Enhancing spinal cord repair with connexin43 antisense oligodeoxynucleotides

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Abstract

Spinal cord injury is comprised of the initial injury and the secondary effects including injury spread, swelling, inflammation, and scarring. Connexin43 (Cx43) gap junctions and hemichannels contribute significantly to spinal cord injury by remaining open in dying cells, spreading toxic substances to neighbouring cells and the surrounding extracellular environment, and exacerbating inflammation. Peripheral nerve grafts and self-assembling IKVAV-PA nanoparticles have been used successfully to promote axon outgrowth from damaged spinal cord but such regeneration is limited by new scarring resulting from the intervention itself. We have investigated the effect of Cx43 down-regulation in preventing the onset of secondary injury spread and enhancing these spinal cord injury repair strategies by promoting axon regeneration and behavioural improvements. An ex vivo spinal cord segment culture model was established using rat spinal cord segments cultured for five days. The segments were treated with Cx43 antisense oligodeoxynucleotides (Cx43 AsODN) resulting in viable, organotypic, air-liquid interface cultures that were then used for repair studies. Fresh peripheral nerves were grafted into the spinal cord segments with Cx43AsODN applied concomitantly, followed by five days of culture and subsequent processing. Results showed that spinal cord segments cultured in the presence of Cx43 AsODN had significantly less swelling than control spinal cords, indicating the efficacy of this treatment in reducing initial cellular and tissue swelling. Improved neuronal survival, tissue preservation and axon regeneration from the injured spinal cord segments into the grafted peripheral nerve was seen in this Cx43 AsODN treated organotypic culture model. In vivo peripheral nerve grafting and Cx43 AsODN treatment was then carried out following complete transection injury in adult rats; axonal regeneration and behavioural improvement was assessed. Cx43 AsODN promoted rostral axon regeneration and behavioural improvement, and reduced the extent of the lesion. The physical properties of self-assembling IKVAV-PA nanoparticles were then investigated in vitro. The nanoparticles were found to be suitable as a stable and injectable implant material. Cx43 AsODN was combined with the IKVAV-PA gel implant and applied following an in vivo complete transection injury and behavioural and axon regeneration assessments undertaken. Although the complete transection model proved difficult, long term behavioural improvement was evidenced with signs of enhanced axon regeneration. In conclusion,
Cx43 AsODN treatment may offer an innovative combination therapy to enhance other spinal cord repair strategies that are applied at later time points following injury.
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Glossary

BBB: Basso, Beattie, Bresnahan locomotor rating scale

Cx43 AsODN: Connexin 43 antisense oligodeoxynucleotides

CMFDA: 5-chloromethylfluorescein diacetate

DAPI: 4',6-diamidino-2-phenylindole

EDTA: Ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

FR: Fluoro-ruby

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFAP: Glial Fibrillary Acidic Protein

HBSS: Hank’s Balanced Buffer Solution

IKVAV-PA: Peptide amphiphile nanoparticles with the laminin motif IKVAV

MEM: Minimum Essential Medium

OSP: Oligodendrocyte Specific Protein

PBS: Phosphate Buffered Saline

PN: Peripheral nerve

PVDF: Polyvinylidene fluoride

SCI: Spinal cord injury

TBS-T: Tris Buffered Saline with Tween-20
Chapter 1. Introduction

1.1. Introduction to spinal cord injury

Spinal cord injury (SCI) affects approximately 12,000 people per year in the US alone, with a current average age of 40.2 years at the time of injury; approximately 80% of victims are male (National Spinal Cord Injury Statistical Center, 2009). SCI is caused mainly by motor vehicle crashes (42.1%), falls (26.7%), violence (15.1%), sports (7.6%), and others (8.5%) (National Spinal Cord Injury Statistical Center, 2009). SCI is classified by the neurological level of injury and completeness of injury (Jallo & Vaccaro, 2009). The neurological level of injury corresponds to the most caudal segment of the spinal cord with normal motor function. The completeness of injury refers to either a complete lack of function or presence of function in the lowest segment (Jallo & Vaccaro, 2009). Cervical injuries (cervical level one, C1 to thoracic level one, T1) comprise approximately 55% of all SCI (Jallo & Vaccaro, 2009). C5 is the most commonly injured level (Jallo & Vaccaro, 2009). SCI usually results in paraplegia (paralysis of the lower body) after thoracic injuries or tetraplegia (paralysis of both the upper and lower body) after cervical injuries, and a lower life expectancy (National Spinal Cord Injury Statistical Center, 2009). The lifelong financial costs per individual are estimated to be between half a million to three million US dollars depending on the level and age at the time of injury (National Spinal Cord Injury Statistical Center, 2009). Despite the significance of this trauma there is only one neuroprotective treatment available to patients, methylprednisolone (and this is controversial) (Sayer, Kronvall, & Nilsson, 2006), and no treatment aimed at promoting axon regeneration and improving function of the damaged nerve tracts, let alone effecting any form of cure (Sayer et al., 2006). A review of the anatomy of the spinal cord is provided below to help us understand the pathological processes that result after SCI and the potential treatments, with a focus on the experimental aspects of SCI.
Introduction

1.2. Spinal cord anatomy

1.2.1. Gross anatomy

The spinal cord is a long cylindrical tube that extends from the base of the brain, the foramen magnum, to the first or second lumbar vertebra, and provides a pathway for motor, sensory and autonomic information trafficking between the brain and the body (Afifi & Bergman, 2005). It is the only other organ besides the brain that is completely encased in bone, signifying its importance and vulnerability (Afifi & Bergman, 2005).

The spinal cord can be divided into 31 segments encased within 31 vertebrae according to the 31 pairs of spinal nerves that emerge from each segment (Figure 1.1). It is further divided into four groups according to parts of the body the spinal cord innervates; the cervical region innervates the neck, arms, and respiratory muscles; the thoracic region innervates the muscles involved in posture and the internal organs; the lumbar region innervates the legs; the sacral and coccygeal region regulate the bladders, bowel, and sexual function. Facial organs are innervated by cranial nerves that originate from the brain directly. There are 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal spinal cord segments and accompanying spinal nerves. Some important regions to note with regard to spinal cord injury are C1-C4 that control breathing, C4-C6 that control heart rate, T11-L2 that control sexual function, and S2-S3 that control bladder and bowel activities (Afifi & Bergman, 2005). An injury at a high cervical level is, therefore, usually fatal because control of breathing and heart rate is frequently lost (Stein et al., 2010). Quality of life of spinal cord injured patients greatly depends on heart and lung functions, in addition to the obvious locomotor paralysis; therefore, any ‘improvement’ in the level of damage, even over less than one segment, still confers significant benefit even though locomotion may not be restored (Charlifue et al., 2011). This degree of improvement provides motivation for many spinal cord injury scientists, even though the kind of regeneration achieved so far is usually small. One point to note is that the vertebral column grows faster and is, therefore, longer than the spinal cord in both humans and rats, so the level of vertebra usually does not correspond to the level of spinal cord encased within (Afifi & Bergman, 2005). For example, a laminectomy (removal of the dorsal arch of vertebra) at T10 vertebra of the rat reveals the L1 spinal cord (Afifi & Bergman, 2005), whereas one at T10 vertebra of the human reveals the T11-T12 spinal cord (McDonald, 1999). This is very important when interpreting the literature, because most animal work refers to the vertebra level as the injury level. In this thesis, we will also follow that convention. Each pair of spinal nerves travels down
the spinal column to reach the respective vertebra level and emerges from the vertebra foramen. The lower the segment, the longer these spinal nerves have to travel (Afifi & Bergman, 2005).
The spinal cord is encased within the hard casing of the spinal column or vertebra, and grouped into cervical, thoracic, lumbar and coccygeal sections. Thirty one pairs of spinal nerves emerge from the spinal cord, and spinal cord segments are divided and numbered accordingly. The spinal cord is encased within the spinal column or vertebra, which is also divided into 31 segments. Due to the discrepancy between the length of the spinal cord and the spinal column, the levels do not match up from mid thoracic levels. Therefore, a laminectomy (removal of the dorsal spinous process) at T10 vertebra reveals the T11 spinal cord. Modified from McDonald, 1999.
1.2.2. Meninges

The spinal cord itself is encased within three layers of sheaths called meninges - from the outside to inside the dura mater, arachnoid, and the pia mater (Afifi & Bergman, 2005). The outer-most dura mater is the toughest, and adheres to localised places on the vertebra, the base of the skull and the sacrum, and is continuous with the epineurium of spinal nerves. The loose connective tissue between the dura mater and the arachnoid carries the blood supply, fat and loose connective tissue. The arachnoid is a simple sheath that overlies the subarachnoid space. It contains collagen and elastin fibres laid down by fibroblasts, and encloses cerebrospinal fluid that provides physical buffering and a diffusion medium for nutrient, gas, messengers, and waste products. The pia mater is an elastin and collagen fibre rich sheath that adheres closely to the spinal cord surface, locked in place by astrocyte processes that line the outermost surface of the spinal cord to form a glial limitans (Afifi & Bergman, 2005). In surgeries requiring a close apposition of the spinal cord to another structure, be it a peripheral nerve graft, a synthetic matrix graft, or the other end of the damaged spinal cord, the tough and fibrous dura mater provides a point of anchor for attaching and suturing, allowing the soft spinal cord tissue to be spared (Dinh et al., 2007).

1.2.3. Grey matter and white matter composition

In cross section, the spinal cord can be roughly divided into 2 areas - grey and white matter (Afifi & Bergman, 2005). These areas can be distinguished by their colour with the un-aided eye; this is possible even in rat spinal cords that are only 2 mm in diameter (experimental observation). The white coloured lipid-rich myelin of axon fibres in the white matter gives it its colour. A central canal carrying cerebrospinal fluid can be found in the middle of the spinal cord (Afifi & Bergman, 2005). An immunohistochemically labelled spinal cord cross section is shown in Figure 1.2, with neuron cell bodies in the grey matter (some large ones indicated by arrows), axons (some indicated by arrowheads) and bundles of neuronal processes in the white matter labelled with anti-SMI32 antibody. One dorsal and one ventral nerve emerge from the left or right hand side of the spinal cord and join to form one spinal nerve that exits the spinal column via the intervertebral foramen. The dorsal and ventral nerve roots can be seen in Figure 1.2.
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Figure 1.2. Rat Spinal Cord Cross Sectional Anatomy.
This cross section has been labelled with anti-SMI32 antibody for neuron cell bodies and axons. The grey matter contains neuron cell bodies located in the dorsal, lateral or ventral horns. Some large neurons in the lateral and ventral horns are marked (arrows). The white matter contains axon bundles (hence much brighter labelling intensity) located in the dorsal, lateral or ventral funiculi. Axon connections made by grey matter neurons with white matter are seen (arrowheads). The dorsal and ventral roots emanating from this level of the spinal cord have also been identified.
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The grey matter contains the cell bodies of neurons that reside in the spinal cord that are organised in groups called nuclei, named according to their inputs and outputs (Bear, Connors, & Paradiso, 2001, Chapter 7; Martini, Timmons, Tallitsch, & Ireland, 2003, Chapter 14). Unlike white matter fibre tracts that are grouped in bundles, the grey matter is anatomically homogeneous, so these nuclei are not clearly distinguishable from each other in cross section. In general, however, sensory and motor nuclei can be separated by an ‘imaginary line’ crossing the central canal from left to right (Figure 1.3) (Martini et al., 2003). The grey matter includes large diameter motor neurons (seen in Figure 1.2, arrows) in the ventral and lateral horns that convey centrally generated motor information to peripheral organs, sensory neurons in the dorsal horn that convey sensory information from peripheral and somatic receptors to the brain, and interneurons that analyse sensory inputs and coordinate motor outputs locally in the spinal cord (Martini et al., 2003). These also form reflex circuitries including the central pattern generator (Rossignol, Barriere, Alluin, & Frigon, 2009). It is of note that the interneurons in the brain and spinal cord outnumber all other neurons combined both in number and type (Martini et al., 2003, Chapter 13), and these interneurons contribute greatly to recovering reflex motor activities after spinal cord injury when connections to the brain are cut off (Rossignol et al., 2009). Interneurons are also important targets for induction of axon regeneration (Rossignol et al., 2009). The grey matter also contains some relatively short distance axon fibres called the grey commissures that cross over from one side of the spinal cord to the other before reaching their target (Martini et al., 2003, Chapter 14).
Figure 1.3. Schematic Representation of Major White Matter Descending and Ascending Pathways and Grey Matter Nuclei Organisation. For clarity tracts are shown on only one side of the spinal cord but they exist on both sides. Modified from Martini et al., 2003.
Introduction

The white matter contains axon tracts that send motor and sensory information from and to the brain in descending and ascending tracts respectively (Figure 1.3) (Martini et al., 2003). Most tracts contain axons that cross over from the left to the right and vice versa so the left brain, therefore, controls and senses the right hand side of the body and vice versa (Martini et al., 2003). The tracts are named according to their origin and destination. For example, the spinocerebellar tract originates in the spinal cord and terminates in the cerebellum. This is also the case for descending tracts such as the cerebrospinal tract. When two tracts share the same origins and destinations, they are named according to their location in the spinal cord cross section, such as the lateral cerebrospinal tract and the ventral cerebrospinal tract. The tracts are located in one of three funiculi on each side named according to location; the dorsal, lateral and ventral funiculi (Martini et al., 2003).

The dorsal funiculus contains the fasciculus gracilis and fasciculus cuneatus (Figure 1.5) that carry ascending axons that send discriminative fine touch, vibration and proprioception (position and movement) information from the lower and upper half of the body, respectively, to the nucleus gracilis and nucleus cuneatus in the medulla oblongata (Martini et al., 2003). Cell bodies of first order sensory neurons are located in the dorsal root ganglia just outside the spinal cord; they receive sensory input in the form of action potentials from an organ via a long sensory arm, and output to the local spinal cord circuitry via interneurons to generate reflexes; some sensory neurons also send collaterals to the dorsal horn to modify pain impulses. The first order neurons also send another long axon to the medulla oblongata, where they synapse with a second order sensory neuron for simple integration of information (Figure 1.4). These then synapse with a third order neuron that reaches the sensory cortex for central processing (Figure 1.5). Only 1% of all sensory information reaches the brain, the rest is processed in the spinal cord and the brainstem (Martini et al., 2003). The axons of second order neurons cross over (decussate) to the other side in the medulla oblongata. A lesion to the dorsal funiculus, therefore, results in loss of sensation including fine touch, vibration, and proprioception ipsilateral to the lesion (Paxinos, 2004, Chapter 7). Sensations from specific parts of the body are relayed to a specific place in the sensory cortex, organised according to a map called the ‘sensory homunculus’ (Paxinos, 2004, Chapter 7). Mis-projections can result in the sensation being felt at a different part of the body than its origin (Paxinos, 2004, Chapter 7).
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Sensory neurons located in the dorsal root ganglion receive sensory information from peripheral organs and send collaterals to interneurons in the dorsal horn of the spinal cord. These inputs are then integrated in a local integration centre before an output is generated to lower motor neurons to elicit a reflex motor response. The sensory neurons can also send a long axon to the nucleus cuneatus in the medulla oblongata, where it synapses with a second order sensory neuron that in turn sends inputs to the ventral nucleus of the thalamus. For clarity tracts are only shown on one side of the spinal cord but they are mirrored on the other side. Modified from Martini et al., 2003.

Figure 1.4. Local Integration of Sensory Information in the Spinal Cord.
Sensory neurons located in the dorsal root ganglion receive sensory information from peripheral organs and send collaterals to interneurons in the dorsal horn of the spinal cord. These inputs are then integrated in a local integration centre before an output is generated to lower motor neurons to elicit a reflex motor response. The sensory neurons can also send a long axon to the nucleus cuneatus in the medulla oblongata, where it synapses with a second order sensory neuron that in turn sends inputs to the ventral nucleus of the thalamus. For clarity tracts are only shown on one side of the spinal cord but they are mirrored on the other side. Modified from Martini et al., 2003.
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Figure 1.5. Schematic Diagram of Dorsal and Ventral Ascending Pathways to the Brain. The fasciculus cuneatus (purple) and the ventral spinothalamic tracts (dark blue) are used as examples. First order sensory receptors in the dorsal root ganglion convey fine touch, vibration and proprioception information (purple) via axons in the fasciculus cuneatus on the ipsilateral side of the spinal cord to the medulla oblongata, where they synapse with second order sensory neurons in the nucleus cuneatus. Their axons decussate before continuing up as a bundle in the medial lemniscus to reach the ventral thalamus. There a second synapse is made and thalamic neurons send information via their axons to the sensory cortex.

By contrast, crude touch, pressure, pain and thermal information in first order sensory neurons (dark blue) synapse with second order neurons in the dorsal horn. Axons of second order neurons decussate to the contralateral side of the spinal cord before ascending in the ventral spinothalamic tract, where they reach the ventral thalamus. Thalamic neurons then send their axons to the sensory cortex. For clarity each of these tracts are shown on only one side of the spinal cord but they are mirrored on the other side. Modified from Martini et al., 2003.
The lateral and ventral funiculi contain a mix of descending and ascending tracts. Descending tract systems deal with control of movement, and modulation of sensory activity, posture and head position (Martini et al., 2003). They include the lateral and ventral corticospinal tracts, tract of Barnes, rubrospinal tract, lateral and medial vestibulospinal tracts, reticulospinal tract, and tectospinal tract (see Figure 1.3 for their location in the spinal cord cross section) (Martini et al., 2003). The lateral and ventral corticospinal tracts (Figure 1.6) are responsible for voluntary motor control, and they are the main targets for spinal cord injury recovery after injury. In both human and rats, the lateral and ventral corticospinal tracts originate from upper motor neurons located in the primary motor cortex (50%), pre-motor cortex (30%), primary sensory cortex, supplementary motor area and the other cortical areas (Purves, 2008). Eighty-five percent of the descending fibres from upper motor neurons decussate in the medulla to continue in the lateral corticospinal tract (Purves, 2008). Fifteen percent of the descending fibres continue to travel on the ipsilateral side down the spinal cord in the ventral corticospinal tract until they reach the appropriate level, where they decussate to the dorsal horn of the contralateral side (Purves, 2008). The axons synapse with lower motor neurons, which then innervate target muscles via the ventral root (Martini et al., 2003). Therefore, lesions to the spinal cord result in mainly ipsilateral paralysis and some degree of contralateral paralysis. The motor cortex is also organised into a ‘motor homunculus’, similar to the sensory homunculus, with specific areas innervating corresponding areas of the body (Martini et al., 2003). The rubrospinal tract originates from the red nuclei of the mesencephalon and controls muscle tone and precise movement of distal parts of the limbs (only the upper limbs in humans), and can contribute significantly to recovery after corticospinal tract lesions (Kanagal & Muir, 2009; Martini et al., 2003). The vestibulospinal tracts originate from the vestibular nuclei and maintain posture and balance (Martini et al., 2003). The tectospinal tracts originate from the superior and inferior colliculi and produce reflex changes in head, neck and upper limbs in response to bright light, sudden movements or loud noises (Martini et al., 2003). Reticulospinal tracts originate from the reticular formation in the brainstem. The reticular formation is very unique because it receives almost all ascending and descending information conveyed in other tracts and connects with the cerebellum and brainstem nuclei. Activation of this tract can generate movements of any area of the body depending on region of activation (Martini et al., 2003).
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Figure 1.6. Schematic Diagram of Representative Descending Pathways from the Brain. Lateral and ventral corticospinal tracts have been used as examples. Upper motor neurons in the primary motor cortex (shown), pre-motor cortex, primary sensory cortex, supplementary motor area and the other cortical areas (not shown) send descending axons in a bundle. At the level of the mesencephalon the bundle is called a ‘cerebral peduncle’. At the medulla oblongata level, 15% of these axons continue on the ipsilateral side in the ventral corticospinal tract which then decussate at the destination level of the spinal cord before synapsing with lower motor neurons. The majority (85%) of the axons decussate at the medulla; the bundle of fibres crossing over is called a ‘pyramidal decussation’. After the decussation the axons travel in the lateral corticospinal tract, and terminate at the dorsal horn of each spinal cord level, where the axons synapse with lower motor neurons. Lower motor neurons then send movement commands to target muscles. For clarity tracts are shown on only one side of the spinal cord but they are mirrored on the other side. Modified from Martini et al., 2003.
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Ascending tracts in the lateral and ventral funiculi include the dorsal and ventral spinocerebellar tracts, and the lateral and ventral spinothalamic tracts (Paxinos, 2004, chapter 7). The spinothalamic tracts convey crude touch, pressure, pain, thermal sensations to the brain (Paxinos, 2004, chapter 7). The spinocerebellar tracts convey conscious and unconscious proprioception information, providing the location of all the muscles and joints of the body. The first order sensory neurons normally synapse with a second order neuron in the dorsal horn of the spinal cord (an example is shown using the ventral spinothalamic tract in Figure 1.5); axons then decussate before ascending in the respective tract (e.g. ventral spinothalamic tract) to the ventral thalamus. There they synapse with third order neurons that send information to the sensory cortex. The main anatomical differences between the spinocerebellar and spinothalamic tracts and the dorsal column pathways are the location of the decussation and the location of the second order neurons (Paxinos, 2004, chapter 7). This means that lesions to spinocerebellar and spinothalamic tracts results in loss of crude touch, pressure, pain and thermal sensations contralateral to the lesion, whereas lesions to the dorsal column pathways results in loss of fine touch, vibration and proprioception ipsilateral to the lesion.
1.3. Cells of the spinal cord

The cells that make up the spinal cord can be categorised into two types – neurons and glia. Glia can be further categorised into astrocytes, oligodendrocytes, microglia and ependymocytes. Cells of the vasculature are also present in the spinal cord (Bear et al., 2001, Chapter 2).

1.3.1. Neurons

Neurons are the fundamental units of the nervous system and communicate with each other mainly by forming synapses (Bear et al., 2001, Chapter 2). They differ from all other cells of the body by possessing an axon (Figure 1.7) that has the special ability to transmit electrical impulses (action potentials) down its length, transfer this information to another neuron by releasing neurotransmitters at a synapse, which then interact with receptors in dendritic spines and the cell body of the recipient cell and induce electrochemical changes to produce a propagating electrical impulse or other changes such as contraction of a muscle (Bear et al., 2001, Chapter 2). This is called synaptic transmission. A neuron has only one axon which branches to form telodendria that end in synaptic terminals, and this allows it to synapse with multiple neurons (Martini et al., 2003, Chapter 13). A neuron also possesses one to many dendrites, which branch to form numerous dendritic spines, allowing a neuron to receive information from multiple neurons (Martini et al., 2003, Chapter 13). Dendrites of a neuron transmit information to the recipient neuron cell body and represent 80-90% of the surface area of a neuron (Martini et al., 2003, Chapter 13). Axons can send collateral branches to innervate different effector organs including neurons, muscles or glands (Hagg, 2006). Axons and dendrites together are called neuronal processes or neurites (Martini et al., 2003, Chapter 13). One feature that distinguishes an axon from dendrites is the axon hillock (Figure 1.7 red arrows), an area at the beginning of the axon that contains a concentrated amount of neurotubules and neurofilaments (Martini et al., 2003, Chapter 13). Dendrites on the other hand do not contain so many neurotubules and filaments. Therefore, neuronal cell bodies are the ‘hubs’ of the body’s communication system, and axons and dendrites are the ‘wiring’. Axons can be quite long (for example the upper motor neuron that innervates the lower extremities can be over one metre in length in humans) and often appear in bundles in the white matter of the spinal cord, such as the corticospinal tract (Martini et al., 2003, Chapter 13). If this communication pathway is cut as in a spinal cord injury, effector organs downstream to the injury become
denervated, and sensory information from those organs cannot reach the brain, producing paralysis and loss of sensation of the corresponding organs.
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Figure 1.7. Structure of a Neuron Illustrated by Diagram, and using Immunohistochemistry and Dye Tracing Techniques.
A. A neuron contains three main parts: dendrites, cell body and an axon. Dendrites branch into dendritic spines and the axon branches into telodendria which end in synaptic terminals. The synaptic terminals can act on effector cells including neurons (contacting dendrites or the cell body), muscle cells, and gland cells. B. Neurofilament labelling using anti-SMI32 antibody shows spinal cord neuron cell bodies and the axon hillock (red arrows) that distinguishes the axon (arrowhead). The axons contain a greater concentration of neurofilaments compared to dendrites (which contain little or no neurofilament material). C. Fluoro-Ruby dye uptake by spinal cord neurons shows accumulation of the dye in granules in the cell body and major processes. The axon hillock (red arrow) can be distinguished in one neuron.
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1.3.2. Astrocytes

Astrocytes are the most numerous glial cell type and the main support cells for neurons (Martini et al., 2003, Chapter 13). The processes (GFAP positive) and end plates (GFAP negative) of an astrocyte fill the space between neuron cell bodies, axons, synapses and between neurons and blood vessels, shielding neurons from each other and blood capillaries constituting part of the blood-brain or spinal cord-barrier (Martini et al., 2003). Processes and end plates of an astrocyte take on a three dimensional polyhedron shaped space that barely overlaps with that of other astrocytes, possibly creating a functioning domain of their own (Nedergaard, Ransom, & Goldman, 2003). Views on the precise function of astrocytes have been changing rapidly in recent years, with emerging roles in all aspects of neuronal function including synaptogenesis, neurogenesis and neuronal signaling (Escartin & Bonvento, 2008; Ransom, Behar, & Nedergaard, 2003). Established functions include buffering of potassium ions built up during neural activity, removal of neurotransmitters glutamate and GABA from the synaptic cleft to ensure the transient nature of excitation, and guidance to developing neurons (Ransom et al., 2003). Probable functions include maintenance of the blood-brain barrier and blood-spinal cord barrier, regulation of extracellular pH, providing nutrients to neurons, and sharing energy substrates derived from glycogen with neurons (Ransom et al., 2003). Astrocytes are electrically unexcitable, but they can communicate with each other in a different way from neurons; that is via gap junction channels (Nedergaard et al., 2003). Processes from the same astrocyte can also be connected via gap junctions (Nedergaard et al., 2003).
Figure 1.8. Schematic Drawings of Glial Cells of the Central Nervous System.
Astrocytes have many processes and end plates that take on a three dimensional polyhedron shape that barely overlaps with other astrocytes. The processes and endplates fill the space between neuron cell bodies, axons, synapses and blood capillaries.
Oligodendrocytes wrap a lipid-rich insulating membrane called myelin around axons, facilitating fast action potential propagation in myelinated axons. Each oligodendrocyte can myelinate multiple axons.
Microglia also have many processes that continuously sense changes in the extracellular environment and detect cellular debris and pathogens.
Ependymocytes line the central canal of the spinal cord and assist in production, circulation and monitoring of cerebrospinal fluid.
Modified from Martini et al., 2003.
1.3.3. Oligodendrocytes

Oligodendrocytes provide axons with a lipid-rich, high-resistance, insulating spiral membrane called the myelin sheath; one axon is myelinated by multiple oligodendrocytes, leaving short spaces between segments of myelin called ‘nodes of Ranvier’. One oligodendrocyte can myelinate multiple axons hence tying them together (Martini et al., 2003, Chapter 13). The nodes of Ranvier contain voltage gated sodium channels that become open in response to an action potential to let sodium enter the axon, depolarising the region adjacent to the channel and resulting in action potential propagation (Poliak & Peles, 2003). The inter-nodal areas do not contact the extracellular fluid and do not contain voltage gated sodium channels (Poliak & Peles, 2003). As a result, action potential ‘jumps’ from one node to another and sodium channels only open in the nodes of Ranvier; therefore, the time taken for action potential propagation down the length of an axon is greatly reduced (Wu & Ren, 2009). If the axon becomes demyelinated, nerve impulse propagation is impeded (Wu & Ren, 2009). Oligodendrocytes only myelinate axons in the CNS; their PNS functional equivalents are Schwann Cells (Martini et al., 2003). Not all axons in the CNS are myelinated; some sensory neurons that sense slow pain, temperature and mechanical stimuli are not myelinated. These sensory neurons have slow-conducting unmyelinated axons that have voltage gated sodium channels along their entire length and are protected by astrocyte processes in the CNS or ensheathed in bundles by Schwann cells in the PNS (Martini et al., 2003).

1.3.4. Microglia

Microglia are the resident immune cells of the CNS. They have processes that continually extend and retract to sense changes in the extracellular environment, and to detect cellular debris and pathogens (Wirenfeldt, Babcock, & Vinters, 2011). The CNS is free from circulating macrophages and monocytes because of the blood-brain-barrier except at times of injury and inflammation. Therefore, microglia are the only cells that function to clear cellular debris, waste and pathogens in the normal CNS (Wirenfeldt et al., 2011).

1.3.5. Ependymocytes

Ependymocytes are cells lining the central canal and have been found to possess stem cell properties (Hamilton, Truong, Bednarczyk, Aumont, & Fernandes, 2009; Marichal,
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Garcia, Radmilovich, Trujillo-Cenoz, & Russo, 2009). In juvenile turtles, some ependymocytes possess neurogenic precursor properties and support repair of transected spinal cords (Marichal et al., 2009). Ependymocytes in rats have been found to express neuronal development markers and there is a possibility that some of these cells can mature under injury conditions as neurons (Marichal et al., 2009; Meletis et al., 2008).
1.4. Pathology of spinal cord injury

Spinal cord injury is a two-step process – the initial mechanical injury and a secondary injury cascade. The initial injury can be one or any combination of contusion (hemorrhage within meninges), compression (squeezed or distorted), laceration (tearing) or transection (cut or severed) depending on the type of injury suffered (V. W. Lin & Cardenas, 2003, Chapter 56; Martini et al., 2003, Chapter 14). For example, gun shots always produce a laceration whereas falls are less likely to do so, but instead produce contusion and compression.

The secondary injury cascade is characterised by expansion of damage, an inflammatory reaction and reactive gliosis (Renault-Mihara et al., 2008). Within a few hours of human SCI, the injury site expands rapidly as evidenced by the presence of hemorrhagic necrotic tissue in several segments rostral and caudal to the injury (V. W. Lin & Cardenas, 2003, Chapter 56). Following inevitable necrotic and apoptotic cell death and tissue disruption, the expanded lesion area becomes infiltrated with neutrophils, macrophages and other immune cells that will eventually clear the lesion area leaving a fluid-filled cavity over the following months in humans (V. W. Lin & Cardenas, 2003, Chapter 56), or 8-15 weeks in adult rats (Guizar-Sahagun et al., 1994). This cavity is usually lined with a thick glial scar and potentially dysfunctional but anatomically intact axon fibres, and a collagenous scar if there was damage to the dura (V. W. Lin & Cardenas, 2003, Chapter 56). In a rat complete transection, the same process takes place but at both cut ends, resulting in cavities on both sides separated by a collagenous scar tissue in place of the original gap (Spilker et al., 2001). In human SCI, new cavities can form and expand rostral-caudally years later due to subtle damages to the relatively vulnerable spinal cord, vertebral instability, or spinal stenosis (constriction of spinal cord volume due to vertebra overgrowth), resulting in further functional loss after a period of recovery (Eismont, Green, & Quencer, 1984; V. W. Lin & Cardenas, 2003, Chapter 56). Knowledge of the timeline of involvement and fate of each spinal cord cell type in the secondary injury cascade is essential for treatment development.

1.4.1. Neurons and necrosis

Both necrosis and apoptosis is involved in neuron death (M. S. Beattie, Farooqui, & Bresnahan, 2000; Yong et al., 1998). Necrosis is not limited to neurons and the process
is common between all cell types affected. Mechanical damage and lipid peroxidation damage to cell membranes inevitably results in necrosis of the cell, and is characterised by a swollen and disrupted morphology (M. S. Beattie et al., 2000). Other mechanisms also contribute to necrosis of partially damaged or nearby intact cells (Michael S. Beattie, Hermann, Rogers, & Bresnahan, 2002). Free radicals and excitatory amino acids such as glutamate and aspartate are released from disrupted cells, accompanied by hypoxia produced by a disrupted blood supply (Michael S. Beattie et al., 2002; Nicotera & Melino, 2004). Over-activation of glutamate receptors on neurons and oligodendrocytes leads to excessive intracellular Ca^{2+} and water accumulation and cell rupture (Michael S. Beattie et al., 2002; McDonald, Althomsons, Hyrc, Choi, & Goldberg, 1998). This process has been termed excitotoxicity (Choi, 1992). Blocking glutamate receptors reduced grey and white matter damage after rat contusion SCI (Wrathall, Choiniere, & Teng, 1994). The resultant release of enzymes into the extracellular matrix triggers a cascade of inflammatory processes (Kajta, 2004).

Whereas necrosis starts minutes after injury, apoptosis of neurons is evident from day 1 to day 30 in a rat contusion injury model, peaking on day 3 (Kajta, 2004; Yong et al., 1998). By 1 week, there is already massive tissue loss in the lesion centre, leaving a rim of spared white matter (M. S. Beattie et al., 2000). Apoptotic cell loss away from the lesion at later time points maps to oligodendrocytes in the white matter, accompanying axon death (M. S. Beattie et al., 2000). Apoptosis is characterised by cell shrinkage, chromatin condensation, nucleus and DNA fragmentation without loss of membrane integrity, and without triggering an inflammatory reaction (Kajta, 2004). Apoptosis happens via the mitochondrial pathway or the death pathway (Kajta, 2004). The mitochondrial pathway of apoptosis is triggered by a lack of neurotrophic factors and non-specific insults leading to Ca^{2+} entry, though the level of Ca^{2+} does not reach that of necrosis (Kajta, 2004; Nicotera & Lipton, 1999; Nicotera & Melino, 2004). The death pathway is triggered by activation of a death receptor in the cell membrane, for example Fas (CD-95) (Kajta, 2004). Both of the mitochondrial and death pathways lead to activation of the caspase pathway, which then initiates DNA fragmentation and cell death via initiators such as caspase-9 (mitochondrial pathway) or caspase-8 (death pathway), converging in ‘executioners’ such as caspase-3 (Kajta, 2004).
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While neuron loss during the first two weeks of SCI is extensive, mature neurons in the CNS do not possess the centrosome complex required for mitosis and proliferation (Martini et al., 2003, Chapter 13). The mature SC neuron pool is, therefore, unable to renew itself (Martini et al., 2003, Chapter 13). However, uninjured neurons are able to send axon collaterals, potentially to denervated targets to re-establish lost connections (Hagg, 2006). This intrinsic property of the axon implies that reinnervation after SCI is possible, but unlike the PNS, axons in the spinal cord encounter the glial scar in their path which is inhibitory and hostile to regeneration (Fawcett & Asher, 1999). Astrocytes are the main cellular constituent of the glial scar (Fawcett & Asher, 1999).

1.4.2. Astrocytes

Besides the inevitable initial necrotic event, astrocytes in the injury penumbra in rat SCI do not suffer an apoptotic fate after injury, unlike neurons and oligodendrocytes (M. S. Beattie et al., 2000). Instead they proliferate and at the same time become hypertrophic, characterised immunohistologically by increased production of the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, and contribute to glial scar formation to limit the injury (Renault-Mihara et al., 2008). They do this along with activated microglia and macrophages, and fibroblasts if the dura is damaged (Renault-Mihara et al., 2008). As discussed earlier, astrocytes support and possibly guide neurons acting like ‘parents’, so it is no surprise that they become over-protective by proliferating and turning hypertrophic when neurons are injured (Nedergaard et al., 2003). Astrocyte proliferation seems to be a consequence of disruption of the blood-spinal cord-barrier; areas of most glial scarring correlate with areas of most blood-spinal cord-barrier disruption (Renault-Mihara et al., 2008). The astrocytes in the scar are tightly packed with dense processes connected via gap junctions and tight junctions (Fawcett & Asher, 1999).

The glial scar presents a great challenge for regenerating axons since reactive astrocytes secrete proteoglycans such as chondroitin sulfate proteoglycans and other molecules that inhibit axon growth (Renault-Mihara et al., 2008). Reactive gliosis also has beneficial effects including restoration of normal function and compaction of the lesion (Renault-Mihara et al., 2008). Astrocytes are necessary for the removal of glutamate from the extracellular environment, pH buffering, and maintenance of the blood-spinal cord barrier. These functions are all essential for restoring normal neuron function.
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(Renault-Mihara et al., 2008). Inhibition of astrocyte proliferation after mild or moderate SCI in mice can lead to further demyelination and functional deficits, and prolonged and increased immune cell and macrophage infiltration (Faulkner et al., 2004b). Inhibition of astrocyte hypertrophy can lead to impaired lesion area compaction (Okada et al., 2006). Therefore, for SCI treatment purposes, careful manipulation of astrogliosis at the proper time, such as down-regulation at the initial stages of injury but recovery at the later stages is required to allow for axon regeneration and lesion compaction in parallel (Renault-Mihara et al., 2008).

1.4.3. Oligodendrocytes

Oligodendrocytes have been found to undergo necrosis due to damage and over-activation of AMPA glutamate receptors, and subsequently apoptosis, with the number of apoptotic oligodendrocytes peaking at days 3 and 14 after severe contusion injury in rats (Yong et al., 1998). The early oligodendrocyte apoptotic events accompany the degeneration of distal portions of cut axons (McDonald & Belegu, 2006). The causes of oligodendrocyte apoptosis in the apparently normal functioning white matter away from the lesion centre in the longer term (weeks after injury) are, however, not yet known (Michael S. Beattie et al., 2002). Microglia can be found associated with apoptotic oligodendrocytes, but it is not clear whether they are mediators of apoptosis or simply cleaning up debris (Michael S. Beattie et al., 2002). There is considerable axon demyelination in the lesion epi-centre and distal to the lesion along white matter fascicles, peaking at 24 hours and lasting for up to 45 days after injury in rats, or up to a decade in humans (Wu & Ren, 2009). Some white matter fibres are spared in the lesion area in most human SCI cases, but demyelination as a consequence of oligodendrocyte death and inflammation renders these white matter fibres ineffective in impulse conduction (Wu & Ren, 2009). This prolonged oligodendrocyte apoptosis and demyelination contributes to long term deterioration of neural function after human SCI (Wu & Ren, 2009) and is similar in pattern to neurodegenerative disease processes such as in lateral sclerosis (Matute et al., 2001). Oligodendrocytes secrete stabilising molecules including Nogo-A, myelin associated glycoprotein and oligodendrocyte-myelin glycoprotein (Huber, Weinmann, Brosamle, Oertle, & Schwab, 2002; X. Wang et al., 2002). These molecules act to stabilise the complex wiring of the spinal cord but are growth-cone collapsing and neurite growth inhibiting after SCI (Rossignol, Schwab, Schwartz, & Fehlings, 2007). Therefore, remyelination of residual fibres and
neutralisation of these myelin inhibitory molecules are two of the important goals for achieving functional regeneration.

1.4.4. Activated microglia, macrophages and the immune system

The immune response after spinal cord injury is very important for repair and protection of the spinal cord (Schwartz, 2000). The repair function is carried out by activated microglia, macrophages, and neutrophils removing cellular debris and dead cells, while the protection from pathogens is carried out by T-cells acting against self-antigens displayed by damaged CNS tissue (Schwartz, 2000). Resident microglia are activated immediately following rat SCI to become activated microglia together with infiltration of neutrophils and macrophages and a rise of cytokine levels (Hermann, Rogers, Bresnahan, & Beattie, 2001). Macrophages from blood begin infiltrating the lesion from day one (Michael S. Beattie et al., 2002). It has been found, via flow cytometry for immune cells, that there is a multi-phasic immune response, with the first peak of neutrophils at 1 day, a second peak of macrophages, microglia and T cells at 7-9 days, with a trough at 14 days, and a much later peak of macrophages and microglia at 60 days (Beck et al., 2010). Removal of axon, myelin, and cellular debris distal to the lesion, which is termed Wallerian degeneration, appears to be essential for regeneration, but this process happens much more slowly in the CNS than the PNS, hindering regeneration but at the same time slowing down damage spread to healthy neurons (Schwartz, 2000). Although traditionally viewed as destructive, T-cells that display immunity to a CNS antigen were found to be neuroprotective. Passive immunisation by transfer of T-cells against myelin basic protein and active immunisation by vaccination with the relevant antigen reduced axon loss after CNS injury. Since the CNS is an immune-privileged site, such T-cell activity is limited to avoid auto-immune diseases, but this also limits recovery. Therefore, both microglial and T-cell responses could potentially be amplified carefully to help with the repair and protection of injured spinal cords (Schwartz, 2000).

