Connexin43 following partial optic nerve transection

Shenton Sen Lee Chew

ABSTRACT

Retinal ganglion cell (RGC) loss is the common pathway of optic nerve injury, a leading cause of blindness worldwide. This thesis investigates the relationship between glial connexin43 (Cx43), the most ubiquitously expressed gap junction protein in the central nervous system, and RGC loss in an in vivo model of optic nerve injury. Partial, unilateral superior optic nerve transection was performed in Wistar rats. Optic nerves and retinas were evaluated at various timepoints up to 56 days after injury.

In the optic nerve, an increase in Cx43 was observed around the transection site as early as 4 hours after injury that was still evident by 8 and 24 hours. At 3 and 7 days, a decrease in Cx43 was observed at the cut edges of the injury site, progressing to decreased Cx43 levels at the lesion centre at 14, 28 and 56 days. In the optic nerve head, a transient increase in Cx43 occurred in the lamina region at 3 and 7 days post-injury. In the retina, superior Cx43 peaked at 3 days (192.1% of control; P = 0.0002) and 28 days (212.1% of control P < 0.0001), and troughed at 14 days (73.8% of control; P = 0.0028) and 56 days (72.5% of control; P = 0.0232) after injury. Inferior Cx43 was elevated at only 28 days (127.4% of control; P = 0.0481) after injury. Superior RGC loss began at 3 days (84.0% of control; P = 0.0454) and continued to decline by 56 days (18.8% of control; P < 0.0001). Inferior RGC loss began at 28 days (73.4% of control; P = 0.0021). The initial increase in superior retinal Cx43 preceded significant superior RGC loss, with superior RGC counts 66.9% of controls at 7 days following injury. By comparison, inferior retinal Cx43 only significantly changed at 28 days post-injury, paralleled by significant inferior RGC loss at 28 days.

The timing of the Cx43 changes suggests that RGC and glial interplay has a highly significant role in the regulation of RGC death following injury. The data provided in this thesis supports the participation of astrocytic upregulation of Cx43 in the early phenomena of the injury process. Retinal astrocytes may well be reacting to local signals and events generated by RGC body injury. Further studies should be directed at the modulation of Cx43 to determine its definitive impact on RGC survival.
ACKNOWLEDGEMENTS

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<td>GFAP</td>
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<td>GLAST-1</td>
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<td>GS</td>
<td>Glutamine synthetase</td>
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<td>Poly (ADP-ribose) polymerase</td>
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SECTION 1:
INTRODUCTION
Injury to the optic nerve can result from several mechanisms such as direct trauma, ischaemia, raised intraocular pressure, compression from tumours and inflammation from autoimmune disorders.\textsuperscript{1-3}

Common to all injury mechanisms is a consequential loss of retinal ganglion cells (RGCs).\textsuperscript{4} Of the seven main cell types of neurons within the retina, RGCs are of particular interest as they connect with the brain.\textsuperscript{4} RGCs project axons from their cell bodies in the retina, through the optic nerve, optic chiasm and optic tract, to their targets in mainly the contralateral midbrain, namely the superior colliculus and lateral geniculate nucleus.\textsuperscript{5} The aetiology, location and extent of the optic nerve injury govern the pattern, speed and quantity of RGC loss. For example, the degenerative pattern of RGC loss by glaucoma or compressive tumours is much different from acute trauma.\textsuperscript{1}

Traumatic optic nerve injury represents a widely used experimental paradigm to study RGC degeneration in experimental animals and is particularly well characterised with respect to the kinetics of RGC demise, mode of RGC death and factors regulating RGC degeneration. A major advantage is that the anatomy and topography of the projection as well as many of the factors determining them are well known. Moreover, the optic nerve in rodents is easily accessible to microsurgical manipulation under microscopic visual control and the structure of the retina allows for quantitative pathological analysis both on whole mounts and in retinal sections. Traumatic optic nerve injury can be performed in several ways, with the most common being an axotomy of the optic nerve via a transection or crush injury.

It has been suggested that the principles of optic nerve axotomy-mediated RGC death may apply also to other neurodegenerative conditions of the central nervous system (CNS), such as chronic neurodegenerative diseases, and even acute cerebral ischaemia.\textsuperscript{4} Therefore, work on optic nerve
lesion-induced RGC pathology may be of broad value for CNS neuronal pathology in a much more general sense, yet bearing the advantages of easy accessibility, and reliable quantification of both pathological changes and neuroprotective effects of therapeutic approaches.\textsuperscript{6}

1.1 Patterns of RGC Loss following axotomy

There is a delayed RGC loss following axotomy with minimal change in RGC numbers at 4-5 days post injury\textsuperscript{7}, but massive degeneration between 7-10 days following axotomy.\textsuperscript{8} The speed and quantity of RGC loss is dependent on both injury type and distance of injury from the globe.\textsuperscript{8} Considerable variability in axotomy technique, RGC identification technique, observation times and tissue preparation mean that an accurate comparison between studies observing patterns of RGC loss following axotomy is difficult.

1.1.1 RGC identification

One of the challenges of understanding and monitoring RGC death is the identification of RGCs following injury. Techniques include histological identification of cells in the ganglion cell layer, retrograde labelling and immunolabelling of proteins expressed by RGCs. Each technique has its strengths and limitations. Quantification of cells in the ganglion cell layer histologically or by nuclear markers such as 4′,6-diamidino-2-phenylindole (DAPI) is a rapid and easy technique, but is limited by its inability to differentiate RGCs from the equally numerous population of displaced amacrine cells in the same layer.\textsuperscript{9}

Retrograde labelling of RGCs by application of tracer dyes to an axotomy site or a central target provides a significant improvement in RGC identification.\textsuperscript{10} Dyes such as Fluorogold, carbocyanine marker Dil (1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate), horseradish
peroxidase (HRP), true blue, nuclear yellow, fast blue and rhodamine B-isothiocyanate have been used.\textsuperscript{10} This can be performed at the axotomy site or via injection/application to the central targets. Simultaneous axotomy and dye labelling at the axotomised stump relies on retrograde labelling of all RGCs that are connected to the transected axons. Subsequent degeneration of RGCs is paralleled by loss of its’ intracellular dye. Care must be taken in dye selection, as most do not persist in neurons for long periods of time and some do not stain the entire viable RGC population. HRP is metabolised thus does not persist and labelling with true blue or fast blue can be complicated by diffusion from neurons into extracellular spaces and neighbouring cells with prolonged survival and during the processing of tissues.\textsuperscript{10} While DiI persists for extended periods of time, labelling from the axotomy site has been shown to label only one third of the RGC population.\textsuperscript{11} Fluorogold appears to be the best choice for retrograde labelling as it is stable for up to 18 months and shows no significant trans-synaptic diffusion.\textsuperscript{12} Simultaneous axotomy and dye labelling at the optic nerve stump is a viable and accurate option if the injury model is a complete axotomy, though would not be possible in a partial axotomy as the non-transected axons would be unable to take up dye.\textsuperscript{12} Similarly this technique would seem to be inappropriate in a model of optic nerve crush where the meninges remain intact and preventing direct application of dye to nerve tissue. A way to allow for dye uptake in both these situations would be to perform a second complete axotomy a couple of days prior to sacrifice. In this way, only viable axons would take up dye and the short timeframe prior to sacrifice means that the second axotomy should have no impact on RGC degeneration.\textsuperscript{10} Variations of these principles have been used by Yoles et al\textsuperscript{13}, in an attempt to separate primary from secondary RGC loss following optic nerve crush in rodents. They applied the neurotracer dye 4-Di-10-Asp distal to the crush site immediately following injury and stated that viable axons not involved in the crush injury were those that survived primary injury. To assess secondary RGC loss, they performed a second complete axotomy at delayed timepoints after the crush injury and backlabelled from the optic nerve stump, stating that only axons unaffected by the primary injury and secondary RGC loss would transmit the dye. However, as discussed above, a limitation would have been their immediate RGC labelling distal
to the crush site, as presumably they simply applied dye to the meninges-covered optic nerve distal to the crush site.

Perhaps a more elegant and accurate technique that can be performed in any model of RGC injury is retrograde labelling at the central targets, which are the superior colliculi in rodents.\textsuperscript{10} In this way, an animal’s entire RGC population can be labelled well in advance to any experimental injury model and as RGC loss occurs, so too will intracellular dye labelling. This has successfully been performed with Dil and Fluorogold with good concordance in control RGC numbers observed in studies by Villegas-Perez et al\textsuperscript{6} and Berkellar et al\textsuperscript{7}. However, because of increased leakage out of tissue sections seen with Dil, Fluorogold has become the gold standard.\textsuperscript{12} In a computerised analysis of the entire RGC population and its spatial distribution in adult rats, Salinas-Navarro et al\textsuperscript{14} compared numbers of RGCs backlabelled with Fluorogold from transected optic nerve stumps to those from the superior colliculi. Assuming that those backlabelled from optic nerve stump labelled 100% of the RGC population, bilateral superior colliculi retrograde Fluorogold administration labelled just 1.6% and 2.2% less RGCs in albino and pigmented rats respectively. The authors felt this confirmed that the majority of RGCs project to the superior colliculi and provided additional evidence for the massive retinotectal projection in the retinofugal system of adult rats.

An alternative to trace RGCs is the immunodetection of proteins specifically expressed by these cells, or in situ hybridization to detect RGC-specific mRNAs. Several markers, such as Thy1\textsuperscript{15-18}, Bex1/2\textsuperscript{19}, \(\gamma\)-synuclein\textsuperscript{20}, have been trialled but each has problems that restrict their viability as a substitute for Fluorogold retrograde labelling. While Thy1 has been shown to decrease prior to Fluorogold RGC loss\textsuperscript{16,18}, there is concern its decrease may not correlate with RGC death. Thy1 expression has been shown to shut down in Bax knockout mice whose RGCs are resistant to intraorbital optic nerve crush\textsuperscript{18} and upon retinal injury it is expressed by Müller cells\textsuperscript{21}. In fact, little is known about the role of Thy1 in RGCs and there no not appear to be reports linking Thy1 to survival. Bex1/2 and neurofilament antibodies are expressed in cell bodies and axons of RGCs\textsuperscript{19} making it more difficult to
use them for RGC quantification analysis due to inability to discern RGCs from one another. In-situ hybridisation of γ-synuclein is a good approach\textsuperscript{20}, though the technique impairs double labelling using antibodies and its’ protocols are more difficult than standard immunohistochemistry.

