The average number of endogenous reactive astrocytes varied in the syrinx group, being 46, 34, 50, and 53 cells/cross-section at 14, 28, 42, and 56 days, respectively. The number of endogenous BrdU positive astrocytes was low and relatively consistent in controls, being 6-9 cells per cross-section.

Location of astrocytes

Increased expression of GFAP was observed in the experimental group at 1 day after syrinx induction (Figure 6-6 A). Reactive fibrous astrocytes were located predominantly in the pial zone, and extended throughout the white matter towards the grey matter. Reactive protoplasmic astrocytes were located at the edge of the grey matter (Figure 6-6 B). Seven days after syrinx induction, reactive fibrous astrocytes were located at the border between the white matter and grey matter on the white matter side (Figure 6-6 C). GFAP expression was less along the pial zone and the surrounding white matter than at 1 day. Reactive protoplasmic astrocytes were on the grey matter side of the border (Figure 6-6 D). Compared to 1 day post syrinx induction in Figure 6-6 B, reactive protoplasmic astrocytes were located away from the centre of the grey matter (Figure 6-6 E and F).

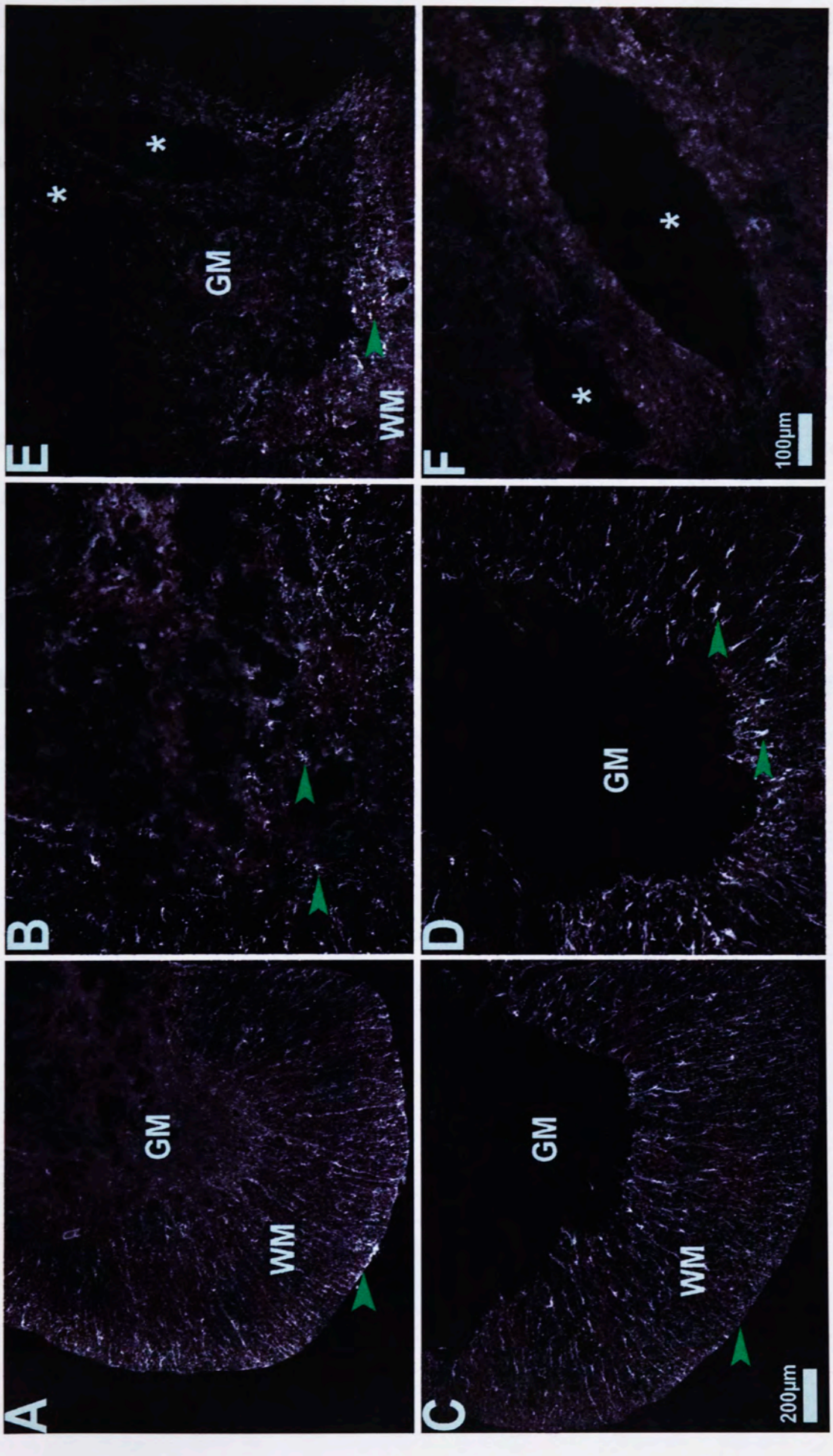

Figure 6-6. Migration of reactive astrocytes 1 (A and B) and 7 days (C, D, E and F) post-syrinx induction. A: Syrinx animals showed an induction of GFAP expression