1.4.5. Ependymocytes

Ependymocytes are the major source of neural stem cells residing in the spinal cord (Hamilton et al., 2009). A minimal needle injury to the SC is sufficient to induce approximately 3% of ependymocytes around the central canal to proliferate within one day, migrate from the central canal to the grey matter, and differentiate mainly into GFAP-expressing astrocytes by 14 days (Mothe & Tator, 2005). They also give rise to
some myelin-forming oligodendrocytes, as discovered by genetic fate mapping (Meletis et al., 2008). This proliferative response is not sufficient for spinal cord regeneration, although it can be enhanced by growth factors (Mothe & Tator, 2005). Ependymocytes at the dorsal surface of the central canal express markers for both ependymocytes and neural precursors, suggesting their potential for neural stem cell activity after injury (Hamilton et al., 2009).

1.4.6. Fibroblasts and mesenchymal cells

When SCI involves damage to the dura mater and other meninges such as in a transection or laceration, fibroblasts and mesenchymal cells from the meninges proliferate profusely to form a dense collagen deposit in the middle of the injury sandwiching the glial scars between it and the spinal cord (V. W. Lin & Cardenas, 2003, Chapter 56). In a rat model of complete transection, the fibrous scar tissue can span the entire distance between transected spinal cord stumps in a dense crimp pattern (Spilker et al., 2001). The scar is devoid of GFAP labelling and constituent cells were identified as myofibroblast, indicating a connective tissue origin and possible role in wound closure using mechanical contraction (Spilker et al., 2001). The fibrous scar was found to be axon regeneration permissive (Spilker et al., 2001) or inhibitive (Brazda & Muller, 2009; Klapka et al., 2005) in different studies and agreement has not been reached yet.
1.5. Spinal cord injury repair

1.5.1. Endogenous repair of spinal cord injury

Despite the continuing secondary injury cascade, there is a certain level of endogenous repair in the spinal cord. Following incomplete injury of the spinal cord in quadruped mammals such as rats, mice and cats, a considerable amount of functional locomotion can be recovered because of the Central Pattern Generator (CPG) located at approximately L1-L4 (Rossignol et al., 2009). The CPG is defined as ‘dedicated networks of nerve cells that generate movements and that contain the information that is necessary to activate different motor neurons in the appropriate sequence and intensity to generate motor patterns’ (Grillner, 2003). In animals, the CPG response can be enhanced by locomotor training and perineal region skin stimulation (Rossignol et al., 2009). In one experiment, cats that had received a T10-T11 hemisection recovered good quadrupedal walking after several weeks of treadmill locomotor training (Rossignol et al., 2009). After L1 complete transection; it was found that they could still walk bipedally with their hind limbs just a few hours after complete transection (Rossignol et al., 2009). This suggests that the locomotor rhythm is generated in the CPG with a great deal of independency, with multiple descending pathways from the brain or brainstem providing modulation (Rossignol et al., 2009). Direct evidence for the existence of CPG in humans is scarce, but indirect evidence including presence of involuntary stepping in SCI patients and movements elicited by vibration or electrical stimulation point to a role of CPG in human SCI (Molinari, 2009).

The molecular mechanisms involved in CPG function are not clear, but the cortex, brainstem and spinal circuit axonal reorganisation and neurogenesis in quadrupedal mammals can contribute to the spontaneous functional recovery (Florence M. Bareyre, 2008; Rossignol et al., 2009). Motor cortex representation changes can lead to development of alternative movement strategies (Florence M. Bareyre, 2008). At the level of the brainstem, the rubrospinal and bulbospinal tracts compensate for the lesioned corticospinal tract (CST) (Florence M. Bareyre, 2008). In the spinal cord, the minor dorsolateral and ventral CSTs can send sprouts to compensate for the lesioned main lateral CST, and spared axons in incompletely lesioned CST can increase their terminal branching. It is still not clear what molecular cues trigger these re-organisations (Florence M. Bareyre, 2008). In addition, the ependymocytes in the
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central canal region can give rise to astrocytes and oligodendrocytes after SCI, and aid in replenishment of lost cells and remyelination of axon fibres.

The existence of endogenous repair does not eliminate the need for external repair and regeneration of the spinal cord through pharmacological or surgical methods. The kind of fast functional recovery seen in quadrupedal mammals has not been seen in humans; locomotor training may be able to help but this is not yet proven (Molinari, 2009). The CPG relies on spared descending motor pathways and healthy spared propriospinal neuron pools in the lumbar spinal cord, which are also affected by the continuing secondary injury cascade and ongoing cavity formation as discussed before (Fawcett, 1998). Moreover, axonal sprouting is greatly dependent on the position of the cut (Fawcett, 1998). Axons cut closer to their cell body would attempt to regenerate whereas those cut far from the cell body would not, making long distance regeneration of cortex neurons or dorsal root ganglion sensory neurons difficult (Fawcett, 1998). Therefore, modulation of the secondary cascade should be beneficial, both for the CPG and re-establishing long distance functional connections between the spinal cord and the brain.

1.5.2. Repair and regeneration goals

The goals for repairing and regenerating the spinal cord are not unrealistic, given that the spinal cord possesses endogenous repair abilities. Axon regeneration from the brain to the appropriate level of spinal cord is an ambitious challenge that will take many more years of research to achieve. However, a small amount of axon sprouting or reconnection translates into a significant functional improvement (Fawcett, 1998). The amount of spared white matter and resulting functional recovery does not follow a linear pattern, but instead follows a higher harmonic function (Rossignol et al., 2009). Therefore, a realistic goal would be to promote axon regeneration across one or two more segments, as this could make a huge functional difference to patients. For example, a patient recovering from C5 to C6/C7 tetraplegia has much higher chances of achieving functional independence, including ambulation by gaining wrist extension, and such an improvement then has major effects on depression and social integration (Jallo & Vaccaro, 2009; McKinley, Silver, Santos, & Pai, 2008; Yarkony, Roth, Lovell et al., 1988; Yarkony, Roth, Heinemann, & Lovell, 1988). The difference between T9 to T10 injury is that of standing to walking with assistive devices (McKinley et al., 2008).
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Axon regeneration across this distance is most often achieved by regeneration of interneurons or local circuitry (Rossignol et al., 2009).

1.5.3. Current treatments

Management of spinal cord injury has advanced considerably in recent years. Prehospital management including evaluation, resuscitation, immobilisation, extrication, and transportation makes sure the patients arrive at a hospital in the best neurological state possible (V. W. Lin & Cardenas, 2003, Chapter 6). Then either non-surgical or surgical management by decompression stabilises the spinal cord. Long term management of respiratory, cardiovascular, gastrointestinal, bladder and skin systems aims to reduce neurological damage caused by the primary damage and to minimise secondary injury spread caused by hypoperfusion, ischemia, biochemical and inflammatory changes. Rehabilitation ensures patients have the highest quality of life possible long term relative to their level of injury (V. W. Lin & Cardenas, 2003, Chapter 46).

To date there has only been one pharmacological treatment approved for treatment of SCI and this has been controversial. Since the 1990s, high dose methylprednisolone (30mg/kg initial bolus within 3 hours and 5.4mg/kg/hour continued infusion over 23 hours, or initial bolus within 3-8 hours and continued infusion over 48 hours) has become a wide-spread standard treatment for spinal cord injury following controlled multicenter clinical trials under the recommendation of the National Acute Spinal Cord Injury Studies (NASCIS II and III) (Bracken, 1991; Bracken et al., 1997; Bracken et al., 1998; Sayer et al., 2006). The initial NASCIS II trial examined motor and sensory scores but no functional assessment. The latter has been addressed in NASCIS III but this study showed no improvement in Functional Independence Measure (Sayer et al., 2006). The authors made recommendations based on motor scores alone, although the clinical relevance is questionable since no difference was seen in the Functional Independence Measure (Sayer et al., 2006). Due to various short-comings of the NASCIS studies, the efficacy of methylprednisolone is still being reviewed. A literature review by Sayer et al. found 34 pro versus 14 con methylprednisolone studies in animals, and 3 pro versus 8 con results in clinical trials (Sayer et al., 2006). The ultimate goal of regrowth and regeneration is not addressed by standard treatments and research in these areas is still high on the agenda.
1.5.4. Current research

Current research aimed at restoring the functional connections and restricting damage can be grouped into three areas. (i) Biomolecular therapies aimed at protecting cells in the penumbra from lesion spread, and to induce neuronal outgrowth to reconnect truncated connections. (ii) Cellular therapies using implants such as fetal cells and multipotent cells aimed at providing sources of new neurons and glial cells to fill the gap, and at the same time providing a permissive environment. (iii) Guidance therapies aimed at providing a physical conduit and a permissive environment to guide regenerating axons to their destinations.

1.5.4.1. Biomolecular therapies

Treatment of SCI with nerve growth factor (NGF), neurotrophin-3 (NT3) and glial derived neurotrophic factor (GDNF), but not brain-derived neurotrophic factor (BDNF), has been shown to induce functional reconnection of axons (M. S. Ramer, Priestley, & McMahon, 2000). In general, neurotrophic factors like these are effective in inducing axonal regrowth. For example, NGF promotes the survival and development of neurons and NT-3 selectively promotes axon regeneration from the brain to the spinal cord (Grillner, 2003; McDonald, 1999). These neurotrophic factors may, however, result in neurogenic pain because the brain tends to interpret the impulses coming from newly synthesized synaptic connections as ‘pain’ (McDonald, 1999) and this remains an issue of serious concern for all repair therapies. Proper synaptic reconnection is the next goal following axon regeneration and new synapse formation.

Neutralisation of inhibitory molecules produced by the glial scar and oligodendrocytes has also shown promising results. Enzymatic digestion of chondroitin sulfate proteoglycans secreted by astrocytes with chondroitinase ABC promoted axon regeneration of Clarke’s nucleus axons from the brain into an implanted peripheral nerve graft (Yick, Cheung, So, & Wu, 2003). Inhibition of Nogo-A by antibody, receptor blocking, gene knockout, and effector pathway enhanced sprouting of injured fibres, compensatory growth of un-injured fibres and tracts, and long distance regeneration of some fibres with some functional improvement (Rossignol et al., 2007).
1.5.4.2. Cellular therapies

Given the large loss of neurons and glia in the injury site, it is logical to try to replenish this pool of cells by introducing cells that are capable of proliferating. These include fetal cells and multipotent cells. Fetal brain and spinal cord have been grafted to provide denervated axons with an embryonic permissive environment. These grafts could act as a relay station within the injured site and replace some neurons lost in SCI (L. M. Ramer, Ramer, & Steeves, 2005). Although multipotent cells have the potential to differentiate into neurons, in previous studies most of the cells have differentiated into glia instead of neurons when grafted (Cheng, Cao, & Olson, 1996).

1.5.4.3. Guidance therapies

If axons are to regenerate, a permissive environment is needed to support growth, to allow axons to pass, and there needs to be some protection from inhibitory molecules. Researchers have made use of the peripheral nervous system for this purpose since it has the intrinsic ability to heal itself and guide regenerating axons, hence the name ‘guidance therapy’. This ability is thought to be provided by Schwann cells that myelinate axons in the peripheral nervous system. Schwann cells secrete neurotrophic factors and cell adhesion molecules that together promote axon regeneration, and decrease the expression of various inhibitory myelin proteins (Fukunaga et al., 2004). Peripheral nerves, Schwann cells, olfactory endothelial cells, and man-made scaffolds have been tested extensively as guiding material, and are generally implanted some time after injury when the patients and their wounds are stable enough to be operated on.

Autologous peripheral nerve grafts are the ‘gold standard’ of repair for peripheral nerve injuries in humans and have so far been the most successful in all guidance therapies for SCI repair (Schmidt & Leach, 2003). Peripheral nerve grafts are sometimes carried out in combination with the addition of acidic fibroblast growth factor (aFGF) (L. M. Ramer et al., 2005), which is thought to prevent dieback and promote outgrowth of neurons (Cheng et al., 1996). In the pioneering work of Richardson et al, dorsal root ganglion and intrinsic spinal cord axons grew inside a single peripheral nerve autograft both rostrally and caudally in a 1.5 cm transection gap after 2-4 months, but no axon regenerated across the graft-spinal cord junction (Richardson, McGuinness, & Aguayo, 1982). Motor improvements and increases in axonal action potential conduction were
found starting as soon as two weeks after allografting into a hemisection site (Itohara et al., 2004).

Compared to cellular therapies where cells are simply injected to fill the injury site, implantation of a peripheral nerve is timed to be implanted in patients after the glial scar has formed; this is, therefore, more complicated. It requires resection of the glial scar and microsurgical techniques to properly suture the epineurium of the peripheral nerve with the dura mater of the spinal cord. Improvements to study design such as mechanical pre-conditioning of the graft, reconstructing pathways using multiple grafts to direct axons, and using minced grafts for better surface contact have all achieved better outcomes (Cheng et al., 1996; Feng, Zhou, Rush, & Ferguson, 2008; Rasouli, Bhatia, Suryadevara, Cahill, & Gupta, 2006). The use of autologous grafts instead of allografts also seemed to help (Feng et al., 2008; Hermelinda et al., 2003). Pre-conditioning of sciatic nerve allografts by mechanical compression promoted axon regeneration and axon sprouting past a moderate contusion injury site around the graft at six weeks while the fresh nerve did not; both fresh and pre-degenerated nerve grafts promoted functional recovery (Rasouli et al., 2006). In a similar model that used autologous grafts, both fresh and pre-degenerated grafts one month after weight-drop injury promoted myelination and collateral axon sprouting one and three months after treatment (Hermelinda et al., 2003). Work done on a complete transection model using 18 intercostal nerve implants stabilised in fibrin gel containing acidic fibroblast growth factor (aFGF) to bridge the rostral white to distal grey matter, and caudal white to rostral grey matter found functional improvement from three weeks to one year (Cheng et al., 1996). Another variation on the traditional grafting method used minced preconditioned autologous grafts, and found better functional recovery with a neurotrophic factor cocktail containing BDNF, NT-3 and GDNF (Feng et al., 2008). Some restoration of function has also been shown in humans (Cheng et al., 1996). In this human study, four pieces of peripheral nerve were grafted in conjunction with aFGF to direct white to grey matter pathways. This led to one paraplegic patient being able to walk two and a half years after the surgery.

While peripheral nerve grafting has shown promise for induction of axon regeneration from the brain and spinal cord, the extent of axon regeneration and functional
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Improvement is limited by the surgical procedure, astrogliosis and cyst formation (Benfey & Aguayo, 1982; Richardson, et al., 1982). In complete transection cases, huge cysts and a fibrous scar were found between the transection stumps (Cheng et al., 1996; Richardson et al., 1982). The simple procedure of producing a myelectomy, which is removing a small area of spinal cord tissue before graft insertion, created astrogliosis around the myelectomy region, with the extent of astrogliosis found to be the same with or without a graft (Dam-Hieu et al., 2006). Excision of the glial scar in preparation for grafting triggered severely impaired functional and axon recovery, very likely due to creation of another spinal cord injury induced in the repair process (Rasouli et al., 2006). This becomes a serious problem for successful grafting or alternative treatment outcomes, as the grafting procedure itself creates astrogliosis and cell loss that then impedes axon regeneration; little research has addressed this issue.

Cultured Schwann cells have been used as an alternative to peripheral nerve grafts because they exhibit the desired functions of a peripheral nerve, and are well characterised. To act as a guiding material, they are usually seeded in a synthetic conduit placed in the injury site (L. M. Ramer et al., 2005). They have been shown to proliferate and migrate in response to injury of associated axons, but the axons that have generated from the central nervous system (CNS) to the graft do not re-enter the CNS (L. M. Ramer et al., 2005). This is possibly due to CNS myelin inhibition and Chondroitin Sulfate Proteoglycan (CSPG) production (L. M. Ramer et al., 2005).

Olfactory ensheathing cells also express adhesion molecules and neurotrophic factors, but do not migrate in response to axonal injury. However, in olfactory ensheathing cell grafts, the regenerating axons do successfully extend from the CNS to the graft then back into the CNS again (Ramon-Cueto, Plant, Avila, & Bunge, 1998).

Autologous grafts such as those listed above are biocompatible and have low toxicity, but all suffer from an important drawback - tissue has to come from the patient (Schmidt & Leach, 2003). Therefore, attention has turned towards non-autologous grafts and natural or man-made scaffolds. Non-autologous grafts from cadavers, animals, or human placenta require either immune suppression of the host, or killing off
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the cells of the graft, leaving behind the extracellular matrix. Natural or man-made scaffolds such as poly-glycolic acid, poly-lactic acid or silicone tubing have shown some promise in nerve regeneration. One particularly interesting scaffold material, investigated in Chapters 6 and 7, is peptide amphiphile particles displaying a laminin epitope (IKVAV-PA gel) (Tysseling-Mattiace et al., 2008). The molecules self assemble into cylindrical nanofibers once they contact cations in blood or cerebrospinal fluid. The nanofibers then assemble a supra-molecular matrix scaffold, taking on a gel-like nature (Tysseling-Mattiace et al., 2008). This scaffold is very useful as a grafting material because it is injectable as a liquid and sets to the contour of the site, and is then stable for at least 2 weeks before being degraded.

Nanoparticles are the newest advancement in medicine, and have shown great versatility regards development for the desired medical purposes. Within the range of nanoparticles available, peptides make an excellent choice of particle building block for nanostructures in medicine because of their bio-compatibility and bio-degradability into nutrients (Cui, Webber, & Stupp, 2010). A team of researchers at the Northwestern University, Chicago, U.S.A. have developed a way to self-assemble peptide nanoparticles into a matrix of nanofibers by making them amphiphilic, i.e., incorporating a short hydrophobic block into a relatively hydrophilic peptide sequence (Beniash, Hartgerink, Storrie, Stendahl, & Stupp, 2005). The individual assembly nanoparticles of the IKVAV-PA gel are made up of four subunits (Figure 1.). The first component is a hydrophobic alkyl tail that remains inside the assembled structure when in aqueous solution. The second is a short sequence of hydrophobic peptides that tend to form hydrogen bonds with other such peptides to form beta sheets, thus forming longitudinal cylindrical structures when numerous such sheets are formed. The third component is charged amino acids that allow self-assembly of the nanoparticles when ions are introduced. The fourth component is the pentapeptide sequence isoleucine-lysine-valine-alanine-valine (IKVAV), which is a sequence from the protein laminin and possesses bioactive properties, but can be substituted with other sequences with different biological activities.
Figure 1.9. Molecular Composition and Structure of IKVAV-PA Nanoparticles and the Assembled Nanofibers and Matrix.
A. Molecular structure of an IKVAV-PA nanoparticle constitutes of 1) a hydrophobic alkyl tail, 2) a short sequence of hydrophobic peptides, 3) charged amino acids, and 4) the pentapeptide IKVAV sequence. B. Illustration of an IKVAV-PA nanoparticle and self-assembly of many nanoparticles into a cylindrical nanofiber. C. Transmission electron microscopy and D. scanning electron microscopy images of the assembled IKVAV-PA nanofiber matrix. Modified from Webber, Kessler & Stupp, 2009.
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These four components make the particles amphiphilic, which means that particles have a hydrophilic end that is water soluble and a hydrophobic region at the other end that tends to aggregate together in an aqueous solution. Peptide amphiphiles combine the structural features of amphiphilic surfactants with the functions of bioactive peptides, and can form stable hydrophobic, ionic and hydrogen bonds upon mixing with polyvalent ion solutions such as Ca$^{2+}$ and Mg$^{2+}$ solutions (Beniash et al., 2005; Cui et al., 2010). Furthermore, the amphiphilic nature of the particles allows the presentation of short hydrophilic peptide sequences such as IKVAV on the surface of the assembly (Cui et al., 2010). The IKVAV sequence is found naturally on laminin, and is one of the principle sites for cell adhesion and neurite growth (Nomizu, 1998; Tashiro et al., 1989). This IKVAV-PA gel treatment has already been shown to promote impressive regeneration of descending and ascending fibres compared to the no treatment control (Figure 1.10), reduced astrogliosis, reduced cell death, and increased numbers of oligodendrocytes when injected into a contused mouse spinal cord (Tysseling-Mattiace et al., 2008).
Figure 1.10. Axon Regeneration 11 Weeks after Severe Contusion Injury and IKVAV-PA Gel Treatment in Adult Mouse Spinal Cords.
A. Tracing of descending fibres from rostral spinal cord. B. Tracing of ascending fibres from the caudal spinal cord. When compared to no treatment controls, significantly more descending and ascending axon regeneration was seen entering and leaving the injury site to the opposite spinal cord stump after IKVAV-PA gel treatment. Modified from Tysseling-Mattiace et al., 2008.
1.5.4.4. Current experimental models

Most spinal cord injury research has been done using *in vivo* animal models, in which the spinal cords were contused, compressed, or transected (Onifer, Rabchevsky, & Scheff, 2007). Treatment effects can be studied in a natural physiological environment for as long as required and behavioural improvements measured, but problems exist that call for an alternative model of SCI repair. Spinal cord injured animals require extensive post-injury care including weight management, health management and bladder expression, and there is also inherent variability between animals that can make research data difficult to interpret. In addition, surgeries are relatively complex and experiments time consuming. In contrast, an *ex vivo* model is ethically advantageous, does not require post surgical animal care, enables more reproducibility between lesions, and provides a tightly controlled artificial environment that can be reliably compared between studies. Published *ex vivo* spinal cord models include the culture of several hundred micron thick transverse slices maintained for up to three weeks (Cho et al., 2009; Guzman-Lenis, Vallejo, Navarro, & Casas, 2008; Krassioukov et al., 2002), unfixed longitudinal 16 µm cryostat sections of spinal cord maintained for one week (Pettigrew, Shockley, & Crutcher, 2001), and spinal cord segments maintained for up to four hours (Hamann et al., 2008; Saruhashi, Matsusue, & Hukuda, 2002). There is a lack of segment culture models maintained for a relatively longer period of time; thus establishing a segment culture model became the first objective of this thesis.

The most common level of injury in humans is C5, but most *in vivo* rodent research on spinal cord injury, especially complete transection studies, involve the thoracic region only. This is because the level of animal care that is possible after cervical injury is not comparable to that of humans without the use of ventilators and intensive care. Thoracic injuries are much more manageable thus allowing larger study group size and better survival rates. For these reasons thoracic level T10 injuries were carried out in the *in vivo* experiments described in this thesis.
1.6. Connexins in the central nervous system

1.6.1. Introduction to gap junctions

1.6.1.1. Functions

Many cells of the central nervous system possess gap junctions, which are clusters of a few to hundreds of tightly packed intercellular channels that, in the simplest assessment, function to allow small molecules and ions with a size of less than 1 kDa to be directly exchanged between adjoining cells (W. Howard Evans & Patricia E. M. Martin, 2002), thus facilitating synchronous activity such as in the glial synctium by distributing the neurotransmitter and K⁺ gradients and creating waves of elevated ions, metabolites and currents (I. H. Lee, Lindqvist, Kiehn, Widenfalk, & Olson, 2005). There have also been recent reports of neurons communicating to each other in the form of electrical ‘synapse’ by passing ions through gap junctions between axons in addition to the standard chemical synapses (W. Howard Evans & Patricia E. M. Martin, 2002), allowing fast recruitment of neurons for oscillation activity in hippocampal pyramidal cells (I. H. Lee et al., 2005). The range of substances that are permeable via gap junction channels include ions, amino acids including glutamate, ATP, signalling molecules such as Ca²⁺, cAMP, IP₃, and larger linear molecules such as polypeptides and RNAi (I. H. Lee et al., 2005; Spray, Ye, & Ransom, 2006).

1.6.1.2. Constituents, formation and degradation

Each gap junction channel is made up of two hemichannels, also called connexons, on opposite cell membranes, and each hemichannel is made of six connexin (Cx) subunits (W. Howard Evans & Patricia E. M. Martin, 2002). They comprise a family of 21 (human) or 20 (mouse or rat) connexin types, each named according to their predicted molecular weight. For example, Cx43 cDNA has a predicted molecular weight of 43kDa. Connexins are synthesized in the ER, and assembled into a hexameric hemichannel in the ER or the endoplasmic-Golgi-intermediate compartment before being transported to the Golgi and integrated to the cell membrane via vesicular or direct (as in the case of Cx26) transport (Figure 1.11). The inserted hemichannels then diffuse laterally to the site of the gap junction plaque and become incorporated by docking with a hemichannel from the opposite cell (W. Howard Evans & Patricia E. M. Martin, 2002). The union between two hemichannels on opposite membranes is irreversible and of high affinity, forming a tight seal between the hemichannels with no
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leak to the extracellular space (Spray et al., 2006). A connexon or hemichannel can contain the same connexins (homomeric) or different connexins (heteromeric); a gap junction channel can contain two identical connexons (homotypic) or different connexons (heterotypic) (W. Howard Evans & Patricia E. M. Martin, 2002). Gap junctions have different half-lives, but that of Cx43 is approximately one and a half to three hours (Evans & Leybaert, 2007; W. H. Evans & Patricia E. M. Martin, 2002) so when it is time for a gap junction channel to be degraded, double membrane structures containing patches of the gap junction plaque, called a ‘connexosome’, form in one of the cells, carrying gap junction channels (Laird, 2006). These then get directed to lysosomes and proteasomes for degradation (W. Howard Evans & Patricia E. M. Martin, 2002).
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Figure 1.11. Schematic Representation of Gap junction Structure, Hemichannel Structure, Connexin Structure, and Life Cycle.
Connexin proteins are transmembrane proteins that possess intracellular amino (N) and carboxyl (C) terminals, four transmembrane domains and two extracellular loops. The structure of a Cx43 protein is shown. Modified from Laird, Puranam & Revel, 1991. Connexins are synthesised in the ER, assembled in the Golgi apparatus into a hexamer, and inserted into the membrane via direct or vesicular transport. The inserted hexamer is called a connexon or a hemichannel, which then diffuses laterally to dock with a connexon from an opposing cell to form a gap junction channel. Most gap junction channels exist in a plaque, which is identifiable with electron microscopy or immunohistochemistry. The gap junction channels are turned over constantly by forming ‘Connexosomes’ which are double membrane vesicles containing gap junction channels destined for degradation by lysosomes and proteasomes. Modified from Laird, 2006.
1.6.1.3. Hemichannels

Until the last 10 years, hemichannels not yet incorporated into gap junction plaques were considered to be non-functional because their opening to the extracellular space was hypothesised to cause loss of ionic gradients and metabolites, energy sources and secondary messenger molecules owing to their predicted large conductance (Spray et al., 2006). Recently, controlled and brief opening of hemichannels has been proposed to play a role in the release of large molecules such as ATP, glutamate, prostaglandin, and glutathione from cells (Evans & Leybaert, 2007). Under normal physiological conditions, hemichannels account for the release of small molecules from the cell (Saez, Contreras, Bukauskas, Retamal, & Bennett, 2003). Convincing evidence now supports the existence of functional connexin hemichannels; Cx46 expression in isolated Xenopus oocytes resulted in cell swelling and death (Paul, Ebihara, Takemoto, Swenson, & Goodenough, 1991); Cx43 expression in HeLa cells resulted in non-junctional currents characteristic of that expected of hemichannels (Spray et al., 2006). Hemichannel conductance of Cx30, Cx43, Cx45 and Cx46 is approximately twice that of a gap junction and the substances that permeate are sometimes different to that of the respective gap junctions (Saez et al., 2003).

1.6.2. Connexins in each CNS cell type

Although controversy still remains as to what connexin genes are expressed and proteins translated in which cell type of the CNS, there is agreement that Cx43 is mainly expressed in astrocytes and ependymocytes in the grey and white matter, Cx36 in neurons, and Cx32 in oligodendrocytes and Schwann cells mainly in the white matter in the normal SC and peripheral nerves (Rouach et al., 2002). These connexin types are the main targets for pharmacological manipulation of gap junction functions in the CNS.
Figure 1.12. Summary of Connexin (Cx) Expression in CNS Cell Types. Cx43 was found to be expressed in every cell type (highlighted in red). Neuronal connexins enclosed in brackets were found only occasionally and may not, therefore, be representative of all neurons.
1.6.2.1. Astrocytes and the glial syncytium

Due to the limits of resolution of light microscopy, the expression of connexins by a particular cell type, distinct from its neighbours, have not yet been agreed upon (Rouach et al., 2002). A study in 2004 demonstrated that the connexin composition of gap junction channels in the glial syncytium formed by astrocytes and oligodendrocytes was quite complex (Altevogt & Paul, 2004). Astrocytes were found to express Cx43, Cx30, and Cx26. Two distinct forms of gap junction plaques between astrocytes were found: plaques consisting of both Cx43 and Cx30 and plaques consisting of Cx26 only (Altevogt & Paul, 2004). Cx40, Cx45, Cx46 can also be expressed in astrocytes (Rouach et al., 2002). Besides inter-cellular junctions, astrocytes also possess junctions between the processes of a single cell, which may lead to the organisation of cellular compartments that are in turn coupled to neighbouring astrocytes to form functional units (Rouach et al., 2002). Cx43 is the major constituent of astrocyte connexins both in number and distribution. Astrocytes in Cx43 knockout have only 5% coupling compared to control (Dermietzel et al., 2000; Dermietzel, Hertberg, Kessler, & Spray, 1991; Rouach et al., 2002). Cx43 is found in astrocytes of all regions, whereas Cx30 is not found in the white matter, and Cx26 has a preference for subcortical regions, or more specifically subependymal, subpial and perivascular regions (Rouach et al., 2002). Cx43 is also found in all other cell types of the CNS (Figure 1.12). Therefore, interventions designed to modulate astrocyte gap junctions mainly target Cx43.

It is well established that astrocytes and oligodendrocytes form an extended glial syncytium via gap junctions. The syncytium functions to provide metabolic support by clearing K\(^+\) accumulation from the extracellular space after neuronal activity, and distributing K\(^+\) to areas of low concentration as seen in a brain slice culture (Holthoff & Witte, 2000). Homogeneous distribution of intracellular Na\(^+\) ion concentrations and cell volume regulation by redistribution of ions, amino acids and osmotically regulated water also depends upon astrocytic gap junctions (Rose & Ransom, 1997). Gap junctions provide intercellular signalling between glial cells by spreading Ca\(^{2+}\) waves to modulate glutamate release from astrocytes, that in turn impacts on synaptic activity (Altevogt & Paul, 2004; W. Howard Evans & Patricia E. M. Martin, 2002; Haydon, 2001). Nutrient supply to neurons also depends upon astrocyte gap junctions (Rouach et al., 2002).
1.6.2.2. Oligodendrocytes

Oligodendrocytes express Cx32, Cx29, Cx47, and Cx45 specifically; astrocyte to oligodendrocyte gap junctions may consist of either astrocytic Cx32 and oligodendrocyte Cx26 proteins, or astrocytic Cx43/Cx30 and oligodendrocyte Cx47 proteins (Altevogt & Paul, 2004). Cx29 does not colocalize with any of these five connexins (Altevogt & Paul, 2004). Oligodendrocyte to oligodendrocyte and intermyelin sheath gap junctions are rarely found suggesting that oligodendrocytes form gap junctions primarily with astrocytes (Altevogt & Paul, 2004; Rouach et al., 2002). Cx45 has also been reported in oligodendrocytes (Rouach et al., 2002).

1.6.2.3. Neurons and electrical synapses

Neurons are reported to express Cx36, Cx32, Cx26, and Cx43 in the order of the descending number of studies, that are also supported by a single cell RT-PCR study (Rouach et al., 2002). Cx37, Cx40, Cx45 and Cx47 expression was only very occasionally found (one study each). Cx36 is exclusive to neurons and possesses special properties such as low voltage sensitivity, small unitary conductance, and permeability to large anions, making them well suited to electrical and biochemical communication functions (Srinivas et al., 1999). Cx36 is also unique in that it is encoded by two exons instead of one (Saez et al., 2003). Various reports have shown that GABAergic interneurons in the CNS were indeed coupled via electrical synapses (Rouach et al., 2002). Neuron-glial gap junctions have only been found in a very limited number of cases and under certain conditions, such as in locus coeruleus slice culture and neuron-astrocyte co-cultures at 24-72 hours (Rouach et al., 2002). This kind of gap junction, however, was not found in a vigorous screening of more than 5000 gap junctions in several brain regions using immunogold freeze-fracture techniques (Rash, Yasumura, Dudek, & Nagy, 2001).

1.6.2.4. Other cells

In the normal rat cerebral cortex, resting microglia express Cx43 in less than 5% of their population with a diffuse cytoplasmic localisation, but when activated, Cx43 expression increases and becomes localised at the interface between cells coupled by gap junctions (Rouach et al., 2002). Fibroblasts and mesenchymal cells of the meninges express high levels of Cx43, Cx26, and Cx30 similarly to astrocytes (Rouach et al., 2002). Ependymocytes and CNS blood vessel endothelial cells also express Cx43 (Farahani et al., 2005).
The strength of gap junction channel mediated intercellular coupling is determined by the number of channels in the membrane, their functional state and unit permeability, rather than merely passive diffusion (Herve, Bourmeyster, Sarrouilhe, & Duffy, 2007). The number of channels can be regulated at the transcriptional level as well as at each step of channel insertion and removal (Herve et al., 2007), which takes place over hours or days (Rouach et al., 2002). The functional state and unit permeability are regulated by various molecular processes and interaction with other proteins in a multiprotein complex (Herve et al., 2007) over the time scale of minutes (Rouach et al., 2002). Membrane depolarisation, dephosphorylation, high intracellular pH, hyposmolarity, and extracellular Ca\(^{2+}\) all increase astrocyte gap junction permeability. ATP and nitric oxide decreases permeability. Intracellular Ca\(^{2+}\) concentrations in the 500 – 2000 nM range close gap junctions (Rouach et al., 2002). The multiprotein complex that gap junctions interact with involves regulatory proteins, signalling enzymes, substrates, protein effectors, scaffolding proteins, and the cytoskeleton (Herve et al., 2007).

The number of hemichannels in the cell membrane is likely regulated by the same mechanisms as those that regulate the number of gap junctions, since they are an intermediate step in gap junction formation. Membrane depolarisation, dephosphorylation and high intracellular pH (the same processes that increase gap junction permeability) also increase the ‘open probabilities’ of hemichannels. Metabolic inhibition, oxygen, tension, intracellular redox potential, oxidative stress, cytoplasmic signals, quinine and quinidine all increase the open probability of hemichannels (Evans & Leybaert, 2007; Saez et al., 2003). A major difference in gap junction and hemichannel regulation is the differential Ca\(^{2+}\) regulation. Intracellular Ca\(^{2+}\) concentrations of 500 – 2000 nM close gap junctions but open hemichannels and decreases in extracellular Ca\(^{2+}\), such as in ischemic conditions, result in lower gap junction permeability and higher hemichannel ‘open probability’ (Evans & Leybaert, 2007). An extracellular Ca\(^{2+}\) concentration higher than 1 mM and negative membrane potentials, such as in normal physiological conditions, close hemichannels (Evans & Leybaert, 2007; Saez et al., 2003). Application of Ni\(^{2+}\), Co\(^{2+}\), Mg\(^{2+}\), La\(^{3+}\), various gap junction blockers, and retinoic acid decreases the ‘open probability’ of some hemichannels (Saez et al., 2003).
1.7. Cx43 in spinal cord injury

Following complete transection SCI in adult rats, Cx43 mRNA and protein levels as assessed by *in situ* hybridisation and immunohistochemistry increased from 4 hours after injury to at least 3 times normal levels by 4 weeks (I. H. Lee et al., 2005). The levels of neuronal Cx36 and oligodendroglial Cx32 did not show significant changes (I. H. Lee et al., 2005). The effects of this Cx43 upregulation are still controversial, but it is hypothesised that glial gap junction communication after injury can be both detrimental and beneficial depending on the specific circumstances at a precise time point (Perez Velazquez, Frantseva, & Naus, 2003), such that the effects of increased Cx43 expression can be speculated to have different effects.

Up-regulation of Cx43 is detrimental to SCI in the acute phase of the injury. Death signals can be propagated via Cx43 gap junctions; Cx43 expression in injury-resistant cells leads to decreased survival once Cx43 was expressed and gap junctions were formed (J. H. Lin et al., 1998). Excessive intracellular Ca$^{2+}$ and water accumulation is a direct trigger for the initial necrotic cell burst following SCI but astrocytic gap junctions then contributed to propagation of a Ca$^{2+}$ wave both directly and indirectly (Rouach et al., 2002). Evidence for direct involvement includes the following: gap junctions were permeable to Ca$^{2+}$; Ca$^{2+}$ wave spread was not directed by speed and direction of any Ca$^{2+}$ gradient; gap junction uncoupling agents blocked Ca$^{2+}$ propagation; cells devoid of gap junctions spread Ca$^{2+}$ waves once Cx43 was expressed (Rouach et al., 2002); intracellular messenger of the Ca$^{2+}$ cascade IP$_3$ diffuses through Cx43 gap junctions (Rodriguez-Sinovas et al., 2007). Besides direct propagation of Ca$^{2+}$ ions, this Ca$^{2+}$ wave can also spread indirectly via the extracellular messenger ATP (Rouach et al., 2002) that is released from Cx43 hemichannels (Stout, Costantin, Naus, & Charles, 2002). Given that Cx43 is the main constituent of astrocytic gap junctions and is capable of coordinating synchronous activity over large populations of glial cells, Cx43 up-regulation and subsequent enhanced gap junction communication may play a role in propagating death signals and Ca$^{2+}$ waves extensively and rapidly in the glial network in the acute phase (J. H. Lin et al., 1998).
When examining the role of Cx43 after spinal cord injury, it is necessary to consider both gap junctions and hemichannels. Hemichannels have increased open probability under ischemic conditions such as metabolic inhibition, positive membrane potentials and low extracellular Ca\(^{2+}\) concentration (Evans & Leybaert, 2007), all possible after SCI. There are several mechanisms proposed by which hemichannels can contribute to cell death and survival. First of all, Cx43 hemichannels are permeable to ATP, NAD\(^+\), glutamate, and IP\(_3\) (W. Howard Evans & Patricia E. M. Martin, 2002). After CNS injury, opening of astrocyte hemichannels could lead to propagation of Ca\(^{2+}\) waves by releasing ATP, NAD\(^+\) and glutamate from astrocytes, and spread of IP\(_3\) between neighbouring astrocytes hence spreading the injury (W. Howard Evans & Patricia E. M. Martin, 2002; Rodriguez-Sinovas et al., 2007; Rouach et al., 2002; Verma, Hallett, Leybaert, Martin, & Evans, 2009). Furthermore, astrocytes have a yet unidentified mechanism for sensing low extracellular Ca\(^{2+}\) concentrations and respond by releasing Ca\(^{2+}\) from intracellular stores, increasing intracellular Ca\(^{2+}\) concentration and enhancing hemichannel opening (Saez et al., 2003). Secondly, hemichannels have been implicated in cell swelling, which is a hallmark of ischemic cell death by a mechanism not yet understood (Rodriguez-Sinovas et al., 2007). Over-expression of Cx46 in Xenopus oocytes and Cx43 in single cells resulted in cell swelling and lysis when the extracellular Ca\(^{2+}\) concentration was lowered, a condition promoting hemichannel opening (Rodriguez-Sinovas et al., 2007). Therefore, pathological Cx43 hemichannel opening may contribute to lesion spread in several ways.