The Brn3 family of POU-domain transcription factors are emerging as much more reliable cell-specific markers for RGCs.\textsuperscript{22,23} These factors have been shown to play important roles in the differentiation, survival and axonal elongation during development of mice RGCs.\textsuperscript{24} Nadal-Nicolas et al\textsuperscript{22} compared Brn3a immunoreactivity with Fluorogold retrograde labelling following optic nerve cut and crush in rats. Brn3a-positive cells were only present in the GCL and showed a spatial distribution comparable to that of Fluorogold-positive cells. In radial sections, 92.2% of Fluorogold-positive cells were also Brn3a-positive, 4.4% were Brn3a-positive but Fluorogold-negative, and 3.4% were Fluorogold-positive and Brn3a-negative. The authors had no clear explanation for the 7.7% discrepancy in Brn3a and Fluorogold labelling though postulated reasons are a clearer differentiation between RGCs with Brn3a as it is a nuclear stain\textsuperscript{23} and uptake of Fluorogold by microglial cells that have phagocytosed dying RGCs\textsuperscript{25}. In addition to the high concordance with Fluorogold retrograde labelling, Brn3a has advantages over other RGC markers. Since it labels nuclei and not cell bodies and axons like Bex 1/2, quantification analysis is more reliable due to easier discrimination of individual RGCs. Compared to Thy1 which is a cell surface marker, Brn3a is a transcription factor that has a survival function antagonising p53 activation of two pro-apoptotic protein\textsuperscript{26}, Noxa and Bax, and through the activation of survival genes\textsuperscript{27}, Bcl-2 or Hsp27. While it is difficult to link loss of Thy1 to RGC death, a clearer link exists with Brn3a with its loss signalling a commitment to death by the RGC. Like Thy1, Brn3a loss following axotomy preceeds that seen with Fluorogold retrograde labelling of RGCs. It is postulated that the reduction of Brn3a expression happens earlier than Fluorogold loss because it reflects a commitment to death that precedes the disappearance of Fluorogold-labelled RGCs by phagocytic clearance.\textsuperscript{22}
1.1.2 Effect of Injury Type and Site

Early qualitative studies suggested that the distance between axotomy site and retina influenced the amount of retrograde RGC loss in adult animals. Histological studies indicated that intraorbital lesions lead to greater and faster death of RGCs than intracranial lesions and that ongoing RGC loss occurs with extended periods of time. In these studies intraorbital crush led to RGC densities of 65% by 7 days and 32% by 18 days, whereas intracranial crush led to stable RGC densities until 3 months, dropping to 60% by 230 days.

Misantone et al also raised the concept of two degeneration stages. The first lasting up to 1 month postoperatively, where neurons in the GCL shrink but some may then partially recover and a second phase where regressive changes in axons, RGC numbers and organisation predominate. This concept was reinforced by Villegas-Perez et al who used the improved retrograde RGC labelling technique with DiI for long-term quantitative studies following both intraorbital cut and intracranial crush injuries at various distances from the globe. They observed a phase of rapid RGC loss within 2 weeks, and a protracted phase that occurred over the subsequent months up to their final 20 month timepoint. A small population of RGCs (~5%) have displayed persistence up to 20 months following lesion to the optic nerve. Intraorbital cuts closest to the globe produced the more rapid loss of RGCs with just 24.7% of control RGCs remaining at 15 days, while intracranial crush produced the most pronounced RGC loss in the protracted phase. With all lesion types, RGC loss was more pronounced in retinal areas closer to the disc in the protracted phase. The authors postulated that the differences seen with type and site of injury may be related to a more severe disruption of RGCs whose axons are severed near their perikarya, greater trophic support from a longer ocular stump and a more prominent inflammatory reaction and greater loss of nonneuronal components after optic nerve cut compared to crush.
The rapid phase of RGC loss was more closely examined by Berkelaar et al.\textsuperscript{7} who used the same injury model of intraorbital optic nerve transection and intracranial optic nerve crush. Using Fluorogold retrograde labelling at the superior colliculus, they showed that virtually all RGCs survive axotomy for several days. Subsequently, and at predictable times that can be correlated with site of lesion, large numbers of RGCs are abruptly lost. Specifically, intraorbital transection 0.5mm from the globe led to a loss of RGCs that started at day 5, with RGC numbers dropping to 5% of control values by day 14. In contrast, intracranial transection led to a more delayed onset of RGC loss at 8 days, with RGC numbers of only 54% compared to controls by 14 days. Following intracranial crush, RGC loss was even less and RGC numbers only decreased to 90% of controls at 14 days.

Other studies have confirmed the large loss of RGCs by 14 days after injury. Mansour-Robaey et al.\textsuperscript{31} transected the optic nerve 0.5mm from the globe and showed that RGC densities, retrogradely labelled by Fluorogold at the superior colliculi, were 57% of controls 7 days after axotomy, 23% at 10 days and 12% at 14 days. Studies by Kermer et al.\textsuperscript{32-34} utilised Fast Blue, Fluorogold and Dil as retrograde tracers. They consistently showed that RGC numbers dropped to 17% of controls at 14 days following an optic nerve transection 2mm from the globe. However, it is not completely clear from their methods whether retrograde tracing was performed from the ocular stump or from the superior colliculi.

As described above, Nadal-Nicolas et al.\textsuperscript{22} compared Brn3a immunoreactivity with Fluorogold retrograde labelling following optic nerve cut and crush in rats. The temporal course of RGC loss followed a similar course with both Brn3a and Fluorogold, however Brn3a loss was detected earlier. At 2 days post optic nerve transection, Brn3a-positive RGCs were 79% of control numbers, but it was not until 5 days post lesion that Fluorogold-positive RGC numbers were significantly lower, measuring at 84% of controls. At this 5 day timepoint, Brn3a RGC numbers had decreased to just 36% of controls. There was no statistical difference in RGC numbers between Brn3a and Fluorogold
groups at 9 days post lesion, but by 14 days the Brn3a RGC population was down to 6% of controls, compared to the Fluorogold population which was 13% of controls.

1.2 Mechanism of RGC Degeneration

Retrograde cellular death of RGCs subsequent to optic nerve transection has been mainly attributed to apoptosis. While the mechanisms of this apoptotic RGC degeneration have been described in some detail, the primary causes for RGC death remain elusive. As described above, the time-course of retrograde degeneration is particularly intriguing, as cell death occurs after a period of survival and can continue several months after acute axonal injury.

At present, it is not known which initial signals trigger the degeneration and what physiological disturbances eventually escalate to cellular death, though possibilities include retrograde death signals from the site of injury and lack of retrograde trophic support. The latter is supported by a body of data indicating that RGCs, like other neurons, receive retrograde trophic support from their central targets, which is thought to be crucial for neuronal survival. The notion that the kinetics of RGC degeneration following adult optic nerve transection depends on the length of the remaining optic nerve stump is considered compatible with these hypotheses. Surprisingly however, a direct lesion of the central target (i.e. rodent superior colliculus) without axonal damage may not necessarily cause RGC death. Furthermore, the presence of a persisting group of RGCs months after axotomy is puzzling, and may represent a population of cells with different survival requirements, or alternatively the maximum number of RGCs sustained by retinal trophic support.

1.2.1 Apoptosis
Apoptosis is a form of programmed cell death that actively involves the cell and requires gene activation and the expression of proteins to cause an internal degradation. Apoptosis involves a reduction in cellular size and chromatin condensation followed by fragmentation of nuclear DNA. The integrity of the cell membrane remains until late stages in the process, resulting in the absence of an inflammatory response. Identification and labelling of nicks present in the fragmented DNA (terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)) is commonly used as an indicator of programmed cell death and has been demonstrated in RGCs following optic nerve injury on both retinal wholemounts and sections. Maximum TUNEL labelling of cells was reported between two and seven days post-injury to the optic nerve.

Apoptosis programmes can be activated intrinsically, via the mitochondrial pathway, or by extracellular pathways involving activation of cell surface death receptors. These pathways converge in the modulation two major gene families, namely the Bcl-2 family and the caspases. A number of factors have been described that are regulated following optic nerve axotomy, and prior to RGC death. The caspase family, a collection of cysteine proteases, have been linked to the signalling, induction and execution of apoptosis. The activation of caspase-3, a key protein in the apoptosis pathway, was demonstrated in degenerating rat RGCs following optic nerve axotomy. The pro-apoptotic Bax protein and anti-apoptotic Bcl-2 and Bcl-xL proteins have been implicated in caspase-independent cell death. Following transection of the rat optic nerve, Bcl-2 and Bcl-xL expression progressively declines accompanied by an upregulation of Bax protein.

1.2.2 Excitotoxicity

In addition to apoptosis, some RGC death following axotomy may also occur by necrosis, which is characterised by cell swelling and linked to rapid energy loss and generalised disruption of ionic homeostasis. In fact, apoptotic and necrotic cell death may not be completely separate entities,
but rather represent a continuum, since both apoptotic and necrotic death can share a number of features.\textsuperscript{55,56} Both apoptosis and necrosis can be induced by oxidative stress, with low levels of oxidative stress inducing apoptosis and high levels promoting necrosis.\textsuperscript{57} Excitotoxic damage can also lead to either apoptosis or necrosis depending on the intensity of the insult.\textsuperscript{58,59}

Excitotoxicity is characterised by excessive activation of excitatory amino acid receptors and is typically induced by high extracellular glutamate concentrations. The N-methyl-D-aspartate (NMDA) receptor plays a prominent role because of its high permeability to calcium ions.\textsuperscript{59} The interaction of glutamate with excitatory amino acid receptors initiates a cascade of events involving excessive calcium entry\textsuperscript{60} and activation of several enzymes including phospholipases, proteases and nitric oxide synthetase (NOS).\textsuperscript{56} The resultant formation of oxygen free radicals can lead to oxidative stress and, together with the activation of Ca\textsuperscript{2+}-dependent catabolic processes, can lead to either necrosis or apoptosis depending on the intensity of the insult.\textsuperscript{58,59} In addition, it has been shown that nitric oxide, generated by activation of NOS, can react with O\textsubscript{2}\textsuperscript{-} to reactive nitrogen intermediates\textsuperscript{61} which are neurotoxic.\textsuperscript{62}

Ca\textsuperscript{2+} influx also results in mitochondrial depolarization and decreased neuronal adenosine-5'-triphospate (ATP) levels.\textsuperscript{63} It is suggested that energy generation and mitochondrial function are critical factors in the decision for the mode of execution of neuronal death.\textsuperscript{56} If excitotoxic damage leads to irreversibly dissipated mitochondrial membrane potentials, necrosis rapidly ensues. If on the other hand, death occurs when the surviving neuronal population has recovered both mitochondrial membrane potential and energy levels, it is through delayed apoptosis. Subsequent to depolarization, the release of mitochondrial proteins such as endonuclease G or apoptosis inducing factor (AIF) leads to neuronal death that does not produce classical apoptotic features such as internucleosomal DNA fragmentation, somal shrinkage or nuclear condensation.\textsuperscript{64}
Research also indicates that the tumour suppressor gene p53 is an important upstream initiator of apoptosis following neuronal injury. Specifically, it has been shown that p53 is upregulated in response to excitotoxins, hypoxia and ischaemia, and neurotrauma. Following oxidative DNA damage, p53 levels increase and trigger apoptosis through the upregulation of several regulatory enzymes, including Bax, CD95 and DR5, which are all classical members of the core apoptosis pathways.