throughout the white matter (WM), long slender processes of fibrous astrocytes extended towards the grey matter (GM) and the pial zone showed intense staining (arrowhead). B: Protoplasmic astrocytes gathered at the edge of the GM. Reactive astrocytes (arrowheads) showed stronger GFAP staining in the periphery of the GM than the central part of the GM. C: GFAP positive fibrous astrocytes accumulate in the WM near the GM (arrowhead). D: Reactive astrocytes accumulated at the border between the WM and GM (arrowheads). E: Multiple small cysts (*) are apparent in the GM. There were GFAP positive reactive astrocytes near the border between WM and GM (arrowhead), with few in the GM. F: There was no GFAP positive reactive astrocyte around the cysts (*). Scale bar (A, C and E) = 200μm. Scale bar (B, D and F) = 100 μm.

Glial scar formation and thickening is shown in Figure 6-7. A thick layer of hypertrophic astrocytes surrounded the entire syrinx at day 14 (Figure 6-7 A and B). A densely packed glial scar surrounded the syrinx at day 28 (Figure 6-7 C). A glial scar surrounded syrinxes at day 56 (Figure 6-7 D and E).

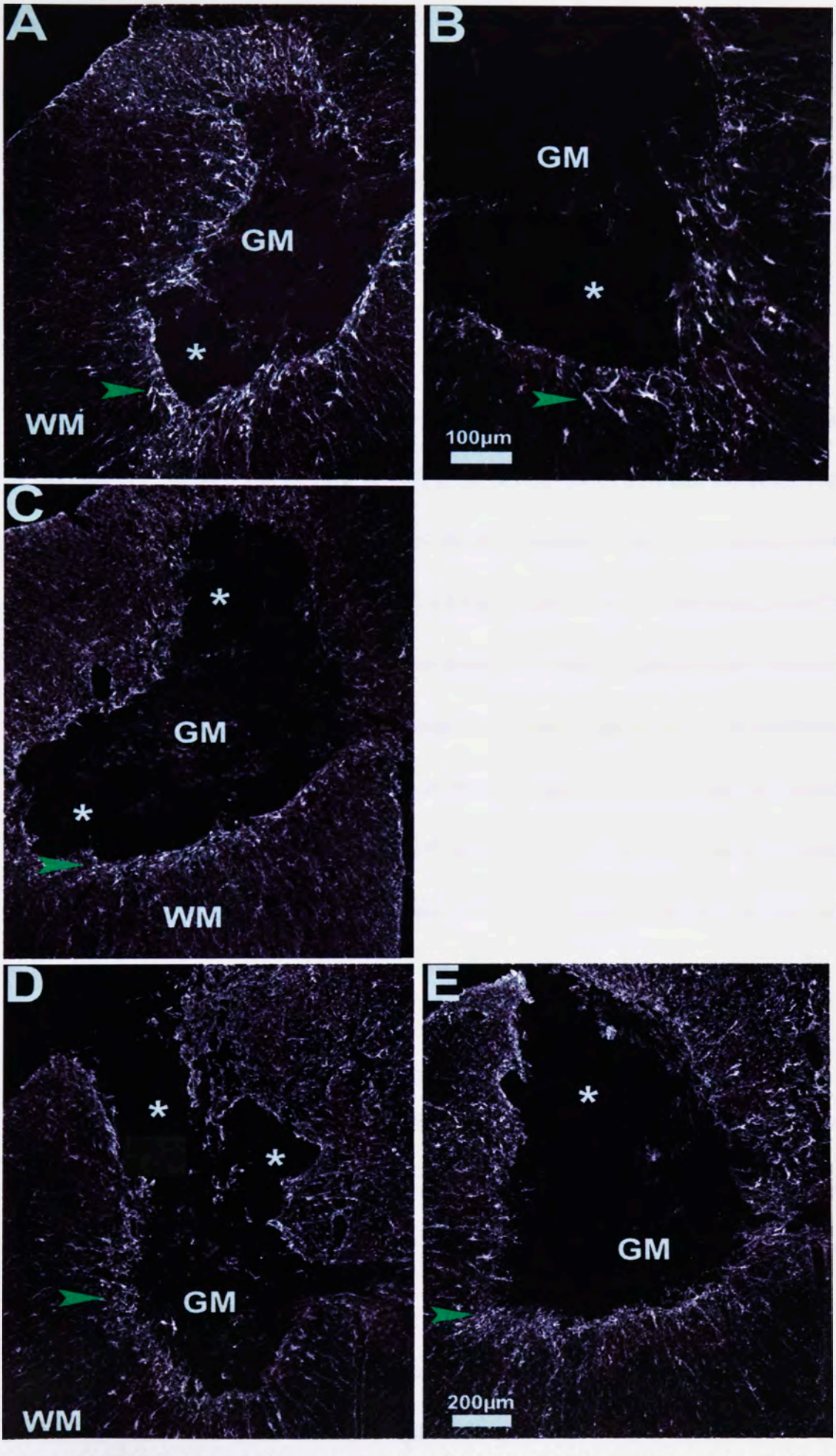

Figure 6-7. Glial scars in syrinx animals 14 to 56 days post-syrinx induction. A: Reactive astrocytes formed a glial scar barrier (arrowhead) at the border between

the WM and GM. B: Tissue loss and formation of cysts (*) were observed in the GM. Reactive fibrous astrocytes (arrowhead) accumulated at the border of the WM side. C: By day 28, a glial scar (arrowhead) surrounded the GM, and cysts (*) enlarged. D and E: by day 56, a glial scar (arrowhead) surrounded a loose mesh of glial fibers in the GM, and a cyst (*) extended towards the WM. Scale bar (A, C, D and E) = 200μm. Scale bar (B) = 100μm.

6.4 Discussion

In our animal model of post-traumatic syringomyelia [26, 54], there is a period of cyst enlargement followed by stability, or even a reduction in cyst size. The histopathological features of this model closely resemble those of human syringomyelia: the syrinxes are extracanalicular and lined by gliotic tissue rather than ependymal cells [12, 26]. We hypothesize that gliosis