Up-regulation of Cx43 could be beneficial at later stages. CNS injury has been shown to induce Cx43 expression in microglia, and this enhanced Cx43 gap junction communication has been postulated as a pathway for microglia activation signals (Eugenin et al., 2001). Activation of microglia can be beneficial to recovery since their activation and removal of lesion debris is essential preparation for subsequent regeneration. The native microglial activation response is long term and bi-phasic, with a first peak at 7-9 days and a second peak at approximately 60 days after injury (Beck et al., 2010). Insufficient activation and infiltration of immune cells can be an issue after SCI because of the blood-spinal cord-barrier, although the barrier is usually damaged initially to some extent (Schwartz, 2000), suggesting that promoting microglial activation by up-regulating Cx43 gap junction communication at later stages might promote recovery and regeneration.
Despite conflicting findings on the effects of up-regulated Cx43 after SCI, work done on in vivo models supports the idea that the detrimental effects outweigh benefits, at least in the first 24 hours after injury (M. Cronin, Anderson, Cook, Green, & Becker, 2008). Transient knockdown of Cx43 protein expression using antisense oligodeoxynucleotides immediately following rat compression and partial transection injuries resulted in better locomotor recovery, reduced swelling, less tissue disruption, and reduced astrocyte hypertrophy in the long term. Reduced blood-spinal cord-barrier leakiness, and reduced neutrophil recruitment and microglial activity around lesion sites was also observed (M. Cronin et al., 2008). Functional recovery was improved by the Cx43 knockdown effects on astrocyte gliosis despite the fact that microglia activation and immune cell infiltration from blood vessels was suppressed (M. Cronin et al., 2008). Work done using organotypic brain slice cultures has also shown that blocking Cx43 expression with antisense oligodeoxynucleotides rescued cells and synaptic function after impact (Frantseva, Kokarovtseva, Naus et al., 2002) and hypoxic injury (Frantseva, Kokarovtseva, & Perez Velazquez, 2002). Similar effects were achieved with knockdown of Cx32 and Cx26 which are expressed in neurons (Frantseva, Kokarovtseva, Naus et al., 2002; Frantseva, Kokarovtseva, & Perez Velazquez, 2002). Non-specific gap junction inhibitors such as octanol and halothane have also shown neuron protective effects in focal and global brain ischemia (Perez Velazquez et al., 2003).
1.8. Connexin43 antisense oligodeoxynucleotides

To counter the detrimental effects of Cx43 upregulation, a way to specifically and effectively modulate Cx43 protein expression was developed using Cx43 antisense oligodeoxynucleotides (Cx43 AsODN) (Green, Law, Lin, & Becker, 2001). This Cx43 AsODN is a single strand DNA of 30 deoxynucleotides with an unmodified backbone and binds specifically to complementary sequences on an accessible region of rat Cx43 mRNA, halting protein translation (Green et al., 2001). Once bound to the mRNA, RNase-H degradation of the mRNA occurs (Green et al., 2001). Antisense oligodeoxynucleotides (AsODN) typically have a half life of only approximately 20 minutes, therefore, a Pluronic F-127 gel (Pluronic gel) delivery system was employed to provide sustained delivery (Law, Zhang, Stott, Becker, & Green, 2006). Pluronic gel is liquid at cold temperatures (4°C), sets into a soft gel at physiological temperatures, and stays as a gel for several hours in culture conditions depending on the level of medium. The gel is also a mild surfactant so aids AsODN penetration into cells. The sustained delivery of Cx43 AsODN to tissue avoids the need to use high doses or modified backbones that have lower affinity, lower efficiency at entering cells and reduced specificity (Green et al., 2001).
Introduction

Figure 1.13. Mechanism of Action of Cx43 Antisense Oligodeoxynucleotides (Cx43 AsODN).
In the process of protein production, DNA is transcribed into RNA, processed into messenger RNA (mRNA) which then exits the nucleus into the cytoplasm. There the mRNA is translated on a ribosome to produce Cx43 protein. When Cx43 AsODN is applied, it binds to the mRNA to stop its translation, and promotes the mRNA's degradation by RNase-H, resulting in inhibition of translation.
Introduction

Up until now, the same Cx43 AsODN has been used in several experimental disease models including optic nerve ischemia (Danesh-Meyer, Huang, Nicholson, & Green, 2008), skin wound healing (Mori, Power, Wang, Martin, & Becker, 2006; Qiu et al., 2003; C. M. Wang, Lincoln, Cook, & Becker, 2007), skin burn healing (Coutinho et al., 2005), and spinal cord injury (M. Cronin et al., 2008), demonstrating anti-inflammatory and wound healing-promoting effects. Rapid penetration (4-8 hours) of Cx43 AsODN in all areas, continued replenishment of Cx43 AsODN (1-6 hours) to overcome the 20 minute short half-life, cellular breakdown of Cx43 AsODN, and recovery of Cx43 levels (48-72 hours) was demonstrated in vivo after 250 µL of 1 µM Cx43 AsODN was applied to intact rat spinal cords with intact dura mater (Michael Cronin, Anderson, Green, & Becker, 2006). Connexins have a half life of around 1.5-2 hours (Leithe, Brech, & Rivedal, 2006) and the delayed and differential rate of recovery of Cx43 expression (48-72 hours) after Cx43 AsODN clearance by 24 hours indicates that there is a slower turn over of Cx43 in dorsal white matter and ventral grey matter than dorsal grey matter, possibly reflecting differences in the number of astrocytes and astrocytic Cx43 expression (Michael Cronin et al., 2006).
1.9. Objectives

Transient regulation of Cx43 expression after SCI has been shown to be very beneficial (M. Cronin et al., 2008; Michael Cronin et al., 2006). A one-off application of the same Cx43 AsODN immediately after compression injury in rats resulted in better recovery in locomotion, less swelling and tissue disruption, less blood vessel leakage and less glial up-regulation starting from one day after injury and lasting for at least 28 days (M. Cronin et al., 2008). Reduced glial up-regulation, reduced neutrophil recruitment and reduced microglia activity was seen after partial transection spinal cord injury and acute Cx43 AsODN treatment in rats (M. Cronin et al., 2008). In reality, however, the majority of SCI patients are unlikely to present early enough, and existing patients cannot be cured. Therefore, the specific aim of my study was to investigate the application of Cx43 AsODN in conjunction with spinal cord repair strategies, overcoming the limitations of existing protocols (whether that be cell implants, nerve grafts or scaffolding implants) by reducing astrogliosis and bystander cell loss, and promoting peripheral nerve grafting efficacy. These limitations exist owing to the fact that the intervention in itself creates a new lesion reducing repair efficacy.

The first objective of my study was to establish an *ex vivo* spinal cord segment culture model for study of repair strategies. The *ex vivo* model was then used to test the effects of Cx43 AsODN in promoting axon regeneration into peripheral nerve grafts before translating the technology into an *in vivo* model. *Ex vivo* testing of the properties of a synthetic implant IKVAV-PA gel was then carried out with the final objective of *in vivo* testing of Cx43 AsODN treatment in inducing axon repair in conjunction with this synthetic implant. This set of studies was designed to enable comparison between peripheral nerve graft therapies in conjunction with Cx43 AsODN, and nanofiber technology in conjunction with Cx43 AsODN treatments.
1.10. Hypothesis

The hypothesis to be tested through this thesis is that regulation of gap junction channels in conjunction with spinal cord injury repair strategies can prevent lesion spread and formation of scar tissue separating the host tissue from the donor graft, leading to more effective outcomes.
Chapter 2. Methods and materials

2.1. *Ex vivo* spinal cord segment culture

2.1.1. Animals

Postnatal day 7 Wistar rats were used in this study. Protocols and use of animals were approved by the University of Auckland Animal Ethics Committee in accordance with guidelines provided by the National Animal Ethics Advisory Committee of New Zealand.

2.1.2. Spinal cord removal

Animals were decapitated at the cervical C2-C3 region and a longitudinal medial incision made in the skin down the back of the animal. A horizontal cut was then made at the lumbar L1 region of the vertebrae, and all the dorsal vertebrae rostral to the cut (C1-L1) were excised, exposing the spinal cord. Ice cold Hank’s Balanced Salt Solution (HBSS, Gibco 24020-117) was then applied to keep the spinal cord moist during removal. The peripheral nerves and connective tissue beneath the exposed spinal cord were cut with spring scissors and the spinal cord removed from the vertebrae with a blunt curved probe. The tissue was washed briefly in ice cold HBSS and placed onto wet filter paper. Six 0.5 cm segments for culture establishment, or two 1.5 cm long segments for peripheral nerve grafting experiments, were cut from one spinal cord with a tissue chopper (Starrett No.263M), and transferred to 30 mm Millicell culture inserts with a 30 mm diameter and 0.4 µm pore size (Millipore PICMORG-50) and placed in pre-warmed Neurobasal-A medium (Gibco 12349-015) in 100 mm petri dishes (BD Falcon 353003).

2.1.3. Spinal cord culture

The spinal cord segments and peripheral nerve grafted segments were cultured in an air-liquid interphase culture using porous membrane inserts; each insert contained one segment. Inserts were placed in 100 mm culture dishes and incubated at 37 °C, 5% CO₂. Neurobasal-A medium (8 mL) supplemented with 2% B27 (Gibco 17504-044), 1% of 100 U/mL Penicillin and 100 µg/mL streptomycin (Penicillin and streptomycin Gibco 15140-122), and 1 mM L-Glutamine (Gibco 25030-081) was used for the first 18 hours of culture. After that inserts were transferred to Minimum Essential Medium with
Hank’s salts and 2 mM L-Glutamine (Gibco 11575-032) (9 mL) with 25% heat-inactivated horse serum (Gibco 26050-088), 25% HBSS, 100 U/mL Penicillin, 0.1 mg/mL streptomycin, 25 mM HEPES buffer solution (Gibco 15630-080), 1 mM L-Glutamine, and 25.6 mg/mL D-Glucose (Sigma G8769) and cultured for another four days (Stoppini, Buchs, & Muller, 1991).

2.1.4. Connexin43 antisense oligodeoxynucleotide delivery and dose response
To determine the optimum concentration of Cx43 AsODN to be used for subsequent experiments, a dose response experiment was performed using 0, 0.1, 2, 5, 10 and 25 μM Cx43 AsODN (sequence 5’ GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC 3’, unmodified backbone) on 0.5 cm spinal cord segments. Ten segments were included for each dose. Results showed that 2 μM was the most effective dose as measured by the reduction in segment swelling; 2 μM was, therefore, used in all subsequent experiments. A total volume of 30 μL (for 0.5 cm segment culture establishment) of Cx43 AsODN at a concentration of 2 μM in 30% Pluronic F-127 gel (Sigma, 30% w/v in KH₂PO₄ 1.1mM, Na₂HPO₄ -7H₂O 4.5mM solution) was applied to the spinal cord segment forming a coating on top and around the sides of the tissue. Unmodified backbone nucleotides give greater specificity and reduce potential toxicity effects but are prone to serum-born nuclease degradation. Serum-free Neurobasal-A medium was, therefore, used for the first 18 hours. While modified backbone nucleotides have been used by others, in general these have a weaker affinity for the message of interest, can be less efficient at entering cells, and can cause non-specific inhibition by binding to essential proteins (Milligan et al., 1993; Wagner, 1994; Shimmings, 1998). Pluronic gel remains liquid at low temperature (4 °C), sets as it warms to tissue temperature, and dissolves after 6 to 8 hours of immersion in culture medium. The same volume of Pluronic gel or medium alone was used for control segments or grafts.

2.1.5. Measurement of spinal cord swelling
For analysis of spinal cord swelling, twenty segments each received 30 μL of Cx43 AsODN in Pluronic gel or Pluronic gel alone and ten segments received 30 μL of culture medium. In order to analyse the amount of swelling that had taken place in the first 18 hours post dissection and before the culture medium change from Neurobasal-A to Minimum Essential Medium, both ends of the 0.5 cm spinal cord segments were photographed with a digital camera (Nikon Digital Sight DS-5Mc) attached to a stereo microscope (Zeiss Discovery V20). ImageJ software was used for image analysis. The
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*en face* area of swelling was determined by demarcating the outer edge relative to the dura mater wrapped around the original length of the spinal cord segment (Figure 2.1). The diameter of the spinal cord at that end was also measured. Because the area of swelling was greatly influenced by diameter (which varies from 1.4 mm to 3.3 mm), area of swelling cannot be used independently as an accurate measure; diameter differences must be compensated for accordingly.
Two measurements were made at each end of the spinal cord – the en face area of swelling (area enclosed in blue lines) and diameter (black line). The en face area of swelling was determined by demarcating the outer edge relative to the dura wrapped around the original length of the spinal cord. The area of swelling was then divided by the diameter to take account of the variation in cord diameter.
2.1.6. Determination of Cx43 knockdown and short term changes in Cx43 protein levels

To determine Cx43 expression knockdown after low and high dose Cx43 AsODN treatment, spinal cord samples were analysed for protein levels using Western blotting. Six 0.5 cm spinal cord segments from one animal were used in each treatment group (0 µM, 2 µM, and 25 µM Cx43 AsODN). Segments from one animal were homogenised immediately after dissection to serve as a control for normal Cx43 expression (0 hour control). Spinal cord segments were cultured for 18 hours after 0, 2, and 25 µM Cx43 AsODN treatment in Pluronic gel. The segments from one animal were then pooled before homogenisation to represent one whole spinal cord from an animal. The four samples were run in parallel, allowing direct comparison of different groups on a single gel.

To determine the short term changes in Cx43 expression after dissection, spinal cord segments were cultured for 2, 4, or 6 hours after 2 µM Cx43 AsODN or Pluronic gel or medium only treatment, and analysed for Cx43 protein expression. Six 0.5 cm spinal cord segments from one animal were used for each treatment and time point (0, 2, 4, and 6 hours). Western blotting is a semi-quantitative method of visualising proteins of interest; therefore, measurements between different runs cannot be accurately compared. The segments from one animal were pooled before homogenisation to represent one whole spinal cord from that animal. Samples were loaded into the wells of two gels with the same fresh spinal cord sample as control loaded on each. These gels were then run in parallel on the same electrophoresis apparatus and processed in exactly the same way as a single experiment in order to minimise variation.

To prepare samples, tissue was homogenised in ice-cold homogenisation buffer (150 mM sucrose, 50 mM HEPES pH 7.9, 60 mM KCl, 5 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, and one Complete mini protease inhibitor cocktail tablet (Roche 04693124001) per 10 ml) using a syringe with descending diameter needles (18 G, 21 G, 27 G) to break up tissue until the solution could be freely drawn up and down a 27G needle. Samples were then sonicated for 30 seconds each while being kept on ice. Samples were incubated on ice for 1 hour with 1% Triton-X100. The sample was centrifuged at
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14,000 rpm for 10 minutes, and the pellets re-suspended in the supernatant; larger particles not homogenised well were broken up. The samples were centrifuged and re-suspended once more, then centrifuged at 14,000 rpm for 3 minutes, and the pellet discarded.

Sample protein concentrations were determined with the Biorad RC DC Protein Assay Kit II (500-0122) following kit instructions. Samples were diluted with homogenisation buffer to a standard concentration of 1 µg/µL for western blotting. A 10 µL sample in 10 µL of sample loading dye, and 10 µL of Benchmark pre-stained protein ladder (Invitrogen 10748-010) were run on a 10% separating bis-acrylamide gel with 4% stacking gel for 50 minutes at 170 mV in SDS-PAGE denaturing buffer (0.2M glycerine, 25 mM Tris pH 6.8, 35 mM SDS). Separated proteins and the ladder on the gel were transferred to PVDF membrane that was activated by pre-soaking in 100% methanol, and assembled with the gel in a wet transfer apparatus. Transfer was performed at 170 mA for 1 hour in cold transfer buffer (25 mM Tris, 192 mM Glycine and 20% v/v methanol). The membrane was blocked with 5% non-fat milk powder in TBS-T (containing 20 mM Tris, 137 mM NaCl, 0.1% Tween-20) for 30 minutes and thoroughly washed (6 times for 5 minutes each in TBS-T). All membranes were labelled with rabbit anti-Cx43 antibody (Sigma C-6219, 1:8000) in antibody solution (TBS-T with 2 mM EDTA pH 8.0 and 1% bovine serum albumin) overnight, thoroughly washed, followed by anti-rabbit Ig Horse Radish Peroxidase linked whole antibody (Amersham Biosciences NA934V, 1:40000) in antibody solution, then thoroughly washed again. The signal was detected using an Amersham ECL Plus Western Blotting Detection System (GE Healthcare RPN2132) and a Fujifilm LAS 3000 Imager with the chemiluminescence function. A visible light photo of the protein ladder was also taken without moving the membrane.

Equal loading was controlled for by the amount of a housekeeping protein GAPDH or beta-actin. This is necessary even though sample protein concentrations were determined and each sample lane should contain 10 µg of protein. After Cx43 detection, the membrane was incubated with stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM beta-mercaptopoethanol) for 30 minutes at 50 ºC, thoroughly washed, blocked and labelled in the same way as before for the house keeping protein. The primary
antibodies used were rabbit anti-GAPDH antibody (Sigma Aldrich G9545, 1:4000) or mouse anti-beta-actin antibody (Abcam ab6276, 1:5000).

The images taken were then analysed with ImageJ. The integrated density of Cx43 bands and GAPDH (or beta-actin) bands was measured, and a relative density calculated by dividing the integrated intensity of Cx43 by GAPDH (or beta-actin).

2.1.7. Determination of optimal culture period

To determine the optimal culture period, two 0.5 cm spinal cord segments per time point received 30 µL of 2 µM Cx43 AsODN treatment, and were cultured for 5, 7, or 14 days. Two fresh fixed spinal cord segments served as controls. Medium was changed twice a week. Two segments each were fixed at the end of the culture period with 4% paraformaldehyde. The fixed segments were processed for cryosectioning (please refer to 2.1.9) and a cross section from the middle of the segment analysed for glial responses and neuron survival using immunohistochemical labelling.

2.1.8. Cell Viability Assay

The viability of the explant tissue was assessed using a Cell Tracker Green CMFDA (Invitrogen C7025) live cell assay. CMFDA only fluoresces when it is cleaved by intracellular enzymes active in viable cells. Six 0.5 cm segments were cultured for each culture period (1 or 5 days) and for each treatment (2 µM Cx43 AsODN or culture medium only). At the end of 1 day or 5 days in culture, 6 segments from each group were detached from the culture inserts and immersed in 200 µL of a 25 µM CMFDA solution in Minimum Essential Medium without horse serum for 3 hours at 37°C, followed by a wash in Minimum Essential Medium without horse serum for 30 minutes at 37°C. The spinal cords were then snap-frozen in Tissue-Tek O.C.T. compound (Sakura Finetek 4583) and cryosectioned at 30 µm using a cryostat (Zeiss Mikrom HM550) and mounted on Superfrost plus slides (Menzel Glazer). An air-dried cross section from the middle of the segment (2.5 mm from either end) was photographed with a confocal laser scanning microscope (Olympus Fluoview-1000) using a 4x objective. All 12 cross sections obtained at each time point were photographed with the same gain, offset, and laser power settings to allow for comparison of intensity of labelling between Cx43 AsODN treated and medium treated groups. Segments
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processed on different days required different settings to allow for intensity analysis using a full grey scale.

2.1.9. Immunohistochemistry

After 5 days of culture, the tissue was fixed in 4% paraformaldehyde (Sigma P6148) in phosphate buffered saline (Oxoid BR0014G) overnight, washed 3 times, 15 minutes each in PBS, and then cryoprotected in 20% sucrose for 3 hours before it was embedded in Tissue-Tek O.C.T. compound. The embedded segments were then rapidly frozen in liquid nitrogen. For neuron survival analysis 12 µm thick cross sections were cut using a cryostat and mounted on Superfrost plus slides. After blocking with blocking solution (1% normal goat serum, 0.2% Triton-X100 in PBS) for 1 hour, sections were incubated with mouse anti- non-phosphorylated neurofilament H monoclonal antibody (SMI32) (Sternberger Monoclonals, diluted 1:1000 in PBS) at 4 ºC overnight to label neurons and their axons. After washing in PBS 3 times, 15 minutes each, slides were incubated in antibody against mouse IgG (H+L) conjugated to Alexa-568 dye (Molecular Probes, A-11031, 1:400) for 3 hours. They were then washed in PBS 3 times, 15 minutes each, and mounted in anti-fade mounting medium (Citifluor AF-1). Rabbit anti-GFAP antibody (DAKO Z0334, 1:1000) single label was used in the experiment to determine the optimal culture time period.

2.1.10. Image quantification

Neuron survival in cultured spinal cord segments was assessed by taking 7-8 cross sections along the length of each spinal cord segments, a distance of 700 µm apart, and labelling them with the neuronal marker anti-SMI32 antibody. Ten segments from each treatment group (2 µM Cx43 AsODN, Pluronic gel, or culture medium only) were analysed after five days of culture. Spinal cords freshly fixed after dissection were used as controls. Two confocal images (Olympus Fluoview-1000, 20x objective) were taken from the left and right grey matter areas of each cross section and the number of healthy neurons counted. Healthy neurons were defined as having normal cytoplasm and extended neurites, as apposed to a dense and pyknotic cytoplasm with no extending neurites. The total number of healthy neurons from each cross section was calculated as the sum of the number of healthy neurons in the left and right grey matter areas; the data was analysed with SPSS.
2.1.11. **Statistical analysis**

The degree of swelling and the average number of healthy neurons in spinal cord cross sections was compared between treatments using SPSS (version 14.0) and one-way ANOVA analysis. Homogeneity of variance test was performed to test the assumption of equal variances. If group variances were not equal, the Brown-Forsythe test was performed instead of a one-way ANOVA and the ‘equal variances not assumed’ results of planned comparisons were read. If group variances were equal, a one-way ANOVA test was performed. If this produced a p-value of less than 0.05, a conclusion was reached that there was a significant difference between these three groups, and planned comparisons were made to find out which pairs contributed to this difference. To test the hypothesis that Cx43 AsODN treatment produces a significant improvement in outcome compared to both Pluronic gel and medium, planned comparisons were made between Cx43 AsODN and Pluronic gel, Cx43 AsODN and medium, Cx43 AsODN with both control groups, and Pluronic gel and medium using the contrast analysis function. One-tailed significance was used for the first three comparisons.
2.2. **Ex vivo peripheral nerve grafting**

2.2.1. **Sciatic nerve ex vivo culture**

Before performing *ex vivo* fresh peripheral nerve grafting, the phenotypic changes of sciatic nerves in culture were characterised by immunohistochemistry. Two sciatic nerves were analysed after culturing for different periods of time (0, 1, 2, 3, 4, 5, 6 and 7 days). P7 Wistar rats were decapitated. An incision down the thigh of the animal exposed the biceps femoris and the gluteus maximus muscles. The muscles were separated by blunt dissection and the sciatic nerve identified in the groove between the muscles. The rostral end of the sciatic nerve was traced to its origin in the spinal cord, and the caudal end traced to the knee joint. A 5 mm section of sciatic nerve between the two ends was excised, and briefly washed in ice cold Liebovitz’s L-15 medium (Invitrogen 11415-114) before being transferred to 1 mL pre-warmed D-10 medium (Dulbecco’s Modified Eagle Medium High Glucose supplemented with 10% fetal calf serum, 1% of 100 U/mL Penicillin and 100 µg/mL Streptomycin, 2 mM L-Glutamine) in a six-well culture plate. At the end of culture, sciatic nerves were fixed by immersion in 4% paraformaldehyde overnight, cryoprotected with 20% sucrose for 3 hours, and then frozen in liquid nitrogen in OCT compound.

Cross sections of 12 µm were immunolabelled for mouse anti-non-phosphorylated neurofilament H monoclonal antibody (SMI32) (Sternberger Monoclonals, 1:1000) or mouse anti-S100 antibody (Chemicon MAB079-1, 1:200) at 4°C overnight to label axons (SMI32) and Schwann cells (S100). After washing in PBS 3 times, 15 minutes each, sections were incubated in antibody against mouse IgG (H+L) conjugated to Alexa-568 dye (Molecular Probes, A-11031, 1:400) for 3 hours. Sections were then washed in PBS 3 times, 15 minutes each, and mounted in anti-fade mounting medium (Citifluor AF-1). Fluorescent images were taken with a fluorescence microscope (Leica DMRA) and recorded with a digital camera (Nikon digital sight DS-U1) and the imaging software NIS-Elements BR (version 2.10).

2.2.2. **Ex vivo peripheral nerve grafting procedures**

For *ex vivo* peripheral nerve grafting, two 1.5 cm long segments of spinal cord were obtained from each animal. Using the same animal, the left and right sciatic nerves were exposed. Approximately 5 mm long sciatic nerve segments from both thighs were
dissected out, and washed briefly in HBSS. The petri dish containing the freshly
dissected 1.5 cm spinal cord segments was placed under a stereomicroscope (Zeiss).
Using a 3.00 mm straight Ophthalmic slit knife (Alcon Surgical 8065-922961) an
incision was made in the centre of the spinal cord segment and the tool then held
obliquely in the lesion site to create a cavity for peripheral nerve insertion. One end of
the unconditioned sciatic nerve was gently squeezed inside the space between the slit
knife and the spinal cord using fine tweezers. The peripheral nerve was positioned at
least half way into the depth of the spinal cord so that it traversed both the grey and
white matter. The slit knife was then gently removed. The second sciatic nerve, from the
same animal, was inserted, in the same way, into the other segment. For each 1.5 cm
segment with peripheral nerve grafting 80 µL of Cx43 AsODN at a concentration of 2
µM in 30% Pluronic gel F-127 (Sigma) was applied to the spinal cord / peripheral nerve
complex forming a coating on top and around the tissue. The same amount of Pluronic
gel or culture medium was applied to control segments.
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Figure 2.2. Illustration of ex vivo Peripheral Nerve Grafting. A stab wound was created in the middle of the spinal cord segment, and one end of a sciatic nerve inserted into the wound and held tight by the surrounding spinal cord tissue. The graft traversed both the grey and white matter.
2.2.3. Peripheral nerve grafting experiment design

Because 0.5 cm spinal cord segments were not long enough to withstand the surgical manipulation and lost tissue integrity during a peripheral nerve grafting procedure, the suitability of 1.5 cm spinal cord segments was investigated by examining tissue integrity and neuron survival after tissue dissection, single unconditioned fresh peripheral nerve grafting and culture. Four segments were used in each treatment group (Cx43 AsODN, Pluronic gel and culture medium only control). After five days of culture, the spinal cord segments were fixed and cryosectioned longitudinally at 12 μm. Sections were immunohistochemically labelled for neurons and their axons using mouse anti- non-phosphorylated neurofilament H monoclonal antibody (SMI32) (Sternberger Monoclonals, 1:1000) followed by secondary antibody against mouse IgG (H+L) conjugated to Alexa-568 dye (Molecular Probes, A-11031, 1:400). Nuclei of neurons were labelled for using anti-Neuronal Nuclei antibody (Chemicon MAB377, 1:60) followed by secondary antibody against mouse IgG (H+L) conjugated to Alexa-568 dye (Molecular Probes, A-11031, 1:400). This experiment confirmed that 1.5 cm segments treated with Cx43 AsODN survived as well as 0.5 cm segments treated with Cx43 AsODN in terms of tissue integrity and neuron survival. Owing to deficiencies in surgical techniques while inserting the graft, a gap was present between the graft and the spinal cord and no axon outgrowth was found. The experimental design was improved in the major experiment by keeping the horizontal slit created as the graft site to a minimum width, so that the grafts could maintain tight contact with the spinal cord. The major peripheral nerve grafting experiment was then performed using this improved method.

The major peripheral nerve grafting experiment was carried out with 6 segments treated with Cx43 AsODN, 3 segments with Pluronic gel, and 3 segments with medium only control. Axon regeneration was labelled for using anti-SMI32 antibody (Sternberger Monoclonals, 1:1000). The peripheral nerve grafts were identified using anti-laminin antibody labelling (Sigma L-9393, 1:60) on the same sections. The two primary antibodies were applied as a mixture. A mixture of antibodies against mouse IgG (H+L) conjugated to Alexa-568 dye and antibodies against rabbit IgG (H+L) conjugated to Alexa- 488 dye (Molecular Probes, A-11034, 1:400) was used in the second step. Sections were screened to identify the section from each graft that displayed the greatest
amount of outgrowth from the spinal cord into the peripheral nerve graft, and confocal microscopy images were then taken of the area surrounding the graft using a confocal laser scanning microscope (Olympus Fluoview 1000). Axons crossing the border between the spinal cord and the peripheral nerve graft were traced with ImageJ. The number of axon outgrowths and total length of these axons were determined and the data was compared using SPSS. The total length of axon outgrowth and the number of axons outgrowing from each grafted spinal cord segment of the major experiment was compared with SPSS (version 14.0) and one-way ANOVA analysis.
2.3. In vivo peripheral nerve grafting

2.3.1. Animals

Adult male Sprague Dawley rats weighing 200-250g were used for this part of the study; the use of animals was approved by the University of New South Wales Animal Ethics Committee in accordance with The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). These experiments were carried out in the Neural Injury Research Unit at the University of New South Wales, Sydney, Australia. Thirty-one animals received T10 transection and a single preconditioned peripheral nerve graft. 10, 11, 10 animals were treated with 2 µM Cx43 AsODN in Pluronic gel, 2 µM Sense ODN in Pluronic gel, and no treatment respectively. Behavioural tests were performed on all surviving animals at a given time point (2, 10, 20, and 30 days). At the end of the experiment, 6, 6, and 5 animals survived in each treatment group. Their spinal cords were removed and labelled immunohistochemically.

2.3.2. Pre-operative preparations

Ketamine/Xylazine (90/10 mg/kg) intra-peritoneal injection was given to each animal before surgery. The temperature, forepaw reflexes, hindpaw reflexes, and respiratory rate of the animal were monitored every 15 minutes after initiation of anaesthesia. Temperature was monitored by a rectal thermometer. Breathing was monitored closely by assessing respiratory rate every 15 minutes and close observation of chest movements during surgery; when occasionally the animal stopped breathing resuscitation of the animal was performed by compression of the rib cage and blowing air into its nostrils via a plastic pipe. Each animal was kept on a surgical drape on a heating mat with a thermostat, and lubricant eye drops were placed on its eyes. A top-up of 27 mg/kg Ketamine was applied after 45 minutes or when the animal started recovering hindpaw reflexes. In most cases, surgery was completed within 45 minutes and no top-up was given. The anatomical area of the animal undergoing surgery (upper back for spinal cord surgeries, lower left back for left sciatic nerve ligation) was shaved and Betadine solution (Betadine, 10% povidone-iodine solution) applied to sanitise the surgery area. Surgery commenced when the animal had lost all forepaw and hindpaw reflexes.
2.3.3. Sciatic nerve ligation

The left sciatic nerve was exposed using the minimally invasive gluteal-splitting approach. A line was drawn between the left femur head and the dorsal midline of the rat and a horizontal incision was made. The biceps femoris and the gluteus maximus muscles were separated by blunt dissection and the sciatic nerve found in the groove between the two muscles. The sciatic nerve was traced to the most rostral point exposed by the groove and to the most distal point before it divides into the peroneal and tibial nerves, and ligated at these two points using 4-0 silk sutures. The muscles and skin were closed with silk sutures. Each animal received 0.02 mL Carprofen (analgesic, trade name Rimadyl, 50 mg/mL), 0.1 mL Cephalothin sodium (antibiotic, trade name Keflin, 100 mg/mL), and 5 mL lactated Ringer’s solution (0.9% NaCl) subcutaneously. Carprofen (0.02 mL) was administered once daily thereafter. Each animal was allowed to recover and was euthanased after 7 days. The ligated sciatic nerves were then used subsequently as donor peripheral nerve grafts. 12 animals had the left sciatic nerve ligated.

2.3.4. Spinal cord transection

Local anaesthetic Bupivacaine (0.5%) 0.05 ml was injected subcutaneously on the cutting line. T12 was located at the joining point between the caudal end of the rib cage and the spinal column. A vertical incision in the skin was made from 3-4 cm rostral to T12. T8 was identified as the caudal end of a visible fat pad underneath the skin. The superficial muscles covering the spinal cord were exposed by slicing open the space between connective tissues covering the muscles. Muscles attached to T9-T11 spinous processes were dissected away, and held to one side with artery clips to expose the spinous processes. The T10 spinous process was identified by its small size compared to the others, and by its morphology; T10 points straight up while T9 and T11 spinous processes are curved or lean towards T10. The ligaments between T10 and other processes were cut, and the dorsal lamina was cleared of attachments. Bupivacaine (3-5 drops) was applied to prevent reflex action. A T10 laminectomy was performed under the dissecting microscope with rongeurs, removing the spinous process and the lamina until the width of the spinal cord was seen. The spinal cord was transected with iris scissors followed by a scalpel blade to ensure a complete cut, creating a gap of approximately 1 mm wide between the cut stumps.
2.3.5. Peripheral nerve grafting

Before transecting the first animal in a session of 3 animals, the sciatic nerve donor animal was euthanased with an overdose of Lethobarb (sodium pentobarbital, 100 mg/kg, intra-peritoneal injection) and the ligated sciatic nerve removed between the two ligation points. The 5 mm sciatic nerve was kept in D-10 medium (Dulbecco’s Modified Eagle Medium High Glucose supplemented with 10% fetal calf serum, 1% of 100 U/mL Penicillin and 100 µg/mL Streptomycin, 2 mM L-Glutamine). After transection of the recipient animal, several minutes was usually required for blood to fill the transection gap and to clot; this was then removed before grafting. During this time a 1 mm segment was cut from the proximal end of the sciatic nerve, and kept in its original rostral-caudal orientation while being transferred to the transection gap and slid into position. Each sciatic nerve provided graft material for the three recipient animals operated in a single two hour session. Great effort was made to align the rostral cut end of the sciatic nerve to the rostral transection face and the caudal cut end to the caudal transection face. This was, however, only achieved in 40% of surgeries because the sciatic nerve was very slippery and soft, and therefore, difficult to manipulate. In some cases the orientation of the sciatic nerve was not confirmed.

2.3.6. Cx43 AsODN application

A volume of 100 µL of 2 µM Cx43 AsODN in Pluronic gel was applied to the transection area containing a piece of sciatic nerve. The amount of gel applied was enough to fill the gap between the transected spinal cord stumps and the space between T9 and T11 spinous processes. For control animals, Cx43 sense oligodeoxynucleotides (Sense ODN, Agilent Technologies) containing the complementary sequence of Cx43 AsODN in Pluronic gel, or no treatment at all was used. The animals were randomly allocated a treatment group. A small piece of gel foam was inserted between T9 and T11 spinous processes to help keep the gel in place. The muscles and connective tissue were then stitched back with 6-0 absorbable sutures, and the skin wound closed with 4-0 silk sutures or skin clips. Each animal then received 0.02 mL Carprofen, 0.1 mL Cephalothin sodium, and 5 mL Lactated Ringer’s solution subcutaneously and was monitored until waking.

2.3.7. Post-operative care

The animals were weighed once daily; a weight loss of more than 20% resulted in the animal being euthanased. Manual bladder expression and 0.02 mL Carprofen, 0.1 mL
Cephalothin sodium, and 5 mL Lactated Ringer’s solution was injected subcutaneously twice daily for the first 3 days. Following this Cephalothin was administered twice daily until the urine became clear. Carprofen was given if the animal showed any sign of pain or discomfort. Manual bladder expression was continued until the animal recovered spontaneous bladder voidance, usually within 2 weeks. Silk sutures on the skin were removed without anaesthesia after approximately 2 weeks.

2.3.8. Fluoro-Ruby labelling

At one week before perfusion the animals received Fluoro-Ruby (FR) injection bilaterally into the T8 spinal cord region. The same pre-operative preparations were carried out and the spinous process and lamina of T8 exposed. A partial laminectomy was performed, exposing approximately 0.5mm of spinal cord left and right of the dorsal midline. A volume of FR solution (0.5 µL, 10% in water, Fluorochrome, LLC) was injected into the spinal cord at a depth of 0.5 mm, 0.5 mm either side of the dorsal midline over a time course of 1 minute, using a pulled-glass needle with 0.5 µL markings with a fine metal rod as the piston. To ensure proper absorption of the dye the needle was left in place for another minute before being pulled out. The wound was closed with 6-0 absorbable sutures and animals monitored until awake. Injections of 0.02 mL Carprofen, 0.1 mL Cephalothin sodium, and 5 mL Lactated Ringer’s solution were given subcutaneously. The animals should theoretically start gaining weight from the second day with no apparent deficits, and should not require any more post-operative injections.

2.3.9. Behavioural testing

Animals were recorded for the Basso, Beattie, and Bresnahan score (the BBB score) on days 2, 10, 20, and 30 (Basso, Beattie, & Bresnahan, 1995) post-surgery. On day 2 (one day after transection surgery) any animal that showed hindlimb movement was excluded from the study, since this should not be possible after a T10 complete transection. The BBB score is a 21-point open field locomotor rating scale assessing joint movement, weight support, limb coordination, foot placement and gait stability. For T10 complete transection rats, only joint movement can be assessed within 6-11 weeks since none of the other functions are recovered. Each animal was recorded alone for 2 minutes in a circular open field. If they became stationary for more than 10-20 seconds, they were induced to move by lightly tapping on the side of the field. If this failed, they were moved to the centre of the field by picking them up under the arms;
this usually lead to them moving towards the sides. The recording was analysed by at least two people independently, and the scores averaged and plotted on a graph. Reflex movements induced by urinating, defecating, or after their first touch-down on the field were not scored.

2.3.10. Perfusion

At 6 weeks (42 days) after the initial transection animals were killed and spinal cord dissected out. Animals were given an overdose of Lethobarb (sodium pentobarbital, 100mg/kg, intra-peritoneal injection), perfused with saline (0.9% NaCl, 0.1% of 500 IU/mL heparin) for 3 minutes at a rate of approximately 20 mL per minute, and then 4% paraformaldehyde (in 0.1 M phosphate buffer) for 3 minutes at 20 mL per minute, and a further 10 minutes at 5 mL per minute. The dorsal surface of the animal was then opened and laminectomy performed. The spinal cord was dissected out from 3 cm rostral to T8 to approximately 5 cm caudal, and post-fixed in 4% paraformaldehyde for 2 hours before being transferred to 30% sucrose with 0.1% sodium azide for storage.

2.3.11. Immunohistochemistry

A 1.8 cm long piece of spinal cord (with FR labelling) containing T8 and T10 in the middle of the segment was cut from the rest of the spinal cord. This was frozen in OCT in the cryostat prior to cutting, and 30 µm longitudinal sections collected every 300 µm from dorsal to ventral. Each slide contained a series of 7 to 8 sections at 300 µm separation dorsal to ventral distance apart. After blocking with blocking solution (1% normal goat serum, 0.2% Triton-X100 in PBS) for 1 hour, sections were incubated with mouse anti-non-phosphorylated neurofilament H monoclonal antibody (SMI32) (Sternberger Monoclonals, diluted 1:1000 in PBS) at 4 ºC overnight to label neurons and their axons. After washing in PBS 3 times, 15 minutes each, sections were incubated in antibody against mouse IgG (H+L) conjugated to Alexa-488 (Molecular Probes A-11001, 1:400) for 3 hours before being washed in PBS 2 times 15 minutes each. A 0.1 µg/mL DAPI solution was applied to sections for 10 minutes before being washed off in PBS 2 times for 5 minutes each. The sections were mounted in anti-fade reagent (Citifluor AF-1). To label glial cells, slides were incubated in rabbit anti-GFAP antibody (DAKO Z0334, 1:1000) overnight and then, after extensive washing, anti-rabbit IgG conjugated to Alexa-488 (Molecular Probes A-11001, 1:400). After three hours, the slides were mounted in anti-fade reagent (Citifluor AF-1).
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2.3.12. Image quantification

The longitudinal section with the widest or largest cavity size, which was usually the middle section, was imaged at 10 times and 20 times magnification with a confocal laser scanning microscope (Olympus Fluoview-1000). Montages at 10 times of DAPI (blue)/ SMI32 (green) / FR (red) triple labelled (Figure 2.3) or GFAP/ FR labelled (Figure 2.4) images were made and measurements of these images recorded. Measurements included:

1) Lesion length. The cavity area after 6 weeks was characterised by the presence of one or multiple cavities rostral and/or caudal to the lesion. Lesion length was measured from the most rostral end of the cavities to the most caudal end of the cavities present after 6 weeks, using ImageJ software (Figure 2.3).