RGCs also die by glutamate excitotoxicity and is thought to be largely mediated by NMDA receptors. Intraocular glutamate levels appear to be elevated 3 to 7 days following optic nerve injury and remained elevated up to 4 weeks after injury. NMDA antagonists have been shown to decrease RGC death following optic nerve injury. Furthermore, prolonged oral glutamate intake in rats increased intravitreal glutamate levels and evoked retinal pathology including RGC death, gliosis and shrinking of the retina.

1.3 Protection of RGCs from Death

Protection of RGC cell bodies from death is a prerequisite to achieving anatomical and functional restoration of the retinofugal pathway following axotomy. Various substances through different routes of administration have been tested for their ability to rescue RGCs from death. These attempts can be divided into different groups depending on the target or kind of neuroprotection.

1.3.1 Neurotrophic and growth factors

Axotomised RGCs are responsive to a variety of neurotrophic factors in vivo. Brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4), ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), neurturin and fibroblast growth factors (FGFs) all provide some degree
of protection of RGCs after optic nerve axotomy. In all cases however, protection is only partial and transient, even when continuous BDNF expression is granted by a viral vector\textsuperscript{69} or when NT-4 is administered continuously by osmotic minipumps\textsuperscript{70}.

A body of evidence exists to support the notion that BDNF protects RGCs from apoptosis following optic nerve lesion.\textsuperscript{31,71,72} RGCs express BDNF themselves, and BDNF expression is upregulated after optic nerve transection.\textsuperscript{73} Recently, it was shown that the BDNF gene can also be electroporated \textit{in vivo} into RGCs, causing endogenous BDNF secretion.\textsuperscript{74} This protected against axotomy induced apoptosis of RGCs for at least 6 weeks. A more conventional method of increasing BDNF levels is via intravitreal injections. Intriguingly, BDNF rescues the same number of RGCs irrespective of whether injected early or late after axotomy.\textsuperscript{31} In fact, the application of BDNF exhibited RGC protection when administered up to six days prior and five days post-injury. BDNF, in addition to rescuing RGCs from apoptosis, also appears to partly prevent large RGCs from shrinking, a sign observed prior to RGC degeneration.\textsuperscript{75} Combination therapy involving BDNF has also been shown to protect RGCs from axotomy-induced degeneration. Gene transfer of the BDNF receptor and tyrosine kinase receptor B (TrkB) in RGCs, followed by intravitreal BDNF administration, led to 76\% of axotomised RGCs remaining viable 14 days after injury as opposed to about 10\% without treatment.\textsuperscript{76} In another study, a combination of BDNF with a free radical scavenger gave improved RGC survival rates from 40\% to 63\% fourteen days following optic nerve transection.\textsuperscript{77}

Compared to BDNF, NT-4 seems less potent in mediating RGC survival in vitro.\textsuperscript{72} NT-3 and nerve growth factor (NGF) reveal only moderate, or no, capacity to rescue RGCs from retrograde degeneration. Intraocular GDNF administration also delays and prevents RGC death in a concentration dependent manner.\textsuperscript{78} GDNF mediated RGC rescue under saturated conditions is comparable to that of NT-4.\textsuperscript{31,72} Several further GDNF family members have now been identified including neurturin, which increases RGC survival after axotomy and potentiates the effects of
GDNF. CNTF is thought to be a lesion factor released from Müller glia or astrocytes after injury. Intravitreal or retrograde adenoviral transduction of a CNTF expressing minigene rescued a higher number of RGCs after optic nerve transection than recombinant GDNF from a similar vector. Finally, insulin and insulin-like growth factor 1 (IGF-1) also protected axotomised RGCs, mediated by downstream signalling routes of TrkB and inhibition of caspase-3 activation.

1.3.2 Intervention into apoptotic pathways

Activation of caspase-3 or its suspected activator protein, caspase-9, was directly demonstrated in degenerating axotomised RGCs, with maximum caspase-3 activity displayed between 4 and 7 days following optic nerve axotomy. Inhibition of either caspase-3 or -9 by peptide inhibitors was found to be neuroprotective in vivo. Caspase-3 inhibition, like neurotrophin administration, failed to afford long-term RGC survival. It appears caspase-1 and -8 are of less importance in neuroprotection as the caspase inhibitor, cytokine response modifier A (crmA), which is more specific for these caspases, exerted no protective effect. As described above, treating an injured optic nerve with IGF-1 or BDNF provides some neuroprotection and this is thought to partly be due to blockade of caspase-9 activity, with enhanced RGC survival from 18% to 37% at 14 days post-injury.

Blockade of the pro-apoptotic protein Bax, with antisense oligonucleotides, rescued axotomised RGCs locally and temporarily and thus the authors postulated that axotomy-induced Bax overexpression is causally involved in RGC apoptosis. In another approach, the availability of anti-apoptotic Bcl-XL was increased in RGCs by fusion protein injections intravitreally that rescued RGCs from axotomy-induced death.

1.3.3 Excitotoxic blockade
NMDA antagonists appear to decrease RGC death following optic nerve injury.\textsuperscript{13,66} Confirmation that glutamate toxicity to RGCs is largely mediated by NMDA receptors came when Kermer et al.\textsuperscript{65} showed that modification of metabotropic glutamate receptors did not mitigate RGC death following optic nerve axotomy. Combination of NMDA receptor antagonist, MK801, with caspase inhibitors showed synergistic neuroprotective effects.\textsuperscript{89}

Poly (ADP-ribose) polymerase (PARP) is a tightly bound nuclear enzyme that is thought to play an important role in the repair of DNA strand breaks which result from DNA damage mediated by reactive oxygen species (ROS) and nitric oxide (NO). However following optic nerve axotomy, excessive PARP activation by free radical damaged DNA can result in excitotoxic RGC death. Blockade of PARP activation by intravitreal 3-aminobenzamide led to increased RGC survival compared to control animals 14 days after injury.\textsuperscript{90}

\subsection{1.3.4 Other mechanisms}

α-adrenergic agonists, but not β-adrenergic antagonists, have been shown to induce neuroprotective effects on RGCs following optic nerve crush.\textsuperscript{91} One suggested mechanism of neuroprotective action for α-adrenergic agonists is an increase in BDNF expression in rat RGCs.\textsuperscript{73} BDNF responsiveness is also thought to be increased by electrical activity and electrical stimulation at the transected optic nerve stump enhanced survival of RGCs after 7 days.\textsuperscript{92}

Lens injury has been found to increase the number of RGCs surviving optic nerve axotomy and while the molecular signals mediating this effect remain elusive, macrophage activation appears to be involved.\textsuperscript{93} In keeping with this concept, macrophage inhibitory factor retards the onset of RGC death following optic nerve crush through prevention of microglial activation.\textsuperscript{94} It was thought that
activated microglia may not only remove degenerated cells, but may themselves act as killers and contribute to RGC demise.

Blockade of the neuron specific Cdk5, a cell cycle gene that upregulates in degenerating neurons and appears to activate cell death pathways upstream of mitochondria, was shown to protect axotomised RGCs.\(^9^5\)

Melatonin may have a role in RGC survival following optic nerve axotomy. Pinealectomy, thus stopping endogenous melatonin production, further decreased the number of surviving RGCs following optic nerve axotomy and this effect was reversed with melatonin substitution.\(^9^6\)

### 1.4 Partial optic nerve transection model

There is increasing evidence that death of neurons can be associated with damage to other neurons that were not injured by the primary insult, a process referred to as secondary degeneration.\(^9^7^-^9^9\) Attempts have been made to identify the mediators of secondary degeneration, with the aim of neutralising them and/or their effects. Studies are increasingly aimed at the protection of neurons which, following acute nerve insult, do not sustain direct injury, but are adjacent to or surrounded by a damaged milieu and will consequently undergo secondary degeneration unless adequately treated.

Distinguishing neurons that have degenerated as a result of the primary injury and those that have degenerated as a result of a secondary process is not as simple as in other parts of the CNS. The first requirement is a partial injury to the optic nerve to allow for a population of surviving RGCs and axons post-injury. Yoles et al\(^1^0^0\) used a partial crush injury and retrograde labelling from the optic nerve to distinguish secondary death. They backlabelled with a neurotracer dye at the time and site of injury to determine the number of RGCs not injured by the primary lesion under the assumption
that only intact axons are able to transport dye back to their cell bodies. Then at various timepoints after injury, they transected the optic nerve distal to the primary lesion and backlabelled to determine the number of surviving RGCs. Any RGC loss more than the primary loss measured from the lesion site was considered to be secondary cell loss. While this study was conceptually sound, the labelling of RGCs with certain neurotracer dyes from the ocular stump does not always result in the complete labelling of the entire RGC population as discussed above.\textsuperscript{10,11}

It appears difficult to clearly differentiate primary and secondary cell death on the basis of temporal changes. While there appear to be rapid and protracted phases of RGC loss\textsuperscript{8}, there is no clearly defined timepoint which separates these phases. Furthermore, the mechanism of both phases appears to be predominantly apoptosis thus not helping with differentiation. Levkovitch-Verbin et al have since developed a more precise model that may demonstrate both primary and secondary RGC degeneration following optic nerve injury in both monkeys and Wistar rats.\textsuperscript{101,102} By partially transecting the optic nerve from the superior aspect, they were able to create a primary injury to the superior axons, leaving the inferior axons intact. Because retinotopic organisation is maintained in the optic nerve, they reasoned that any loss of inferior RGCs would therefore be as a result of secondary degeneration. They showed that at nine weeks following partial superior optic nerve transection in rats, RGC numbers in the inferior retina were 34.5\% less than fellow eyes. This model provides an anatomical means of isolating a subset of RGCs undergoing secondary degeneration.\textsuperscript{100} Since its development, the model has successfully been used by groups to determine the benefits of lomerizine in prevention of secondary degeneration\textsuperscript{103} and in immunohistochemical studies of early events following secondary degeneration\textsuperscript{104}.