produced by reactive astrocytes lining post-traumatic syrinxes acts as a limiting factor for syrinx expansion. The purpose of this study was to examine the proliferation of reactive astrocytes and gliosis formation adjacent to syrinxes in the animal model and determine whether the formation of a glial scar lining the syrinx cavity corresponded with the stage of stability or cyst size reduction.

6.4.1 Reactive astrocytes

Following syrinx induction, there was a rapid proliferation of endogenous progenitor cells within the spinal cord (Figure 6-5 A and B). Up to 55% of these differentiated into astrocytes that were demonstrated with positive staining for GFAP (Figure 6-5 C). Hypertrophic reactive astrocytes can be identified by an increase in expression of GFAP [321, 366]. Up-regulated GFAP expression in response to syrinx induction was observed in both protoplasmic and fibrous astrocytes within 24 hours in this model. These astrocytes subsequently became hypertrophic or reactive. Reactive

astrocytes are the principle cells responding in a non-specific manner to CNS injuries. After trauma, they increase expression of intermediate filaments (such as GFAP) and proliferate. Fibrous astrocytes increase the length of their processes and protoplasmic astrocytes increase the number of their process side branches. One of the known functions of reactive astrocytes is removal and metabolism of glutamate, the principle excitatory transmitter, from the vicinity of firing neurons.

6.4.2 Astrocyte location

Astrocytes are able to sense changes in the CNS and react by migrating towards a lesion [184, 185]. In this study, reactive fibrous astrocytes migrated from the pial zone, crossing the white matter towards the injury site (Figure 6-6). Interestingly, reactive protoplasmic astrocytes moved away from the region of injury towards healthy tissue. The astrocytes gathered and merged on the border between surviving healthy and damaged grey matter to form a glial scar (Figure 6-7). Glial scar is different from fibroblast scar, as it does not consist of collagen. Glial scar is formed by the cytoplasmic extensions of the reactive astrocytes themselves.