2) Regenerating axon band width. The original lesion area usually contained a complete or partial band of strongly SMI32-positive fibres in the area between the rostral and caudal spinal cord. Because this was originally a blank area with no cells, this represents a regenerating axon band. The width of this band was measured.

3) Cavity area. Cavity area was also measured by combining all the areas of the cavities on any one section. The lesion areas of 6 to 7 longitudinal sections representing one spinal cord were averaged. The average lesion area gave an estimate of lesion extent that is indirectly equivalent to lesion volume.

4) Width of the subpial white matter rim. Although the lesioned spinal cord was characterised by cavities rostral and/or caudal to the lesion, it was still continuous as a whole when dissected out of the body. The combined width on the left and right hand side of the rostral main cavity divided by the width of the spinal cord at the main cavity level was expressed as a measure of the width of this subpial rim.
5) Rostral grey matter to caudal grey matter distance. Fibrous FR and neuronal SMI32 labelling usually stopped a variable distance from the border of the cavities, signifying the existence of a dieback zone between the grey matter and the border of the cavity. The distance between the edges of the rostral and caudal grey matter was measured to account for the extent of this dieback.
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Figure 2.3. A Marked-up Image to Illustrate Image Quantification Based on Neuronal and Cell Nuclei Labelling.

Longitudinal sections of transected spinal cords 6 weeks after transection, peripheral nerve grafting and Cx43 AsODN or control treatments were measured. Neuron cell bodies and processes have been labelled with anti-SMI32 antibody (green), cell nuclei have been labelled with DAPI (blue) and axons that originated from T8 and neurons that have axons at T8 have been labelled with FR (red).

As indicated, cavity length was measured from the most rostral tip of the major cavities to the most caudal tip. Cavity area is the sum of the areas of all the cavities. At the original site of the transection, there is usually a band of regenerating SMI32 and DAPI positive axon fibres wrapping around the lesion, sometimes partly consumed by invading cavities. The rostral-caudal width of this regenerating axon band was measured. The width of the subpial white matter rim was measured at the thinnest point and combined to be expressed as a proportion of the diameter of the spinal cord at that point. The rostral to caudal grey matter distance was also measured, using large motor neurons as an indicator of the start of remaining grey matter.
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6) Length of area devoid of GFAP. In the middle of the lesion, there was always an area that was GFAP-negative that represents an area of necrosis cleared by activated microglia and infiltrating immune cells, and subsequently invaded by fibroblasts activated by dural damage. The rostral-caudal length of this area was measured from 10 x montages of GFAP/FR images (Figure 2.4).

7) GFAP intensity rostral and caudal to the lesion. GFAP intensity immediately rostral and caudal to the lesion was measured in 10 x montages by taking 3 rectangular areas from the immediate area rostral or caudal to the lesion, each measuring 250 x 250 pixels (0.32 x 0.32 mm), and measuring the integrated density of GFAP label. The rostral or caudal average integrated density was normalised to that of three 250 x 250 pixel areas taken of normal spinal cords away from (2500 pixels or 3.2 mm rostral to) the lesion.
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Figure 2.4. A Marked-up Image to Illustrate Image Quantification Based on Glial Cell Labelling. Longitudinal sections of transected spinal cords 6 weeks after transection have been labelled for astrocyte cell bodies and processes with anti-GFAP antibody (green). Axons that originated from T8 and neurons that have axons at T8 have been labelled with FR (red). The transection site is characterised by strong GFAP labelling of hypertrophic astrocytes on both sides of the lesion. The intensity of GFAP labelling in the areas immediately rostral and caudal to the lesion was measured and compared with areas away from the lesion as controls. The area in between the hypertrophic astrocytes is usually clear of any GFAP labelling except the occasional dot. This area has presumably been cleared by activated microglia and cells of the immune system, and is now invaded by regenerating axons as indicated by FR labelling and SMI32 (Figure 2-3).
2.3.13. Control experiments

To observe changes in the ligated sciatic nerve and to be sure of Schwann cells proliferation, sciatic nerve ligated animals were sacrificed at 1, 3, 5 or 7 days and their ligated sciatic nerves dissected out and fixed in 4% paraformaldehyde. One sciatic nerve was analysed on each day of 1, 3, 5, and 7 days after ligation. Two un-ligated sciatic nerves from the right legs were also fixed as controls. Overall morphology, Schwann cell phenotype and peripheral nerve basal lamina were visualised by Haematoxylin and Eosin staining, mouse anti-S100 labelling (section 2.2.1) and rabbit anti-laminin labelling (section 2.2.2), respectively. Images were taken with a Leica DMRA fluorescence microscope and recorded with a digital camera (Nikon digital sight DS-U1).

To confirm that FR injected at T8 could travel to the level of T10 (1 cm caudal to T8) or beyond within one week, animals without any injury received bilateral T8 FR injection, and were perfused one week later. Two animals received bilateral T8 FR injections into the intact spinal cord and the spinal cords were analysed after one week. The extent of FR labelling up to 2 cm caudal to T8 was assessed on longitudinal sections.

To gain a better understanding of the course of normal spinal cord healing, scar tissue deposition and peripheral nerve graft survival at earlier time points, two transected spinalised rats received FR injections at 1 week and were perfused at 2 weeks. No Cx43 AsODN was applied at the time of transection and grafting. Longitudinal sections were cut and sections labelled with either mouse anti-SMI32, rabbit anti-laminin, mouse anti-S100, or rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (Dako Z0334) antibodies, followed by either goat anti-mouse antibody conjugated to Alexa-488 dye (Molecular Probes A11001, 1:400) or goat anti-rabbit antibody conjugated to Alexa-488 dye (Molecular Probes A11008, 1:400). Images were taken with a Leica DMRA fluorescence microscope and images recorded with a digital camera (Nikon digital sight DS-91).
2.4. Ex vivo IKVAV-PA gel testing

2.4.1. Requirements for IKVAV-PA gel to set

In order to determine the ionic strength required for IKVAV Peptide Amphiphile compound (IKVAV-PA) (NPA16 Potassium Salt, Nanotope Inc, U.S.) to self-assemble into a gel *ex vivo*, 100 µL of 1% IKVAV-PA solution (20 mg powder in 2 mL water) was mixed with equal volumes of different solutions, including CaCl₂ solutions at different concentrations (60, 30, 15, 7.5, 3.75, 1.875 mM), artificial cerebrospinal fluid (124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO₄, 1.25 mM KH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 4 mM sucrose, 2.5 mM CaCl₂), Neurobasal-A medium, and Pluronic gel (10% w/v in KH₂PO₄ 1.1 mM, Na₂HPO₄ -7H₂O 4.5 mM solution). A droplet of 100 µL of one of the above solutions was placed on a petri dish, and 100 µL of 1% IKVAV-PA solution was then pipetted into the centre of the droplet, swirling from inside out. 1% IKVAV-PA solution was also combined with Pluronic gel, before mixing with 60 mM CaCl₂. The mixtures were observed by eye for the structural change from solution to gel.

2.4.2. Stability of IKVAV-PA gel

Reports in the literature showed that IKVAV-PA gel is stable for 2-4 weeks *in vivo* when injected in small quantities (2.5 µL) into rat spinal cords (Tysseling-Mattiace et al., 2008). The stability of the gel, when used in an *ex vivo* culture in large quantities (100 µL), had however not been determined. A solution of 50 µL of 1% IKVAV-PA solution was mixed with 10 µL of 0.1% Alcian green solution to aid visualisation of the gel once it forms. This mixture was added to 60 µL of 60 mM CaCl₂ on a petri dish. After the green coloured gel had formed, water was added to the petri dish to prevent it from drying out. The petri-dish was incubated at 37°C for 4 weeks, and observed once daily for the first week, then once weekly for signs of dispersion of the green colour.

2.4.3. Cx43 AsODN-CY3 retention and release from IKVAV-PA gel

250 µL of 1% IKVAV-PA solution was mixed with 6 µL of 100 µM Cx43 AsODN conjugated to Cy3 at the 5’ end (Cx43 AsODN-CY3, Sigma Genosis) to achieve a final concentration of 2 µM (566 e⁻⁶ µmol of Cx43 AsODN-Cy3 in total). The mixture was pipetted into 50 µL of 60 mM CaCl₂ solution at the bottom of an eppendorf tube. Gelling occurred almost instantly. Then 600 µL of water was added on top taking care not to disturb the gel. A 200 µL sample on top of the gel was taken out immediately as a
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0 hour control, and the 200 µL volume of water was replaced with water. The eppendorf tube was incubated at 37°C. A 200 µL sample was then taken out at 0.5, 1, 2, 3, 4, 24, 48, and 72 hours, with 200 µL replenished after each sampling. A control gel was made with 250 µL of 1% IKVAV-PA gel and 50 µL of 60 mM CaCl₂, omitting the Cx43 AsODN-CY3, and samples were removed at the same time points. The concentration of Cx43 AsODN-CY3 in each 200 µL sample was determined using a fluorophotometer (excitation 543 nm, emission 570 nm), using known concentrations of Cx43 AsODN-CY3 (2, 1.5, 1, 0.5, 0.1, 0.05, 0.01 µM in water) as calibration controls. The amount of Cx43 AsODN-CY3 released into 600 µL of solution above the gel is equal to the concentration x 600 µL. The total amount of Cx43 AsODN released from the gel at each time point was obtained by adding this number to the amount taken out by sampling previously, and plotted on a graph. For example, the total amount of Cx43 AsODN released from the gel in 2 hours is equal to concentration (at time 2 hours) x 600 µL + concentration (at time 1 hour) x 200 µL + concentration (at time 0.5 hour) x 200 µL + concentration (at time 0 hour) x 200 µL.
2.4.4. Active trapping of Cx43 AsODN-CY3 by IKVAV-PA gel

To investigate how pre-formed IKVAV-PA gel interacts with Cx43 AsODN-CY3 penetrating the nanofibers, a device was constructed with a vertical column made of the trunk of a plastic pipette, superglued to a porous membrane insert that was then placed in a dry petri dish (Figure 2.5). A solution of 400 µL of 1% IKVAV-PA solution was added to 80 µL of 60 mM CaCl$_2$ to form a gel, filling up the column. Then 100 µL of 2 µM Cx43 AsODN-CY3 in Pluronic gel was added forming a dome on top of the column. The petri dish was kept covered. Because Cx43 AsODN-CY3 solution is pink, any changes in colour and whether colour disappeared from the column over time could be observed. Active trapping of Cx43 AsODN-CY3 by IKVAV-PA gel was indicated by retention of the pink colour for 3 days or more. The IKVAV-PA gel had shrunken to approximately 2/3 its original volume due to water evaporation. More water was then added inside the column in an attempt to flush out the Cx43 AsODN-CY3, and any change in distribution of the pink colour was recorded.
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Figure 2.5. Illustration of Experimental Set-up to Demonstrate Active Trapping of Cx43 AsODN-CY3 by IKVAV-PA Gel.

The experiment apparatus is comprised of a vertical plastic column made of the trunk of a plastic pipette superglued to a porous membrane insert that was then placed in a dry petri dish. 400 µL of 1% IKVAV-PA solution was added to 80 µL of 60 m CaCl$_2$ to form an IKVAV-PA gel (clear), then 100 µL of 2 µM Cx43 AsODN-CY3 (pink) in Pluronic gel was added on top of the column. Disappearance of the pink coloured Cx43 AsODN-CY3 from the column was observed. Three days later, the Cx43 AsODN-CY3 was diffuse in the IKVAV-PA gel, which had shrunk to approximately 2/3 its original volume due to water evaporation. Water was then added inside the column to see if Cx43 AsODN-CY3 can be flushed out from the IKVAV-PA gel.
2.4.5. Effectiveness of IKAV-PA gel in ex vivo spinal cord segment culture

To determine if Cx43 AsODN delivered in Pluronic gel can penetrate through IKAV-PA gel, an ex vivo spinal cord segment culture was set up. After dissection of 5 mm spinal cord segments, these were randomly assigned to one of 5 treatment groups. In one group, 50 µL of Neurobasal-A medium was applied to cover the spinal cord segment, then 50 µL of 1% IKAV-PA gel was pipetted into the droplet (PA group). Once the IKAV-PA droplet had set into a gel, 100 µL of Pluronic gel (PA-PG group) or 100 µL of Pluronic gel containing 2 µM Cx43 AsODN (PA-AS group) were applied on top. A solution of 100 µL of 2 µM Cx43 AsODN in 30% Pluronic gel (AS group) was used as a positive control and 100 µL culture medium only (Medium group) as a negative control. The segments were cultured as described previously and spinal cord segment swelling was analysed on the second day of culture.
2.5. In vivo IKVAV-PA gel grafting

To investigate the regeneration-promoting effects of Cx43 AsODN treatment on spinal cord completely transected animals receiving an IKVAV-PA gel implant, *in vivo* experiments were carried out in the Neural Injury Research Unit at the University of New South Wales, Sydney, Australia. Ten animals each received Cx43 AsODN or Sense oligodeoxynucleotide (Sense ODN) treatment after T10 complete transection and IKVAV-PA gel grafting. Three Cx43 AsODN and four Sense ODN treated animals remained at the end of 11 weeks and the spinal cords were analysed using immunohistochemistry.

2.5.1. Animals and pre-operative preparations

Male adult 200-250 g Sprague-Dawley rats were used in this study. Pre-operative preparations were as outlined in sections 2.3.2, but using a mixture of Isoflurane and O₂ inhalation as the general anaesthetic (4% / 2% Isoflurane/ O₂ for induction of anaesthesia in a plastic box, followed by1.5% / 2% for maintenance via mouth piece during surgery). This allowed a tighter and spontaneous control on the level of anaesthesia, reduced post-surgery recovery time, and greatly reduced intra-operative casualties due to animals stopping breathing.

2.5.2. Spinal cord transection

T10 spinal cord transection was performed as described in section 2.3.4., with slight modifications. After laminectomy, a small round roll of cotton was placed under the chest of the rat so that T10 was more elevated than nearby spinal cord tissue. This reduced the accumulation of blood in the transection gap, the time taken for blood clot to form, and subsequently reduced the delay time between transection and IKVAV-PA gel implant.

2.5.3. IKVAV-PA gel and Cx43 AsODN application

Once the blood clot was removed from the transection gap, 5 µL of 1% IKVAV-PA solution was pipetted into remnant blood and cerebrospinal fluid at the bottom of the transection cavity so that it contacted only the two cut faces of the spinal cord. One minute was allowed for the gel to set. Then 50 µL of Pluronic gel containing 5 µM Cx43 AsODN or 5 µM sense oligodeoxynucleotide (Sense ODN) was applied on top of the gel, covering the dorsal surface of the two cut ends and filling up the space between
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T9 and T11 vertebrae. A higher concentration of Cx43 AsODN was used here because it was expected that some of it could be taken up (results of Cx43 AsODN retention and release experiments, section 2.4.3) and trapped inside the IKVA-PA gel, reducing the availability to spinal cord segments. No gel foam was inserted into the gap between T9 and T11 vertebrae. Instead of 4-0 silk sutures, skin was closed with special skin staples for rats, that were usually removed by the animals themselves as the skin healed, eliminating the need for silk suture removal.

2.5.4. Post-operative care

The animals received normal post-operative care. Since some animals exhibited autophagia after approximately 5 to 7 days, commercial nail-bite stopper solution was applied to the lower body, legs, feet, and hands of the animals concerned twice a day. There were 10 animals in each group originally. However, at the end of 11 weeks only 3 Cx43 AsODN and 4 Sense ODN treated animals remained. Animals were lost owing to burst bladders during bladder expression, bladder infections, and autophagia and unknown reasons but these were all unrelated to the IKVAV-PA gel and Cx43 AsODN.

2.5.5. Behavioural testing and perfusion

Animals were recorded for the Basso, Beattie, and Bresnahan behavioural assessment (the BBB score) on weeks 0 (first day after surgery), 0.5, 1, 2, 3, 4, and 11. Animals were killed and perfused at 11 weeks, which was 5 weeks longer than the in vivo peripheral nerve grafting experiment, to allow regenerating axons to extend for longer distances.

2.5.6. Immunohistochemistry

Following cryoprotection in 30% sucrose and subsequent freezing, 30 µm longitudinal sections containing the T10 transection site in the middle were cut on a cryostat. Sections were immunohistochemically labelled with the following antibodies: mouse anti-SMI32 antibody for neuron cell bodies and processes, rabbit anti-GFAP antibody for astrocyte cell bodies and processes, rabbit anti-Oligodendrocyte specific protein (OSP) for oligodendrocytes double labelled with SMI32 antibody, and mouse-anti Vimentin antibody (Sigma V6630, 1:1000) for fibroblasts. Isolectin-B4 conjugated to Alexa-568 (Molecular Probes I-21411, 1:100) dye was also used to label activated microglia and macrophages. The appropriate goat anti-mouse or goat anti-rabbit antibody conjugated to Alexa-488 or Alexa-568 was used as the secondary antibody.
Methods and materials

ProLong Gold antifade reagent with DAPI (Invitrogen P36935) was used to mount the sections.

2.5.7. Image analysis

Images of the spinal cord longitudinal section containing the most extensive cavity formation were taken with a confocal laser scanning microscope (Olympus Fluoview-1000). Cavity area was measured as the sum of all cavity areas on the section. GFAP intensity immediately rostral and caudal to the lesion was measured in 4 x montages by taking 5 rectangular areas from the immediate area rostral or caudal to the lesion, each measuring 100 x 100 pixels (0.2 x 0.2 mm), and measuring the integrated density of GFAP label. The rostral or caudal average integrated density was normalised to that of five 100 x 100 pixel areas taken of normal spinal cords away from (3200 pixels or 6.4 mm rostral or caudal to) the lesion. Because of the limited sample size at the end of the experiment, focus was placed on qualitative analysis of axon fibre outgrowth through the lesion instead of quantitative analysis. All sections from dorsal to ventral of the spinal cord were examined, the presence and quantity of axon fibres in the area of the lesion was recorded.
Chapter 3. *Ex vivo* spinal cord segment culture optimisation results

3.1. Cx43 AsODN dose response

An *ex vivo* spinal cord segment culture model was established for study of repair strategies using Cx43 AsODN. The optimal dose of Cx43 AsODN to be used for the culture was first established in a dose response experiment (Figure 3.1). Ten 0.5 cm spinal cord segments were used for each dose (0, 0.1, 2, 5, 10 and 25 µM Cx43 AsODN in Pluronic Gel). The amount of swelling in spinal cord segments cultured for 18 hours with increasing doses of Cx43 AsODN treatment was measured. Inhibition of spinal cord swelling was evident at 2 µM Cx43 AsODN compared to control. None of the other doses within this range (0 - 25 µM) produced a significant difference compared to control (Figure 3.1). Therefore, 2 µM was determined to be the most effective dose and was used in all subsequent experiments except determination of Cx43 protein knockdown (section 3.2) where 25 µM was also used, and *in vivo* spinal cord injury and IKVAV-PA gel grafting experiment (Chapter 7) where 5 µM was used to allow for IKVAV-PA gel absorption and retention of the Cx43 AsODN.
Figure 3.1. Cx43 AsODN Dose Response Curve. Spinal cord segment swelling after 18 hours in culture was measured by *en face* area of swelling divided by diameter at that end of the segment. Group averages +/- standard error of the mean are presented. Maximum inhibition of spinal cord swelling was evident at 2 µM, and this was significantly different from 0 µM (Pluronic gel only) and 10 µM, therefore determined to be the most effective concentration.

*p < 0.05. n = 10 per treatment dose.*
3.2. Cx43 protein knockdown *ex vivo* by Cx43 AsODN

In the last experiment, 2 µM, but not 25 µM, Cx43 AsODN was effective in inhibiting spinal cord segment swelling. To determine whether Cx43 expression had been effectively knocked down at 2 µM and 25 µM, the expression of Cx43 protein after 2 µM and 25 µM Cx43 AsODN application and 18 hours in culture was analysed using Western blotting (Figure 3.2). Six 0.5 cm spinal cord segments from one animal were used in each treatment group (0 µM, 2 µM, and 25 µM Cx43 AsODN). The membrane was labelled with anti-Cx43 antibody and anti-GAPDH antibody; both antibodies labelled bands on the gel at the appropriate molecular weight; Cx43 at 43 kDa and GAPDH at 36 kDa. Cx43 appeared as a doublet representing different phosphorylation states of Cx43. Cx43 protein levels were adjusted for the amount of total protein in each sample by dividing the level of Cx43 protein by the level of the house keeping protein GAPDH. There was a decrease in total Cx43 levels during the first 18 hours of culture with Pluronic gel only (0 µM) compared to a fresh spinal cord owing to inevitable cell death after dissection. However, Cx43 levels went up in surviving tissue but 2 and 25 µM Cx43 AsODN inhibited this new Cx43 expression to approximately 40% of 0 µM treatment. Based upon immunohistochemistry and cell viability analyses (section 3.4) extensive cell death occurred within the first 18 hours of culture with control treatments, but Cx43 AsODN treated segments contained a significantly higher number of surviving cells after 18 hours. Thus any decrease in Cx43 levels was not due to cell death in Cx43 AsODN treated spinal cord segments.
Figure 3.2. Knockdown of Cx43 Protein Expression after 2 μM and 25 μM Cx43 AsODN Treatment.
A. Western blots of Cx43 and GAPDH proteins in spinal cord segments treated with 0, 2, and 25 μM Cx43 AsODN and cultured ex vivo for 18 hours. Fresh spinal cord segments served as controls. Both Cx43 (MW 43 kDa) and GAPDH (MW 36 kDa) bands were located at their respective molecular weights. Cx43 appeared as a doublet representing different phosphorylation states of Cx43.
B. Relative density of Cx43 calculated by dividing integrated density of Cx43 by that of GAPDH. There was a general decrease in total Cx43 levels after 18 hours of culture compared to a freshly homogenised spinal cord but 2 μM and 25 μM Cx43 AsODN both inhibited Cx43 expression to approximately 40% that of untreated. N = 6 segments per sample.
The short term time course of Cx43 expression after dissection and 2 µM Cx43 AsODN treatment was analysed using Western blots. Control segments received Pluronic gel treatment or culture medium. Segments were cultured for a period of 2, 4 or 6 hours. Fresh segments served as controls. Six 0.5 cm spinal cord segments from one animal were used for each of the three treatments and for each of the three culture periods; therefore, a total of 10 animals were used (Figure 3.3). The blot membrane was labelled with anti-Cx43 and anti-beta-actin antibody as a house keeper protein control. Both blots were labelled in bands of the appropriate size. Cx43 appeared as a double band representing different phosphorylation states. Cx43 protein levels were adjusted for equal loading by dividing the integrated density of Cx43 by that of beta-actin. A general decrease in Cx43 level was evident within 2-4 hours in all groups (Pluronic gel, culture medium or Cx43 AsODN treated). Cx43 levels then increased from 4-6 hours with culture medium or pluronic gel treatments. With Cx43 AsODN treatment Cx43 level, however, remained low compared to the controls. Therefore, 2 µM Cx43 AsODN was ‘controlling’ Cx43 expression in cultured spinal cord segments very rapidly after application and certainly well before the up-regulation seen in controls (over and above initial cell death) at 4-6 hours after dissection or spinal cord injury.
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Figure 3.3. Knockdown of Cx43 Protein Levels at Different Time Points after 2 µM Cx43 AsODN Application.

A. Western blots of Cx43 protein. Because one gel cannot accommodate all samples, two gels were used; 0 hour fresh spinal cord control and 4 hour sample duplicates were loaded onto each gel to act as internal controls for this Western blot run. Cx43 appeared as a doublet representing different phosphorylation states of Cx43.

B. Western blots of beta-actin protein. Both Cx43 (MW 43 kDa) and beta-actin (MW 42 kDa) bands were located at their respective molecular weights.

C. Relative density of Cx43 calculated by dividing integrated density of Cx43 by that of beta-actin. There was a general decrease of Cx43 levels in the first 2 - 4 hours because of inevitable necrotic cell death within the dissected spinal cord tissue. Cx43 levels increased from 4-6 hours with culture medium only or pluronic gel only treatments. With Cx43 AsODN treatment Cx43 level remained low compared to the controls.

AS = 2 µM Cx43 AsODN. Gel = Pluronic gel only control. Medium = Culture medium only control.
3.3. Optimal culture time period

To examine the extent of glial activation with time in the Cx43 AsODN treated spinal cord segment culture, GFAP immunohistochemical labelling for astrocytes was carried out (Figure 3.4). Two 0.5 cm spinal cord segments per time point received 30 µL of 2 µM Cx43 AsODN treatment, and cultured for 5, 7, or 14 days. Two fresh fixed segments served as normal spinal cord controls. In normal spinal cords (Figure 3.4 A), astrocytes were found mainly in the glial limitans locking the pia mater in place, oriented radially. Some weaker labelling astrocytes were also found in the grey matter. Following culture and Cx43 AsODN treatment, the glial processes became thicker and more robust at the air exposed side; the thickness of the glial limitans and the intensity of labelling increased with time in culture from 5, 7, to 14 days. No untreated controls were included in this experiment. Limited glial up-regulation within the spinal cord interior was found at any time point, suggesting that Cx43 AsODN has a role in inhibiting glial up-regulation after spinal cord dissection. The other side of the segment in contact with culture medium tended to have decreased GFAP labelling after culture.
Figure 3.4. Glial Up-regulation in Spinal Cord Segments Cultured for 0, 5, 7, and 14 Days after Cx43 AsODN Treatment. Astrocytes have been immunohistochemically labelled using anti-GFAP antibody.

A. In a fresh fixed spinal cord, astrocytes were found mainly in the glial limitans (arrows) lining the pia mater. The thickness of the glial limitans varied along the circumference of the spinal cord. Some lighter-labelling astrocytes (arrowheads) were found in the grey matter.

B. After 5 days of culture, there was a slight thickening of glial processes in the glial limitans (arrow).

C. After 7 days, there was increased intensity of labelling and increased thickness of the glial limitans (arrows) at the air exposed side of the spinal cord. The side of the spinal cord in contact with the culture medium had greatly reduced astrocyte labelling.

D. After 14 days, the thickness of the glial limitans was further increased (arrows). The side of the spinal cord in contact with the culture medium had almost no astrocyte labelling.

Scale bar = 1mm.
To examine the extent of neuron survival with time in Cx43 AsODN treated spinal cord segments, immunohistochemical labelling for neuron cell bodies and processes was carried out. Figure 3.5 shows the morphology of normal uninjured spinal cords in cross section and the morphology of healthy neurons. The non-phosphorylated form of the heavy component of neurofilament protein (NF-H), one of the intermediate filaments found in neuron cell bodies, dendrites, and some thick axons, was labelled using an anti-SMI32 antibody (antibody product information, Sternberger Monoclonals). The grey and white matter of a normal spinal cord was easily distinguished at low magnifications. The white matter contained bundles of axon fibres shown in cross section in the descending and ascending tracts; the grey matter was mainly occupied by neurons in a background of short axon fibres. Smaller sized neurons found in the dorsal horn mainly represent interneurons that connect neurons in different regions of the grey matter and adjacent levels of the spinal cord. The ventral horn contains large neurons representing lower motor neurons, and small interneurons such as Renshaw cells. Healthy neurons were defined as having a large cell body with extended processes, and a clearly visible but not labelled nucleus.
Figure 3.5. Neuron and Axon Labelling in Fresh Fixed P7 Rat Spinal Cord. Neurons and axons have been labelled immunohistochemically using anti-SMI32 antibody.

A. A low magnification image of a spinal cord cross section shows the outer white matter containing ascending and descending tracts, which consisted of axon fibre bundles shown in cross section. The grey matter contained neuron cell bodies and processes in a background of numerous short axon fibres. The dorsal horn contained mainly small sized neurons, which are supposedly interneurons (arrows). The ventral horn contained large neurons, which can be lower motor neurons (arrowheads).

B. Higher magnification image shows the morphology of fresh fixed healthy neurons with immunohistochemically labelled neurofilaments. These cells are characterised by having a large cell body and multiple extended processes with an unlabelled nucleus. Scale bar = 100 µm.
The tissue organisation of the Cx43 AsODN treated spinal cord cross section remained essentially intact at 5 and 7 days, with clearly distinguishable grey and white matter (Figure 3.6). After 5 days, the white matter was still clearly distinguishable from the grey matter with visible groups of white matter fibres. Numerous surviving healthy neurons could be found in the grey matter. No untreated control segment was included in this experiment, but later experiments (section 3.4, page 101) showed that untreated segments rapidly degenerated and large numbers of healthy neurons were lost within 5 days. After 7 days, the white matter started to become less distinguishable indicating that some white matter fibres had degenerated. Some healthy neurons can still be found. After 14 days, the whole section had very high background fluorescence, the white and grey matter organisation was completely disturbed and dead tissue constituted most of the segment. Only pyknotic neurons with condensed cytoplasm remained and the white matter had lost almost all axon fibres. Five days was, therefore, determined to be the most suitable culture period for optimal culture conditions, along with results of glial labelling showing minimal glial activation by 5 days.
Figure 3.6. Spinal Cord Segments Cultured for Different Lengths of Time Display Different Morphologies.
Neurons and axons have been labelled immunohistochemically with anti-SMI32 antibody.
A. After 5 days, the white matter was still clearly distinguishable from the grey matter with apparent groups of white matter fibres cut at cross section. Many surviving healthy neurons remained in the grey matter.
B. After 7 days, the white matter began to be less distinguishable indicating some white matter fibre degeneration. Some healthy neurons remained.
C. After 14 days, the whole section had very high background fluorescence, the white and grey matter organisation was completely disturbed and dead tissue constituted most of the segment. Only pyknotic neurons with condensed cytoplasm remained.
Scale bar = 100 µm.
3.4. Segment swelling, cell viability, microglial response, and neuron survival

The amount of swelling in 0.5 cm Cx43 AsODN treated spinal cord segments was measured and compared to Pluronic gel treated and medium only control segments (Figure 3.7) after 18 hours in culture. Twenty segments each received 30 µL of Cx43 AsODN or Pluronic gel treatment and ten segments received 30 µL of culture medium. A one-way ANOVA analysis of the area of swelling showed that the difference between these three groups was significant (p = 0.002), and the differences between Cx43 AsODN and Pluronic gel (p = 0.014), Cx43 AsODN and medium (p < 0.001), and Cx43 AsODN and the other two groups combined (p < 0.001) were also significant. Although Pluronic gel treated segments appeared to have less swelling than those in culture medium alone, this effect was not significant (p > 0.05). Therefore, Cx43 AsODN treatment produced a significant reduction in spinal cord segment swelling after one day in culture, and the effect was independent of the delivery vehicle Pluronic gel.
Figure 3.7. Spinal Cord Segment Swelling after 18 Hours in Culture. *En face* area of swelling was divided by diameter at that end of the segment. Group averages +/- standard error of the mean are presented. A one-way ANOVA (p = 0.028) and contrast pairwise comparisons show that Cx43 AsODN had a significant effect in inhibiting segment swelling.

* p < 0.05, ** p < 0.01. n = 20 / 20 / 10 for each treatment.
The overall viability of segments in culture with and without Cx43 AsODN treatment was first assessed using the CMFDA live cell assay. Six 0.5 cm segments were cultured for each culture period (1 or 5 days) and treatment (2 µM Cx43 AsODN or culture medium only). The intensity of CMFDA 2.5 mm from either cut end of 1 and 5 day cultured segments was higher after Cx43 AsODN treatment than medium only control (Figure 3.8: p = 0.057 for 1 day cultured segments and p = 0.03 for 5 day cultured segments). The differences (statistically significant by day 5) indicated a higher level of cell survival in the middle of the spinal cord segments cultured after treatment with Cx43 AsODN. This provided an overview of cell viability but did not take into account possible changes in cell population (for example astrocyte proliferation but neuronal loss).
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Figure 3.8. CMFDA Intensity (Mean Grey Value) of Cross Sections from the Centre of 1 and 5 Day Cultured 5 mm Spinal Cord Segments. A direct comparison cannot be made between days that were sampled separately. It is, however, clear that Cx43 AsODN treated segments had a higher intensity than medium only control segments indicating greater cell viability. The difference between Cx43 AsODN treated and medium only controls was statistically significant at 5 days. 

\[ p = 0.057 \text{ for 1 day cultured segments and } ^* p < 0.05 \text{ for 5 day cultured segments.} \]
The viability of neurons, the main functioning units in the spinal cord, was also examined after culture. Figure 3.9 shows representative images from the middle of 5 day cultured spinal cord segments after Cx43 AsODN and Pluronic gel only treatments. Following Cx43 AsODN treatment, most neurons in the grey matter areas of the spinal cord cross section remained healthy with clearly visible cytoplasm and long extended neurites. Following Pluronic gel and medium only treatment, neurons became pyknotic and had shrunken cytoplasm and neurites. The average number of healthy neurons in each spinal cord cross section was calculated and analysed by a one-way ANOVA (Figure 3.10); the difference between groups was statistically significant (p = 0.046). Following 2 µM Cx43 AsODN treatment the average number of healthy neurons with normal morphology was 29.6 +/- 3.1 per cross section (mean +/- SEM), compared to 20.7 +/- 4.3 and 18.1 +/- 1.7 following Pluronic gel or culture medium only treatment, respectively. Contrast pair-wise comparisons showed that the difference between Cx43 AsODN and Pluronic gel (p = 0.031), Cx43 AsODN and medium (p = 0.01) were all significant. The difference between Pluronic gel and medium (p = 0.579) treatment was not significant. Therefore, Cx43 AsODN had a significant and specific effect on increasing neuron survival in spinal cord segments in culture.
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Figure 3.9. Representative Images Showing Neuron Cell Bodies and Axon Fibres in Spinal Cord Segments after Five Days in Culture. Longitudinal sections have been immunohistochemically labelled for neuron cell bodies and axons using anti-SMI32 antibody.

A. Following Cx43 AsODN treatment, neurons remained healthy with a large cytoplasm and multiple extended neurites (arrow).

B. Following Pluronic gel treatment, neurons were small and had become pyknotic with a shrunken cytoplasm and either a single neurite or none at all (arrow). The number of visible healthy neurons was significantly decreased.

Scale bar = 100 µm.
Figure 3.10. The Average Number of Healthy Neurons in Spinal Cord Cross Sections after Five Days.
The number of healthy neurons was the greatest with Cx43 AsODN treatment followed by Pluronic gel only and medium only treatment. Group averages +/- standard error of the mean are presented. One-way ANOVA (p = 0.046) and contrast pair-wise comparisons showed that Cx43 AsODN had a significant and specific effect on promoting neuron survival. * p < 0.05.
3.5. Discussion of ex vivo optimisation results

In summary, results of the ex vivo spinal cord segment culture optimisation experiments showed that:

- A viable ex vivo spinal cord segment culture model was established by using Cx43 AsODN to reduce inflammatory processes after spinal cord dissection, and maintained in a viable condition for at least 5 days.

- Cx43 AsODN generated an inverse hyperbolic dose response curve with the most responsive dose at 2 µM when delivered in Pluronic gel.

- Both low (2 µM) and high (25 µM) dose Cx43 AsODN inhibited Cx43 protein expression one day after treatment, but only the low dose was effective in inhibiting segment swelling.

- Eighteen hours after culture, segment swelling was significantly reduced by 2 µM Cx43 AsODN.

- Following a decrease in Cx43 protein levels due to the initial necrosis within 2 hours of dissection in all treatment groups, control spinal cords showed increasing Cx43 levels from 4 hours after dissection; after 2 µM Cx43 AsODN treatment Cx43 level remained steady from 4 to 6 hours. The inhibition of Cx43 expression after Cx43 AsODN treatment was also seen after 18 hours of spinal cord culture.

- Immunohistochemical labelling of spinal cord tissue cultured from 5 to 14 days showed that 5 days was the optimal culture time period, with very good neuron survival, intact tissue architecture and minimal glial activation.

- Segment swelling 18 hours after culture was significantly inhibited by 2 µM Cx43 AsODN. Overall tissue viability and neuronal viability was significantly promoted by Cx43 AsODN treatment.

- This model has considerable advantage over in vivo models in that it provides open access to pharmacological agents, and is, for some studies, more useful than spinal cord in vitro cell preparations because it preserves both the intact tissue architecture and cell populations.
3.5.1. Cx43 AsODN affected both intracellular and extracellular mechanisms of segment swelling

Results show that Cx43 AsODN at 2 µM inhibited spinal cord segment swelling. Segment swelling or oedema occurs as a result of both intracellular and extracellular events, the first being more major (Ito, Marmarou, Barzo, Fatouros, & Corwin, 1996; Marmarou, 2007). Cx43 AsODN treatment can act on both the intracellular and extracellular pathways of oedema or swelling.

The most extreme manifestation of intracellular oedema is necrosis, characterised by a swollen morphology. Necrosis is the major mechanism of cell death immediately following spinal cord injury, resulting from altered ion gradients and passive water entry (Marmarou, 2007). Irreversible mechanical damage, energy failure and oxidative peroxidation of the membrane leads directly to necrosis (M. S. Beattie et al., 2000). Therefore, direct causes of necrosis are not amenable to Cx43 AsODN treatment. However, necrosis or intracellular oedema can also occur indirectly in cells not mechanically damaged via a mechanism known as excitotoxicity (Choi, 1992), which is amenable to Cx43 AsODN treatment. Membrane deformation and depolarisation can lead to excessive release of free radicals, glutamate, and aspartate from dying cells and astrocytes (Marmarou, 2007). In astrocyte membranes, the abnormal release of excitatory amino acids can occur via Cx43 hemichannels (Rodriguez-Sinovas et al., 2007; Rouach et al., 2002), that also have a higher probability of opening under conditions of membrane depolarisation and low extracellular Ca$^{2+}$ concentration (Saez et al., 2003). Release of excitatory amino acids leads to over-stimulation of glutamate receptors in neurons and oligodendrocytes and excessive accumulation of intracellular Ca$^{2+}$ and water (Michael S. Beattie et al., 2002; Rodriguez-Sinovas et al., 2007; Rouach et al., 2002). Membrane deformation and depolarisation can also lead to activation of voltage-sensitive ion channels that allow ions to move down their electrochemical gradients, increasing extracellular K$^+$ and decreasing extracellular Na$^+$ and Ca$^{2+}$ concentrations (Marmarou, 2007). Chloride ions and water then follow Na$^+$ and Ca$^{2+}$ passively into the cells (Marmarou, 2007). Furthermore, the resultant low extracellular Ca$^{2+}$ concentration seems to be coupled to the release of Ca$^{2+}$ from intracellular stores in astrocytes. Here, we postulate that by down-regulating Cx43 expression after injury, the
number of Cx43 hemichannels in the membrane was dramatically decreased, therefore limiting glutamate release leading to a significant reduction in swelling (Beardslee, Laing, Beyer, & Saffitz, 1998; Laird, Puranam, & Revel, 1991).