1.5 Summary
Traumatic optic neuropathy results in the loss of RGCs, the pattern, speed, and quantity of which is determined by aetiology, location and extent of the injury. RGC loss following axotomy begins by at least 5 days following injury. Intraorbial lesions lead to a greater and faster death of RGCs than intracranial lesions, and loss can be separated into a rapid phase within 2 weeks and a protracted phase occurring over subsequent months. RGC death may be triggered by death signals from the site of injury or perhaps loss of trophic support from their central targets. This results in apoptosis, the most common form of RGC death, though excitotoxicity has more recently been implicated as an important process. Protection of RGCs from death involves targeting of these various mechanisms and is an area of high interest. It has now been recognised that some RGCs, whose axons are not involved in the primary injury, are at risk of secondary degeneration and perhaps represent a key subset that may be amenable to neuroprotection. Difficulties accessing this subpopulation have existed until the development of the in vivo partial transection model of optic nerve injury.

1.6 References


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Chapter 2: Connexin43 and Central Nervous System Injury

Gap junctions are specialised cell-to-cell contacts that provide direct intercellular communication of small molecules (less than 1200 Daltons), which can include nutrients, metabolites, second messengers, cations and anions.\(^1\) A single gap junction channel consists of two hemichannels, or connexons, each of which is composed of six connexin proteins. The connexin protein itself consists of four plasma membrane spanning sections, two extracellular loops, one intracellular loop and an intracellular N- and C-terminal (Figure 2-1). At least 20 connexin genes have been described in mammals and are most commonly named by their respective proteins’ molecular mass in kiloDaltons.\(^2\)

Figure 2-1. A connexin protein consists of four plasma membrane spanning domains, two extracellular loops, one intracellular loop and an intracellular N- and C-terminal. Six connexin proteins form a connexon, or hemichannel, which interacts with a hemichannel on a neighbouring cell to produce a gap junction.
In the CNS, gap junction coupling occurs between neurons, astrocytes, oligodendrocytes, microglia and ependymal cells, as well as between different cell types. Eleven connexins have been identified in various CNS cell types and during different stages of development. Of these, connexin43 (Cx43) is the most ubiquitously expressed and is predominantly found in astrocytes, activated microglia, developing neurons, and the smooth muscle and endothelial cells of blood vessels. In astrocytes, they are abundant at end-foot processes along blood vessels thus impacting on the blood-brain barrier, as well as within astrocytic processes that surround chemical synapses.

The functional syncytium formed by astrocytes via Cx43 gap junctional intercellular communication has been implicated in maintaining the homeostasis of the extracellular milieu of neurons. In particular, astrocytes are involved in the spatial buffering of extracellular potassium ions, glutamate and other signalling molecules, energy sources and mediation of intercellular calcium ion signalling.

2.1 Response of Cx43 to CNS injury

It is the response of Cx43 to CNS injury that first highlighted Cx43 as an important mediator of CNS injury. In vitro work confirmed that astrocytic Cx43 gap junctions remain functionally open following injury and in vivo work has shown significant changes in both spatial and temporal Cx43 protein expression observed following various models of CNS injury.

2.1.1 In vitro

Models of in vitro ischaemia have been used to study Cx43 gap junctional communication in astrocyte cultures. Cotrina et al observed that while ischaemia led to a decrease in Cx43 gap junction coupling, the gap junctions did remain functional meaning intercellular communication could still occur under ischaemic conditions. Specifically, astrocyte death evoked by either ionophore exposure or metabolic inhibition resulted in increased cytosolic calcium and a subsequent
decrease in gap junctional coupling. This reduction in astrocytic Cx43 gap junction coupling was confirmed following iodoacetate-induced hypoxia by Li and Nagy\textsuperscript{11,12}, who also observed an associated dephosphorylation of Cx43, thought to contribute to the uncoupling process. The idea that decreased but functional Cx43 gap junction communication can occur in ischaemic conditions is significant as it means the intercellular transfer of ions and metabolites from dying astrocytes to healthy ones can occur.

A further noteworthy change following chemical ischaemia by inhibition of glycolytic and oxidative metabolism was the permeabilisation of astrocytes to external Lucifer yellow and ethidium bromide. This was thought to be due to opening of Cx43 hemichannels since astrocytes were impermeable if gap junction blockade with 18α-glycyrrhetinic acid (AGA) was performed or Cx43-knockout were used.\textsuperscript{13} This experiment raised the concept of Cx43 hemichannels as another theoretical route of entry for extracellular ions and metabolites that may be released by dying cells.

\subsection*{2.1.2 In vivo}

\subsubsection*{2.1.2.1 Ischaemia}

Models of CNS ischaemia affect the expression of Cx43 in many neural areas, particularly in the hippocampus where increases in Cx43 immunoreactivity (Cx43-ir) have been observed in the CA1/2 pyramidal subfields following transient forebrain ischaemia.\textsuperscript{14} Consequently, many of the \textit{in vitro} and \textit{in vivo} models used to assess modulation of Cx43 utilise hippocampal tissue.

A similar increase in Cx43-ir in hippocampal and striatal areas was observed 2 days after mild to moderate global cerebral ischaemia induced by bilateral carotid artery occlusion in rats.\textsuperscript{15} However following severe ischaemia, there was an area of reduced Cx43-ir surrounded by a zone of increased Cx43-ir. Immuno-electron microscopy confirmed a greater preponderance of gap junctions among astrocytic processes in the vicinity of degenerating neurons and increased intracellular Cx43 in astrocytic processes and cell bodies. However, no change in Cx43 protein levels was detected by
Western blot. From these results the authors inferred that a process of gap junction reorganisation from a pool of Cx43, normally undetectable by immunohistochemistry, was involved following ischaemia and that the qualitative nature of the response was dependent on the severity of neuronal damage or loss.

The same group employed a rodent model of focal cerebral ischaemia by transient occlusion of the middle cerebral artery to demonstrate that astrocytic Cx43 epitope masking, dephosphorylation and cellular redistribution occurs after a focal ischaemic brain injury.\(^{16}\) After 1 hour of ischaemia and 24 hours of reperfusion, unphosphorylated Cx43-ir was absent in the ischaemic core within the hypothalamus, but persisted in a thin corridor at the ischaemic penumbra which contained presumptive apoptotic cell profiles. Similar results were obtained in ischaemic striatum and cerebral cortex, though with a delayed time course that depended on severity of the ischaemic insult.

Another rodent model of focal cerebral ischaemia utilised photothrombosis to induce small and defined ischaemic lesions restricted to the cortex.\(^{17}\) In addition to assessing Cx43-ir, \textit{in-situ} hybridisation was applied to study Cx43 mRNA expression. In the cortex of the injured hemisphere, there was a transient downregulation of Cx43 mRNA on day 1 post-ischaemia, followed by an increase of Cx43 mRNA positive cells by days 3 and 7 that subsequently normalised. This was accompanied by a generalised reduction in Cx43-ir at all timepoints. The difference in Cx43 mRNA and protein expression was explained by the possible internalisation and degradation of Cx43 protein molecules following ischaemia. The underlying hippocampus followed a similar Cx43 mRNA and protein pattern to that displayed in the cortex, except in the stratum oriens subjacent to the injury, which showed elevated Cx43-ir.

In a separate experiment\(^{18}\), the group looked more closely at the lesion site, surrounding cortex and developing glial scar following photothrombosis. In the cortex directly flanking the lesion, Cx43-ir was increased as early as 1 day after injury, becoming denser by 7 days and remaining elevated through until day 60. This was accompanied by an increase in glial fibrillary acidic protein (GFAP), a
marker for astrocytes, over the same time course. Of note was the appearance of a band of highly GFAP reactive cells and increased Cx43-ir, likely to represent reactive astrocytes at the bottom of the lesion by day 14.

The observations from these experiments raise the concept of a penumbra of increased Cx43-ir surrounding a focal lesion following two rodent models of cerebral ischaemia. Nakase et al\textsuperscript{19} confirmed a similar pattern in post-mortem human brain samples with ischaemic damage. Those brains that had suffered embolic strokes were considered as an acute ischaemic model, while multiple infarction brains were considered as a chronic ischaemic model. Cx43-ir was elevated in the lesion penumbra of both acute and chronic models, as well as in the normal white matter of the chronic model. The distribution of increased Cx43-ir was mirrored by a similar pattern of increase in astrocyte numbers, which also showed expansion of their processes indicative of activation, as measured by GFAP-ir. In fact, Cx43-ir was predominantly observed in GFAP positive cells in both models; however a subset of Cx43 positive but GFAP negative areas existed in the acute model. Double immunolabelling for microglia, with CD68 and Cx43, revealed that microglia expressed Cx43 more abundantly in the acute model than in the chronic model.

2.1.2.2 Excitotoxins

In support of their cerebral ischaemia work, Hossain et al\textsuperscript{20-22} showed that following stereotactic injection of both kainic acid (KA) and NMDA into rat brain striatum, there was decreased Cx43-ir at the lesion site where there was a depletion of neurons surrounded by a zone of increased Cx43-ir. They demonstrated altered immunohistochemical recognition of Cx43 epitopes and purported that Cx43 molecular modification occurs in excitotoxin-lesioned tissue.

2.1.2.3 Trauma

Following acute compression injury of the rat spinal cord, alteration in Cx43-ir was observed in the white and gray matter area of spinal cord for up to 7 days.\textsuperscript{23} At 1 and 3 days post injury, Cx43-ir was
decreased in the lesion epicenter, whereas immediately adjacent regions both rostrally and caudally exhibited intensified staining. By 7 days, all Cx43-ir had returned to normal/resting levels. Reactive astrocytes displaying GFAP appeared by day 1 and were prominent by day 3. Their distribution in white and gray matter corresponded closely to that of Cx43 staining at day 1 and 7, but less so at 3 days when GFAP-positive profiles were present at sites where Cx43 labelling was absent.

Ultrastructural observations of the areas of intensified Cx43-ir on nonjunctional astrocytic membranes led the authors to believe these areas represented injury-induced gap junction disassembly and Cx43 dispersal in plasma membrane leading presumably to an uncoupling of astrocytes immediately adjacent to the site of injury.