6.4.3 Glial scar and PTS

In contrast to the response of other tissues to injury, death of CNS tissue can result in a cyst or a loose mesh of glial fibers. Formation of a cyst at the site of spinal cord injury is thought to be the first stage in post-traumatic syrinx formation. This initial cyst formation is believed to be caused by resolution of hematoma, infarction, inflammation, or the overproduction of excitatory amino acids after trauma [48-51]. In our model, an excitatory amino acid triggers neuronal death, inflammation, and contributes to initial cyst formation [25, 26]. A subsequent imbalance between CSF inflow and outflow causes syrinx enlargement [54-58]. After the phase of enlargement,

there is stabilization, or even reduction, in syrinx size. Once formed, glial scar may inhibit further syrinx enlargement by acting as a barrier to inflammatory cell migration and further tissue damage [46, 367, 368]. The scar may also increase the syrinx wall tensile strength, limiting further enlargement.

Additional potential roles of reactive astrocytes in syringomyelia include the regulation of the microenvironment for progenitors to survive, proliferate and differentiate [203, 312]. Reactive astrocytes induce and release vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), and neurotrophins. These trophic factors promote proliferation of progenitors [129, 202, 219, 233, 350-352] and neurogenesis [352-354]. Apart from growth factors, reactive astrocytes also produce some inflammatory mediators, which contribute to the regulation of micrenviroment. It has been reported that chemokines (eg. CCL5 and CX_3CL1) are vital to the proliferation of progenitors [238-240] and interleukins (eg. IL-1β and IL-6) are important to the differentiation of progenitors [242, 243, 259, 260]. Manipulation of reactive astrocytes to express stem cell regulators, such as those growth factors, chemokines and interleukins, can be an important part of the future stem cells therapy to treat PTS by building an ideal microenviroment in the injured spinal cord.

The aetiology of PTS in human is not clear. The pathological features of human PTS are similar to those found in our PTS model. We assume that similar 2 steps are involved in the formation of human PTS. They are the initial cyst formation and the enlargement of the syrinx (details refer to Chapter One: Introduction). The enlargement of syrinx is thought to be caused by increased pressure in the syrinx due to CSF inflow [5, 10, 48, 49, 54, 56-58, 369]. The high pressure overcomes the resistance from glial

scar and pushes glial scar and its surrounding tissue backwards, leading to the syrinx enlargement.

It is reasonable to assume that the syrinx enlargement may be limited or even stopped if the gliosis lining the syrinx is strong enough to resist against the pressure in the syrinx. Thus, a strategy of enhancing the gliosis formation in the early stage after SCI should be considered. This strategy potentially not only limits the syrinx enlargement but also restricts the infiltration of inflammatory cells. Further studies can be designed to examine the effects of reactive astrocytes and gliosis. PTS model can be developed in genetically modified animals, in which the cell division of reactive astrocytes is ablated, such as GFAP-TK mice [370]. The size of syrinx and neurological function can be compared between them and normal PTS animals (with normal reactive astrocyte division).

Stem cell therapy using endogenous NSCs is a possible direction to fulfil the strategy of enhancing the gliosis formation. Some factors, such as bone morphogenetic protein, Notch and interleukin-6, have been found to guide the NSCs to differentiate into glial cells [234, 235, 237]. Further studies are necessary to examine the PTS model for pathological changes of syrinx and possible improvement of neurological function after delivering the above factors into the lesion.

6.5 Conclusions

This study demonstrates activation, proliferation and migration of endogenous astrocytes in post-traumatic syringomyelia. The development of a glial scar corresponds with the stage of cyst stability or reduction in size. Our findings suggest that one role of reactive gliosis is to seclude the injury site from healthy tissue, preventing a cascading wave of uncontrolled tissue damage.

Chapter Seven Summary and Future Directions

7.1 Summary

7.1.1 Proliferation of endogenous progenitors in PTS

Our knowledge about endogenous progenitors in the adult spinal cord is poor and no study has been reported about NPCs in PTS. Endogenous progenitors, including multipotential progenitors and glial restricted progenitors, exist in PTS from the acute stage up to 8 weeks post injury. These NPCs respond to injury by proliferating as early as 24 hours post injury. A large number of progenitors appear in PTS at this time and their number is around 30 times larger than that in the control animals. Moreover, these NPCs proliferate much faster in PTS than in normal conditions. It takes NPCs less than 2 days to double their number in PTS, while the NPCs in control animals require at least 6 days doubling their number. With time, newly divided progenitors continuously appear in PTS and their number is stable at the same level as that in the acute stage (around 30 times larger in PTS groups than in control groups) at least 2 weeks post injury. The proliferation of progenitors decreases significantly from 4 weeks after injury, but their number is still around 10 times that in the control animals. This is the first report about the proliferation of endogenous progenitors in the PTS model. It also extends our knowledge in the biology of NPCs under pathological condition.