Extracellular retention of water follows the breakdown of the blood-spinal cord-barrier (BSCB), accumulation of proteins released from necrotic cells, and breakdown of extracellular proteins (Marmarou, 2007). Breakdown of the BSCB is initiated and regulated by proinflammatory mediators including oxidative mediators, adhesion molecules, cytokines and chemokines, resulting in increased blood vessel endothelial barrier permeability and vasogenic oedema (Stamatovic, Dimitrijevic, Keep, & Andjelkovic, 2006); Cx43 AsODN applied to compression injured spinal cords in vivo resulted in less extravasation of fluorescently labelled bovine serum albumin and neutrophils within one day therefore BSCB breakdown was inhibited (M. Cronin et al., 2008). Cx43 AsODN applied to spinal cords injured by partial transection in vivo resulted in reduced neutrophil recruitment and microglial activation therefore reducing the amount of proinflammatory mediators and hence BSCB breakdown (M. Cronin et al., 2008). Release of proteins from necrotic cells can be reduced owing to the effect of Cx43 AsODN on necrosis as discussed. Breakdown of extracellular proteins and extravasation of proteins from blood in vivo has been shown to retard water clearance due to an increased osmotic gradient (Marmarou, 2007), but does not seem to be related to Cx43 expression. Hence, it was postulated that by down-regulating Cx43 expression after injury, the extent of BSCB breakdown and accumulation of proteins from necrotic cells was reduced, resulting in reduced extracellular retention of water hence extracellular oedema.

### 3.5.2. Cx43 AsODN has an optimum dosage

Results show that the effect of Cx43 AsODN on spinal cord segment swelling followed an inverted hyperbolic curve with the most effective dose at 2 µM, ineffective at 25 µM, although both doses inhibited Cx43 protein expression after one day in culture. This dose dependent effect on spinal cord segment swelling was also seen when mimetic peptides against Cx43 were continuously applied to cultured spinal cord segments for a period of 4 days, effective only at 5-50 µM in a range of 0-500 µM (O'Carroll, Alkadhi, Nicholson, & Green, 2008). Interestingly, all mimetic peptide concentrations within the range of 5-500 µM were effective when only applied for one day, creating a sigmoidal
dose response curve in the range 0-500 µM. The cumulative dosage of 5 - 50 µM applied for four days possibly created the same dosage as 50 – 500 µM over one day (O'Carroll et al., 2008). Therefore, a possible explanation to the inverted hyperbolic curve of Cx43 AsODN dose response could be that at 2 µM (and possibly a range of other concentrations between 0.1 and 10 µM yet to be tested) the number of newly synthesized hemichannels in the membrane was reduced to an optimal level for an optimal amount of time. This reduction in hemichannel number rescued spinal cord segments from excessive oedema that in turn delayed the inflammatory reaction and blood vessel breakdown. At 10- 25 µM, however, the knockdown of Cx43 constituting hemichannels and gap junctions may have lasted for a longer time than necessary.

Prolonged Cx43 expression knockdown could result in not only hemichannel depletion but also gap junction uncoupling by depleting the number of hemichannels available for insertion into gap junction plaques. Application of a mimetic peptide designed to impair Cx43 hemichannel and gap junction function demonstrated protection from neurodegeneration in an ex vivo model of epileptiform activity when applied at low doses only (Yoon, Green, O'Carroll, & Nicholson, 2010). In this model 5 – 50 µM mimetic peptide applied during epileptiform activity significantly reduced cell death but 500 µM exacerbated the lesion, suggesting that gap junction communication, blocked at 500 µM, was essential for tissue survival while hemichannel opening was damaging (Yoon et al., 2010). Prolonged Cx43 knockdown could also result in delayed microglial activation to the level of being detrimental, ultimately affecting neuronal survival. Studies have supported a beneficial effect of microglial up-regulation in preparing tissue for regeneration, although there is a delicate balance between too little and too much up-regulation (Schwartz, 2000). On one hand microglial activation is responsible for clearing up cellular debris after necrotic and apoptotic events (Schwartz, 2000; Schwartz & Yoles, 2005), but microglial activation also appears to be associated with ongoing cavity formation and long term enlargement of the lesion, as observed in the in vivo spinal cord injury and IKVAV-PA gel grafting experiments discussed later in Chapter 7. Cx43 gap junction signalling was postulated as a pathway for microglial activation signals; therefore, prolonged Cx43 knockdown could result in delayed microglial activation. In the model used in the study here, however, only short term (approximately 24 hours) regulation of Cx43 expression was anticipated and achieved
with a low dose (2 µM). The number of hemichannels in the membrane is likely to be reduced but gap junction communication is unlikely to be completely blocked.

3.5.3. Cx43 AsODN decreased Cx43 protein levels

Western blotting data showed that Cx43 protein levels began to decrease as soon as culture started, indicating the immediate start of necrotic cell death after spinal cord injury resulting from spinal cord dissection and segmentation. Cx43 up-regulation in control segments began as soon as 4 hours after injury, which is in agreement with studies on Cx43 expression after in vivo spinal cord injury (I. H. Lee et al., 2005). Our results showed that 2 µM Cx43 AsODN acted to maintain a steady Cx43 level at 4, 6 and 18 hours by preventing new Cx43 synthesis and minimising necrosis. This is in agreement with results of a study performed on intact adult rat spinal cords in vivo (Michael Cronin et al., 2006). It was shown that following 250 µL of 1 µM Cx43 AsODN applied to an exposed spinal cord segment, in vivo knockdown of Cx43 levels in the dorsal white matter and ventral grey matter of that segment began within 2 hours, and reached almost undetectable levels at 4, 8, and 24 hours, with recovery at 72 hours not quite reaching normal levels (Michael Cronin et al., 2006). The knockdown in the dorsal grey matter started at 4 hours and Cx43 was almost undetectable at 8 hours, with recovery from 24 hours, reaching normal level at 48 hours. These data suggest that a one-off application of Cx43 AsODN in spinal cords can have 2 to 3 days of effective knockdown in vivo.

3.5.4. Optimal period of culture was shorter compared to slice cultures

An optimal culture period of 5 days was determined. In spinal cord segments treated with Cx43 AsODN, neuronal survival, determined by examining the number of large motor neurons in the ventral horn, was excellent compared to after 7 or 14 days. In addition, the amount of glial activation was minimal in the interior of the segment and activation was limited to the formation of the glial limitans on the air-exposed side of the segment.

Thick air-liquid interphase slice cultures (350 - 400 µm thick) of neonatal rat spinal cord using a very similar MEM formulation as that used in this study could generally be kept in vitro for up to 3 months depending on the age of the animal, with intact grey and white matter organisation and good culture viability (Corse et al., 1999; Rothstein, Jin,
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Dykes-Hoberg, & Kuncl, 1993; Stavridis, Dehghani, Korf, & Hailer, 2005). Researchers have noted the importance of an air-liquid interphase for viable slice cultures in ensuring oxygen and nutrient diffusion, where generally the air-side of the membrane does not contain any liquid. In my experimental paradigm for segment culture, besides incubation in a moist chamber, the top of the segment was kept moist by a film of culture medium or Pluronic gel that could have partially limited access to oxygen. The shorter viable time of this segment culture reflects impaired diffusion of oxygen and nutrients limiting penetration into the segment.

3.5.5. Ventral motor neuron survival in segment culture was better than in slice cultures

Several earlier studies have reported that immature ventral root motor neurons in the early postnatal stage are particularly susceptible to denervation and are lost within the first day of spinal cord injury and spinal cord slice culture (Stavridis et al., 2005). This pattern is seen in even the well-established entorhinal-hippocampal slice culture, with selective neuronal loss in the hippocampus and adjacent cortical regions (Pozzo Miller, Mahanty, Connor, & Landis, 1994; Stavridis et al., 2005). A similar ventral motor neuron loss was also observed in the middle of control segments after 5 days in our model. The exceptional survival of large motor neurons in the ventral horn in the Cx43 AsODN treated group suggests that the spinal cord segment culture together with Cx43 AsODN application provided a better environment than that of slice cultures for the interior of the segment. Neuronal damage was limited to the two cut ends of the segment only, isolating the middle of the segment from initial damage, with Cx43 AsODN delaying secondary injury progression.

3.5.6. Astrocyte up-regulation was limited compared to slice cultures

In spinal cord slice cultures, astrocyte GFAP immunoreactivity up-regulation was found throughout the white and grey matter areas after 3 days in culture in both embryonic and neonatal spinal cords (Munoz-Garcia & Ludwin, 1985; Rakowicz, Staples, Milbrandt, Brunstrom, & Johnson, 2002; Stavridis et al., 2005). An interesting observation was that of astrocyte over-growth on the top surface in a 250 – 300 µm thick slice culture after one week (Rakowicz et al., 2002), which is supported by the observation of the formation of a glial limitans on the air-exposed side only of the spinal cord segment in this study. This differential growth of astrocytes could be caused by a gradient of inflammatory mediators due to a nutrient gradient away from the liquid
interphase or be a direct response to oxygen levels by astrocytes near the air surface. There was limited glial activation in the interior of the segment after Cx43 AsODN treatment, suggesting that inhibiting Cx43 up-regulation after injury was effective in ‘dampening’ the glial reaction by limiting glial syncytial communication, and perhaps limiting the spread of proliferative and hypertrophic signals.

3.5.7. Cx43 AsODN effects on necrosis, apoptosis and inflammation

In our study, it was found that Cx43 AsODN treated spinal cord segments had significantly higher neuron viability after 5 days, with evidence suggesting higher overall cell viability after 1 and 5 days. Down-regulating Cx43 levels could have effects on necrosis, apoptosis, and inflammation, all of which affect cell viability.

Neuronal and other cell death happens via necrosis and apoptosis. Necrosis of cells in the injury zone starts immediately after injury (or dissection in my model), and is caused by either mechanical damage to the cell membrane or glutamate-mediated Ca^{2+} and water entry. At the early stages, Cx43 AsODN treatment could reduce glutamate release via Cx43 hemichannels, therefore rescuing cells that were not mechanically damaged from excitotoxicity-induced necrosis. Cx43 AsODN treatment can also reduce the amount of intercellular coupling, which is implicated in necrotic signaling; convincing support for this theory comes from studies in cardiac myocytes that are normally extensively coupled via Cx43 gap junctions to achieve synchronisation (Shintani-Ishida, Unuma, & Yoshida, 2009). After ischemia, an increase in Cx43 gap junction numbers at intercalated disks and enhanced gap junction intercellular spread of Lucifer yellow was found, and thought to mediate the spread of ischemia-reperfusion injury in a defined band of necrosis (Shintani-Ishida et al., 2009). Therefore, down-regulating Cx43 expression could act to reduce necrotic processes in the spinal cord by reducing glutamate release from Cx43 hemichannels and the extent of intercellular coupling.

Apoptosis is a chronic event that results in controlled or programmed cell death (Kajta, 2004). Apoptotic gradual motor neuron loss over weeks to months in spinal cord slice cultures could happen via glutamate-induced excitotoxicity mediated by non-NMDA receptors on neurons, at a level of glutamate less than that required for induction of
necrosis (Rothstein et al., 1993), and glutamate can be released via Cx43 hemichannels in the spinal cord as discussed before. Apoptosis happens via activation of one of two pathways, the external death cascade or the internal mitochondrial pathway (Kajta, 2004). Studies of Cx43 in other organs suggest that increased Cx43 expression was linked to apoptotic processes (Naiki-Ito et al., 2010; Y. Wang et al., 2010). In acetaminophen-induced liver toxicity, induction of Cx43 expression was found along with elevated caspase-3, a main effector protein in the apoptotic pathway, indicating the possible involvement of Cx43 in apoptotic signalling (Naiki-Ito et al., 2010). Ischemia and reperfusion of rat hearts caused an increase in Cx43 dephosphorylation and myocyte apoptosis, resulting in increased permeability and reperfusion arrhythmias, respectively (Y. Wang et al., 2010). Gap junctions may also represent a new mechanism for antigen presentation in apoptotic cells, by allowing transfer of apoptotic bodies to a presenting dendritic cell, eliciting a phagocytic reaction leading to elimination of the apoptotic cell (Pang et al., 2009). The key between Cx43 gap junctions and apoptosis could lie in the spread of cell death signals from one cell to another; recent work on rat C6 glioma cells found that apoptotic events could spread to neighbouring cells through gap junction channels, with hemichannels spreading this damage even further (Decrock et al., 2009). Although apoptosis is mostly an unwanted phenomenon in pathological processes, it can lead to a desirable outcome in the treatment of cancer where the up-regulation of Cx43 expression could be beneficial. In human prostate cancer cells, treatment with Bowman-Birk inhibitor, a soybean-derived serine protease inhibitor, resulted in higher Cx43 expression and caspase-3 (apoptotic executioner protein) expression, and subsequent apoptosis of the cancer cells (Tang et al., 2009). In the injured spinal cord, however, Cx43 AsODN treatment could act to reduce apoptosis in neurons and oligodendrocytes in the injured spinal cord by reducing glutamate release via Cx43 hemichannels and the spread of cell death signals via Cx43 gap junctions and hemichannels.

Cx43 is also linked to inflammatory processes in a number of ways; a delayed microglial response was observed in previous studies after Cx43 AsODN treatment. Down-regulating Cx43 expression using the same Cx43 AsODN has been shown to reduce inflammatory responses, including fewer activated microglia and reduced capillary breakdown following optic nerve ischemia (Danesh-Meyer et al., 2008), decreased blood-spinal cord-barrier permeability and neutrophil infiltration in spinal
cord injury (M. Cronin et al., 2008), and reduced macrophage and neutrophil recruitment in skin wound healing (Mori et al., 2006). A potent immune reaction inducer, bacterial lipopolysaccharide, when injected into rats induced expression of Cx43 in the kidneys and lungs, suggesting that Cx43 expression forms part of the inflammatory response (Fernandez-Cobo, Gingalewski, & De Maio, 1998). In mouse lung subjected to inflammation, up-regulated Cx43 expression can lead to neutrophil recruitment to the airspace, causing a spread of inflammatory signals (Sarieddine et al., 2009). The cellular mechanism underlying the increased blood capillary leakiness after Cx43 expression could be the propagation of elevated Ca^{2+} levels through Cx43 gap junctions in the capillary bed; focal increases in Ca^{2+} levels can spread from one capillary endothelial cell to a vascular location as much as 150 µm away from the target site (Parthasarathi et al., 2006). The Ca^{2+} spread in capillary endothelial cells was not observed in mice lacking endothelial Cx43 (Parthasarathi et al., 2006). Therefore, down-regulating Cx43 expression have reduced inflammatory responses after spinal cord injury by reducing blood-spinal cord-barrier breakdown via reduction of Ca^{2+} spread through Cx43 gap junctions in endothelial cells, and by reducing immune cell recruitment and microglia activation.

Necrosis, apoptosis and inflammation exist within an intricate network. The effect of Cx43 AsODN on delaying microglial responses can feed back into the inflammation-apoptosis cycle, which features microglia at its centre (M. S. Beattie, 2004). CD95 and CD95 ligands and activated microglia are important steps in the inflammation-apoptosis cycle (M. S. Beattie, 2004). Mechanical injury to the CNS causes hemorrhage, which releases pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α) into the environment, acting on TNF receptors including CD95 on all cells to cause apoptosis (also known as FAS or the death receptor, a receptor for TNF-α). TNF-α also acts upon TNFR-1 in microglia, the binding of which in turn releases more TNF-α and CD95 ligand to activate the death cascade of apoptosis in surrounding neurons and glia including astrocytes (M. S. Beattie, 2004). Apoptotic astrocytes can aggravate the cascade by releasing inflammatory molecules and not providing adequate buffering of glutamate that stimulates microglia and causes necrosis of other cells (M. S. Beattie, 2004). Anti-inflammatory drugs including minocycline (a microglial activation reducer), blockade of glycosphingolipid-induced nitric oxide synthase, and CD95 ligand blockade (M. S. Beattie, 2004) all achieved effective reduction of neuron and glia
apoptosis. The down-regulating effects of Cx43 AsODN on necrosis and inflammation can inhibit the feed-forward towards apoptosis via microglia. Therefore, necrosis, inflammation and apoptosis are all intricately linked to each other, and the down-regulating effects of Cx43 AsODN on these processes are likely to be multi-faceted and non-linear.
3.6. Summary of experiment findings

- An \textit{ex vivo} spinal cord segment culture model was established by using Cx43 AsODN to inhibit spinal cord segment swelling, promote overall cell viability, promote neuron (especially ventral horn motor neuron) survival, and limit astrocyte up-regulation. The cultured segments can be kept in an optimal condition for at least 5 days.

- Spinal cord segment swelling was inhibited by Cx43 AsODN treatment. This observation can be explained by the hypothesised effects of Cx43 AsODN in intracellular and extracellular swelling.
  - By down-regulating Cx43 expression after injury, the number of Cx43 hemichannels in the astrocyte membrane are dramatically decreased, limiting glutamate release leading to a significant reduction in intracellular Ca$^{2+}$ and water accumulation, and subsequent swelling.
  - Following Cx43 AsODN treatment, reduced breakdown of the blood-spinal cord-barrier (results of another SCI studies) and reduced accumulation of proteins as a product of necrotic cell burst are postulated to result in reduced extracellular swelling.

- Necrotic, apoptotic and inflammatory processes were dampened down by Cx43 AsODN treatment \textit{ex vivo}.
  - Necrotic processes in the spinal cord were limited by reducing glutamate release from Cx43 hemichannels and decreasing the extent of intercellular coupling via Cx43 gap junctions.
  - Apoptotic processes in neurons and oligodendrocytes in the spinal cord were limited by reducing glutamate release via Cx43 hemichannels and reducing the spread of cell death signals via Cx43 gap junctions and hemichannels.
  - Inflammatory responses after spinal cord injury were limited by reducing blood-spinal cord-barrier breakdown via reduction of Ca$^{2+}$ spread through Cx43 gap junctions in endothelial cells, and by reducing immune cell recruitment and microglia activation.
Ex vivo spinal cord segment culture optimisation results

- Necrosis, inflammation and apoptosis are all intricately linked to each other, and the down-regulating effects of Cx43 AsODN on these processes are likely to be multi-faceted and non-linear.

- Cx43 AsODN at 2 µM was determined to be the optimal dose in inhibiting segment swelling, although both 2 µM and 25 µM Cx43 AsODN were effective in inhibiting Cx43 protein expression. Up-regulation of Cx43 expression levels from 4 hours after spinal cord dissection was inhibited by 2 µM Cx43 AsODN treatment. Higher doses may have resulted in prolonged inhibition of gap junction communication, which seems to be essential for tissue survival.
Chapter 4. *Ex vivo* peripheral nerve grafting results

4.1. Morphology of fresh and cultured sciatic nerves

The morphology of fresh sciatic nerves was characterised using immunofluorescence labelling. Two sciatic nerves were analysed after culturing for different lengths of time (0, 1, 2, 3, 4, 5, 6 and 7 days). Peripheral nerves are made of bundles of axons; axons labelled with anti-SMI32 antibody can be seen in a mono- or di-fascicular structure (Figure 4.1); axons are wrapped in membrane coverings provided by Schwann cells (labelled using anti-S100 antibody) called myelin and neurilemma for myelinated and unmyelinated axons, respectively. The axon-myelin and axon-neurilemma units are in turn enclosed in basal lamina (labelled using anti-laminin antibody) laid down by Schwann cells. Schwann cells in their resting state are only weakly labelled by anti-S100 antibodies. The endoneurium surrounds all nerve fibres and fills up the space inside a fascicular compartment bound by the perineurium. The perineurium is not evident in this sciatic nerve section because compartmentation into fascicles has not yet occurred at seven days postnatal (Morris, Hudson, & Weddell, 1972). A tough fibrous epineurium encloses these axons and sheaths to form a nerve.
Figure 4.1. Morphology of a Peripheral Nerve.
A and C. Low power magnification images of a sciatic nerve cut in oblique and cross sections, labelled with the antibodies to SMI32 to profile axons. The sciatic nerve appears as a mono- or di-fascicular structure at this P7 postnatal age.
B and D. Higher power magnification image showing the morphology of SMI32 labelled axons in longitudinal and cross section. Axons lie longitudinally in neatly packed bundles inside the faintly fluorescent basal lamina laid down by Schwann cells.
E. Anti-laminin labelling for the basal lamina of the peripheral nerve. (This label was later used to distinguish the boundaries of the grafted nerves because spinal cord tissue does not contain laminin, except in blood vessels.)
F. Anti-S100 labelling for Schwann cells. In the normal peripheral nerve the Schwann cells are not very abundant as indicated by the scarcity of brightly labelled cells. In their resting state, Schwann cells express little S100 as indicated by the low intensity of labelling.
Scale bar = 100 µm.
Ex vivo peripheral nerve grafting results

Sciatic nerves in culture were also analysed for changes independent of grafting into spinal cord segments. During *ex vivo* culture, the 5 mm peripheral nerve segments underwent a series of inherent changes, marked mainly by proliferation of Schwann cells and degeneration of axons. Proliferation of Schwann cells was evident within one day of culture, and was characterised by an increase in the number of strongly S100 positive cells and the overall intensity of labelling, resulting in a peripheral nerve packed with spindle-like Schwann cells starting at 5 days consistent with their proliferated phenotype (see Figure 4.2). Normal sciatic nerves contain densely packed organised arrays of axons as demonstrated by anti-SMI32 immunohistochemical labelling (see Figure 4.3). Loss of organisation and degeneration of axons, seen as axon fragments, was evident within one day of culture. After seven days the number of axons had decreased to approximately half the original number and most that remained were degenerating fragments.
Ex vivo peripheral nerve grafting results

Figure 4.2. Proliferation of Schwann Cells in ex vivo Sciatic Nerve Culture Demonstrated using Anti-S100 Immunohistochemical Labelling.

A. Schwann cell labelling (arrows) in sciatic nerves after 1 day in culture shows proliferation of Schwann cells and increased intensity of S100 staining when compared to fresh sciatic nerves (Figure 4.1 F).

B-F. Schwann cell labelling (arrows) 2-6 days in culture shows the number of S100 positive cells and intensity of S100 labelling gradually increasing as Schwann cells proliferated.

G-H. Longitudinal views of Schwann cells after 7 days of culture show that the Schwann cells had a spindle-like shape (arrowheads) consistent with their proliferative phenotype. The whole peripheral nerve was filled with Schwann cells.

Scale bar = 100 µm.
Ex vivo peripheral nerve grafting results

Figure 4.3. Degradation of Axon Fibres in ex vivo Sciatic Nerve Culture Demonstrated using Anti-SMI32 Immunohistochemical Labelling.
A. A cross section of a fresh sciatic nerve shows densely packed, organised arrays of axons (arrows).
B-C. Axon labelling in sciatic nerves 1 and 3 days after culture shows increasingly disorganised axons that have undergone degeneration and are now seen as axon fragments (arrowheads).
D. After 7 days the number of axons had decreased to approximately half the original numbers and most that remained were degenerating (arrowheads).
Scale bar = 100 µm.
4.2. *Ex vivo* peripheral nerve grafting

*Ex vivo* spinal cord segments are subject to surgical damage from the two cut ends but our results have shown that 0.5 cm long segments treated with Cx43 AsODN retained high cell viability and neuronal survival. Peripheral nerve grafting creates another injury in the middle of the 0.5 cm long segment that was not able to withstand the surgical manipulation and exhibited loss of tissue integrity. Therefore, 1.5 cm long spinal cord segments were trialled for their suitability as peripheral nerve graft recipients by examining tissue integrity and neuron survival. Four segments were used in each treatment group (Cx43 AsODN, Pluronic gel and culture medium only control). A horizontal slit was created in the middle of each spinal cord segment and a single peripheral nerve graft was inserted into the slit followed by treatment with Cx43 AsODN, Pluronic gel, or culture medium only. After five days of culture, the spinal cord segments were immunohistochemically labelled for neurons and their axons using anti-SMI32 antibody and anti-Neuronal Nuclei antibody for neuronal nuclei staining. Results from this grafting experiment showed that after Cx43 AsODN treatment the 1.5 cm spinal cord segments generally survived well, with intact grey and white matter organisation retained (Figure 4.4), an abundance of large motor neurons in the grey matter, and long axon fibres in the white matter. Control grafted segments showed an absence of surviving neurons, a decrease in the number of large diameter axons labelled in the white matter indicative of axon degeneration, and signs of lost grey-white matter organisation.
Figure 4.4. Neuron Cell Body and Processes in Spinal Cord Segments Cultured for 5 Days after Receiving a Fresh Sciatic Nerve Graft Inserted. Longitudinal sections were immunohistochemically labelled for neuron cell bodies and processes using anti-SMI32 antibody.

A. Longitudinal section of a spinal cord grafted segment after Cx43 AsODN treatment at a lower magnification shows intact grey - white matter organisation. No axon outgrowth from the spinal cord into the sciatic nerve graft was found in any treatment group.

B. A Cx43 AsODN treated grafted segment shows an abundance of large viable neurons with extended neurites (arrows) in the grey matter and strongly-labelled long axons (arrowheads) in the white matter.

C. Pluronic gel treated grafted segment shows a lack of neurons (arrows) and weakly labelled white matter consisting of fine fibres (arrowheads), indicating a loss of neurons and degeneration of large diameter axons.

D. Culture medium treated grafted segment shows similar labelling to the Pluronic gel treated segment with loss of neurons (arrow), axon degeneration (arrowhead) and disrupted grey - white matter organisation.

GM = grey matter. WM = white matter.
Scale bar = 100 µm.
Ex vivo peripheral nerve grafting results

Labelling of neuronal nuclei further substantiated the findings of neuron survival in the 1.5 cm long spinal cord segment culture after Cx43 AsODN treatment (Figure 4.5). Cx43 AsODN treated segments contained a combination of large and small neuronal nuclei. Large neurons contained clearly labelled nuclei and cytoplasm with a central nucleolus. Pluronic gel treated segments contained small nuclei with little cytoplasmic labelling indicating a decline in neuronal health. Culture medium treated segments showed a decrease in the number of nuclei, most of which were small with no cytoplasmic labelling. The Cx43 AsODN treatment has, therefore, maintained the 1.5 cm segments in a viable condition ex vivo for 5 days, despite the longer segment length and resultant decrease in nutrient and oxygen diffusion efficacy, a cut in the middle of the segment and the insertion of ‘foreign’ tissue as a result of peripheral nerve grafting.
Ex vivo peripheral nerve grafting results

Figure 4.5. Neuronal Nuclei in the Grey Matter of Peripheral Nerve Grafted Spinal Cord Segments after Five Days of Culture. Nuclei and some cytoplasm of neurons have been immunohistochemically labelled with anti-Neuronal Nuclei antibody (red).

A. A lower magnification view of a Cx43 AsODN treated spinal cord segment shows that the segment contained a combination of large and small neuronal nuclei.

B. An example of neuronal nuclei labelling in Cx43 AsODN treated segments shows clearly labelled large nuclei and cytoplasm with a central nucleolus.

C. Pluronic gel treated control segment at the same magnification contained mostly small neuronal nuclei with little cytoplasmic labelling, indicating a decline in neuronal health.

D. Culture medium treated control segment at the same magnification contained a decreased number of nuclei that were mostly small with no cytoplasmic labelling.

Scale bar = 100 µm.
In the initial experiments, no axon outgrowth from the spinal cord into the graft was found in any treatment group mainly due to the presence of a ‘gap’ between the graft and the spinal cord as a result of surgical techniques when inserting the graft. In a subsequent unconditioned peripheral nerve grafting experiment the horizontal slit in the cord was reduced to a minimum width so that the peripheral nerve graft maintained tight contact with the spinal cord during culture. Six segments were treated with Cx43 AsODN, three segments with Pluronic gel, and three segments with culture medium only. Axon outgrowth was observed in most segments. In all of the Cx43 AsODN treated segments, very good graft to spinal cord integration was seen, defined by labelling for neurons and axons with anti-SMI32 antibody and for basal lamina of the graft with anti-laminin antibody (Figure 4.6A, representative image). In all of the grafts the tubular channels demarcated by laminin-positive basal lamina staining was retained. At these places of contact, there were usually large aggregates of SMI32-positive axon fibres, some of which crossed the border and entered the peripheral nerve graft. After five days in culture, the amount of axon regeneration after Cx43 AsODN treatment was marked. In Pluronic gel treated or medium only control segments, axon aggregates at the border were rarely seen, and there was a decrease in the number of axon extensions into the graft in these segments. The amount of axon outgrowth into grafted spinal cord segments was quantified by measuring the total length of outgrowth and the number of axons crossing the graft interface (Figure 4.7). On average, the total length of axon outgrowth was 0.39 +/- 0.12 mm (mean +/- SEM) after Cx43 AsODN treatment compared to 0.038 +/- 0.013 mm and 0.045 +/- 0.035 mm after Pluronic gel and culture medium only treatment. The average number of axons crossing the graft interface after Cx43 AsODN treatment was 12.1 +/- 2.9 compared to 2.3 +/- 0.9 and 1.7 +/- 1.2 after Pluronic gel and medium only treatments respectively. Because the variances of the groups were not equal, Brown-Forsythe analysis was undertaken instead of a one-way ANOVA. Results showed that both the length and number of axon outgrowths from the spinal cord segments into the peripheral nerve grafts were greater after Cx43 AsODN treatment with a significant difference (p=0.024, p=0.01 respectively). Pair-wise comparisons were performed for both length and number of axons crossing the graft between Cx43 AsODN treated and Pluronic gel (p=0.0165, 0.01 respectively), Cx43 AsODN and medium (p=0.0175, 0.0075), and Pluronic gel and medium (p=0.88, 0.68). Therefore, Cx43 AsODN treatment promotes axon regeneration from spinal cords into peripheral nerve grafts.
Ex vivo peripheral nerve grafting results

Figure 4.6. Representative Immunohistochemistry Images from Cx43 AsODN Treated (A, B) and Culture Medium Only (C, D) Fresh Peripheral Nerve Grafted Spinal Cord Segments. Regenerating axons have been labelled with anti-SMI32 antibody (red) and the peripheral nerve graft labelled by anti-laminin antibody (green).
A. After Cx43 AsODN treatment, the junction between the spinal cord and peripheral nerve graft (demarcated by the white line) was tight with no gap between the spinal cord and the grafted tissue. Axon aggregates were commonly seen (asterisks). This phenomenon was observed in all Cx43 AsODN treated segments.
B. Image showing an enlargement of the area enclosed in A. Regenerating axons were seen extending from the spinal cord and entered the graft (arrows).
C. With culture medium only treatment, axon aggregates were rarely seen.
D. Image showing an enlargement of the area enclosed in C. No axon regeneration was seen even at areas of higher axon density.
Scale bar = 100 µm.
Figure 4.7. Analysis of the Total Length and Number of Axon Outgrowth from a Spinal Cord Segment into a Peripheral Nerve Graft.

A. Total length of axon outgrowth that crossed the spinal cord segment - peripheral nerve graft border was analysed. Group averages +/- standard error of the mean are presented. After Cx43 AsODN treatment the total length of axon outgrowth was much greater compared to the two control groups (Brown-Forsythe test p = 0.024). This difference is attributed to Cx43 AsODN treatment only and not to the Pluronic gel.

B. The number of axon outgrowths was also analysed and the mean +/- standard error is presented. After Cx43 AsODN treatment the number of axon outgrowths was much greater compared to the two control groups (Brown-Forsythe test p = 0.01). This difference was attributed to Cx43 AsODN treatment alone and not to the Pluronic gel.

* p < 0.05, ** p < 0.01.
4.3. Discussion

We have investigated the effectiveness of Cx43 AsODN in promoting axon regeneration *ex vivo* in combination with unconditioned peripheral nerve grafting. In summary, results of the *ex vivo* peripheral nerve grafting experiments showed that:

- Proliferation of Schwann cells and degeneration of axons began within one day of peripheral nerve segment culture.
- Cx43 AsODN treatment maintained the 1.5 cm spinal cord segments in a viable condition with intact tissue organisation and good neuronal survival despite insertion of ‘foreign’ tissue as a result of peripheral nerve grafting.
- The number and length of axon outgrowth from the spinal cord into grafted peripheral nerves within five days of culture was significantly increased by Cx43 AsODN treatment.

4.3.1. Peripheral nerves in culture retained axon regeneration promoting capacity

Peripheral nerves have an anatomical organisation comprising either one myelinated or several non-myelinated axons wrapped around by the membrane extensions of one Schwann cell, each axon-Schwann cell unit being enclosed in a basal lamina tube, with bundles of axon-Schwann cell units comprising a peripheral nerve. The nerves are surrounded by three layers of connective tissue, the endoneurium, perineurium and epineurium (R. P. Bunge, 1987). Schwann cells and fibroblasts produce components of the endoneurium including type I, III, IV, V collagen, entactin and a proteoglycan (R. P. Bunge, 1987). Laminin is a major component of the basal lamina actively laid down by Schwann cells, and has been implicated in promoting axon outgrowth, Schwann cell migration, differentiation and myelination (Dubovy, 2004). Here, laminin labelling was used to distinguish peripheral nerve tissue from spinal cord tissue, as it was evident that anti-SMI32 labelling alone did not allow clear identification of the border of the peripheral nerve. The ambiguity in anti-SMI32 labelling made it difficult to tell if growing axons had crossed the spinal cord-graft border. At seven days postnatal, the rat sciatic nerve structure is quite variable. It appears as a mono- or di-fascicular structure; compartmentation to form multiple fascicles happens from seven days to six weeks when Schwann cells and endoneurial fibroblasts undergo changes to become perineurial cells (Morris et al., 1972). Therefore, in my P7 postnatal studies, the perineurium was not evident in some of the cross sections shown.
During culture, peripheral nerve axons cut off from their cell bodies degenerated as part of the Wallerian degeneration response, leaving the basal lamina tubes they had formerly occupied available for new regenerating axons. An increase in the intensity and number of S100 immunolabelled Schwann cells was seen, along with changes in Schwann cell shape to spindle-like form, consistent with previous observations of Schwann cell de-differentiation and proliferation \textit{in vivo} (Corfas, Velardez, Ko, Ratner, & Peles, 2004). The tubular organisation of the basal lamina was intact after grafting and \textit{ex vivo} culture. The proliferation of Schwann cells distal to the transection in response to degradation of myelin has been well documented, and results in migration of Schwann cells from cut stumps to bridge a certain size transection gap, before axons can grow across the gap (R. P. Bunge, 1987). Proliferating Schwann cells become aligned inside the basal lamina tubes after degeneration of axons, allowing regenerating axons to grow in the innermost surface of the tube in contact with Schwann cells (Dubovy, 2004). It was found that Schwann cells and the extracellular matrix components were essential for growth of axons regenerating from the CNS, but for regenerating axons from sensory neurons that reside in the dorsal root ganglion outside the CNS, Schwann cells alone or the extracellular matrix alone were sufficient for induction of axon growth (R. P. Bunge, 1987). Therefore, it is reasonable to expect that peripheral nerves in my \textit{ex vivo} culture would have the same capacity for supporting axon regeneration as \textit{in vivo}, and should be able to do so after grafting.

4.3.2. \textbf{Cx43 AsODN promoted grey and white matter survival in long segment culture}

I have shown improved grey and white matter tissue survival, better graft integration and axon outgrowth after \textit{ex vivo} peripheral nerve grafting in combination with Cx43 AsODN treatment compared to graft only. The extent of survival with Cx43 AsODN treatment in these 1.5 cm segments was comparable to the shorter 0.5 cm spinal cord segment cultures despite a longer segment length, requiring more extensive diffusion of nutrients and oxygen, and despite having a further injury site in the middle of the segment and ‘foreign’ tissue inserted into the segment as a result of peripheral nerve grafting. Because the stab wound in the middle of the segment and peripheral nerve grafting creates considerably more damage to the segment, a short 0.5 cm segment did not ‘survive’ to allow axon regeneration. An allograft was used instead of an autograft, but no signs of rejection were observed, consistent with results obtained in \textit{in vivo} experiments using allografts (Cheng et al., 1996; Rasouli et al., 2006). Better neuronal
Ex vivo peripheral nerve grafting results

survival in the grey matter after Cx43 AsODN treatment supports similar results obtained in short segment culture, with reduced Cx43 expression effecting necrosis, apoptosis and inflammatory reactions in a network manner thus promoting overall tissue and neuron survival.

White matter removal in traumatic spinal cord injury happens in two distinct processes known as Wallerian degeneration and axon dieback distal and rostral to the lesion respectively. Wallerian degeneration is the deterioration of axons disconnected from their cell bodies, and their phagocytosis by activated microglia and blood-borne macrophages; it is essential preparation for future axon regeneration. Wallerian degeneration is of little real interest for clinical interventions, however, since decentralised axons with no cell body cannot persist (Martini et al., 2003; Misgeld, 2005). Dieback of the axons still connected to neuron cell bodies is, however, a target for pharmaceutical intervention because it produces long-term neurological deterioration. Dieback has been shown to be attributed to microglia activation and blood-borne macrophage infiltration, both of which also prevents axon regeneration later by causing growth cone retraction upon contact, an effect exerted via matrix metalloproteinase-9 and chondroitin sulfate proteoglycans (Busch, Horn, Silver, & Silver, 2009). Smaller axons are more resistant to degeneration than larger axons greater than 5 µm in diameter (Smith & Jeffery, 2006). This was a feature also observed in my study with larger diameter axons being lost in the control segments after 5 days. Since the spinal cord segments were cut at both ends, both Wallerian degeneration and axon dieback could occur, with both involving microglia activation. Macrophages cannot infiltrate the lesion as the blood supply was absent in the ex vivo culture model. Therefore Cx43 AsODN prevented or hindered axon degeneration in the injured segments most likely through its effects in delaying microglia activation.

Anti-SMI32 antibody only labels the non-phosphorylated form of the neurofilament protein and has a restricted localization to a minority of neurons (King, Canty, & Vickers, 2001). There is evidence that SMI32 labelling is much stronger in regenerating axons because they are usually non-phosphorylated (King et al., 2001). Therefore the SMI32 label in both the grey and white matter after Cx43 AsODN treatment and the prominence of small diameter axons in this culture may also suggest axon regeneration.
Ex vivo peripheral nerve grafting results

Other neurofilament markers such as NF-H and NF-L (antibodies towards the heavy and light chains of the neurofilament protein) should be considered for future studies as these would label all existing neurofilaments irrespective of the phosphorylation state of the protein and hence the age of the filament.

4.3.3. Cx43 AsODN promoted axon regeneration into grafted peripheral nerves

We hypothesised that Cx43 AsODN treatment could promote axon regeneration into a peripheral nerve graft within a 5 day culture period, and found significantly enhanced axon penetration both in number and length. Peripheral nerve grafting is promoted as a strategy for regeneration following peripheral nerve injuries (Politis, Ederle, & Spencer, 1982). Peripheral nerve grafts have shown considerable promise and prompted much spinal cord injury research in the 1980s after researchers realised the potential for regeneration in the spinal cord (Benfey & Aguayo, 1982; Cheng et al., 1996; Dam-Hieu et al., 2006; Feng et al., 2008; Hermelinda et al., 2003; Houle et al., 2006; Oudega & Xu, 2006; Rasouli et al., 2006; Richardson, McGuinness, & Aguayo, 1980; Richardson et al., 1982; Tanigawa, Saito, Ogawa, & Iida, 2005). However, there are still limitations to peripheral nerve graft repair, including the inability to promote regeneration of corticospinal tract axons, the inability of regenerating axons to exit the graft, and the requirement of autologous tissue which can lead to functional deficits elsewhere in the body (Oudega & Xu, 2006). In addition, signs of poor graft integration was seen in my Pluronic gel and culture medium only groups with big gaps between the peripheral nerve graft and the spinal cord. The lesion spread and inflammatory response is a barrier to axon penetration, despite the peripheral nerve grafts being held firmly in place. This was entirely consistent with my hypothesis that the intervention itself will lead to injury spread and inflammation. This was improved by Cx43 AsODN treatment. The graft was inserted in an end-to-side position, which was expected to attract axon growth into the graft from the cut cross section. However, as was observed in the spinal cord longitudinal sections, axon outgrowth entered the graft side-on as well, and the number and length of these axons were significantly enhanced by Cx43 AsODN treatment. In this study axon regeneration was not only enhanced by the provision of a better regenerative environment for a peripheral nerve graft, but also by reducing bystander cell loss and inflammation using Cx43 AsODN, providing a more permissive environment in the spinal cord tissue surrounding the graft.
4.4. Summary of experiment findings

- Proliferation of Schwann cells and maintenance of basal lamina tubes in *ex vivo* culture ensured that the peripheral nerves retain the same capacity for supporting axon regeneration as *in vivo*.