Using a model of rodent spinal cord transection injury, Lee et al.24 examined Cx43, GFAP and OX-42 (microglia marker) immunoreactivity. An increase in Cx43-ir and Cx43 mRNA-positive cells in the gray matter adjacent to the lesion was observed from 4 hours post-injury and remained higher than controls at 4 weeks. Cx43-ir mostly colocalised with GFAP rather than with OX-42, indicating that such upregulation was mainly in astrocytes rather than microglia, though the distribution of OX-42 staining was not described.

Another rodent model of traumatic brain injury utilises lateral fluid percussion, which is thought to be selective for the vulnerable hippocampal CA3 subfield, dentate hilar neurons and cortical neurons. Ohsumi et al.25 observed an initial reduction in Cx43-ir within the hippocampus up to 6 hours after injury, followed by a subsequent increase in Cx43-ir from 24 to 72 hours. The injured cortex showed a similar increase in Cx43-ir by 72 hours compared to controls.

Table 2-1. Response of Cx43 following CNS injury

<table>
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<th>Model and injury</th>
<th>Changes in Cx43/gap junction coupling</th>
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<td>Astrocytic cultures</td>
<td>Permeabilisation of astrocytes thought to be due to opening of Cx43 hemichannels</td>
<td>Contreras, Sanchez et al. 2002</td>
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<td>Global cerebral ischaemia</td>
<td>In hippocampus and striatum, increased Cx43 levels following mild-moderate insult, but decreased Cx43 levels with surrounding increased Cx43 following severe insult</td>
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<td>Decreased Cx43 levels in ischaemic core, but increased in a thin corridor at the ischaemic penumbra</td>
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<td>Spinal cord trauma -transection</td>
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### 2.2 Role of Cx43 to CNS injury

#### 2.2.1 Neuroprotective or neurodestructive?

While there is little controversy surrounding the great body of evidence outlining changes in Cx43 expression following CNS injury, the role of Cx43 gap junctions in these disease states is of some debate. This can be distilled down to two seemingly disparate views of the role of gap junctional communication in response to CNS injury.
The “good samaritan effect” put forward by Farahani et al., proposed a number of possible mechanisms by which gap junction communication may be beneficial following injury (see Figure 2-2). Cellular injury results in accumulation of deleterious metabolites in the cytoplasm, such as calcium ions and glutamate, key players in excitotoxicity. Gap junction communication would allow the passage of these factors into neighbouring healthy cells. By allowing the buffering of toxic metabolites by healthy cells, injured cells may be prevented from dying. In addition to the idea that astrocytes may buffer neurons from excitotoxicity and hypoxic depolarisation by taking up extracellular glutamate and potassium, they also secrete various neurotrophic factors and cytokines that may stimulate the survival of neighbouring neurons and protect neurons from excitotoxic, metabolic and oxidative insults. Ischaemia-induced elevation of endothelin-1 and norepinephrine may lead to enhanced astroglial glycogenesis and glycolysis producing pyruvate and lactate that can pass via gap junction channels to neighbouring neurons to be used as an energy substrate. Reduced glutathione, synthesised in astrocytes, may pass via gap junctions to neighbouring neurons to protect them from oxidative stress.

The opposing view of the role of gap junction communication following CNS injury is that by allowing the spread of death messengers from injured cells to otherwise healthy neighbouring cells, the zone of damage extends. This “gap junction-mediated bystander effect” has been demonstrated by the ability of herpes simplex virus type 1 thymidine kinase (HSV-tk)-expressing cells incubated with ganciclovir to induce cytotoxicity in neighbouring HSV-tk-negative (bystander) human glioblastoma cells in primary culture. Asklund et al further demonstrated that ganciclovir treatment led to the death of 90% of cells in culture within 1 week, but that the loss could be inhibited by the addition of AGA, a gap junction inhibitor that was shown to inhibit intercellular fluorescent dye transfer and immunodetection of Cx43 by Western blot.
Figure 2-2. Cx43 can contribute to cell death or survival through gap junction communication, hemichannel activation or action independent of either channel’s activity. (1) Gap junction communication promotes cell survival by allowing passage of toxic molecules out of the injured cell and neuroprotective substances into the cell, a process known as the ‘good samaritan effect’. (2) Gap junction communication can also harm healthy neighbouring cells by distribution of these toxic materials, a process known as the bystander effect. (3) Hemichannels contribute to the bi-directional movement of neuroprotective or neurodestructive substances into or out of the injured cell. (4) Substances, or breakdown products, released from injured cells via hemichannels act on neighbouring cells’ receptors. (5) Cx43 may act directly at a genetic level, independent of gap junction or hemichannel activity.

Another concept specific to damage spread in the CNS is that of spreading depression, a slowly propagating depression of cerebral neuronal activity and transmembrane ionic gradients, that arises in response to a variety of noxious stimuli. It is experimentally evoked by applying potassium chloride or glutamate to exposed cortical tissue, or by electrical stimulation. Spreading depression bears a strong resemblance to gap junction-mediated calcium waves triggered by glutamate among cultured astrocytes. Experimental evidence now supports the notion that astrocytic calcium waves constitute the leading edge of a propagating, spreading depression wave. It has been demonstrated...
that astrocytic calcium increments precede the depolarising wave of spreading depression by several seconds in acutely prepared hippocampal slices.\textsuperscript{32}

In order to determine the role of Cx43 in the CNS response to injury, investigators have studied the effect of altering the expression of Cx43 and thus astrocytic gap junctional communication in the CNS.

2.2.2 \textit{Methods of altering Cx43 expression}

A wide range of neurotransmitters, growth factors, and cytokines regulate or alter Cx43 expression and gap junction permeability in astrocytes.\textsuperscript{33, 34} For example, treatment of cultured astrocytes with interleukin-1 beta (IL-1) for several hours reduced Cx43 levels and cell to cell communication.\textsuperscript{35, 36} Reactive astrocytes, as well as activated microglia, have been shown to produce IL-1 and activated microglia in cultures have been shown to reduce astrocytic gap junctional communication.\textsuperscript{37, 38} Chemical regulation of Cx43 by insulin-like growth factor and insulin uncoupling has been demonstrated to occur via a particle-receptor mechanism.\textsuperscript{39} Endothelins have been shown to decrease the expression of phosphorylated Cx43 forms and are potent inhibitors of gap junction communication in astrocytes.\textsuperscript{40} Even human marrow stromal cells have been shown to increase Cx43 expression in co-culture with astrocytes.\textsuperscript{41} Of particular note is that halothane, used in numerous animal studies as inhalational anesthesia, has also been shown to influence gap junctional communication in cultured cells.\textsuperscript{16}

While many factors have been shown to influence Cx43 expression, experimental blockade of Cx43 expression is the key to understanding its role in CNS injury. This has been performed using three methods: knockout mice models, global gap junction blockade, and specific transient blockade.

Connexin knockouts permanently delete a gene, but permit compensatory changes in gene expression. Inducible knockouts allow for greater time and tissue specificity. Homozygous Cx43-knockout transgenic mice, with permanent deletion of the Cx43 gene, do not survive beyond the
perinatal period due to malformation of the conus region overlying the pulmonary outflow tract. Failure at the pulmonary gas exchange, heart embryonic alteration and delayed ossification and osteoblast dysfunction has been noted. While homozygous Cx43-knockout mouse tissue has been used for in vitro studies, it is the heterozygous Cx43-knockout mice, which have normal survival, that are used for in vivo studies. Siushansian et al. confirmed that astrocytic cultures from homozygous and heterozygous Cx43-knockout mice had lower levels of Cx43, a more dispersed Cx43 distribution, and decreased dye-coupling compared to wild-type animals. A key issue with Cx43-knockout models is their permanent effect on Cx43-gap junction communication, which is known to be necessary for normal astrocytic homeostasis.

Global gap junction blockade is usually induced by global gap junction blockers such as carbenoxolone, a derivative of glycyrrhetinic acid, which is extracted from the licorice root. Carbenoxolone is thought to uncouple gap junctions by disrupting connexon particle arrangements. It is relatively specific for gap junctions and nontoxic compared with more harmful agents such as octanol and heptanol. The major criticism of these global gap junction blockers are that while they appear relatively specific for gap junctions, they are not specific for different connexins and that some can reduce transmission at chemical synapses by a direct effect.

Specific transient connexin blockade can be induced by agents such as antisense oligodeoxynucleotides (AS-ODNs) or mimetic peptides. The advantage of these agents is not only their specificity for Cx43, but their transient knockdown of gap junction communication allows for quick recovery and return of normal astrocytic homeostasis. AS-ODNs are short chain nucleotides that temporarily block the expression of a target gene by binding to its mRNA, preventing translation and thus production of a specific protein. Because unmodified AS-ODNs have a short half-life of about 20 minutes, after which they are degraded by cell nucleases, incorporation of a Pluronic gel delivery system aids penetration of AS-ODNs into cells and acts as a reservoir to provide sustained release. Knockdown of Cx43 expression can be achieved for 24 to 48
hours using this gel delivery system. Connexin mimetic peptides are small peptide sequences designed against the extracellular regions of the connexin molecule. They may impair the interactions of the extracellular loops by binding to recognition sites on the hemichannel. Mimetic peptides have been shown to regulate both hemichannels and gap junctions independently of each other dependent on administration concentration.\textsuperscript{50}

While each of these methods results in decreased Cx43 expression, contradictory results obtained must be interpreted with caution due to key differences in specificity and duration of action.

2.2.3 In vitro/ex vivo studies

2.2.3.1 Knockout

Hippocampal organotypic slice cultures were used by Frantseva et al\textsuperscript{52} in a traumatic model of weight-drop injury. Hippocampal slices were taken from newborn homozygous, heterozygous and wild-type Cx43-knockout mice. Cell death was evaluated in the neocortical region, and was found to be significantly lower in slices from Cx43-knockout animals 24 hours after the impact when compared to wild-type and heterozygote littermates.