It is hard to measure the proliferation of NPCs in PTS accurately, because cell death can not be ruled out. Moreover, we are unable to measure the lifespan of these proliferating NPCs in this *in vivo* study. A possible way to solve the above problem is to develop new strategies to mark cell division separately.

7.1.2 Location of endogenous progenitors in PTS

The major stem cell pool in the adult spinal cord is located throughout the white matter, especially the areas close to the pial zone [186]. In the PTS model, the majority of the endogenous progenitors appear in the white matter at the very acute stage, which is similar to that under physiological conditions. Endogenous NPCs do not change their position obviously under physiological conditions. In comparison with that, the majority of progenitors in PTS appear in and around the lesion site shortly after injury. We are not able to decide whether the above changes are caused by movement of the progeny of the NPCs only. It is possible that the mother cells also move towards the lesion site, although there is no evidence to support it. However, it is clear that some progenitors migrate to the lesion site after injury.

After the acute stage, the endogenous stem cell pool in PTS is located in the right grey matter, where the lesion is. This change of location of stem cell pools between acute and chronic stages has not been reported before. At least two possibilities need to be considered. One is that the whole stem cell pool moves to the lesion site. The other is that the daughter cells migrating from the original stem cell pool keep proliferating and play a major role in the chronic stage, while the proliferation of mother cells slows down. Again, the techniques to mark mother and daughter cells separately will be very helpful to answer the above questions.

7.1.3 Differentiation of endogenous progenitors in PTS

No endogenous neurogenesis has been found in the adult spinal cord *in vivo*, while differentiation from endogenous NPCs to oligodendrocytes still remains controversial. Our one week follow-up phenotype study did not find differentiation into

oligodendrocyte or neurons, nor did the 8 week follow up study. Thus, it is most likely that NPCs in PTS do not give rise to oligodendrocytes or neurons.

The factors controlling the differentiation of stem cells are unknown. An unsuitable local environment is thought to be a major reason. However, intrinsic factors of endogenous progenitors may also play an important role.

7.1.4 Reactive astrocytes and gliosis in PTS

Gliosis is composed primarily of reactive astrocytes and the extracellular matrix molecules of proteoglycans, which are produced by reactive astrocytes [33-35]. They are mentioned in almost every histological study involving CNS injury, including PTS, but glial scar and reactive astrocytes have not been studied in detail in PTS.

Glial scar formation is comprised of three stages in PTS. The first stage occurs soon after injury. The upgrade of GFAP throughout the spinal parenchyma, especially along the pial zone and surrounding the white matter, is the marker of this stage. The next stage occurs around 1 week after injury. The astrocytes become hypertrophic and migrate to the edge of the lesion site during the second stage. In the last stage, densely packed reactive astrocytes form a thick glial scar surrounding the damaged tissue around 2 weeks post injury. This study extends our knowledge about pathological changes in PTS.

Reactive astrocytes and gliosis are thought to have double edged roles after CNS injury. Glial scar may inhibit the axonal regeneration, which is the major negative side of its double edged nature. Except for that, reactive astrocytes and gliosis may have an important function of neuroprotection in PTS by limiting the injury, regulating inflammation and clearing excitatory amino acids. Furthermore, reactive astrocytes can produce various growth factors and may regulate the local environment for endogenous

regeneration. Also, the glial scar lining the syrinx may help to resist the pressure in the syrinx, which propably contribute to limiting the syrinx enlargement.

7.2 **Future investigations**

The current work might lead to a few areas of investigation. New methods need to be explored to improve the technique of using mitotic markers. This is vital for further study of endogenous progenitor biology *in vivo*, such as measuring the proliferation rate and lifespan of NPCs. The local environment is extremely important to endogenous progenitors, in terms of proliferation, migration and especially differentiation. Further studies should be done to find a suitable environment. This can be carried out at both the protein or molecular level (using various growth factors) and cellular level (using different cells, such as reactive astrocyte). Both *in vivo* and *in vitro* studies should be considered. *In vitro* studies, such as culturing endogenous progenitors from PTS, will provide a controlled condition to test different candidates. *In vivo* studies, such as delivering growth factors and transplantation of genetic modified stem cells which can produce growth factors to the rats with PTS, are the basis of future stem cell therapy in humans.

More studies are also required to further assess the roles of glial scars in PTS. Whether the glial scars help to resist fluid flow into the syrinx remains unknown. Further studies are required to solve it.Studies are also required to observe the PTS animals in a longer term after adopting measures to harness the reactive astrocytes. By comparing the histological features of syrinxes and possible functional recovery, we may have more direct evidence to develop the possible new therapeutic strategy to treat PTS by harnessing reactive astrocytes.

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