- Cx43 AsODN treatment resulted in better grey matter neuron survival in *ex vivo* peripheral nerve grafted 1.5 cm spinal cord segments comparable to the level of survival achieved in shorter 0.5 cm segments by reducing necrosis, apoptosis and inflammation.

- Cx43 AsODN treatment resulted in less axon dieback in the white matter of peripheral nerve grafted spinal cord segments by delaying microglia activation.

- By inhibiting lesion spread and inflammatory responses and reducing bystander cell loss using Cx43 AsODN treatment, axon regeneration from the spinal cord segments into grafted peripheral nerves was significantly enhanced.
Chapter 5. *In vivo* peripheral nerve grafting with Cx43 AsODN results

Following the success of the *ex vivo* peripheral nerve grafting experiment, *in vivo* experiments were carried out to test the hypothesis that axon regeneration following peripheral nerve grafting could be enhanced by Cx43 AsODN treatment. This work was carried out in the Neural Injury Research Unit at the University of New South Wales, Sydney, Australia.

5.1. Peripheral nerve pre-conditioning

For this *in vivo* peripheral nerve grafting experiment, grafted sciatic nerves from donor animals were preconditioned to promote Schwann cell proliferation *in vivo* by ligation of the proximal end of the sciatic nerve one week prior to grafting. One sciatic nerve was analysed on each day (1, 3, 5, and 7 days after ligation). Two un-ligated sciatic nerves from the right legs were also analysed as controls. The histological and cellular changes in ligated sciatic nerves distal to the ligation were characterised. Haematoxylin and eosin labelling showed that the sciatic nerve maintained its integrity and general width and length. The cells within changed from an elongated to a rounder shape within 1 day, indicating de-differentiation; there was also a progressive increase in the number of cell nuclei from 3 to 7 days, indicating proliferation (Figure 5.1). Since these nuclei were arranged in rows, apparent from day 5 (Figure 5.1 D), they were thought to be those of Schwann cells, which are arranged in Bands of Bunger in the original basal lamina tubes after proliferation. They also showed their migratory ability moving into acellular nervous tissue in the centre of the ligation after 7 days (Figure 5.1 F arrowhead). Fluorescence labelling (Figure 5.2) with anit-S100 antibody for Schwann cells (left hand column) and anti-laminin antibody for basal lamina (right hand column) showed the normal organisation of the sciatic nerve consisting of Schwann cell cytoplasm forming neurilemma units with enclosed axons, that were in turn enveloped by a basal lamina, also laid down by Schwann cells. Little change in this organisation was seen after one day of ligation. After 7 days, the intensity of Schwann cell labelling had increased along with fragmentation of the neurilemma. The basal lamina appeared disorganised although most of the basal lamina was evident since staining intensity remained the same.
In vivo peripheral nerve grafting with Cx43 AsODN results

Figure 5.1. Schwann Cell Proliferation Immediately Distal to Sciatic Nerve Ligation Revealed by Haematoxylin and Eosin Staining.
Cell nuclei have been labelled with haematoxylin (dark blue). The peripheral nerve appears light purple due to non-colouring myelin and axons and lightly coloured basal lamina labelling by eosin (red).
A. Normal sciatic nerve longitudinal section shows orderly arrays of flattened Schwann cell nuclei (arrow) between axon units.
B. One day after ligation, the area of sciatic nerve distal to the ligation shows a change in the shape of some Schwann cell nuclei from flattened to round (arrow).
C. Three days after ligation, an increase in the number of Schwann cell nuclei was seen along with changes in shape of Schwann cell nuclei (arrow). The ligation site (asterix) was acellular and appeared red.
D. Five days after ligation, the increase in Schwann cell number was pronounced and Schwann cells (arrow) were arranged in long rows. (asterix = ligation site)
E. Seven days after ligation, the sciatic nerve was packed with both flattened and round Schwann cells (arrow) with very little axon remaining.
F. Seven days after ligation, the squashed ligation site (asterix) became infiltrated with Schwann cells (arrowhead), demonstrating their migratory ability. (arrow = Schwann cells in the sciatic nerve)
Scale bar = 100 µm.
In vivo peripheral nerve grafting with Cx43 AsODN results

Figure 5.2. Schwann Cell and Basal Lamina Labelling Distal to a Ligation in Normal and Ligated Sciatic Nerves 1 and 7 Days After Ligation.
Schwann cells (arrows) have been immunohistochemically labelled using anti-S100 Antibody (red, A, C, E). Sciatic nerve basal lamina (arrowheads) has been immunohistochemically labelled using anti-laminin antibody (red, B, D, F).
A. Normal sciatic nerve consists of Schwann cell membrane extensions wrapping around axons to form neurilemma, with flattened Schwann cell nuclei (arrow) located between these axon units.
B. The axon units were in turn enclosed in laminin-positive basal lamina (arrowhead).
C. One day after ligation, Schwann cell (arrow) labelling intensity increased slightly but no major change in morphology was seen.
D. One day after ligation, little change in basal lamina was seen. The basal lamina tubes (arrowhead) were seen clearly in regions where the section was cut obliquely.
E. Seven days after ligation, an increase in Schwann cell labelling intensity was seen, along with fragmentation of the neurilemma (arrow).
F. Seven days after ligation, dis-organisation of basal lamina tubes (arrowhead) was seen reflecting a loss of axons within. Labelling intensity of laminin was unchanged.
Scale bar = 100 µm.
5.2. Time course of Fluoro-Ruby transport to the transection site

To ensure that 1 week was sufficient time for Fluoro-ruby (FR) to be taken up by neurons around the T8 injection site and to be anterogradely transported to the tips of regenerating axons below the transection site at T10, a control experiment was performed. Two animals received bilateral T8 FR injections into the intact spinal cord and the extent of FR uptake and transport was analysed after one week. Despite the manufacturer’s claims that FR was an anterogradely transported dye, results showed that FR was taken up by both neurons and axons, and transported anterogradely and retrogradely to the corresponding axons and neurons (Figure 5.3). FR was readily taken up by neurons and axons surrounding the injection site identified by high intensity FR labelling in the needle tract and cavities in the dorsal cord. At distances as far as 1.95 cm caudal to injury, neuron cell bodies and axons were found clearly labelled with FR. At approximately 1 cm caudal to T8, where T10 resided, many axons in the white matter were labelled (seen in cross section in Figure 5.3). Therefore, one week was determined to be a long enough time for FR to be anterogradely transported from cell bodies in T8 to the transection site and beyond. No behavioural defects were associated with FR injection.
In vivo peripheral nerve grafting with Cx43 AsODN results

Figure 5.3. Fluoro-Ruby Labelling in Neuron Cell Bodies and Axons Caudal to the Injection Site 1 Week after T8 Bilateral Injection into an Intact Spinal Cord.

Spinal cord cross sections are shown at various distances up to 1.95 cm caudal to the injection site.
A. At T8, the needle injection site was seen as a cavity (asterix) with surrounding grey and white matter labelled with FR. The needle tract is not seen in this section.
B. At 0.15 cm caudal to T8, the needle tract on the other side of the spinal cord was evident (arrow), indicating diffusion of FR along the damanged tract and its uptake by some axons in the white matter. The inconsistency between the right and left spinal cord FR injection sites was determined to be experimental variation due to the slippery texture of the dura mater.
C. At 0.98 cm caudal to T8, where T10 was located, many neurons and axons were labelled in the white matter.
D. At 1.5 cm caudal, the amount of FR labelling was reduced.
E. At 1.95 cm caudal, the amount of FR labelling in neuron cell bodies (arrows) and axons (arrowheads) was further reduced.
F. Higher magnification image of section E shows clearly labelled neuron cell bodies in the grey matter.

Scale bar = 500 µm.
5.3. Two week preliminary peripheral nerve grafting and FR injection experiment

To examine the effectiveness of the transection and peripheral nerve grafting techniques, a preliminary experiment was carried out in which the duration was kept to two weeks. Two rats received T10 transection, peripheral nerve grafting and, one week later, bilateral T8 FR injection. No Cx43 AsODN was applied at the time of transection and grafting. The spinal cord tissue was processed for immunohistochemistry at 2 weeks for general astrocytosis, scar deposition and axon outgrowth. Results from both animals are presented.

FR labelling was found to extend 500-1000 µm rostral to T10 (Figure 5.4 A) and was seldom found immediately rostral (0 – 500 µm) to the injury site (Figure 5.4 B, C), indicating the success of the injection procedure. No FR labelling was found caudal to the injury except outside the dura due to extra-dural diffusion of the dye (Figure 5.5 B). Intense GFAP-positive labelling surrounding the injury site indicates astrocytosis with the pattern rostral and caudal to the transection being different. In both samples the rostral spinal cord – transection junction was rounded up by GFAP-positive astrocyte processes (Figure 5.4C, Figure 5.5A), seemingly ‘barricading’ the rostral spinal cord tissue from that of the transection site, which was itself GFAP-negative. In the caudal transection – spinal cord junction, astrocyte processes were seen extending towards the transection, as though invading the site (Figure 5.4 E, Figure 5.5 E). There was also an interesting pattern of laminin expression. The peripheral nerve graft was still laminin-positive, indicating the persistence of the Schwann cell-derived basal lamina after 2 weeks (Figure 5.4 D); therefore, the regeneration-permissive properties of the peripheral nerve graft should have remained. The peripheral nerve was at its supposed position in the middle of the transection site and was very well integrated with little gap between the graft and surrounding tissue. Tissue surrounding the graft was also laminin-positive, indicating fibrous scar formation, extending a small distance rostral or caudal to the graft (Figure 5.4 B, F). Normal spinal cord tissue is laminin-negative except the occasional labelling in blood vessel basement membranes.
Figure 5.4. Glial Scar and Peripheral Nerve Morphology 2 Weeks after T10 Spinal Cord Complete Transection and Peripheral Nerve Grafting without Cx43 AsODN Treatment.

Astrocytes have been immunohistochemically labelled using anti-GFAP antibody (green, A, C, and E). The basal lamina of the peripheral nerve graft has been labelled using anti-laminin antibody (green, B, D, and F). Axons that originated from T8 or neurons that have processes in T8 have been labelled with FR (red).

A. Long axon fibre and neuronal cell body FR labelling rostral to T10 confirmed that if any fibres had regenerated into the peripheral nerve or the other side of the transection, at least some of them would be FR labelled. There was a moderate degree of GFAP labelling.

B. The spinal cord rostral to the transection consisted of two distinct regions, normal laminin-negative spinal cord with some laminin-positive blood vessels and a fibrous scar containing dense laminin labelling on two sides of the transection.

C. At the rostral SC - graft junction, GFAP-positive astrocytes formed a clearly defined border with the highest intensity at the SC-graft junction. Only a few dots of FR labelling were found in this area.

D. The peripheral nerve graft retained the laminin-positive basal lamina and was found in the middle of the transection site after two weeks of grafting, validating the grafting methodology. The peripheral nerve was very well integrated into the surrounding scar tissue.

E. At the caudal graft - SC junction, astrocyte morphology was different from that seen at the rostral junction. Astrocyte processes extended into the injury site, seemingly still in the process of active invasion.

F. The caudal transection site was also comprised of laminin-positive scar tissue. The occasional blood vessel (arrow) in the spinal cord was also laminin-positive. No FR labelling was found caudal to the transection.

Scale bar = 500 µm. SC = spinal cord.
While the first animal had a perfect complete transection, the second animal showed left leg movement during the second week of recovery, indicative of signs of an incomplete transection. Histological observations showed that the pattern of astrocytosis was also different depending on the rostral and distal location. Approximately one tenth of the spinal cord width on the ventral side was not transected, as evidenced in the continuous dura (Figure 5.5 B arrow) and GFAP labelling (Figure 5.5 C right hand side) on that side of the injury. Interestingly remnant spinal cord tissue (Figure 5.5 B right 1/3) was also laminin-positive but with lower labelling intensity compared to the normal fibrous scar, indicating invasion of fibrous scar components through secondary injury processes although the area was not physically damaged. The areas that were GFAP-positive and laminin-positive did not seem to overlap (Figure 5.5 C, D) indicating the exclusivity of the fibrous and glial scars, although double labelling was needed to confirm this. Signs of axon regeneration into the peripheral nerve graft were seen in this spinal cord. SMI32-positive axons, aligned in the rostral-caudal direction, some as long as 300 μm, were seen extending from the caudal spinal cord into the scar or graft region, which was SMI32-negative after degeneration of denervated axon fibres within the graft (Figure 5.5 F). In later surgeries the ventral side of the transection was scraped with a scalpel blade after producing the transection with iris scissors to make sure all connections were cut.

Results from this preliminary experiment indicate the successful integration of peripheral nerve grafts and the induction of axon regeneration without Cx43 AsODN treatment after a short two weeks. A longer term, six week peripheral nerve grafting experiment, was then performed in conjunction with Cx43 AsODN treatment, aiming to promote more axon regeneration into the peripheral nerve graft.
In vivo peripheral nerve grafting with Cx43 AsODN results

Figure 5.5. Glial Scar and Peripheral Nerve Morphology 2 Weeks after Spinal Cord Incomplete Transection at T10 and Peripheral Nerve Grafting without Cx43 AsODN Treatment.

Astrocytes have been immunohistochemically labelled using anti-GFAP antibody (green, A, C, and E). The basal lamina of the peripheral nerve graft has been labelled using anti-laminin antibody (green, B and D). Neurons and axons have been immunohistochemically labelled using anti-SMI32 antibody (green, F). Axons that originated from T8 or neurons that have processes in T8 have been labelled with FR (red, A-F).

A. Astrocytes formed a clearly defined border with a hyperbola shape in the rostral SC - graft junction with astrocyte processes curving backwards once they reach the junction.

B. Ventral SC shows continuous dura due to an incomplete transection procedure. FR labelling was found outside the dura (arrow). Remnant spinal cord tissue (right 1/3 of image) was less dense with respect to laminin labelling than the actual transection site (left 2/3).

C. The peripheral nerve graft area is GFAP-negative.

D. The same graft area is laminin-positive.

E. Caudal graft - SC junction shows astrocyte processes (arrowheads) extending into the graft region.

F. Caudal graft - SC junction shows SMI32-positive axons extending toward the graft (arrowheads) and long axons within the graft aligned in a rostral-caudal direction, some of them as long as 300 µm (arrows).

Scale bar = 250 µm. SC = spinal cord.
5.4. Six week peripheral nerve grafting experiment

To investigate the effects of Cx43 AsODN in promoting axonal regeneration through a peripheral nerve graft, adult rats were subjected to a T10 complete transection injury and each received a single 0.5 mm long piece of preconditioned sciatic nerve from a donor animal, and either 2 µM Cx43 AsODN, 2 µM Sense ODN or no treatment controls. FR was injected bilaterally into T8 after five weeks. Animals were sacrificed one week later and tissues analysed. The numbers of animals that received Cx43 AsODN, Sense ODN, or no treatment were 10, 11 and 10 respectively. Behavioural tests were performed on all surviving animals at any one time point. At the end of the experiment 5, 6, and 5 animals respectively survived (Table 1); the animals were killed, their spinal cords removed and immunohistochemically analysed.

<table>
<thead>
<tr>
<th>Cx43 AsODN</th>
<th>Sense ODN</th>
<th>No treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Died of unknown reason on day 3</td>
<td>Died of unknown reason on day 4</td>
<td>Died of bowel obstruction on day 3</td>
</tr>
<tr>
<td>Died of unknown reason on day 3</td>
<td>Died of a burst bladder on day 6</td>
<td>Died of right kidney damage on day 7</td>
</tr>
<tr>
<td>Died of unknown reason on day 4</td>
<td>Died of a burst bladder on day 7</td>
<td>Died of inflamed lung on day 7</td>
</tr>
<tr>
<td>Died of unknown reason on day 10</td>
<td>Died of unknown reason on day 13</td>
<td>Died of kidney failure on day 15</td>
</tr>
<tr>
<td>Died of anaesthetic overdose and punctured bladder on day 35</td>
<td>Died of anaesthetic overdose and punctured bladder on day 35</td>
<td>Died of anaesthetic overdose on day 35</td>
</tr>
</tbody>
</table>

Table 1. Causes of death before the anticipated end of the experiment.

In longitudinal section, the spinal cords looked remarkably different from those processed at 2 weeks. The main differences included first, the absence of the peripheral nerve grafts (Figure 5.6). Peripheral nerve grafts were seldom found, and when found were located outside the spinal cord. The second main difference was the formation of one or multiple cavities in the spinal cord rostral or caudal to the fibrous scar. Some smaller cavities were also found in the white matter separate to the main cysts. The cavities invade normal spinal cord until only a subpial rim of white matter was left behind as a cavity wall. The formation of cavities followed a pattern. Each spinal cord had at least one cavity rostral to the transection and approximately 1/3 did not have any cavity caudal to the transection (Figure 5.6 C for example). Signs of rostral, but not caudal, cavity formation were already observed in some spinal cords removed after three weeks at the time of an animal death. The rostral cavity(ies) was almost always
bigger than those caudal to the injury. The cavities started close to the centre of the transection which was easily identified by a dense band of horizontal axon fibres at right angles to the longitudinal axis of the cord. The cavities expanded in all directions away from the centre of the transection. There was one instance (out of 17) in which the rostral and caudal cavities joined up, presumably because the centre of the transection was eroded to such a thin strip that it lost its integrity (see Figure 5.6 B).

Figure 5.6. Cavity Formation in Adult Rat Spinal Cords 6 Weeks after Transection and Peripheral Nerve Grafting with or without Cx43 AsODN Treatment. Neurons and axons have been immunohistochemically labelled using anti-SMI32 antibody (green). Cell nuclei have been labelled using DAPI (blue). Axons that originated from neurons in T8 or neurons that had axon extensions at T8 have been labelled with FR (red).
A. The most frequent form of cavity formation, showing a rostral and a caudal cavity separated by the centre of transection, identified by a dense band of horizontal axon fibres at right angles to the longitudinal axis of the cord (arrows).
B. The only example of rostral and caudal cavities joined up into one single cavity. The centre of the transection has been lost (arrows).
C. An example in which no caudal cavity has formed. Only one rostral cavity was present.
For examples of multiple cavity formation please see Methods section Figure 2.3, page 77 and Figure 2.4, page 79.
Scale bar = 1mm.
Upon closer inspection, the rostral border of the transection sites was infiltrated with large numbers of SMI32-positive axon fibres from rostral spinal cord tissue (Figure 5.7). Some of the axons were FR-positive indicating a T8 origin. Axon fibres from the caudal spinal cord sometimes reached the rostral border (e.g. Figure 5.6 A and B, page 147) and sometimes did not (e.g. Figure 5.6 C). Nevertheless rostral and caudal fibres always followed one of two patterns with remarkable similarity to the pattern of astrocytosis seen in the same region two weeks after transection (Figure 5.4 C and E, page 143, and Figure 5.5 A and E, page 145) suggesting a possible linkage in axon path finding and astrocyte orientation or guidance. Axons from the rostral spinal cords curved around horizontally once they entered the transection site (Figure 5.7 arrows). Axons from the caudal cord extended rostrally, but most stopped abruptly on reaching an invisible division line (Figure 5.7 asterix) between rostral and caudal axons. The number of caudal axons approaching the transection was much less than that of rostral axons. Little FR was found caudal to the lesion, indicating that none of the T8 originating fibres had reached the caudal spinal cord. In some spinal cords such as the one shown in Figure 5.6 C (page 147) the caudal axon fibres stopped much further from the transection than in other cases. Therefore, although many axons regenerated into the scar tissue from both directions, rostral and caudal axons did not reconnect with targets on the other side in all the samples.
Figure 5.7. Centre of Transection of the Spinal Cord Shown in Figure 5.6 A. 
Neuronal cell bodies and processes have been immunohistochemically labelled with anti-
SMI32 antibody (green). Cell nuclei have been identified with DAPI labelling (blue). Axons that 
originated from neurons in T8 or neurons that had axon extensions at T8 have been labelled 
with FR (red).

The centre of the transection has been infiltrated with SMI32-positive and sometimes FR-
positive axons from the rostral spinal cord extending caudally in the rim of white matter forming 
part of the cavity wall. Axons seldom extended into the caudal spinal cord, instead they curved 
around the transection site (arrows), behaving in the same way as astrocyte processes in the 
rostral SC - graft junction seen in the two week preliminary experiment (Figure 5.4 C, page 143, 
and Figure 5.5 A, page 145). FR labelling also stopped abruptly at an invisible division line 
(arrowhead) between rostral and caudal axons. Very few FR dots were seen more caudally. 
Caudal axons also extended rostrally to the transection site, but also seemed to end abruptly 
(asterix), behaving in the same way as astrocyte processes in the caudal SC - graft junction 
seen in the two week preliminary experiment (Figure 5.4 E, page 143, and Figure 5.5 E, page 
145).

Scale bar = 1 mm.
The differences between Cx43 AsODN and control treated animals were identified by behavioural analysis and various measurements based upon analysis of the immunohistochemistry. Analysis of the Basso, Beattie and Bresnahan (BBB) Score on days 2, 10, 20, and 30 after transection and peripheral nerve grafting showed that Cx43 AsODN treated animals (average BBB score on day 30 = 1.6) appeared to have better functional recovery on leg movement than Sense ODN (average BBB on day 30 = 1.5) and no treatment animals (average BBB on day 30 = 1.3) at all time points, although the difference was not statistically significant. On day 2, all animals were completely paralysed (BBB score = 0) confirming complete transection (Figure 5.8). Measurements made on immunohistochemically stained images are described in the methods section 2.3.12, page 75.
Figure 5.8. Mean Basso, Beattie and Bresnahan Scores of T10 Spinal Cord Completely Transected Animals with a Single Preconditioned Peripheral Nerve Graft at Different Time Points. The lesion site was treated with either 2 µM Cx43 AsODN, 2 µM Sense ODN, or no treatment at the time of transection and peripheral nerve grafting. Animals treated with Cx43 AsODN appeared to have recovered better than Sense ODN and no treatment animals at all time points except day 2, although the difference was not statistically significant. On day 2 all animals had a score of 0, confirming complete transection. Standard error of the mean for Cx43 AsODN treatments at days 10, 20 and 30 were 0.35, 0.43 and 0.40 respectively, for sense control were 0.33, 0.44 and 0.19, and for no treatment were 0.34, 0.44 and 0.39 respectively.
Measurements of cavity length (Figure 5.9) showed that Cx43 AsODN treatment appeared to have reduced the rostral - caudal cavity length, giving an impression of an effect in limiting the rostral-caudal expansion of cavities, although not significantly. The width of the regenerating band of axons (Figure 5.10) appeared to be greater after Cx43 AsODN treatment, indicating an effect on axon regeneration correlating with effects on behavioural improvement, although neither was significant.
In vivo peripheral nerve grafting with Cx43 AsODN results

Figure 5.9. Lesion Length 6 Weeks after T10 Complete Transection Injury, Preconditioned Peripheral Nerve Grafting and Cx43 AsODN Treatment, Sense ODN or No Treatment. Lesion length was measured by the rostral-caudal length of the cavities present. Cx43 AsODN treatment resulted in a reduced lesion length compared to Sense ODN or no treatment. Differences showed a trend but this was not statistically significant.
Figure 5.10. Regenerating Axon Band Width at 6 Weeks after T10 Complete Transection Injury, Preconditioned Peripheral Nerve Grafting and Cx43 AsODN Treatment, Sense ODN or No Treatment. Cx43 AsODN treatment resulted in an increased axon band width compared to Sense ODN and no treatment. Differences showed a trend but it was not statistically significant.
Measurements were made of the average cavity area of 7 longitudinal sections. This is a close approximation of cavity volume (Figure 5.11). Length of the GFAP-negative region, that represents the length of the fibrous scar (Figure 5.12), and relative GFAP intensity rostral to the transection, that represents rostral glial scar density (Figure 5.13) was also measured. There appears to be no difference in these measurements between Cx43 AsODN and no treatment, with Sense ODN treatment performing the worst. Sense ODN treated spinal cords had a larger average cavity area over 7 longitudinal sections, a longer length of fibrous scar, and higher GFAP intensity rostral to the transection. These could possibly be due to an unknown effect of the delivery vehicle Pluronic gel, because Sense ODN did not have any specific mRNA binding effect.
Figure 5.11. Average Lesion Area in Completely Transected Spinal Cords 6 Weeks after Surgery based upon 7 Longitudinal Sections from Each Spinal Cord Analysed. Animals were subjected to T10 complete transection injury, preconditioned peripheral nerve grafting and either Cx43 AsODN treatment, Sense ODN treatment or No Treatment. The sections were taken at equal distances from dorsal to ventral. Cx43 AsODN treatment produced the same lesion area as no treatment; Sense ODN produced the largest lesion area.
Figure 5.12. Length of the GFAP-negative Region Representing the Fibrous Scar in Transected Spinal Cords 6 Weeks after Surgery. Cx43 AsODN treatment produced the same length of GFAP-negative region as no treatment; Sense ODN produced the longest GFAP-negative region.
In vivo peripheral nerve grafting with Cx43 AsODN results

![Graph showing relative GFAP intensity rostral to the lesion for different treatment groups.]

Figure 5.13. Relative GFAP Intensity Rostral to the Lesion in Completely Transected Spinal Cords 6 Weeks after Surgery. Cx43 AsODN treatment produced similar results as no treatment. Sense ODN treatment produced a much higher intensity of GFAP labelling rostral to the lesion.
In vivo peripheral nerve grafting with Cx43 AsODN results

There appears to be very little difference between treatment groups in the width of the subpial white matter rim (Figure 5.14), that represents the extent of cavity expansion, and GFAP intensity caudal to the lesion (Figure 5.15), that represents caudal glial scar density. Therefore, Cx43 AsODN did not appear to influence the expansion of cavities in the left-right or dorsal-ventral direction that result in the subpial white matter rim, and had no effect on astrocytosis caudal to the lesion. The distance between rostral and caudal grey matter areas (Figure 5.16), that represents grey matter dieback, appeared to be the longest with no treatment, suggesting that the delivery vehicle Pluronic gel in both Cx43 AsODN and Sense ODN may have an effect in restricting grey matter dieback, corresponding to a shorter lesion length in both Cx43 AsODN and Sense ODN treatment groups.
Figure 5.14. Width of Subpial White Matter Rim Expressed as a Proportion of Spinal Cord Diameter in Completely Transected Cords 6 Weeks after Surgery.
No difference between groups was found.
Figure 5.15. Relative GFAP Intensity Caudal to the Lesion in Completely Transected Spinal Cords 6 Weeks after Surgery.

No difference between groups was found.
**In vivo peripheral nerve grafting with Cx43 AsODN results**

Figure 5.16. Distance between Rostral and Caudal Grey Matter Areas in Completely Transected Spinal Cords 6 Weeks after Surgery.

No treatment group produced the longest grey matter-grey matter distance compared to other groups although the results were not significant.
5.5. Discussion

In this chapter the investigations as to the efficacy of Cx43 AsODN in promoting axon regeneration after in vivo spinal cord injury and peripheral nerve grafting is described. In summary, results of the sciatic nerve ligation and peripheral nerve grafting experiments showed that:

- Ligation of the sciatic nerves promoted Schwann cell proliferation, and the basal lamina remained in the grafted sciatic nerves. The regeneration-promoting properties of Schwann cells should have, therefore, remained after grafting.

- Fluoro-Ruby was transported anterogradely and retrogradely from T8 to sites as far as T12 - L1 region in an intact spinal cord within one week. In a transected spinal cord the dye was not found caudal to the transection site two weeks after transection.

- The peripheral nerve graft remained in place and induced axon regeneration from the spinal cord after 2 weeks, before cavity formation had taken place.

- In the six week term experiment, rostral axon regeneration through the glial and fibrous scars appeared to be further promoted by Cx43 AsODN treatment. Axon outgrowth seemed to be guided by astrocyte processes.

- There appears to be a reduction in cavity length and better behavioural improvement after Cx43 AsODN treatment, although the differences were not statistically significant.

- The main drawbacks were the extrusion of the peripheral nerve graft from the spinal cord during the 2-6 weeks healing period, and the gradual formation and enlargement of cysts accompanying grey matter dieback in the areas close to the transection.

5.5.1. Ligating sciatic nerves promoted Schwann cell proliferation and basal lamina retention

Pre-conditioning of peripheral nerve grafts using techniques such as ligation and continuous compression were found to assist with grafting success when compared to fresh grafts (Feng et al., 2008; Hermelinda et al., 2003; Rasouli et al., 2006). The changes within seven days of left sciatic nerve proximal ligation were examined.
Schwann cells were found to change shape from elongated (a differentiated morphology) to round (a de-differentiated morphology), and to increase significantly in number (proliferate) within 3 days of ligation. These changes are consistent with those previously described and associated with de-differentiation and then proliferation of Schwann cells (Harrisingh et al., 2004; Oudega & Xu, 2006). After peripheral nerve injury, such as a ligation, macrophages phagocytose distal axons. Schwann cells proliferate to form a band within the basal lamina tubes vacated by axons and prepare to guide and myelinate new regenerating axons (Martini et al., 2003). Laminin in the basal lamina was found to be retained instead of degraded; therefore, the requirements for axon regeneration into peripheral nerve grafts were present after 7 days of ligation.

5.5.2. Fluoro-Ruby was anterogradely and retrogradely transported beyond the lesion site within 1 week

FR was found be taken up by neuron cell bodies and axons in T8, and transported anterogradely and retrogradely within the neuron to sites as far as 1.95 cm caudal to T8, which is equivalent to the T12- L1 region. Therefore, the transection site at T10 would have been well-covered after 1 week. At least some regenerating axons caudal to T10, originating at T8 would have been labelled by FR. Fluoro-Ruby (FR, Tetramethylrhodamine dextran-amine, Fluorochrome, USA) is in the family of dextran amines, and has proven to be a reliable anterograde and retrograde tracer taken up by neurons and axons (Zaborszky, Wouterlood, Lanciego, Reiner, & Honig, 2006), despite claims from the manufacturer that it is a retrograde tracer only.

5.5.3. Preliminary peripheral nerve grafting experiments

To validate the study design, a preliminary two week peripheral nerve grafting experiment was performed on two animals.

5.5.3.1. Axon outgrowth into the peripheral nerve graft was induced after two weeks

The peripheral nerve graft was found in the middle of the transection site in both animals after two weeks, with no signs of rejection. The basal lamina remained, similar to the ex vivo grafting experiment. Laminin is implicated in promoting axon outgrowth, Schwann cell migration, differentiation and myelination, and constitutes part of the axon regeneration inducing effects of peripheral nerve grafts by providing a structural scaffold (Dubovy, 2004). SMI32-positive axon outgrowth from the spinal cord into the
peripheral nerve graft was found in one animal, some axons as long as 300 μm, and confirmed the efficacy of the peripheral nerve grafts in inducing axonal outgrowth in this model, at least in this 2-week time frame.

5.5.3.2. Fluoro-Ruby labelling was restricted to a distance rostral to the transection site

Neuron cell bodies and long and short axons were labelled by FR from T8 region to 500 – 1000 μm from the rostral border of the glial scar at T10. FR labelling was restricted to the rostral spinal cord and to some dots of labelling outside the dura possibly due to extra-dural leakage during injection. No FR was detected within the caudal cord; therefore, no leakage of the dye within the cord had occurred. This validates the study design, in that before true axon regeneration had taken place no FR labelling was found caudal to the complete transection site.

5.5.3.3. Glial scarring was found in two distinct patterns

Glial scarring is an intrinsic mechanism of the spinal cord thought to contain the damage to a small area, therefore protecting against progressive retrograde damage (Faulkner et al., 2004a). In our study a different pattern of astrocytosis on either side of the graft or transection after two weeks was found. In the rostral spinal cord, GFAP-positive processes extended downwards and then curved away towards the horizontal plane once they neared the transection site, forming a shield-like structure. In the caudal spinal cord, GFAP-positive processes extended upwards towards the rostral cord at the junction, as if to invade the site. These patterns bear much similarity to axon regeneration seen in the scar region in the longer term six week experiment, suggesting a linkage between the early deposition of astrocyte processes and their roles in regenerating axon path finding later on.

5.5.3.4. A fibrous scar was found between the two glial scars

Whereas glial scarring always occurs in spinal cord injury, fibrous scarring happens only in those injuries involving damage to the meninges (V. W. Lin & Cardenas, 2003, Chapter 56). In this complete transection model, fibrous scarring containing laminin expression was found at approximately 500 μm either side of the peripheral nerve graft. The fibrous scar and astrocytic glial scar do not appear to overlap, consistent with the literature where indications are that no astrocyte labelling is usually found in the collagen IV-positive fibrous scar (Brazda & Muller, 2009).
5.5.3.5. An improvement to study design was needed

In the second animal, an incomplete transection was found after the animal recovered a significant degree of locomotion in one of its legs, with a single side BBB score of seven. The incomplete transection was found on the ventral side with a width of approximately 1/10 of the diameter of the spinal cord. Interestingly, laminin was still deposited, but to a lesser extent in that region. As a consequence, later surgeries included scraping the ventral side of the injury with a scalpel blade immediately after creating the injury with iris scissors.

5.5.4. Six week peripheral nerve grafting experiment

Following the preliminary experiments above, the efficacy of Cx43 AsODN in promoting axon regeneration after spinal cord injury and peripheral nerve grafting was investigated with 10, 11 and 10 animals in each treatment group (Cx43 AsODN, Sense ODN, and no treatment).

5.5.4.1. Cx43 AsODN treatment effects on behaviour and rostral axon regeneration

There appeared to be better behavioural improvement in Cx43 AsODN treated animals, consistent with what appears to be increased axon growth through the scars evidenced by the increased width of a band of axons regenerating in the fibrous scar after Cx43 AsODN treatment. These findings suggest that Cx43 AsODN did have an effect in providing a better post-injury environment by decreasing the amount of Cx43-mediated injury spread, ultimately resulting in axon regeneration and better functional improvement. This finding of a connection between Cx43 down-regulation and axon regeneration is, however, not confirmed, because there appears to be no difference in astrocytosis, an impediment to axon regeneration that was thought to be down-regulated by Cx43 AsODN treatment. Astrocyte or glial scar density analysed by anti-GFAP labelling intensity rostral and caudal to the lesion showed no difference between groups. Some subtle undetected changes in astrocyte phenotype may be responsible. Improvements in hindlimb locomotor behaviour after a complete transection was mainly attributed to the re-organisation of the spinal cord circuitry and activation of the central pattern generator, that relies on the availability of healthy spinal tissue in the lumbar region caudal to the transection and can be damaged by secondary injury spread from T10 (F. M. Bareyre et al., 2004). Therefore, Cx43 AsODN treatment could have attenuated the secondary injury spread resulting in better functional improvements.
**In vivo peripheral nerve grafting with Cx43 AsODN results**

Cx43 AsODN treated animals seemed to have performed worse than other treatments in some measurements but not all. This could be due to an effect of the Pluronic gel used as a delivery vehicle. Pluronic F127 gel is a non-ionic surfactant that is relatively non-toxic (BASF product information). It has been used to provide sustained delivery of the Cx43 AsODN in another spinal cord injury model and was demonstrated to be non-cytotoxic, aiding penetration of AsODN (M. Cronin et al., 2008; Green et al., 2001). The gel may have an osmotic effect and reduce spinal cord swelling when administered immediately after injury (Nout et al., 2009). The mechanism of Sense ODN performing worse than no treatment control is not yet clear.

### 5.5.4.2. Axons regenerated into the glial and fibrous scars

Two weeks after injury, the FR dots in the rostral cord were seen mainly at a distance (500 – 1000 μm) from the rostral most border of the glial scar. In contrast, another four weeks later, the FR-positive axons with cell bodies in T8 had advanced 500 – 1000 μm and infiltrated the whole rostral spinal cord – transection site junction, which was a GFAP-negative fibrous scar, along with more axons with origins elsewhere. Therefore, axon elongation had occurred irrespective of the disappearance of the peripheral nerve graft. This kind of intrinsic repair from interneurons and descending central axons has been reported previously (Fenrich & Rose, 2009; Hagg & Oudega, 2006; von Euler et al., 2002) and the fibrous scar is known to allow axon regeneration in some spinal cord injury models (Spilker et al., 2001). In other studies, however, the fibrous scar was found to be regeneration-inhibiting. The corticospinal tract axons were found to retract approximately 1-2 mm initially, as they did in my study at 2 weeks, and to subsequently regenerate to the edge of the fibrous scar where they abruptly stopped (Brazda & Muller, 2009). This was resolved by applying a fibrous scar suppressing iron chelator and cyclic adenosine monophosphate after dorsal column transection, thus encouraging corticospinal tract axon growth beyond 1 cm distal to the lesion (Klapka et al., 2005). Time wise, it appears that the invasion of axons happens after the formation of cavities evidenced by the apparent continuity of the axons in the walls of the cavities. If the invasion of axons occurred first, most of the axons would be cut off by cavity formation. Both axon regeneration and cavity formation were still ongoing at six weeks after injury. Therefore, axons regenerated through the glial and fibrous scars to reach the rostral-caudal lesion border after cavity formation.
Although there were many regenerating axons on either side of the lesion, they did not form functional connections by crossing the lesion to the other side of the spinal cord transection. The incomplete axon regeneration can be explained in part by the properties of the fibrous scar and the glial scar, which have complementary roles. The fibrous scar consists of mainly collagen IV, I, III, fibronectin and laminin fibres, which all support axon growth in vitro (Brazda & Muller, 2009). The cell types that reside in the fibrous scar include meningeal fibroblasts, inflammatory cells, endothelial cells and NG-2 positive oligodendrocyte precursor cells (Brazda & Muller, 2009). Therefore, the fibrous scar itself is not inhibitory per se, but collagen contains binding sites for inhibitory factors including NG-2, phosphacan, syndecan-2, tenascin-C, ephrins, TN-R, Sema 3A, 3B, 3C, 3E, 3F, repulsive guidance molecule, versican, keratin sulphate proteoglycan, heparin sulphate proteoglycan and chondroitin sulphate proteoglycans, making the fibrous scar a sticky matrix that is non-permissive (Brazda & Muller, 2009). Suppression of fibrous scar formation has generally been successful at promoting axon regeneration (Brazda & Muller, 2009). Many experiments have also investigated the non-permissive nature of the glial scar (Moon & Fawcett, 2001; Ramon-Cueto et al., 1998), that can be attributed to the inhibitory factors within it including N250, myelin associated glycoprotein, tenasin-R (secreted by oligodendrocytes), NG-2, phosphacan, versican (secreted by oligodendrocyte precursors), tenascin, brevican, neurocan (secreted by astrocytes), free radicals, nitric oxide, arachidonic acid derivatives (secreted by activated microglia), many of which overlap with those present in the fibrous scar (Fawcett & Asher, 1999). Furthermore, astrocytes have been found to inhibit migration of oligodendrocyte precursors and Schwann cells, limiting the extent to which regenerating axons can be myelinated (Fawcett & Asher, 1999). The final form of the glial scar consists of astrocyte processes tightly woven, connected with tight junctions and gap junctions (Fawcett & Asher, 1999). Unlike the fibrous scar, astrocytes themselves are only occasionally axon-growth supporting, changing their growth-supportive states in response to the environment (Fawcett & Asher, 1999). Therefore, partial attenuation of the inhibitive fibrous and glial scar environment was achieved to allow axon regeneration, but axon regeneration was not allowed across the rostral-caudal lesion border.
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Taken together, regenerating axons face two inhibitory environments; one provided by the numerous inhibitory factors bound to the fibrous scar and the second secreted by the glial scar. By modulating Cx43 gap junction and hemichannel communication in relation to astrogliosis and glial scarring, the production of these molecules could be reduced by reducing the number of cells constituting the glial scar and also by a change of phenotype of microglia and astrocytes to a growth-supporting state. A reduced production of these ‘inhibitory’ molecules can then render the fibrous scar more regeneration-permissive by having less in the way of collagen-adhering inhibitory molecules. The treatments used in our experiments were clearly not enough to attenuate astrocytic and fibrous scarring to allow axons to reconnect with their targets, although some attenuation was achieved resulting in axon regeneration through the scars. Cx43 AsODN treatment stimulated axon growth resulting in enhanced rostral axon regeneration.