2.2.3.2 Global blockade

Several lines of evidence using global blockade of gap junctions suggest that gap junction coupling between cells play a role in bystander killing. Lin et al\textsuperscript{53} generated two different glial lines to evaluate the role of gap junction coupling following metabolic injury. The first line was transfected with cDNA for Cx43 (Cx+) and demonstrated functional gap junction coupling, but remained susceptible to metabolic injury, oxidative stress and calcium ionophore. The second line underwent double transfection with Cx43 and bcl2 (bcl+Cx+), an anti-apoptotic gene allowing increased resistance to injury. As expected, when bcl+Cx+ cultures were exposed to the calcium ionophore, less than 5\% of cells died. However when mixed cultures were exposed to the calcium ionophore, death of the vulnerable Cx+ cell line occurred but more importantly, 95\% of the bcl+Cx+ line also
died. The death of the injury resistant cells indicated that gap junction coupling between the Cx+ cells and the bcl+Cx+ cells contributed to the increased death of the previously resistant cells. This spread of injury from the Cx+ cells to the bcl+Cx+ cells was confirmed by blockade with a global gap junction blocker, AGA, which significantly reduced the incidence of bcl+Cx+ cell death in co-culture. Death of bcl+ cells could also be modified by varying the degree of Cx43 coupling, with intermediate levels inducing less death than their high-expression clones. The potency of bystander killing was also a direct function of the coupling index between resistant and non-resistant cells, with lowering of the concentration of non-resistant Cx+ cells resulting in a reduced amount of bystander death. Increase in death of bcl+Cx+ cells was also demonstrated by substituting the Cx+ cells for primary astrocytes. Annexin V staining confirmed that death of bcl+Cx+ cells was not occurring by the primary exposure, but by delayed secondary mechanisms. Gap junction coupling was shown to raise calcium levels in resistant cells, which usually had reduced calcium levels, to levels of surrounding non-resistant cells.

Other investigators have also evaluated the role of gap junctions using global gap junction blockers with various in vitro hippocampal tissue models. Frantseva et al.\textsuperscript{52} incubated wildtype organotypic hippocampal slices with global gap junction blockers, carbenoxolone or octanol, following traumatic weight drop injury. This resulted in significant neuroprotection, measured over 72 hours, with improved synaptic function and decreased cell death in the CA1-3 pyramidal layers if the blocker was added up to 2 hours after the traumatic insult. However, preincubating and removing carbenoxolone 5–10 minutes after the impact resulted in no neuroprotection. While gap junction blockade was observed to offer neuroprotection, promotion of gap junction communication with intracellular alkalisation by bicarbonate incubation enhanced the cell loss.

The same group\textsuperscript{54} repeated the experiment with a different injury model of in vitro ischaemia-reperfusion with deoxygenated glucose-free culture medium. Incubating the organotypic cultures with carbenoxolone again resulted in significant neuroprotection with cell loss in the pyramidal cell
body layers being 36% of that displayed in untreated slices subjected to identical insults. Significant neuroprotection was observed if the blocker was added 2 hours after the ischaemic episode, though in this case cell death was higher than that found when the drug was present throughout the experiment. Addition of carbenoxolone 24 hours after the ischaemic injury did not result in appreciable neuroprotection in any hippocampal area. From these results, the global blockade of gap junction communication was seen to have a neuroprotective role. These results suggest that there is a critical time, between 2 and 24 hours after injury, in which gap junction coupling results in the spread of injury. Blockade of gap junction communication during this time may be neuroprotective.

In agreement with these findings is work by De Pina-Benabou et al\textsuperscript{55} who used a different oxygen-deprivation (OGD) regime on organotypic hippocampal slice cultures. When a global gap junction blocker, carbenoxolone, was added to the culture medium 30 minutes before, during or 60 minutes after OGD, markedly delayed death of CA1 pyramidal neurons was observed by propidium iodide labelling 24 hours following the insult.

Nodin et al\textsuperscript{56} induced chemical ischaemia on primary rodent hippocampal astrocyte cultures and studied the effects of carbenoxolone, glycyrrhizic acid, heptanol and octanol. Their model utilised iodoacetate to induce ATP depression and subsequent apoptosis. Initiation of apoptosis was defined by the number of Annexin V positive cell groups and the spread of apoptosis by how much these Annexin V cell groups enlarged. Following gap junction blockade, the number of apoptotic processes remained similar to controls, but did not enlarge indicating that gap junction blockade inhibited the spreading but not initiation of apoptosis. In separate experiments employing calcium chelation to prevent the rise in intracellular calcium from iodoacetate, they observed an earlier apoptosis initiation by slower progression of ATP decline and no decrease in Annexin V positive cells. Similarly, broad spectrum caspase inhibition also did not alter the Annexin V positive cells.

Other investigators have demonstrated that gap junction inhibition with octanol and carbenoxolone significantly reduces bystander killing in other in vitro CNS models. Cusato et al\textsuperscript{57} induced retinal cell
death with cytochrome c (Cc) and examined the induced cells and their neighbours for apoptotic morphology or caspase-3 cleavage. Specifically they used a scrape-loading technique to introduce Cc into the cytoplasm of retinal cells in situ. Rhodamine dextran (RD) served as a marker for these cells. While the majority of RD-labelled, Cc-scrape loaded cells were pyknotic, bystander killing was observed in the form of neighbouring, unlabelled pyknotic nuclei. Similarly, caspase-3 cleavage was detected in both RD-labelled and unlabelled bystander cells, confirming the presence of bystander cell apoptosis.

While the above studies demonstrate the neuroprotective effects of global gap junction blockade, other investigators have observed an increase in neuronal damage and extent of injury after blocking gap junctions. Blanc et al. investigated the effect of global gap junction blockade, by AGA or halothane, on neuronal vulnerability to oxidative injury in embryonic rat hippocampal cell cultures. Following oxidative insults with iron sulphate (FeSO₄) and 4-hydroxynonenal, gap junction blockade markedly enhanced the generation of intracellular peroxides, the impairment of mitochondrial function and the amount of cell death (as measure by intracellular lactate dehydrogenase release) in neurons but not astrocytes. Antioxidants (propyl gallate and glutathione) blocked the death of neurons exposed to FeSO₄ and AGA. Neuronal intracellular calcium levels also increased with gap junction blockade following FeSO₄ exposure, and the calcium channel blocker nimodipine prevented impairment of mitochondrial function and cell death, whereas glutamate receptor antagonists were ineffective. In an attempt to best replicate in vivo conditions, they exposed organotypic hippocampal slice cultures to FeSO₄ and kainate oxidative insults, and showed that AGA also exacerbated these injuries. Finally, they excluded nitric oxide and impaired glutamate transport as a mediator of the effects of AGA.

Exacerbation of injury following gap junction blockade was also shown by Ozog et al. in a model of glutamate cytotoxicity in co-cultures of mice cortical astrocytes and neurons. Treatment with
carbenoxolone prior to glutamate exposure led to a 20-25% increase in cell death as assessed by TUNEL and propidium iodide staining, with associated increases in lactate dehydrogenase release.

### 2.2.3.3 Specific transient blockade

Cx43-specific blockade has been shown to have a neuroprotective effect following several in vitro studies. Frantseva et al.\(^{52, 54}\) used Cx43 AS-ODNs to confirm their neuroprotective knockout and global blockade results in both trauma and ischaemia models with organotypic hippocampal slices. Partial knockdown of Cx43, confirmed functionally and biochemically by Western blot, resulted in significantly less pyramidal and dentate gyrus cell death at 48 hours compared to controls or sense oligodeoxynucleotides.

Further work with Cx43 AS-ODNs was performed by Danesh-Meyer et al.\(^{60}\) in an in vitro ischaemic optic nerve model. Results demonstrated that in addition to limiting lesion spread at 24 hours, Cx43 AS-ODNs modulated several facets of the inflammatory process with reduced tissue swelling, improved vascular integrity and slowed differentiation of inflammatory cells (astrocytes and microglial cells). In control tissue, Cx43 upregulation was generalised and persisted for 2 to 3 days post-ischaemia. Cx43 AS-ODNs knocked down this upregulation and any increases in expression were confined to the cut edges of the optic nerve. These findings supported the concept that ischaemic injury to the optic nerve is associated with an upregulation in the local inflammatory response that is at least partially exacerbated by a Cx43 gap junction-mediated bystander effect.

O’Carroll et al.\(^{50}\) used Cx43 mimetic peptides in an ex vivo model of spinal cord injury where cut spinal cord segments were placed in culture. The amount of tissue swelling and extrusion from both dural ends was compared following co-culture with Cx43 mimetic peptides for 24 hours. An almost 50% reduction in swelling compared with controls was observed. A similar reduction in astrocyte activation and neuronal loss with mimetic peptide treatment was demonstrated by both Western blotting and immunohistochemistry. Cx43 analysis showed that the increase seen at 4 hours and
Beyond in response to injury was markedly reduced by mimetic peptide treatment. Gap junction blockade with Cx43 mimetic peptide was confirmed with dye spread studies and the neuroprotective effect was both concentration and time dependent.

2.2.4 In vivo studies

2.2.4.1 Knockout

Nakase et al\textsuperscript{61} performed various experiments using a rodent stroke model by middle cerebral artery occlusion in heterozygous Cx43-knockout mice. Firstly\textsuperscript{62}, the investigators demonstrated that the area of infarction was more extensive in the knockout mice than their wildtype counterparts four days after injury.

Their second experiment\textsuperscript{62} measured markers of apoptosis as well as stroke volume using the same injury model. They observed increased apoptosis by TUNEL labelling in the penumbra, increased caspase-3 levels in the stroke lesion, and again an increased infarct volume in Cx43-knockout mice when compared to wildtype. Interestingly, the number of TUNEL positive cells in the knockout mice was still increased at 4 days despite apoptosis usually reaching a maximum at 24 hours or 48 hours after ischaemia. The authors therefore speculated that Cx43 gap junctions allow removal of pro-apoptotic and cytotoxic agents from the ischaemic lesion. It was also observed that the average length of astrocytic processes was significantly increased in the penumbra of knockout mice. While there was no significant difference between the number of GFAP-positive astrocytes in knockout and wildtype mice, they found a reduced area of astrogliosis by immunohistochemistry but an increased ratio of GFAP by western blot in the knockout mice compared to controls.

A potential limitation of these experiments is the unwanted effects of global Cx43-knockout on other cell types. The investigators addressed this in a third set of experiments\textsuperscript{63} by breeding transgenic mice lacking Cx43 in astrocytes (Cre+), but with normal Cx43 levels in other cell types. In this way, they could specifically analyse the neuroprotective role of astrocytic gap junctions in focal ischaemia.
Animals with astrocyte-directed ablation of Cx43 exhibited a significantly increased infarct volume compared to wildtypes, as well as significantly enhanced apoptosis as detected by TUNEL labelling and cleaved caspase-3 immunostaining. These results additionally supported the notion that a decrease or loss of astrocytic Cx43 increases the vulnerability to ischaemic stroke. An increase in CD11b immunostaining, an inflammatory marker for microglia and macrophages, was also observed in the lesion of Cre+ mice and it was therefore postulated that an increase in cytokine production from activated astrocytes might cause enhanced inflammation after ischaemic insult.