5.5.4.3. The peripheral nerve grafts established a growth-supportive environment

The growth-supporting role of the astrocyte scar is underpinned by the finding that regenerating axons at six weeks followed the same direction as that of astrocyte processes seen at two weeks. This suggests that the glial scar, laid down in a set pattern by 2 weeks, had been in a growth-supporting mode from weeks 3 - 6 with or without Cx43 AsODN treatment, and had been ‘guiding’ regenerating axons. Given the non-permissive nature of the glial scar, this axon regeneration inducing effect could be attributed to the presence of the peripheral nerve grafts even though they were squeezed to the side some time during weeks 3 – 6, although no proof was seen. This speculation is supported by the finding that preconditioned nerve grafts can successfully induce axon growth not only inside, but also alongside the graft, seemingly establishing a regeneration-supportive environment in the spinal cord encompassing a greater area than the graft itself (Rasouli et al., 2006). Collateral sprouting of uninjured axons induced by peripheral nerve grafts was proposed as the mechanism by which axon growth alongside the graft occurred (Rasouli et al., 2006). Nevertheless, the extrusion of the peripheral nerve grafts from the graft site inevitably weakened their effects, since any prior regeneration into the graft as seen at two weeks would be lost.

The growth-supporting effects of peripheral nerve grafts were thought to be provided by Schwann cells. Schwann cells have been heavily studied, and found to self-repair the
injured peripheral nerve by contributing to axon debris digestion, forming Bands of Bunger thereby allowing axon elongation along these bands, and by secreting growth factors to generate a permissive environment (Oudega & Xu, 2006). Schwann cells in spinal cord repair are usually present within a peripheral nerve graft or embedded in a scaffold after culture and purification. Interestingly, transplanted Schwann cells can also invade injured spinal cords and myelinate or ensheath some axons in a contusion injury, although the extent of this intrinsic repair is too minute to cause a significant functional improvement (M. S. Beattie et al., 1997; Bresnahan, 1978; Takami et al., 2002). Many studies have shown the potential of Schwann cells in spinal cord repair when grafted (Benfey & Aguayo, 1982; Cheng et al., 1996; Dam-Hieu et al., 2006; Feng et al., 2008; Hermelinda et al., 2003; Houle et al., 2006; Oudega & Xu, 2006; Rasouli et al., 2006; Richardson et al., 1980, 1982; Tanigawa et al., 2005). They have been shown to induce axon regeneration, myelinate those axons and promote electrical conduction along axons (Felts & Smith, 1992). However, one major drawback has been that even when axons regenerated readily into peripheral nerve grafts enhanced by pre-conditioning, or cultured Schwann cell grafts, none of these studies have shown growth out of the graft back into the spinal cord (Houle et al., 2006). This was thought to be due to the inhibitory environment in the glial scar, the lack of neurotrophic support, and insufficient ability of damaged axons to grow (Oudega & Xu, 2006). Therefore, even though great techniques have been developed to culture autologous human Schwann cells in a 4-5 week period to generate a sufficient number, no human transplantation trials have been performed to spinal cord injured patients (Oudega & Xu, 2006). Combinations of treatments aimed at attenuating the glial scar, such as Cx43 AsODN and / or providing neurotrophic support and / or enhancing the intrinsic abilities of axons to regenerate, need to be investigated as the next step in peripheral nerve grafting / Schwann cell repair research.

5.5.4.4. The fibrous scar is postulated to extrude the peripheral nerve grafts from the lesion site

The success of the peripheral nerve graft in inducing axon outgrowth after 2 weeks originally offered encouragement and support to perform the 6 week experiment, and a much greater magnitude of axon outgrowth was anticipated after Cx43 AsODN treatment. This was offset by the disappearance of grafts from the transection site between 2 – 6 weeks, the cause unknown. It is proposed that during the formation of cavities, the fibrous scar in the transection underwent remodelling and became
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compacted in the rostral-caudal direction, thus squeezing the peripheral nerve graft out to the side of the spinal cord. This speculation is supported by the finding that contractile cells containing α-smooth muscle actin were frequently found in the fibrous scar (Spilker et al., 2001). These cells resembled fibroblasts within an elongated morphology, indicating the possible role of fibroblasts in mechanical contraction and closure of the wound (Spilker et al., 2001). Therefore, it was possible for the fibrous scar to exert contractile forces and squeeze out the graft in the process. Further study is needed to confirm this. In other long term peripheral nerve graft experiments, researchers either secured the peripheral nerve by suturing the epineurium to the dura mater of the spinal cord, by fixing multiple nerves in a fibrin glue matrix (Cheng et al., 1996), or by using a minced peripheral nerve graft mixture (Feng et al., 2008). Suturing is likely to infer further damage to the spinal cords and also requires micro-surgical techniques that were not available to me at that time. The use of preconditioned peripheral nerves from other adult animals could also be a potential trigger for immune rejection, although this did not seem to be a problem in work undertaken by other spinal cord injury researchers (Dinh et al., 2007; Hase et al., 2002; Rasouli et al., 2006; Tom & Houle, 2008; Zurita, Vaquero, Oya, & Montilla, 2001); a majority of other researchers did, however, used autografts.

5.5.4.5. Cx43 AsODN treatment effect on cavity length

Cavities or cysts have been associated with secondary and ascending neurologic deficits in patients (Lohlein, Paeslack, & Aschoff, 1990). The formation of cavities in a spinal contusion injury follows three phases, necrosis from day 1 to weeks 1-2, repair from weeks 1-2 to weeks 8-15, and stability from weeks 8-15 to 1 year (Guizar-Sahagun et al., 1994). There were a number of theories for the origin of cavities, but the reabsorption of necrotic tissue has received the most support (Guizar-Sahagun et al., 1994). Therefore, the extent of necrosis in the initial stages determines the size and extent of cavities formed later on. In the necrotic phase, the affected tissue is characterised by hemorrhage, oedema, neutrophil and later on macrophage infiltration, vascular thrombosis, and axonal segmentation (Guizar-Sahagun et al., 1994). Axonal end bulbs such as those labelled by FR after two weeks were also seen in my preliminary experiment. The repair phase, which is the stage at which this first experiment ended at, involved revascularisation of the spinal cord and resorption of necrotic tissue by macrophages, forming cavities from 2-3 weeks. The stabilisation phase is characterised by large cavities with a trabecular system (Guizar-Sahagun et al.,
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1994). Because my study used a complete transection model, some differences with the Guizar-Sahagun study were found, such as a more delayed repair phase starting from 3-4 weeks, the abundance of large cavities without a trabecular system, and the formation of two cavities either side of the fibrous scar. The abundance of rostral cavities compared to caudal cavities could be explained by a correlation between necrosis and axon dieback, which is more predominant in the rostral cord (Guizar-Sahagun et al., 1994). Microcysts in the white matter have also been found in some spinal cords, seemingly resulting from retraction of axons (Guizar-Sahagun et al., 1994). The cavity wall was found to consist of fibroblasts and collagen (the fibrous scar), astrocytes and their processes (the glial scar), or a combination (Guizar-Sahagun et al., 1994). Ependymal cells usually feature (Guizar-Sahagun et al., 1994).

There appears to be a reduction in cavity (lesion) length after Cx43 AsODN treatment, suggesting that down-regulating Cx43 expression may have beneficial effects in inhibiting the initial necrotic processes, longitudinal lesion expansion and subsequent resorption of necrotic tissue by activated microglia and macrophages (Guizar-Sahagun et al., 1994). Although no difference in average cavity area (a close approximation of cavity volume) was found between treatment groups, the rostral-caudal progression of the cavities, indicated by lesion length, have higher physiological significance. The cavity formation involvement of an additional spinal level would manifest in multi-level loss of function, whereas lateral expansion of the cavity in the same level would be comparatively less detrimental, with further single-level loss of the body’s function. Therefore, cavity length can be weakly correlated with behavioural improvements seen after Cx43 AsODN treatment.

Nonetheless, my results highlighted the value for peripheral nerve graft material, the technical difficulties and variation in graft tissue preparation (preconditioned or minced), technical issues with securing in-place including alignment in some cases (glue or sutures), and subsequent remodelling in vivo. This approach was unlikely to prove optimal and a search for an injectable implant, that has demonstrated long term stability and immune compatibility in the spinal cord without any additional suturing or stabilisation methods in conjunction, was conducted. Such a material would also
provide consistency and greater ease of preparation and application. After extensive searching a novel peptide amphiphile nanofiber with a laminin motif was identified.
5.6. Summary of experiment findings

- Ligation (pre-conditioning) of peripheral nerves promoted de-differentiation and proliferation of Schwann cells while the basal lamina remained. Therefore, the ligated peripheral nerves were at their optimal condition to promote axon regeneration when grafted into transected spinal cords.

- Following spinal cord complete transection, ligated peripheral nerve grafting and no other treatment, the peripheral nerve grafts remained in the transection site and successfully induced axon outgrowth after two weeks. By six weeks, however, the peripheral nerve grafts appeared to be squeezed to the outside of the spinal cord by the contractile actions of the fibrous scar, irrespective of Cx43 AsODN or Sense ODN, or no treatment.

- After the initial dieback over a distance of 500 – 1000 µm away from the transection site, axons regenerated into both the glial and fibrous scars amidst ongoing cavity formation. Early deposition of astrocyte processes in a glial scar in the first two weeks may have a role in guiding regenerating axons. This finding suggests that the usually inhibitive glial scar had been rendered somewhat regeneration-permissive by the Schwann cells in the peripheral nerve graft environment.

- A fibrous scar found in the transection site was permissive to axon outgrowth towards the centre of the transection, but it did not permit regenerating axons to form functional connections across an invisible barrier at the rostral-caudal lesion border. This finding suggests that the inhibitory environment was not sufficiently altered by current treatments.

- Rostral axon regeneration appeared to be further promoted by Cx43 AsODN treatment. Results also suggest a reduction in cavity length and better behavioural improvement, although the differences were not statistically significant.
  - By modulating Cx43 expression, the production of inhibitory molecules bound to the fibrous scar and secreted by the glial scar could be reduced, rendering both scars more regeneration-permissive and allowing more axon regeneration.
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- Reduction in lesion length suggests that Cx43 AsODN one-off application produced long term effects on secondary injury spread and necrotic processes.

- Behavioural improvement is a consequence of axon regeneration and reduced lesion spread.

- The experiment was hindered by the un-foreseen extrusion of the peripheral nerve graft and technical difficulties. A search for an injectable implant to replace peripheral nerve grafts was undertaken.
Chapter 6. *Ex vivo* IKVAV-PA gel testing results

The physical properties of a novel peptide amphiphile (PA) nanofiber with laminin motif (IKVAV) were tested in preparation for *ex vivo* and *in vivo* applications, and applied to *ex vivo* segment cultures to gauge the effects on spinal cord swelling. A literature search was conducted and this nanofiber was chosen because it was the only synthetic implant material that could be injected as a liquid to self-assemble into a solid gel in the spinal cord providing excellent graft integration (Tysseling-Mattiace et al., 2008). Other implant materials did not satisfy one or both of those criteria.

6.1. Physical properties of the IKVAV-PA gel

6.1.1. Gel setting requirements

For *ex vivo* applications of the IKVAV-PA gel as a topical application, instructions from the manufacturer specified the application of, for example, 100 µL of 1% IKVAV-PA gel solution on top of spinal cord tissue, followed by 100 µL of 60 mM CaCl$_2$ solution and 5 minutes incubation at 37°C to accelerate setting of the gel. High extracellular Ca$^{2+}$ is known to close connexin hemichannels and increase gap junction communication, so attempts were made to find a solution with enough ionic strength to allow molecules to assemble into nanofibers without overly perturbing the physiological environment of the spinal cord segment culture. It was found that the gel set into a transparent gel with equal volumes of CaCl$_2$ at concentrations of 60, 30, 15, and 7.5 mM. At 3.75 mM the solution remained half liquid / half gel, and at 1.875 mM the solution was completely liquid. Therefore, it is definitely possible to use a lower ionic strength to induce self-assembly of the nanofibers.

Pluronic gel did not produce the ionic strength required to trigger self-assembly of the nanofibres. Subsequently, the gel was found to set instantly in artificial cerebrospinal fluid and Neurobasal-A medium; therefore, IKVAV-PA alone would gel easily in the transected spinal cord or *ex vivo* segment culture.
**Ex vivo IKVAV-PA gel testing results**

6.1.2. IKVAV-PA gel is stable for at least 4 weeks *in vitro*

The coloured compound, Alcian green, was added to IKVAV-PA gel before the gel was combined with CaCl$_2$ to ensure self-assembly into a gel, and the mixture was observed for diffusion or disappearance of the green colour in water or artificial cerebrospinal fluid. It was found that once the gel was made up, it was stable for at least 4 weeks at 37°C. Hence IKVAV-PA gel offers much better stability than many water-soluble gels that were tested before, such as HPMC (hydroxyl-propyl-methyl cellulose), methyl cellulose, kappa carrageenan, all of which absorbed water and became diffuse within 1 day *in vitro*.

6.1.3. Pluronic gel / IKVAV-PA combinations and Cx43 AsODN release testing from IKVAV-PA gel

IKVAV-PA gel was combined with 10% Pluronic gel in liquid form before 60 mM CaCl$_2$ was added, to see if Cx43 AsODN in Pluronic gel could be delivered in the same gelling mixture. It was found, however, that Pluronic gel prevented setting of the IKVAV-PA gel. Therefore attempts were then made to mix Cx43 AsODN directly onto the IKVAV-PA gel and release of the Cx43 AsODN monitored. The amount of Cx43 AsODN conjugated to a fluorescent tag CY3 (Cx43 AsODN-CY3, a pink coloured fluorescent compound) released into the water portion on top of the IKVAV-PA / Cx43 AsODN-CY3 gel mix was measured with fluorophotometry (Figure 6.1). The total amount of Cx43 AsODN-CY3 mixed into IKVAV-PA gel was 566 pmol (e$^{-6}$ µmol). Approximately 10 pmol was released immediately after water was applied on top of the gel. More tagged Cx43 AsODN was released over the first 4 hours, by which time a plateau was reached and only a minute amount of Cx43 AsODN-CY3 was further released. By the end of 3 days (72 hours), a total of only 52 pmol of the 566 pmol starting quantity (approximately 10%) was released from the IKVAV-PA gel into the surrounding water. The gel appeared to be trapping Cx43 AsODN-CY3 in its matrices and not releasing it freely.
Figure 6.1. Total Amount of Cx43 AsODN-CY3 Released from IKVAV-PA Gel. Release of Cx43 AsODN-CY3 started as soon as water was added on top of the gel in an eppendorf tube, and was sustained over 4 hours. Then a plateau was reached and little more was released at later time points. By the end of 3 days, the total amount released (52 pmol) was approximately 10% of the initial amount (566 pmol). Since only 10% of the total starting Cx43 AsODN-CY3 was released, and equal concentrations between the gel and the solution above was not approached, the gel was trapping the Cx43 AsODN-CY3.
6.1.4. IKVAV-PA gel actively trapped Cx43 AsODN-CY3

The interaction of pre-formed IKVAV-PA gel with Cx43 AsODN-CY3 was investigated, and active entrapment of Cx43 AsODN-CY3 by the gel was found. The pink coloured Cx43 AsODN-CY3 was observed to travel down 1/3 of the column from a Pluronic gel top layer into the IKVAV-PA gel within 1 minute, and diffused through the IKVAV-PA gel within 5 hours. The pink colour remained in the column for 3 days, although the gel had condensed to ½ of the original volume due to water evaporation. The pink colour did not flush away when water was applied to the column after 3 days. This indicated that the Cx43 AsODN-CY3 was trapped as it passed from Pluronic gel into the IKVAV-PA gel, and was not easily released.
6.2. IKVAV-PA gel interfered with Cx43 AsODN delivery *ex vivo*

The effectiveness of IKVAV-PA gel in combination with Cx43 AsODN in inhibiting spinal cord segment swelling was tested *ex vivo* (Figure 6.2). Cx43 AsODN in Pluronic gel was used as a positive control given that reduced swelling when Cx43 AsODN was applied to segments in culture was observed. The medium only was used as a negative control since the segments swelled the most. All other groups with the IKVAV-PA gel applied first on top of the segments fell in between. IKVAV-PA gel alone seemed to have some oedema-suppressing effects, similar to those observed with Pluronic gel, suggesting something in common. IKVAV-PA appeared to have blocked some of the effect of Cx43 AsODN, which suggests the entrapment of Cx43 AsODN by IKVAV-PA gel preventing access to the segments. Therefore, the dosage in later *in vivo* experiments was changed from 2 µM to 5 µM to allow some Cx43 AsODN to be trapped in the gel while the remainder can diffuse freely through the spinal cord on contact.
Figure 6.2. Effectiveness of IKVAV-PA Gel in Inhibiting Swelling in ex vivo Spinal Cord Segment Culture. Positive (Cx43 AsODN in Pluronic gel) and negative (medium only) controls are indicated by solid data points. Cx43 AsODN in Pluronic gel (Cx43 AsODN in PG) was the most effective in inhibiting segment swelling and medium only control the least effective. There was little difference between IKVAV-PA gel followed by Cx43 AsODN in Pluronic gel (PA plus Cx43 AsODN in PG), treatments containing both gels (PA plus PG) or the IKVAV-PA gel alone (PA) groups. All three groups containing IKVAV-PA gel seemed to have some oedema suppressing effects when compared to medium only control. No statistically significant difference was found between any groups.
6.3. Discussion

In summary, results of the in vitro IKVAV-PA gel testing experiments showed that:

- IKVAV-PA gel 1% solution gels easily in artificial cerebrospinal fluid and remains stable for at least four weeks. Therefore, it would gel in the transected spinal cord without external application of ions and it can provide a physical conduit for an extended period of time.

- Cx43 AsODN was trapped by the IKVAV-PA gel, and once trapped only 10% Cx43 AsODN was released in three days.

- IKVAV-PA gel interfered with Cx43 AsODN delivery in ex vivo spinal cord segment culture.

6.3.1. Introduction to peptide amphiphile nano-particles with IKVAV motif

Peptide amphiphile nano-particles with the pentapeptide epitope isoleucine-lysine-valine-alanine-valine laminin motif (IKVAV-PA) that self-assemble were identified after an extensive literature search as an ideal implant material. These peptide amphiphile nano-particles had been tested in a range of life science applications including spinal cord injury, angiogenesis and hard tissue regeneration and replacement (Cui et al., 2010). Self-assembly of nanoparticles is particularly useful because of the simplicity in application. These peptide amphiphile molecules are injectable as a solution to self-assemble into cylindrical nanofibers when in contact with ions present in physiological solution. They form a hydrogel that moulds to the contours of the transected spinal cords creating much better contact than is possible with peripheral nerve grafts (Tysseling-Mattiace et al., 2008). They are stable for 2-4 weeks in the mouse spinal cord when injected in small amounts into a contusion site before being biodegraded and are, therefore, suitable as a graft material (Tysseling-Mattiace et al., 2008).

Furthermore, the bioactive epitope IKVAV has been added on the hydrophilic end of these peptide amphiphile molecules, and when assembled, they are displayed at a density that is approximately 1000 times the density of the epitope on laminin. They have been found to promote neurite sprouting and cell attachment, and to direct neurite growth both in cell culture and in vivo (Silva et al., 2004; Tysseling-Mattiace et al., 2008). Therefore, the graft allows axon regeneration and even promotes this in a manner
similar to the effects of a peripheral nerve graft. There is also the possibility of promoting intrinsic neural progenitor cells in the spinal cord to differentiate into neurons in vivo, since the nanofiber scaffold encourages differentiation of neural progenitor cells into neurons and not astrocytes in vitro (Silva et al., 2004). When 1% IKVAV-PA solution is mixed with equal amounts of a physiological solution, the resultant gel contains 99.5% water, which allows sufficient diffusion of nutrients and oxygen for neural progenitor cell culture in vitro (Silva et al., 2004). The gel should not, therefore, present a problem for diffusion of nutrients in the closed spinal cord environment after injury. Neural progenitor cells do not survive in a denser nanofiber network (99.5% to 98% water) (Silva et al., 2004) and, therefore, a 1% solution was used in my trial experiments and in subsequent in vivo experiments. It was decided to investigate more extensively the gels’ physical properties and its interactions with Cx43 AsODN before performing in vivo experiments.

6.3.2. IKVAV-PA gel was confirmed as a suitable graft material

In these experiments, it was found that self-assembly was triggered by low ionic strengths such as that found in artificial cerebrospinal fluid or Neurobasal-A culture medium, so gelling can definitely occur in physiological situations without the addition of other compounds, such as high levels of Ca\(^{2+}\) which might well affect gap junction and hemichannel function. The gel was stable in vitro for over 4 weeks, consistent with previous observations that the gel, immersed in ionised water 10 times its volume, was stable for at least 14 days (Beniash et al., 2005). It was also found that pre-combining Cx43 AsODN with the IKVAV-PA gel did not work for experiments that require Cx43 AsODN to be available, since the gel trapped approximately 90% of the Cx43 AsODN. Furthermore, even if Cx43 AsODN was applied separately on top of pre-formed IKVAV-PA gel, any Cx43 AsODN that passes into the gel becomes trapped. This was again confirmed in the ex vivo culture experiment, when the ability of Cx43 AsODN to regulate spinal cord swelling was compromised by the IKVAV-PA gel coating on the segments. IKVAV-PA gel alone had some swelling-inhibitory effects, similar to that seen with Pluronic gel alone, suggesting a common mechanism of action. This could be physical inhibition of swelling due to the viscosity of the gel, but further experiments are needed to confirm this.
Therefore, the IKVAV-PA gel was confirmed as a suitable implant material and was predicted to resist degradation for at least 2-4 weeks in vivo. The IKVAV-PA gel was expected to fill the whole transection gap covering both rostral and caudal surfaces of the spinal cord. However it was necessary to apply the Cx43 AsODN on top of the transection site so as to have access to the dorsal surfaces of the cut ends by diffusion through dura mater. Some Cx43 AsODN would be trapped by the IKVAV-PA gel and, therefore, the concentration of Cx43 AsODN used in later in vivo experiments was increased from 2 µM to 5 µM. In vivo experiments were designed and completed, with the above findings in mind, as outlined in the following chapter.
6.4. Summary of experiment findings

- The IKVAV-PA gel is an ideal graft material for spinal cord transection injury repair.

- The IKVAV-PA gel traps Cx43 AsODN; therefore, the concentration of Cx43 AsODN used in *in vivo* experiments was increased from 2 µM to 5 µM, and was applied in Pluronic gel separately to the IKVAV-PA gel (rather than being combined within it).
Chapter 7. *In vivo* IKVAV-PA gel grafting with Cx43 AsODN results

Following *in vitro* testing of IKVAV-PA gel, *in vivo* experiments were performed to test the hypothesis that Cx43 AsODN can promote axon regeneration into the grafted nanofibers and beyond. In this experiment, adult rats were subjected to T10 complete transection, 5 µL of IKVAV-PA gel was injected into the accumulated cerebrospinal fluid in the site after removal of the blood clot. The gel was allowed to set for one minute, and then 50 µL of 5 µM Cx43 AsODN or Sense ODN in Pluronic gel was applied on top, covering the dorsal dura of the two cut ends and filling up the space between T9 and T11 vertebrae. Behavioural improvements were subsequently monitored using the BBB score, and the animals were perfused at 11 weeks for immunohistochemistry to identify neurons and axons, astrocytes, oligodendrocytes, macrophages and fibroblasts. The experiment length was increased from 6 weeks to 11 weeks so that the regenerating axons, seen at 6 weeks in the peripheral nerve graft experiments had a longer time to grow. Lesion area, rostral and caudal GFAP intensity were analysed (for details please refer to methods section). Ten animals each received Cx43 AsODN treatment or Sense ODN treatment after IKVAV-PA gel grafting. Behavioural testing was done on all surviving animals at the time of recording. Due to significant animal loss, only 3 animals receiving Cx43 AsODN and 4 receiving Sense ODN were analysed, and no FR tracing was performed due to potential risk of further animal death. Owing to the small sample size, emphasis was placed on qualitative analysis of axon regeneration rather than quantitative measurements. Animal loss was not attributed to the IKVAV-PA gel (please refer to later discussion).

7.1. Behavioural improvements over 11 weeks

BBB scores for these animals started from zero on the first day after injury, as expected for completely transected animals (Figure 7.1). All animals showed gradual improvements with Cx43 AsODN treated animals having a faster recovery (with a higher gradient) up to 4 weeks, after which time the behaviour scores became dramatically different. Those that received Cx43 AsODN treatment continued to improve up to an average BBB score of 2 while the ones that received Sense ODN control treatment showed reduced scores from a peak of 1.3 to an average of 0.75, the
same score reached at week 2. At 11 weeks, the difference was statistically significant (p-value 0.039).
Figure 7.1. Basso, Beattie, and Bresnahan Scores of Completely Transected Animals after Receiving IKVAV-PA Gel Implants and Cx43 AsODN or Sense ODN Control Treatment. The animals in the Cx43 AsODN treated group had consistently higher BBB scores (except at 4 weeks), and continued to improve from 4-11 weeks to an average of 2. Animals in the Sense ODN treated group had consistently lower BBB scores, which showed improvements from 0 - 4 weeks but deteriorated later.
7.2. Cavity area, astrogliosis analysis and microglia/macrophage distribution

Spinal cord sections revealed far more extensive cavities than those seen in the peripheral nerve graft study did at 6 weeks, although there was considerable variation. The IKVAV-PA gel had disappeared from the spinal cord, but as the gel was only expected to stay in the site for up to 4 weeks this finding was not surprising. Peripheral nerve grafted spinal cords analysed at 6 weeks were dominated by cavities rostral to the lesion while the caudal spinal cord remained intact; in contrast, in 11 week IKVAV-PA grafted samples, major cavities caudal to the lesion were found (Figure 7.2), and some had become even larger than the rostral cavities by this later time point. The cavities formed a ventricular system connected by septa. Although none of the spinal cords were discontinuous in anatomy, the cavities became so big that the walls were sometimes no more than a thin ribbon (Figure 7.2 A, arrowheads) and became discontinuous in places. At such places, peripheral nerves flanking the spinal cord seemed to be providing structural support by increasing the width of the tissue. Therefore, the assessment of axon regeneration in the spinal cords in medial sections became quite difficult owing to the very limited amount of tissue that remained. However, not all spinal cords had the same extensive cavity formation. Figure 7.2 C shows one case where cavity formation was either much slower or less extensive than the others after treatment with Cx43 AsODN, showing much more preservation of tissue in the transection region. The tissue did, however, appear somewhat necrotic, the pre-requisite step prior to cavity formation.

This suggests that Cx43 AsODN treatment was able to delay or reduce cavity formation by delaying or reducing necrotic processes. The rostral and caudal cavities in this spinal cord were all separated by longitudinal septa, suggesting that the formation of cavities occurs in a longitudinal direction first before horizontal expansion. The fibrous scar remnant (asterix) often appeared as a thin strip between rostral and caudal spinal cords, infiltrated with many axon fibres.
Figure 7.2. Examples of Cavity Formation in Spinal Cords 11 Weeks after Complete Transection, IKVAV-PA Gel Grafting, and Sense ODN (A) or Cx43 AsODN Treatment (B, C). Neurons and axons in longitudinal sections have been immunohistochemically labelled with anti-SMI32 antibody (red). Cell nuclei have been immunohistochemically labelled with DAPI (blue).

A. This spinal cord contains one large rostral cavity and three caudal cavities, separated by a thin strip of fibrous scar (*). Caudal cavities were separated by septa (arrows). The rostral cavity was so large that the cavity wall was made up of thin strips of white matter (arrowheads). The spinal cord was flanked on either side by peripheral nerves.

B. This spinal cord contains multiple small rostral cavities that were separated by remnant tissue, and three larger distinct caudal cavities separated by thin septa (arrows). The thin strip of fibrous scar (*) was continuous with the spinal cord only on the right hand side.

C. This spinal cord shows several collapsed rostral cavities, defined by thin strips of spinal cord white matter (arrowheads) on both sides. The fibrous scar (*) was not as defined as in A and B. The caudal spinal cord shows the formation of small cavities in necrotic tissue.

Scale bar = 1mm. PN = peripheral nerve.
The total area of these cavities in the longitudinal section exhibiting the largest cavities was analysed. No difference was found between Cx43 AsODN and Sense ODN treated groups.
*in vivo* IKVAV-PA gel grafting with Cx43 AsODN results

Figure 7.3. Cavity Area in Spinal Cord Longitudinal Sections Exhibiting the Largest Cavities 11 Weeks after T10 Complete Transection Injury, IKVAV-PA gel grafting and Cx43 AsODN or Sense ODN treatment. Cx43 AsODN treated and Sense ODN treated animals did not differ in cavity area.
Intensity of GFAP labelling in tissue surrounding cavities rostral and caudal to the lesion was analysed. The intensity decreased with distance from the fibrous scar or transection site outwards (Figure 7.4, for the same spinal cords shown in Figure 7.2). The regions completely devoid of GFAP represent the fibrous scar (*), while the regions containing GFAP remnants represent extensive cavity formation within necrotic tissue in the original glial scar site. The GFAP-negative region was quite variable; in Figure 7.4 A this region was compacted to a thin strip of approximately 0.5 mm in rostral-caudal distance; in the second example (Figure 7.4 B) this region encompassed the thin strip filled with axon fibres (Figure 7.2 C) and approximately 0.5 mm of necrotic tissue either side of it, constituting 2 mm distance in total; the third one (Figure 7.4 D) was the most extensive, having a fibrous scar approximately 1 mm in the rostral-caudal direction flanked by 2 mm of necrosis either side. The cavity walls were found to be reinforced with GFAP-positive astrocytes that heavily lined almost all sides of the cavities (white arrowheads), indicating their function in sealing off cavities and/or isolating healthy spinal cord from cavity progression. One exception to this was at the rostral or caudal edge of the cavities (A & B, arrows), where GFAP seemed to be absent most of the time, except in smaller well-formed cavities (for example Figure 7.4 C). This implies that cavity formation was still ongoing 11 weeks after injury, enlarging in the direction away from the transection site, with astrocytes infiltrating the cavity walls after the expansion of the cavities. GFAP-rich fibre remnants (blue arrowheads) were seen in the necrosis or cavity region, suggesting the formation of a dense glial scar before the onset of cavity formation.
in vivo IKVAV-PA gel grafting with Cx43 AsODN results

Figure 7.4. Examples of Glial Scar Formation in Longitudinal Sections of Spinal Cords 11 Weeks after Complete Transection, IKVAV-PA Gel Grafting and Sense ODN control treatment (A, B) or Cx43 AsODN treatment (C, D).

Glial cells and processes have been immunohistochemically labelled using anti-GFAP antibody (red). High density astrocyte labelling was found in areas close to the original transection site forming a glial limitans, but the label was absent in the fibrous scar areas (*).

A. In this Cx43 Sense ODN treated spinal cord, intense GFAP labelling (white arrowheads) was found in the walls of almost all sides of the cavities.

B. Magnified view of the area enclosed in A. The most rostral tip of the cavity (arrow) lacked GFAP.

C. An example of a Cx43 AsODN treated spinal cord showing similar intense GFAP labelling lining the walls on three sides of the cavities (white arrowheads); the cavity wall farthest away from the lesion lacked GFAP (arrow). Remnant GFAP labelling (blue arrowheads) was found in the fibrous scar.

D. Another example of Cx43 AsODN treated spinal cord. This spinal cord presents a much longer fibrous scar (*) connected by necrotic tissue containing occasional GFAP fibres (blue arrowheads).

Scale bar = 1mm.
The intensity of GFAP labelling or astrogliosis was measured both rostral and caudal to the lesion, using the level of GFAP in normal spinal cord areas (6.4 mm rostral and caudal to the lesion) as a control. No significant difference was found between Cx43 AsODN and Sense ODN treated animals, although the two treatments seemed to have opposing effects depending on location, with Cx43 AsODN treated animals showing slightly higher intensity rostral to and lower intensity caudal to the lesion.
Figure 7.5. GFAP Labelling Intensity Rostral to the Lesion 11 Weeks after Spinal Cord Complete Transection Injury, IKVAV-PA Gel Grafting and Cx43 AsODN or Sense ODN treatment. No significant difference was found between the two groups, although Cx43 AsODN treated seemed to have a higher intensity of staining than Sense ODN treated animals.
in vivo IKVAV-PA gel grafting with Cx43 AsODN results

Figure 7.6. GFAP Labelling Intensity Caudal to Lesion 11 Weeks after Spinal Cord Complete Transection Injury. IKVAV-PA Gel Grafting and Cx43 AsODN or Sense ODN treatment. No significance was found between the two groups, although Sense ODN treated seemed to have a higher intensity of staining than Cx43 AsODN treated animals.
Activated microglia and macrophages were labelled in spinal cord longitudinal sections, and the staining was found to be similar between all spinal cords examined. An example from a Cx43 AsODN treated spinal cord, the same spinal cord as shown in Figure 7.4 B, is shown below (Figure 7.7). The areas surrounding cavities and necrotic regions were infiltrated with activated microglia or macrophages, sometimes uniformly distributed (as in rostral cavities and necrotic areas) and sometimes clustered (as in caudal cavities). This is suggestive of ongoing phagocytosis of spinal cord tissue in those regions even behind the astrocyte scar, which is immediately adjacent to the cavities, and that the astrocytes were not effective in protecting healthy spinal cord tissue from further necrosis and cavity progression. The labelled microglia and macrophages were seldom found in areas further from the lesion or cavities. One major feature was that microglia/macrophages aggregate in the rostral/caudal tip of the cavities (arrowheads, A, B) corresponded to GFAP-negative regions seen in Figure 7.4 B. This suggests that cavity progression was mainly in the rostral-caudal direction away from the injury site, consistent with the longitudinal shape of many cavities.
Figure 7.7. Microglia and Macrophage Activity in the Injury Site of a Cx43 AsODN Treated Spinal Cord.
Microglia and macrophages have been immunohistochemically labelled using isolectin B4.
A. Labelling on this spinal cord longitudinal section shows that microglia and macrophages (arrows) were distributed around cavities and necrotic regions. In the rostral cavities and the necrotic area, the microglia and macrophages were uniformly distributed. In the caudal cavity the label was present in some places only while missing in others. Aggregates of microglia and macrophages (arrowheads) were seen in the rostral and caudal most tips of the cavities.
B. Enlarged view of the rostral cavity region showing an aggregate of microglia/macrophages (arrowheads) in the rostral-most tip of the cavity and uniformly distributed microglia/macrophages (arrows) in the other walls of the cavity.
Scale bar = 1mm.
7.3. Axon outgrowth across the lesion area

Spinal cord longitudinal sections were screened for axon outgrowth. Obvious axon regeneration reconnecting rostral and caudal spinal cords was found in one Cx43 AsODN treated spinal cord (Figure 7.8); this is the same spinal cord that had delayed or reduced cavity formation (Figure 7.2 C) and a long fibrous scar area (Figure 7.4 D). Because the medial sections taken from all samples have very limited amounts of tissue left as cavity walls and exhibit the most necrosis, more dorsal or ventral sections closer to the dura were also screened for axon regeneration. The section analysed was very close to the dorsal surface. Axon fibres clearly originating from the rostral white matter travelled all the way down and around to the fibrous scar. Some of these axons continued diagonally following the contour of the fibrous scar forming a half-loop. However, the majority of fibres continued to travel downwards into the caudal spinal cord (white, yellow and green arrowheads traced three such axons or bundles of axons). The extending axons seemed to be segmented into short small dots and fragments in the centre of the caudal spinal cord suggesting that the axon is moving in and out of the plane of section. By contrast the rostral spinal cord contained clusters of large diameter swollen axon end bulbs that appeared to be the product of retrograde axon degeneration. However, the presence of these degenerating but nonetheless present axon clusters after 11 weeks suggests that the application of Cx43 AsODN is keeping axons viable.
Figure 7.8. Extensive Axon Regeneration in the Spinal Cord 11 Weeks after Complete Transection, IKVAV-PA Gel Grafting and Cx43 AsODN Treatment.

Neurons and axons have been immunohistochemically labelled with anti-SMI32 antibody (red). Cell nuclei have been immunohistochemically labelled with DAPI (blue).

A. The longitudinal section taken at a dorsal location shows extensive axon elongation on the left hand side of the section traversing the fibrous scar (asterix).

B. The area rostral to the fibrous scar shows the extension of long continuous axons (yellow and green arrowheads) in the white matter cavity wall.

C. The fibrous scar area (*) was infiltrated with regenerating axons. White and green arrowheads trace two axons that extended all the way into the central grey matter of the caudal spinal cord. Yellow arrowheads trace the axon bundle which was continuous from the rostral to caudal spinal cord in the white matter. The caudal spinal cord contained short fragments of axons (arrows) that appear to be segmented suggesting movement of the axons in and out of the plane of section. The rostral spinal cord also contained clusters of larger diameter swollen axon end bulbs (circles).

Scale bar = 1mm (A) or 500 µm (B, C).
in vivo IKVAV-PA gel grafting with Cx43 AsODN results

The only Sense ODN treated spinal cord with continuous axon growth (the same spinal cord shown in Figure 7.2 A) is presented in Figure 7.9. In this example, the left rostral cavity wall had become a very thin ribbon that completely disappeared from the section when reaching the transection site. The right hand side of the transection site contained a peripheral nerve and rostral axon fibres travelling in two directions. The majority of axons followed the contour of the fibrous scar to the centre and left hand side of the scar (white arrows), while a minority made a slight bend but continued to travel down towards the caudal spinal cord (yellow arrows). As these fibres continued down the caudal spinal cord, their numbers decreased because of thinning down of the caudal cavity wall. Towards the middle of the fibrous scar bridge, the axons were cut on a diagonal orientation (arrowheads) suggesting that they were travelling in a direction slightly in and out of the plane of section. The number of regenerating axons was much less than that seen in the Cx43 AsODN treated spinal cord (Figure 7.8).
Figure 7.9. Some Axon Regeneration in the Spinal Cord 11 Weeks after Complete Transection Injury, IKVAV-PA gel grafting and Sense ODN control treatment. Neurons and axons have been immunohistochemically labelled with anti-SMI32 antibody (red). Cell nuclei have been immunohistochemically labelled with DAPI (blue).

A. The spinal cord longitudinal section shows that the left hand side rostral cavity wall had become discontinuous at the site indicated by the arrow. The spinal cord is flanked and/or supported by two peripheral nerves on either side. Long axon regeneration was seen on the right hand side.

B. Higher magnification image of the area enclosed. The junction between rostral and caudal spinal cords contained a piece of peripheral nerve and axon fibres (separated by the white dashed line). Most axons extended from the rostral spinal cord to the middle of the scar by following its contour (white arrows). The axons changed direction when approaching the centre of the bridge to become diagonally oriented (arrowheads). Some fibres made a slight bend while descending down, and curved back down into the caudal spinal cord (yellow arrows).