It must be reiterated that these Cx43-knockout experiments remain limited by the lack of gap junction recovery and thus return of normal astrocytic homeostasis that features in the transient gap junction blockade models.

### 2.2.4.2 Global blockade

There is substantial evidence that global gap junction blockade has a neuroprotective effect following *in vivo* CNS injury. Using the rodent model of focal brain ischaemia by middle cerebral artery occlusion, Rawanduzy et al\(^1\) intraperitoneally pretreated rats with the global gap junction blocker, octanol and compared the sizes of the ischaemic lesions to those that received vehicle dimethyl sulfoxide. Histopathological analysis, performed 24 hours later, showed a significantly decreased mean infarction volume in the octanol group when compared to controls. In a separate set of experiments, octanol treatment was shown to inhibit spreading depression, thought to be involved in lesion expansion, evoked by injections of potassium chloride.

Pre-treatment with intraperitoneal octanol was also performed by Rami et al\(^2\) in a rodent model of transient forebrain ischaemia by bilateral carotid artery clamping. A significant reduction of Cx43-ir and reduced neuronal death by approximately 33% in the more vulnerable CA1–CA2 pyramidal subfields of the hippocampus was observed at 7 days.
A potential drawback of the administration of gap junction blockers by an intraperitoneal route is the ultimate delivery to the site of CNS injury. In fact, following intraperitoneal injections of carbenoxolone, the CSF has been shown to have less than 10% of the plasma concentration. Perez Velazquez et al\textsuperscript{64} worked around this by direct application through chronically implanted hippocampal cannulae. They studied the effects of carbenoxolone, AGA and endothelin in a rodent 4 vessel model of global cerebral ischaemia. A decrease in TUNEL-positive neurons in the treated hemisphere as compared with the control hemisphere receiving saline injection was observed. Both pre and post-treatment resulted in decreased cell death up to 30 minutes after injury. A significant reduction in lipid peroxides was observed in the hippocampi of carbenoxolone treated animals compared to controls.

De Pina-Benabou et al\textsuperscript{55} used an \textit{in vivo} model of perinatal ischaemia to assess the effect of gap junction blockade on neuronal death. Intraperitoneal carbenoxolone, administered immediately after intrauterine hypoxia-ischaemia and 12 hours later, ameliorated the long-term developmental impact of perinatal ischaemia. Carbenoxolone-treated rats were closer to normal weight, showed less signs of abnormal neuronal hypercellularity, had less neuronal death, had lower caspase-3 levels, and overall had reduced histopathological damage in the hippocampus, neocortex and cerebellum.

Finally, in a non-cerebral model, Cusato et al\textsuperscript{57} assessed the patterns of normal programmed cell death in the developing retina and showed this occurred in clusters. Application of carbenoxolone decreased this clustering of dying cells.

\textbf{2.2.4.3 Specific transient blockade}

Cronin et al\textsuperscript{6} used transient Cx43 specific blockade in an \textit{in vivo} model of CNS injury. They manipulated the expression of Cx43 protein in the hours following two rodent models of spinal cord injury with Cx43 AS-ODNs. Within 24 hours following a partial transection injury, spinal cords were
less swollen and inflamed in external appearance, and showed less tissue disruption, than controls.

Cx43 AS-ODNs was confirmed to reduce the elevation of Cx43 and GFAP levels normally seen in response to transection or compression injuries. The functional consequences of Cx43 AS-ODNs application were also assessed by observation of the locomotor ability following injury. Treated rats showed clear improvements in locomotion compared to controls. Assessment of tissue inflammation was conducted with markers for neutrophils (myeloperoxidase), activated microglia (OX42 in injury site) and leakage from damaged vasculature (intravenous FITC-Bovine Serum Albumin). Fewer neutrophils and microglia, along with reduced leakage from blood vessels, was observed at the lesion site in Cx43 AS-ODNs treated animals. Furthermore, the morphology of the microglia in treated animals was more stellate than typical activated, rounded, phagocytic forms seen in controls.

Finally, Li et al.\(^6\) looked at the effects of Gingko biloba extract and nimodpine on Cx43 mRNA and protein expression, and neurological function after rodent cerebral ischaemia. They showed improved neurological function and lower Cx43 mRNA and protein levels (by Western blot) following intraperitoneal nimodipine 1 hour prior to injury, or Gingko biloba extract administered orally for 7 days prior to injury.

Table 2-2. Role of Cx43 blockade following CNS injury - neuroprotective

<table>
<thead>
<tr>
<th>Model and injury</th>
<th>Gap junction blockade</th>
<th>Effects vs controls</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> - neuroprotective</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hippocampal organotypic slices - weight-drop trauma</td>
<td>Cx43 knockout mice</td>
<td>Decreased neocortical cell death at 24 hours</td>
<td>Frantseva, Kokarotseva et al. 2002</td>
</tr>
<tr>
<td>Hippocampal organotypic slices - weight-drop trauma</td>
<td>Carbenoxolone or octanol (post-injury)</td>
<td>Decreased pyramidal cell death and improved synaptic function</td>
<td>Frantseva, Kokarotseva et al. 2002</td>
</tr>
<tr>
<td>Hippocampal organotypic slices - weight-drop trauma</td>
<td>Cx43 AS-ODNs</td>
<td>Decreased cell death at 48 and 72 hours</td>
<td>Frantseva, Kokarotseva et al. 2002</td>
</tr>
<tr>
<td>Hippocampal organotypic slices - hypoxia-hypoglycemia</td>
<td>Carbenoxolone (post-injury)</td>
<td>Decreased pyramidal cell death</td>
<td>Frantseva, Kokarotseva et al. 2002</td>
</tr>
<tr>
<td>Hippocampal organotypic slices - hypoxia-hypoglycemia</td>
<td>Cx43 AS-ODNs</td>
<td>Decreased pyramidal and dentate gyrus cell death</td>
<td>Frantseva, Kokarotseva et al. 2002</td>
</tr>
</tbody>
</table>
Hippocampal organotypic slices
- oxygen-glucose deprivation
Hippocampal astrocyte cultures
- chemical ischaemia
Transfected glial culture
- ionophore exposure/
metabolic inhibition
Retinal whole-mounts
- cytochrome c injury
Optic nerve segments
- oxygen-glucose deprivation
Spinal cord segments
- transaction trauma

In vitro - neuroprotective

Table 2-3. Role of Cx43 blockade following CNS injury - neurodestructive

<table>
<thead>
<tr>
<th>Model and injury</th>
<th>Gap junction blockade</th>
<th>Effects vs controls</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro - neurodestructive</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Hippocampal cell cultures and organotypic slices
  - oxidative injury with FeSO₄ | AGA or halothane | Increased cell death,
generation of intracellular peroxidases and impaired mitochondrial function | Blanc, Bruce-Keller et al. 1998 |
| Astrocyte-neuron co-cultures
  - glutamate cytotoxicity | Carbenoxolone | Increased cell death,
increased LDH release | Ozog, Siushansian et al. 2002; Naus, Ozog et al. 2001 |
| In vivo - neurodestructive |
| Rodent stroke | Cx43-knockout | Increased area of infarction | Siushansian, |
2.3 Cx43 in the Optic Nerve and Retina

2.3.1 Optic Nerve

Given the abundance of astrocytes in the optic nerve, it would be of little surprise to find a similar abundance of Cx43-ir as well. Surprisingly, there is little in the literature about Cx43 in the optic nerve specifically. In 1998, Yukushigawa et al. described Cx43 within the gap junctions of optic nerve astrocytes of the Japanese Macaque monkey. May et al. commented that there was positive Cx43 staining throughout the murine optic nerve that correlated with GFAP-positive cells. As described above, Danesh-Meyer et al. observed that the distribution of Cx43-ir in optic nerve segments was highest at the cut edges but also present throughout the segments.

2.3.2 Retina

Various gap junction have been found between almost all of the approximately 50 types of vertebrate retinal neuron, from photoreceptors through to ganglion cells. Despite the abundance of retinal neuronal connexins, Cx43 itself is minimally expressed in retinal neurons. There are scattered reports of Cx43 in endothelial and epithelial cells of different vertebrates and the outer nuclear and outer plexiform layers in fish species that may correlate with photoreceptor and amacrine cells respectively. The predominant locations for Cx43 are the retinal pigment epithelium, where it is believed to contribute to outer retinal potassium buffering, around blood vessels of the inner...
retina, and in retinal glial cells, where it is the major gap junction protein between Müller cells in lower vertebrates and between astrocytes in higher vertebrates. Müller cells are the only glial cell type in lower vertebrates and are intensively coupled via Cx43 gap junctions. In the higher vertebrate and human retinal glia, astrocytic cell coupling via Cx43 gap junctions has been shown by dye and tracer transfer, but it is less certain as to whether this occurs between Müller cells. While it is certain that there is a predominance of astrocytic Cx43 gap junctions staining the nerve fibre layer of rats and other mammals, studies have more recently shown Cx43-ir does occur in Müller cells of the rat and rabbit, as well as rat Müller cell culture. While functional coupling between mammalian Müller cells has yet to be demonstrated, a shift from the popular belief that there are no gap junctions between mammalian Müller cells has occurred.

Cx43 is primarily expressed in the vitread part of the retina throughout development. It is thought this could potentially be involved in the guidance of RGC axons as Cx43 gap junction plaques have been observed on the growth cones of RGCs. Cx43 gene expression has very low levels early postnatally, but reach adult levels in the rat by P10-P15 and it is postulated this is related to the completion of retinal synaptic circuitry.

2.4 Summary

Cx43 remains the most widely studied connexin protein in the literature. The preceding observations of Cx43 immunohistochemistry following CNS injury indicate that the response of Cx43 varies with severity of injury. Generally, mild to moderate injury appears to lead to increased Cx43-ir in the lesion site or in vulnerable CNS areas, whereas severe injury results in decreased Cx43-ir within the injury site probably due to cell death in that region, and a surrounding zone of increased Cx43-ir in what could be described as the penumbra of the injury in various models.
There can be little debate that Cx43 gap junction communication is an important mediator following CNS injury. In mild injury increased Cx43 expression may be involved in diffusion or spreading of substances such as potassium and glutamate that might otherwise reach toxic levels. For more extensive injury there is, however, evidence in support of both neuroprotection and neurodestruction following Cx43 blockade. In these injuries extensive coupling may exacerbate the injury through ATP release, or by spreading toxins and death signals. The majority of this evidence comes from studies using global gap junction blockade, with most of these results indicating that neuroprotection is achieved. There is more limited evidence of increased neurodestruction following global gap junction blockade in vitro. However, results from experiments using global blockade should be interpreted with caution due to the nonspecific nature of agents such as carbenoloxone and octanol. Cx43-knockout models are another popular choice and interestingly most of the in vivo evidence in support of continued neurodestruction or lesion spread comes from Cx43 knockout mice. While the above data is seemingly disparate, it may not necessarily be mutually exclusive. Cx43-knockout models result in permanent Cx43 modulation and thus it would make logical sense that the lack of gap junction recovery would impact negatively on a return to normal astrocytic homeostasis.