Scale bar = 1mm.
In all the spinal cords analysed at 11 weeks, rostral-caudal axon regeneration was found in one spinal cord from each group only. The other spinal cords contained many axons in the fibrous region, but a continuous axon path could not be traced; an example is shown in Figure 7.10. The rostral axon fibres (arrows) either followed the contour of the fibrous scar horizontally to the other side of the spinal cord, or turned around when they encountered the region of caudal spinal cord.
Figure 7.10. Regenerating Axons Have Difficulty Crossing the Rostral-caudal Spinal Cord Junction Area 11 Weeks after Complete Transection.
In this IKVAV-PA Gel Grafting and Sense ODN control treatment example, neurons and axons have been immunohistochemically labelled with anti-SMI32 antibody (red). Cell nuclei have been immunohistochemically labelled with DAPI (blue).
A. A spinal cord longitudinal section shows axons regenerating from both rostral and caudal spinal cords and meeting at the rostral-caudal junction.
B. Some rostral axons (arrows) followed the contour of the fibrous scar or cavity wall. The other regenerating axons continued to travel downwards, but curved and turned abruptly around at the point when they encountered the caudal spinal cord environment.
Scale bar = 1 mm.
Although most of the other spinal cords did not have significant axon regeneration connecting rostral and caudal spinal cords in medial sections, they exhibited a remarkable pattern of axon growth in dorsal or ventral areas, which was similar between both treatment groups. A swelling has formed in the area of fibrous scar formation centred on the original transection site (Figure 7.11) and longitudinal sections taken either side of the dorsal or ventral surface of the spinal cord were analysed for axon penetration. These sections were labelled according to their distance from the dorsal or ventral surface of the spinal cord.
Figure 7.11. Schematic Representation of Spinal Cord Swelling at the Fibrous Scar Formation Site.

A fibrous scar forms during spinal cord healing to form a swelling that protrudes dorsally and ventrally centred on the original transection site. Longitudinal sections either side of the surface of the original spinal cord were labelled according to their distance from the dorsal or ventral surface. A route originating from the rostral or caudal white matter through which regenerating axons enter the swollen scar formation site is proposed (Route of regenerating axons). Examples of the position of dorsal or ventral sections shown in Figure 7.12 and Figure 7.13 are given.

WM = white matter.
Two extreme examples of very sparse and dense axon fibres in dorsal or ventral sections are shown (Figure 7.12 for sparse axon fibres and Figure 7.13 for dense axon fibres). Axons infiltrated the dorsal or ventral regions to varying degrees. A Cx43 AsODN treated spinal cord showed rostrocaudal axonal regeneration on the left hand side (extensive axon regeneration shown previously in Figure 7.8) but the rostral scar region of the same section contained mostly degenerating axon spots. However, the corresponding location in the dorsal sections contained long sparse axons in 120 µm to 960 µm dorsal sections (Figure 7.12). Most long axons were oriented in the rostral-caudal direction (see arrows for direction of growth) while others appeared randomly oriented. Another Cx43 AsODN treated spinal cord showed no rostrocaudal regeneration in medial sections due to paucity of tissue in the lesion site (Figure 7.13). However, long and abundant fibres with a predominant rostral-caudal orientation were found in 120 µm to 360 µm ventral sections of this spinal cord. These findings suggest that a significant number of axons could have regenerated in the fibrous scar tissue continuous with those found in the medial sections, although medial sections were compromised by the presence of a large cavity diminishning tissue integrity. It is proposed that the axons found in the dorsal or ventral sections originated from the rostral or caudal white matter either side of the injury, and that they followed the shape of the swelling to enter the fibrous scar region (proposed route of regenerating axons shown in Figure 7.11). This form of regeneration was seen in both Cx43 AsODN treated and Sense ODN control treated segments, with the most abundant regeneration being seen after Cx43 AsODN treatment (Figure 7.13). Due to the non-uniformity of the spinal cord in the transection site, the rostrocaudal boundaries could not be readily determined; therefore, a systematic analysis of axons that have crossed the lesion in these sections could not be done. Because this axon outgrowth was quite significant, it is reasonable to assume that the IKVAV-PA gel could have been responsible for inducing this effect. The IKVAV-PA gel may have been squeezed to the side of the cavities in the same way as peripheral nerve grafts in weeks 2 - 6, before the gel was broken down. The gel could have acted in that lateral region to produce the pattern of axon outgrowth seen here. However, spinal cord samples taken at earlier time points are needed to confirm this.
in vivo IKVAV-PA gel grafting with Cx43 AsODN results

Figure 7.12. Axons Present in the Dorsal Sections of a Spinal Cord 11 Weeks after Complete Transection, IKAVA-PA Gel Grafting and Cx43 AsODN Treatment.

Neurons and axons have been immunohistochemically labelled with anti-SMI32 antibody (red). Cell nuclei have been immunohistochemically labelled with DAPI (blue).
A. A longitudinal section level with the dorsal surface of the spinal cord shows regenerating axons (arrows) connecting the rostral and caudal segments. The fibrous scar area was filled with degenerating axon fragments (circle).
B. Long axons (arrows) running longitudinally infiltrated the 120 µm dorsal section, corresponding in location to the circled area in A.
C. Longitudinal axon growth (arrows) was also found in the 480 µm dorsal section.
D. Regenerating axons (arrows) were found in the 960 µm dorsal section, which is approaching the dorsal-most limit of the fibrous scar.
Scale bar = 1mm.
in vivo IKAV-PA gel grafting with Cx43 AsODN results

Figure 7.13. Axons Present in the Ventral Sections of a Spinal Cord 11 Weeks after Complete Transection, IKAVA-PA Gel Grafting and Cx43 AsODN Treatment. Neurons and axons have been immunohistochemically labelled with anti-SMI32 antibody (red). Cell nuclei have been immunohistochemically labelled with DAPI (blue).
A. The size of the dorsal section is comparable to that of the lesion area with a slightly wider diameter. The medial section did not show regenerating axons reconnecting rostral and caudal spinal cords.
B. 120 µm ventral,
C. 240 µm ventral, and
D. 360 µm ventral sections contained many long and robust axon fibres decreasing in number with distance from the ventral surface of the spinal cord.
Scale bar = 1mm.
7.4. Identity of cells accompanying regenerating axons

In order to determine the identity of the cells that were present with axons in the fibrous scar and to confirm if the axons were myelinated, oligodendrocyte and fibroblast labelling was carried out using anti-Oligodendrocyte Specific Protein (OSP) and anti-vimentin antibodies respectively. Labelling patterns with anti-OSP were compared between a negative control tissue, peripheral nerve, and spinal cord tissue. All had high background labelling. Figure 7.14 A and B shows anti-OSP non-specific labelling in the peripheral nerve with positive labelling in the normal white matter of the spinal cord. The peripheral nerve should not contain oligodendrocytes so the labelling pattern seen was regarded as non-specific. Real oligodendrocyte labelling was seen in the normal spinal cord amidst non-specific labelling, with cone-shaped cell bodies and bipolar shaped tapered processes. Only non-specific OSP labelling was found in the scar region accompanying regenerating axons. When labelled with anti-vimentin, it was very clear that all cell nuclei found in this region were from vimentin positive cells (arrowheads) and that the cells were fibroblasts (Figure 7.14 D). Therefore, although many axons were found in all spinal cord transection sites in this experiment after 11 weeks, these appeared to be unmyelinated axons accompanied by fibroblasts.
Figure 7.14. Regenerating Axons in the Scar Region were not Myelinated by Oligodendrocytes. Oligodendrocytes have been immunohistochemically labelled using anti-oligodendrocyte specific protein (OSP, Green, A-C). Fibroblasts have been immunohistochemically labelled using anti-vimentin antibody (Green, D). Cell nuclei have been labelled using DAPI (blue). Neurons and axons have been immunohistochemically labelled using anti-SMI32 antibody (red).

A. A normal un-injured spinal cord with white matter on the right hand side containing individual axons surrounded by oligodendrocytes and the adjacent peripheral nerve containing bundles of axon fibres surrounded by non-specific OSP labelling.

B. In the same region, anti-OSP labelling only shows non-specific labelling in the peripheral nerve region and 'real' labelling in the normal spinal cord. Oligodendrocytes identified (three examples are outlined) are usually bipolar in shape with a cone or triangular shaped cell body (arrows) and two tapered processes (arrowheads).

C. The scar region with numerous regenerating axon fibres shows only non-specific anti-OSP labelling similar to that seen in the peripheral nerve graft region. No cone-shaped cell bodies were identified.

D. In the same scar region as in C labelled with anti-vimentin antibody, a marker for fibroblasts, clear fibroblast labelling (arrowheads) is visible.

Scale bar = 100 µm.
7.5. Discussion

The effectiveness of Cx43 AsODN in promoting axon regeneration through IKVAV-PA gel was evaluated in an *in vivo* spinal cord injury experiment. In summary, results of the experiments showed that:

- The IKVAV-PA gel was not present in all the spinal cords after 11 weeks.
- A significantly improved behavioural outcome was evidenced at almost all time points after Cx43 AsODN treatment, reaching significance at 11 weeks.
- Axon regeneration connecting the rostral and caudal spinal cords was found in one spinal cord from each group with the number of axons forming this connection being significantly greater after Cx43 AsODN treatment. Both groups had axons extending into the swollen dorsal / ventral regions of the fibrous scar formed at the original transection site, where it was thought the IKVAV-PA gel had accumulated after fibrous scar expulsion from the centre of the scar.
- The axons were associated with fibroblasts rather than oligodendrocytes.
- Cavities formed in the transection site and they may have enlarged in the rostral-caudal direction away from the lesion site, as evidenced by the lack of astrogliosis and aggregation of activated microglia/macrophages at the tips of the cavities.
- The results suggest that Cx43 AsODN has been beneficial in promoting axon regeneration through the fibrous scar and potentially through IKVAV-PA gel, with this finding correlating with the behavioural improvements following Cx43 AsODN treatment.

7.5.1. Causes for high mortality rate and future improvements

A higher than usual mortality rate was seen in this experiment. Four animals died of bladder complications (burst bladders) during bladder expression by experienced researchers (with over three years experience in animal care and bladder expression). Two animals were euthanased because of self-mutilating behaviour, which is not uncommon in spinalised animals. One animal died of a bladder infection, which is again common after spinal cord injury. The others died of unknown causes. Several factors
may have been in effect to result in this high mortality rate. First, complete transection injury is extreme for the animals compared to compression and contusion injuries where animals recover sufficiently quickly. Secondly, male adult rats in the weight range 200 – 250g were used in this study to be consistent with a previous study by Cronin et al (M. Cronin et al., 2008). As the animals recovered, they produce an increasing bladder volume thus thinning the bladder wall. The male rats also have a different anatomical system to females. These factors make bladder expression a much more difficult task and thus resulted in a higher rate of bladder infection and higher incidences of bladder bursting. In the future, female rats could be used instead of males and water intake restriction should be put in place. Furthermore, the animals may have suffered temperature fluctuations at night in their housing, although heating mats were used during surgery and for their first night post-surgery. The animal housing unit was in a new animal facility with sub-optimal temperature control, resulting in widely fluctuating temperatures between day and night, sometimes dropping below 18°C and climbing above 25°C even in early summer when the experiments were conducted. In the future all effort would be made to ensure appropriate animal housing conditions. Applying anti-nail bite solution to the lower body of animals after surgery can help prevent self-mutilation.

IKVAV-PA nanofibers have been shown to be non-toxic to mice when injected into a compression injury site in the spinal cord (Tysseling-Mattiace et al., 2008). The animals survived up to 11 weeks after injection (Tysseling-Mattiace et al., 2008). This low toxicity, coupled with its biodegradability (Beniash et al., 2005) and the ability to be metabolised (Beniash et al., 2005), rules out IKVAV-PA nanofibers as a risk factor for a high mortality rate. Cx43 AsODN and Sense ODN treatments delivered in Pluronic gel have been extensively tested in a range of animal models including optic nerve ischemia (Danesh-Meyer et al., 2008), skin wound healing (Mori et al., 2006; Qiu et al., 2003; C. M. Wang et al., 2007), skin burn healing (Coutinho et al., 2005), and spinal cord injury (M. Cronin et al., 2008), demonstrating anti-inflammatory and wound healing-promoting effects with no significant loss in animal numbers in any case. This formulation has passed United States FDA safety testing and clinical trials on corneal wound healing and leg ulcer healing are currently underway. Therefore, Cx43 AsODN delivered in Pluronic gel overlaid onto the IKVAV-PA gel filling the transaction gap is not considered to be a cause for high mortality.
7.5.2. IKVAV-PA gel was degraded by 11 weeks

The first most striking observation when viewing the spinal cord tissue was that the IKVAV-PA gel had disappeared. The gel appeared to have been degraded. This is supported by another in vivo study of spinal cord contusion injury in mice, in which 2.5 µL of 1% IKVAV-PA gel solution was injected into the injury site at 24 hours and degraded by 4 weeks (Tysseling-Mattiace et al., 2008). When a similar type of peptide amphiphile nanoparticle was used in cell culture to trap osteoblast cells, these cells were found to be readily internalised in membranous compartments, likely lysosomes, within 20 days by the surviving and proliferating cells (Beniash et al., 2005). It is indeed advantageous that the nanofibers are biodegraded by cells without triggering an immune cell response or being cytotoxic, as this is important for tissue engineering purposes. Furthermore, the nanofibres have also been shown to be metabolised as nutrients by cells (Beniash et al., 2005). Therefore, the IKVAV-PA gel overcomes the issues of tissue rejection, as in the case of allografts, or the issue of tissue source, as in the removal of autologous nerves resulting in function loss of the innervated organ. However, a longer time of stability in the spinal cord could be more desirable in graft applications, and modifications to the peptide amphiphile molecules could well be performed to achieve this (Cui et al., 2010).

7.5.3. Acute Cx43 AsODN treatment promoted long term behavioural improvement

Significantly better long term behavioural improvement was achieved with the IKVAV-PA gel implant and a single dose of Cx43 AsODN treatment compared to Sense ODN control treatment. The difference between the two treatment groups started from 2 weeks post-injury, although the difference was not significant. After 4 weeks the condition of Sense ODN treated animals appeared to be deteriorated while Cx43 AsODN treated animals continued to improve, achieving a significant difference at 11 weeks (2 ± 0.58 vs 0.75 ± 0.25). The time course of control animal functional recovery is in agreement with other comparable complete transection studies, showing that completely spinalised animals usually reach a recovery plateau of BBB score of 0.5-2 within 1-2 months with either no further recovery or declining performance as was seen in our study (M. B. Bunge, 2008; Fouad et al., 2005; Y. S. Lee, Lin, Robertson, Hsiao, & Lin, 2004; L. Zhang et al., 2009; Y. Zhang et al., 2007).
The long term effect of acute treatments is consistent with functional improvements achieved in other studies in which acute treatments, that alter inflammation and apoptotic processes, were used (M. S. Beattie, 2004). In one particular study using the less severe weight-drop injury model, reducing nitric oxide release immediately after injury was associated with reduced proinflammatory cytokines and iNOS expression in reactive astrocytes, reduced apoptosis within 12 hours, and a better functional BBB score indicative of recovery in 3-15 days (Pannu, Won, Khan, Singh, & Singh, 2004). In other studies acute blockade of the CD95 (FAS) ligand also promoted long term functional recovery; acute treatment with minocycline, an anti-inflammatory and anti-apoptotic drug, reduced long term neural and glial cell death and improved functional recovery (M. S. Beattie, 2004). We have shown that Cx43 gap junction and hemichannel communication affected necrosis, apoptosis and inflammation in the ex vivo spinal cord culture model. The fact that these three processes are clearly linked means that the effects of Cx43 AsODN on spinal cord injury progression can be enhanced both in magnitude and time, and hence lead to more significant and long term functional improvements.

7.5.4. Prolonged and extensive cavity formation was found

The extent of cavity formation in this 11 week experiment was much greater than that found in the 6 week peripheral nerve grafting experiment (Chapter 5). Earlier work done in a severe contusion injury rat model found that cavity formation involved three phases - necrosis produced by contusion in weeks 1-2, cavity formation involving a trabecular system and vascular invasion from weeks 8-15, and a stability phase thereafter (Guizar-Sahagun et al., 1994). According to this hypothesis the extent of necrosis in the first phase determines the extent of cavity formation (Guizar-Sahagun et al., 1994). Our results suggest that complete transection initiates a different progression. There was first a longer necrotic phase of 2 - 3 weeks, during which time solid fibrous and glial scars formed in the injury area prior to degradation of tissue by necrosis; large cavities either side of the original lesion then formed instead of multiple small cavities linked by trabeculae; the cavities frequently became so large that no trabecular system remained; only a thin layer of cavity wall was found after 11 weeks. Delayed necrosis after scar formation suggests delayed recruitment of blood-borne immune cells. Therefore, the original idea of giving axons more time to reach their targets was offset by the ever-enlarging cavities cutting off the potential axonal path. Cavity formation after complete transection was therefore much more prolonged and extensive than in other injury
models. In one Cx43 AsODN treated animal cavity formation was markedly delayed or less extensive than that seen in other animals, and extensive axon outgrowth was found in this spinal cord. This is supportive of the view that the impediment posed by cavity formation inhibits axon regeneration, and hints at the role of Cx43 AsODN in delaying or inhibiting cavity formation although no statistically significant difference in cavity area and extent of astrogliosis was found and this phenomenon was only found in one sample. This suggests that the processes involved in cavity formation and long term astrogliosis were not influenced by the Cx43 AsODN dosage applied, even though Cx43 AsODN was shown to attenuate necrosis and inflammation in the ex vivo spinal cord culture. Cx43 AsODN dosage or treatment frequency in vivo may need to be increased in order to further decrease the necrotic events in the first few weeks.

7.5.5. Activated microglia and macrophages form cavities while astrocytes seal off cavities

The cavity walls contained densely packed astrocytes at the edge of the wall and evenly distributed activated microglia and macrophages a little distance away from the wall. By contrast, the rostral or caudal most edge of the cavities contained no astrocytes but aggregates of microglia and macrophages. This observation again suggests that the cavities were still enlarging rostrocaudally away from the original injury site. Ongoing cavity formation could be related to the insufficient immune recruitment of macrophages, activated microglia and neutrophils in the spinal cord resulting in a prolonged immune reaction (Schwartz, 2000). This theory, however, contrasts with Cronin et al. (2008) who noted significantly reduced neutrophil numbers and OX42+ positive (activated) microglia in their Cx43 AsODN treated compression model. In their model this lead to reduced damage spread and significantly improved behavioural outcomes (M. Cronin et al., 2008). Therefore, ongoing cavity formation is more likely due to excessive microglial activation and neutrophil infiltration. This is in keeping with the necrosis-induced cavity formation hypothesis that predicts concentrations of phagocytic cells to clear necrotic tissue at the areas of cavity formation. The distribution of astrocytes in the cavity wall suggests that astrocytes play a role in stabilising and sealing off the contour of the cavities by forming a structural reinforcement separating them from normal spinal cord tissue.

7.5.6. Rostrocaudal axon regeneration was found and enhanced by Cx43 AsODN treatment
Significant axon regeneration connecting the rostral and caudal spinal cords was found in one sample from each group with the extent of axon regeneration being much greater after Cx43 AsODN treatment compared with Sense ODN control treatment. The axons were associated with fibroblasts rather than oligodendrocytes. Even though only one sample from each group was found with rostral-caudal long distance axon regeneration and care should be taken not to over-interpret results, this kind of regeneration is rare. It was not found after complete transection without any treatment, or with most other single treatments such as Schwann cell transplants or neurotrophin gene transfer (Blits, Oudega, Boer, Bartlett Bunge, & Verhaagen, 2003; Y. S. Lee et al., 2004). Single treatments or no treatment do not afford comprehensive protection against inhibitory factors, scar formation, lack of neurotrophic support, or cavity formation. Only a very limited number of combination treatments have achieved rostral-caudal axon regeneration across the original transection gap (Fouad et al., 2005; Y. S. Lee et al., 2004). Therefore, the axonal outgrowth seen in my experiment after Cx43 AsODN treatment combined with IKVAV-PA gel grafting was remarkable, bearing in mind that the dosage and/or frequency of Cx43 AsODN treatment may not have been optimal. Only a very limited number of combination therapies have shown rostral-caudal axon regeneration across the original transection gap. They include studies involving treatments with multiple peripheral nerve grafts supplemented with acidic fibroblast growth factor (Y. S. Lee et al., 2004), and Schwann cell guidance channels with olfactory ensheathing cell implants supplemented with Chondroitinase ABC (Fouad et al., 2005). In both treatment groups researchers found brainstem serotonergic nuclei axons caudal to the transection (Fouad et al., 2005; Y. S. Lee et al., 2004). Long tract axon regeneration is essential for functional improvement after a complete transection, since lower motor neurons in the caudal spinal cord must receive their innervation from the rostral cord to initiate firing (Y. S. Lee et al., 2004). The presence of long tract axon regeneration in the in vivo experiments suggests that IKVAV-PA gel grafting may have provided a conduit and induced axon regeneration from both the rostral and caudal spinal cords before the gel was degraded, and Cx43 AsODN further modified the usually inhibitive glial and fibrous scar environment by providing neurotrophic support and down-regulation of inhibitory molecules associated with fibrous and glial scar formation.
Axon fibres were found in the dorsal and ventral sections above or below the spinal cord in all samples. Therefore, the fibrous scar and the IKVAV-PA gel present in the first few weeks appeared to be of significant benefit in allowing axon regeneration from both the rostral and caudal spinal cord when axon regeneration was not obliterated by cavity formation. There is evidence that the fibroblast environment is more supportive of axon regeneration than Schwann cell guidance channels under certain circumstances. Following complete transection and olfactory ensheathing cell injection at both spinal cord stumps serotonergic axons grew in the exterior fibrous scar surrounding the Schwann cell channel instead of inside, and grew into the distal spinal cord, crossing both rostral and caudal scars (Ramon-Cueto et al., 1998). While the fibrous scar is regeneration permissive in some models (Spilker et al., 2001), it is not permissive in the majority (Brazda & Muller, 2009). Whether the fibrous scar actively induces axon regeneration is a matter for debate, with the majority of research suggesting that the scar contains too many inhibitory factors such as CSPGs to be inductive (discussed in section 5.5.4.2.). The IKVAV-PA gel could have provided regenerating axons with a permissive conduit. Before the gel was degraded, it could have been either squeezed to the periphery of the spinal cord, as was the case with the peripheral nerve grafts, by the contractile forces of the fibrous scar, or the gel itself became integrated into the fibrous scar before being degraded. Therefore, there was a 2-4 week window of opportunity for axon regeneration to be induced by the IKVAV-PA gel, as is evident in a mouse contusion spinal cord injury model (Tysseling-Mattiace et al., 2008). Therefore, both the fibrous scar and the IKVAV-PA gel could have provided regenerating axons with a permissive conduit.

The effect of IKVAV-PA gel was thought to be due to the bioactive IKVAV laminin motif, that has been shown to induce neurite sprouting, cell attachment and direct neurite growth in cell culture and in vivo (Silva et al., 2004; Tysseling-Mattiace et al., 2008). Laminin is an important component of the basal lamina secreted by Schwann cells, and an essential matrix for new axon extension after the degradation of denervated axons. Laminin is also involved in Schwann cell differentiation and myelination (Dubovy, 2004). The IKVAV sequence was identified as one of the principle active sites of laminin for cell adhesion and neurite outgrowth (Nomizu, 1998; Tashiro et al., 1989). It is perhaps not surprising that the laminin motif, when displayed at very high density, could induce axon outgrowth.
7.5.7. Regenerating axons were not myelinated

The regenerating axons found were unmyelinated and were accompanied by fibroblasts and not oligodendrocytes. Research involving Schwann cell guidance channels found many myelinated axons within the channel, with the ratio of myelinated vs. unmyelinated axons being 1:8 without other treatments, and 1:4 when combined with methylprednisolone or neurotrophins (Chen, Xu, Kleitman, & Bunge, 1996; Xu, Chen, Guenard, Kleitman, & Bunge, 1997; Xu, Guenard, Kleitman, & Bunge, 1995). If myelination of regenerating axons was a goal, combination treatment with Schwann cells or neurotrophins could be beneficial.

7.5.8. Most regenerating axons originated from supraspinal neurons

The origin of the axons seen in our study was unclear because no dye tracing was performed due to the risk of further animal loss. However, studies that found significant regeneration of axons into Schwann cell guidance channels after complete transection found the majority of axons originated from supraspinal neurons (Xu et al., 1997). Neurons that extended axons into the grafts were concentrated at levels near the grafts, and decreased exponentially with distance from the grafts, indicating a higher regenerative response from near-by neurons (Xu, Guenard, Kleitman, Aebischer, & Bunge, 1995). Extension of neurons from the brainstem had to be induced by additional treatments such as neurotrophins, methylprednisolone, olfactory ensheathing cells with Chondroitinase ABC, or a combination of peripheral nerve grafts with acidic fibroblast growth factor, and even then axonal growth from the brainstem was sparse (Chen et al., 1996; Fouad et al., 2005; Y. S. Lee et al., 2004; Xu, Guenard, Kleitman, Aebischer et al., 1995). Therefore, the origin of regenerating axons seen in this experiment is most likely from supraspinal neurons.

7.5.9. Choice of spinal cord injury model

A complete transection model was used in both in vivo studies since this is the only model in which new regenerating axon growth can be distinguished from remnant surviving axons without any ambiguity (Rosenzweig & McDonald, 2004). Complete transection models with neuroanatomical tracing techniques, immunohistochemistry and possibly electron microscopy are the gold standard for studies with axon regeneration as the end goal (Christian Brösamle & Huber, 2006; Rosenzweig & McDonald, 2004). Compression and contusion models are best utilised in translational studies aimed at replicating human injuries. In addition, they provide a greater ease of
in vivo IKVAV-PA gel grafting with Cx43 AsODN results

care and therefore higher animal survival (Christian Brösamle & Huber, 2006). Despite the severity of the complete transection model, constant cavity enlargement and scar formation, application of Cx43 AsODN seemed to have a beneficial effect in promoting axon regeneration through this scar, and this correlated with a higher BBB score. The magnitude of the effect was limited by constant ongoing cavity formation and massive scar formation, both inevitable consequences of complete transection, resulting in tissue degradation and axon regeneration being prevented by the presence of a cavity in the axonal path. The use of a complete transection model was perhaps overly ambitious, but the reasons for its use remain valid. A compression or contusion model could be considered in future studies.

In general, results from this experiment are very encouraging with axon regeneration achieved with the implantation of an IKVAV-PA nanofiber gel and better behavioural outcomes achieved with concomitant treatment application of Cx43 AsODN.
7.6. Summary of experiment findings

- The IKVAV-PA was degraded by 11 weeks. A longer lasting gel may be desirable although prevention of gel extrusion from the lesion site that was occurring may be sufficient.

- Long term behavioural improvements within the range possible for completely spinalised animals were improved by Cx43 AsODN treatment, suggesting that Cx43 AsODN had beneficial effects on injury progression.

- Long distance rostral-caudal axon regeneration most likely from supraspinal neurons, an outcome only achieved by a very limited number of combination treatments by other researchers, was found in two IKVAV-PA gel grafted animals, and the extent of axon regeneration was much greater after Cx43 AsODN treatment.
  
  o This finding seems to suggest that the IKVAV-PA gel, containing a bio-active laminin motif, provided a conduit and induced axon regeneration from both the rostral and caudal spinal cords before the gel was degraded.
  
  o Cx43 AsODN may have modified the usually inhibitive glial and fibrous scar environment.

- Regenerating axons found in the lesion were unmyelinated, suggesting that Schwann cell supplements could be beneficial.

- Axon regeneration was impeded by ongoing cavity formation surrounded by activated microglia and macrophages. Cavity formation was unaffected by the dosage of Cx43 AsODN treatment applied and astrocytes appeared to have a role in stabilising cavities.

- The complete transection model may have been overly ambitious. Compression and contusion models should be considered.
Chapter 8. Summary and conclusion

The hypothesis tested in this thesis was that by regulating gap junction channels in conjunction with spinal cord injury repair strategies, lesion spread and the formation of scar tissue separating the host tissue from the donor graft can be prevented, providing better outcomes. The *ex vivo* spinal cord segment culture and peripheral nerve grafting experiments provided strong evidence for the beneficial effects of Cx43 AsODN application in inhibiting spinal cord inflammation and promoting axon regeneration into the grafted peripheral nerves. The animal numbers surviving in the *in vivo* experiments, however, does not allow statistical significance to be drawn regarding the effects of Cx43 AsODN in promoting behavioural improvements and axon regeneration through peripheral nerve grafts and synthetic implant material IKVAV-PA gel. Limitations and draw-backs of *in vivo* studies will be discussed in later sections. In summary, the findings in this thesis are:

- By using Cx43 AsODN to regulate secondary injury spread after spinal cord dissection, a novel *ex vivo* spinal cord segment culture model was established.

- A significant reduction in segment swelling was achieved after Cx43 expression down-regulation *ex vivo*. The number of Cx43 hemichannels in the astrocyte membrane was dramatically decreased, limiting glutamate release leading to a significant reduction in intracellular \( \text{Ca}^{2+} \) and water accumulation, and subsequent intracellular swelling. Reduced breakdown of the blood-spinal cord-barrier and reduced accumulation of proteins as a product of necrotic cell burst is postulated to result in reduced extracellular swelling.

- Segment viability and neuronal survival was significantly promoted using Cx43 AsODN to dampen down necrotic, apoptotic and inflammatory processes. Necrosis was limited by reducing glutamate release from Cx43 hemichannels and decreasing the extent of intercellular coupling via Cx43 gap junctions. Apoptosis of neurons and oligodendrocytes in the spinal cord was limited by reducing glutamate release via Cx43 hemichannels and reducing the spread of cell death signals via Cx43 gap junctions and hemichannels. Inflammation was limited by reducing blood-spinal cord-barrier breakdown via reduction of \( \text{Ca}^{2+} \) spread through Cx43 gap junctions in endothelial cells, and by reducing immune cell recruitment and microglia activation.
Summary and conclusion

- By applying Cx43 AsODN treatment to peripheral nerve grafted spinal cord segments in the *ex vivo* culture, spinal cord neuronal survival was comparable to the level of survival achieved in shorter segments by reducing necrosis, apoptosis and inflammation. White matter axon dieback was reduced by delaying microglia activation. Axon regeneration from the spinal cord into the grafts was significantly enhanced by inhibiting lesion spread and inflammatory responses, and reducing bystander cell loss.

- *In vivo* T10 complete transection spinal cord injuries were carried out followed by preconditioned donor peripheral nerve grafting. The peripheral nerve grafts successfully induced axon outgrowth after two weeks. By six weeks, however, the grafts were extruded from the spinal cords as a result of the contractile action of the fibrous scar. Large cavities resulting from ongoing lesion spread and necrosis, developed in the transection site. These changes occurred irrespective of Cx43 AsODN treatment (with Sense ODN or no treatment controls).

- Axons regenerated through the glial scar, rendered permissive by the peripheral nerve graft environment before the grafts were extruded from the spinal cord. The fibrous scar was partially permissive to axon regeneration, but still presented a barrier at the lesion border preventing rostral-caudal reconnection. These results suggest that the inhibitory environment was not sufficiently modified by Cx43 AsODN in this severe injury model and modulation of the lesion environment was hindered by the loss of peripheral nerve grafts from the transection site. Cx43 AsODN did, however, further increase the extent of rostral axon regeneration towards the rostral-caudal lesion border, decreased overall cavity length, and subsequently promoted behavioural improvements, although none of these differences was significant. These findings nonetheless support the proposal that Cx43 AsODN has beneficial effects in maintaining a permissive post-injury lesion environment, limiting necrotic processes and secondary injury.

- The experiment regime was made difficult by loss of the peripheral nerve grafts from the graft site, and by technical difficulties. These complications prompted seeking of an axon growth-inducing injectable gel that would completely and evenly fill the cavity that results from nerve retraction after complete transection. Peptide amphiphile nanoparticles with the bio-active IKVAV
laminin motif (IKVAV-PA gel) were identified as the ideal injectable implant material.

- The IKVAV-PA gel was used to fill the cavity after complete transection injury *in vivo* and followed by Cx43 AsODN or Sense ODN treatment. At 11 weeks ongoing cavity formation accompanied by activated microglia and macrophages was evident. Despite this progressive cavity formation unmyelinated axon regeneration connecting the rostral and caudal spinal cords was found in one animal from each group, with the extent of axon regeneration being greater in the Cx43 AsODN treated animals. Axon regeneration in dorsal and ventral regions of the fibrous scar was found in all animals. A significantly enhanced long term behavioural improvement in the Cx43 AsODN treated group at 11 weeks after transection was also found. These findings suggest that the IKVAV-PA gel promoted neurite growth and provided a conduit before being degraded; the fibrous scar provided a permissive conduit; and Cx43 AsODN treatment modified the usually inhibitive glial and fibrous scar environment.

- The IKVAV-PA gel was degraded and immunologically inert, overcoming the issues of implant rejection or loss of function elsewhere due to removal of autologous implant tissue.
The study of spinal cord injury *in vivo*, especially using a complete transection model is challenging, and a number of shortcomings are apparent. The choice of injury model in particular needs further consideration. The complete transection model was used in both *in vivo* experiments for good reasons as it is best for axon regeneration, degeneration and bridge studies (C. Brösamle & Huber, 2007). Transection models are clearer to interpret when carrying out histological and axonal tracing studies for tissue integrity, cytoarchitecture and neural connectivity between graft and host (electrophysiology). Behaviour scoring and modified Tarlov scale and forelimb functional recovery tasks (C. Brösamle & Huber, 2007) are also feasible with the complete transaction model. However, this model is very severe and the level of animal care after surgery is complex. Loss of animals post surgery was disappointing; loss was either directly via illness or indirectly due to autophagia. The high mortality rate also meant that tracing studies were not able to be performed in the second *in vivo* study, and thus the origin and destination of regenerating axons was not determined.

For future work, a less severe contusion, compression or incomplete transection model should be considered to allow better animal survival and recovery, and allowing tracer injections to be performed without animal vulnerability concerns. For a hypothesis involving improving axon regeneration, an incomplete transection model is the next best model to use rather than a complete transection for the reasons discussed above, although ambiguities will exist with the identification of remnant versus regenerating axons. If a research hypothesis is aimed primarily towards promoting behavioural improvement and functional outcomes, compression or contusions models are better suited for the purpose because they mimic human spinal cord injury conditions more closely (C. Brösamle & Huber, 2007). The contusion models (NYU impactor, Ohio State University impactor, Inifinte Horizons Impactor) only offer a maximum compression time of 60 seconds or none at all. Compression models (aneurysm clip, vascular clip, forceps, weight placing) offer initial impact followed by prolonged compression, which better mimic most human SCI conditions (C. Brösamle & Huber, 2007). Within the choice of compression models, the consistency of the aneurysm clip model was proven in a study that showed correlation between force exerted and BBB behavioural score and cavity volume (Poon, Gupta, Shoichet, & Tator, 2007). Furthermore, this model is the only one in which the ventral spinal cord was also compressed, which best represents the ventral compression observed in many human
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SCI cases. Correlation between force and injury extent was also found in the vascular clip model (Marques, Garcez, Del Bel, & Martinez, 2009). Therefore an array of models is available to use depending on the hypothesis of future studies. The use of less severe models would reduce animal complications but would obscure identification of the origin of regenerating axons, and their final destinations, whether using retrograde or anterograde tracing. However, immunohistochemistry could then be used to identify sub-groups such as serotonergic axons.

Axon regeneration through IKVAV-PA gel is suggested by the experimental findings but direct proof is still required. In the peripheral nerve grafting experiment, spinal cords were harvested at two time points, enabling a better understanding of tissue healing, cavity formation, and axon regeneration. In the IKVAV-PA gel grafting experiment the spinal cords were only harvested at one time point, at the end of 11 weeks. This did not allow determination of the time line for IKVAV-PA gel breakdown and did not allow direct assessment of axon regeneration into the gel. Results are, however, encouraging and a full time course analysis, especially during weeks two to four, should be carried out in the future. This would allow one to follow the integration of the IKVAV-PA gel into the damage zone, and to determine the extent of axon regeneration through the gel itself.

With the knowledge gained during my study it is now possible to refine the time course periods that would be optimal for future studies. In my experiments, a two week period after peripheral nerve grafting was insufficient for regenerating axons to reach the spinal cord - graft junction, evidenced by the 500 μm distance between FR labelling and the rostral spinal cord – graft junction. A six week period allowed regenerating axons on both sides to reach the rostral – caudal border, but they were not able to cross the border possibly owing to inhibitory factors in the fibrous scar. Eleven weeks after IKVAV-PA gel implantation growth of some regenerating axons to the opposite side of the lesion was seen, but the number of penetrating axons was greatly restricted by the limited amount of tissue left intact as a result of ongoing cavity formation. Whilst future experiments should cover additional time points, improved treatment paradigms will also be required to further attenuate the necrotic events leading to cavity formation. In
addition, some animals could then be kept for a longer period in order to determine behavioural improvements and extend axon tracing studies.

Although Cx43 AsODN had immediate effects, reducing inflammation and cell loss, and produced some long term benefit for axon regeneration and behavioural improvements, the presence of ongoing cavity formation seen in all six and eleven week spinal cord samples examined suggests that the treatment was insufficient or insufficiently sustained in this model. Ongoing cavity formation was much more extensive in this complete transection model than that seen in compression or contusion injury models, and this is likely due to excessive microglial activation and neutrophil infiltration following an initial necrosis and secondary expansion phase. If the complete transection model were to be pursued, prolonged Cx43 expression modulation using Cx43 AsODN and / or Cx43 specific mimetic peptides may be required. A single dose of Cx43 AsODN appears insufficient to halt the ongoing inflammation and cavity formation characteristic of the severe transection model. It may be that Cx43 AsODN treatment, in combination with systemic delivery of a Cx43 specific mimetic peptide that has also been shown to reduce ex vivo spinal cord segment swelling and inflammation (O'Carroll et al., 2008), or follow up systemic delivery of connexin modulating reagents, might be more effective. In addition, myelination was not promoted by Cx43 AsODN treatment or the space filling IKVAV-PA gel. The axon regeneration promoting effects of IKVAV-PA gel and myelination effects of Schwann cells could be combined to promote both axon regeneration and myelination by incorporating Schwann cells into the gel solution before injection into the injury site.

In conclusion, for non-lacerating contusion or spinal flex injuries to the spinal cord, where the spinal column remains closed, systemic delivery solutions are likely to be most clinically relevant. Where the cord is exposed, Cx43 AsODN treatment is viable as demonstrated by Cronin et al, 2008. However, for spinal cord repair strategies that involve surgical intervention, and especially removal of scar tissue, the cycle of injury is restarted (including Ca\textsuperscript{2+} wave propagation and accumulation in penumbra cells via gap junctions, ATP and IP\textsubscript{3} release via hemichannel-related mechanisms, and intracellular Ca\textsuperscript{2+} release) and poses a severe threat to successful outcomes. Despite the challenges, modulating gap junction communication and connexin hemichannel involvement as a
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component of spinal cord injury repair strategies, including peripheral nerve grafting and IKVAV-PA nanofiber gel fillers, should result in long term improvements in behavioural outcomes and axon regeneration. Whilst the complete transection model was very severe and especially challenging, the previous studies overall involving both ex vivo and in vivo models indicate that the Cx43 AsODN treatment provides an innovative approach to enhance spinal cord repair strategies. It is evident that Cx43 regulation has potential to ameliorate inflammation and ultimately ongoing cavity formation, and to limit scarring that isolates repair tissue from the host cord. The axonal regrowth in the presence of the IKVAV-PA gel is particularly encouraging, and future uses with this material in conjunction with gap junction channel modulation may, with further tuning, provide a realistic approach to spinal injury repair.
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