Combined, the data suggests that permanent blockade of Cx43 may not be neuroprotective, but transient blockade targeting the window of initial Cx43 upregulation observed following injury is potentially therapeutic. Transient Cx43 specific knockdown, by Cx43 antisense oligodeoxynucleotide or Cx43 mimetic peptides, leads to a return to normal Cx43 function and there is building evidence of neuroprotection following their application both in vitro and in vivo.

Cx43 is abundant in the astrocytes of the optic nerve and retina and given the variety of effects on Cx43 following injury in the rest of the CNS, it would be intriguing to determine what optic nerve injury will do to Cx43 expression both at the optic nerve and at its target cells in the retina.

2.5 References


Chapter 3: Glia in the CNS and Retina

3.1 Glia in the CNS

Glia cells significantly outnumber neuronal cells in the nervous system. Broadly, they are classified into macroglia and microglia. Within the CNS, macroglial cell lines include astrocytes, oligodendrocytes, ependymal cells and radial glia, such as Müller cells. Some glial cell lines have long been recognised as having unique, specialised functions such as myelination (oligodendrocytes) and host defence (microglia). More recently, the importance of astrocytes in normal physiology and pathology has become apparent.

3.1.1 CNS Astrocytes

Astrocytes were initially considered to be the ‘brain glue’, providing an inert scaffold necessary for neuronal distribution and interactions. Today, astrocytes are seen as local communication elements of the brain that can generate various regulatory signals and bridge structures (from neuronal to vascular) and networks that are otherwise disconnected from each other.¹

As astrocytes do not generate action potentials, they were initially considered to be non-excitatory and therefore unable to communicate. In fact, astrocytes can be excited non-electrically by internal or external signals, which results in the delivery of specific messages to neighbouring cells.² This so-called “gliotransmission” indicates that brain communication is actually a complex and integrated network of both synaptic and non-synaptic routes.

Astrocyte excitation, which is chemically encoded by calcium ions, can be divided into two forms: neuron-dependent and spontaneous excitation. An important response of astrocytes to their excitation is the release of gliotransmitters, such as glutamate, ATP, adenosine, D-serine and tumour
necrosis factor alpha (TNFα). These chemicals act on adjacent neurons, glial cells and vessels through feedback or feed-forward mechanisms thus forming a complex range of astrocyte-synapse interactions. For example, glutamate release by astrocytes has been shown to modulate neuronal excitability and synaptic transmission, as has ATP and adenosine. Astrocytes also control non-neuronal brain cells. They can signal blood vessel cells to promote neurovascular coupling and can attract cells, such as microglia and lymphocytes during inflammation, through the release of chemokines.

Defects in astrocyte excitation and signalling are now known to have neurodestructive properties. Almost all the steps of astrocyte excitation and gliotransmission are affected in various pathological conditions. Spontaneous calcium ion excitation of astrocytes is abolished in the peritraumatic area of mechanical insults. Loss of astrocyte function can lead to increased neuronal calcium ion permeability, resulting in excitotoxic damage. Conditions such as ischaemia and traumatic injury favour the release of gliotransmitters, through both calcium-dependent and independent pathways. For example, calcium-dependent glutamate release following inflammation results in the clustering of astrocytes together with activated microglia and massive TNFα release. This, in turn, amplifies astrocytic glutamate release leading to neurotoxicity.

Astrocytes can also elicit neuronal mitochondrial damage as a consequence of the interaction between astrocyte-derived reactive nitrogen species and the neuronal electron transport chain (ETC). Astrocytes appear to predominantly synthesise nitric oxide (NO) when stimulated by cytokines and lipopolysaccharide (LPS). While astrocytes have some protection against NO exposure from glycolysis-derived ATP, neuronal cells appear particularly susceptible. NO initially inhibits ETC complexes in a reversible manner by competing with molecular oxygen, thus at low oxygen tensions (such as in ischaemia) modest concentrations of NO could theoretically cause a significant impairment of cellular respiration. Prolonged NO exposure results in irreversible damage to the ETC through peroxynitrite (ONOO) formation.
Co-culture experiments implicate a role for astrocytes in protection of neurons from death. When neurons are co-cultured with astrocytes, or even microglia, they remain viable after brief, but not extended, exposure to NO. A factor in this may be the antioxidant capacity of these cell types. Upregulation of glutathione (GSH) occurs in astrocytes following NO exposure that may combat nitrosative stress. This does not occur in neuronal cells, unless in co-culture with astrocytes where GSH is trafficked from astrocytes to neurons, thus providing a finite amount of neuronal protection from mitochondrial damage.

Co-culturing astrocytes and neurons produces a similar effect on neuronal sensitivity to glutamate toxicity. Ischaemia leads to expression of NMDA receptors on both astrocytes, which normally lack these receptors, and neurons. Oligonucleotide knockdown of these receptors has shown that these NMDA receptors are important in buffering neurons from glutamate toxicity.

3.1.2 CNS Microglia

Microglia make up only 10% of the total brain cell population. They are the brain’s mononuclear phagocytes, or macrophages, and are influenced by their microenvironment. Microglia are bone marrow-derived and present a ramified morphology in the resting stages. The normal phagocytic functions of blood macrophages are considerably downregulated in resting microglia, but in response to injury or upon activation, their morphology becomes amoeboid and their processes retract. Microglial processes rapidly and autonomously converge on the site of injury without cell body movement, establishing a potential barrier between the healthy and injured tissue. Microglia proliferate, become hypertrophic, upregulate a variety of surface receptors involved in antigen presentation and produce a plethora of secreted factors such as growth factors, pro- and anti-inflammatory cytokines, reactive oxygen species (ROS), NO, and glutamate. As would be expected from the variety of factors produced, some can potentially promote neuronal survival whereas others exacerbate neuronal degeneration.
One of the factors that controls microglial phenotype and determines the effect on neuronal survival is the adaptive immune response evoked by injury, via CD4+ T cells. T cells control not only the phenotype but also the microglial population size and the profile of molecules predominantly released by microglial cells. Generally, interaction with T cells leads to the production of fewer neurotoxic factors such as glutamate, increased production of neuroprotective growth factors/anti-inflammatory molecules and decreased production of NO. In normal brain tissue, glutamate buffering is mediated by astrocytes but due to the lack of astrocytes at the site of injury, the role that microglia take in glutamate clearance presumably affects neuronal survival significantly.

Within hours after an acute brain injury such as a stroke, the brain mounts an inflammatory response involving activation of local microglia/astrocytes and infiltration of macrophages from circulation. The inflammatory process is a double-edged sword, because increased production of reactive oxygen and nitrogen species by immune cells can cause secondary damage up to weeks after injury, yet the subsequent remodelling of brain tissue requires the efficient containment and removal of dead tissue. The application of antioxidants has been shown to reduce intracellular release of ROS and reactive nitrogen species (RNS) that occurs with microglial activation.

Astrocytes can also inhibit activated microglial release of NO, and it has been shown that soluble astrocyte-derived factors can induce antioxidant gene expression in microglia in vitro. Thus astrocyte–microglial interactions may well modulate brain inflammation in vivo. In support of this, blockade of astrocytic connexin channels can stop the activation of microglia. The authors postulate the mechanism is via the blockade of ATP, which can mediate a rapid microglial response toward injury.

3.2 Glia in the retina
Similar to the rest of the CNS, retinal glia are comprised of macroglia and microglia. Retinal macroglia consist of Müller cells and astrocytes, of which Müller cells are the most predominant. Both glial cell types have processes that wrap around retinal blood vessels forming a glia limitans.

3.2.1  
**Retinal Macroglia**

3.2.1.1  
**Müller cells**

A single neuroepithelial progenitor cell gives rise to both Müller cells and retinal neurons. Developing Müller cells and neurons migrate inward to their final position and it is thought that the Müller cell processes and trunks guide much of the neuron migrations and direct the neurite differentiations.

Müller cells form architectural support structures stretching radially across the thickness of the retina. Their cell bodies sit in the inner nuclear layer and project irregularly thick and thin processes in either direction to the outer limiting membrane (OLM) and to the inner limiting membrane (ILM), forming the limits of the retina. The surface of the cell membrane facing the vitreous is covered with a mucopolysaccharide material and thus forms a true basement membrane. Müller cell processes insinuate themselves between cell bodies of the neurons in the nuclear layers and envelope groups of neural processes in the plexiform layers. They also form endfeet on the large retinal blood vessels at the inner surface of the retina. Müller cells contain glycogen, mitochondria and intermediate filaments which are immunoreactive for vimentin and, at the inner half of the cells, for GFAP.

3.2.1.2  
**Retinal astrocytes**

Astrocytes do not originate from the retinal neuroepithelium like Müller cells, but are “immigrant” fibrous astrocytes that follow blood vessels into the retina from the optic nerve. They have the characteristic morphology of a flattened cell body and a fibrous series of radiating processes, however this morphology changes from extremely elongated at the optic nerve head to stellate in
the far periphery. In terms of distribution, astrocytes reach their peak on the optic nerve head and have a fairly uniform decline in density in radiating rings from the nerve head. Astrocyte cell bodies and processes are almost entirely restricted to the nerve fibre layer of the retina. Intermediate filaments fill their processes and thus they stain dramatically with antibodies against GFAP. The blood vessels running in and among the ganglion cell bundles are also covered by both processes and even an occasional astrocyte cell body. The function of astrocytes enveloping ganglion cell axons and the relationship to blood vessels of the nerve fibre layer suggests they are axonal and vascular glial sheaths and part of a blood-retinal barrier.

3.2.1.3 Role of retinal macroglia

Müller cells play an essential role in the normal function of the retina. They are intricately involved in the uptake and degradation of the neurotransmitters, glutamate and GABA, shuttling of energy metabolites from the vasculature to neurons and they act as a siphon for the uptake of extracellular potassium. In addition, they maintain both the ion balance and the pH of the retinal milieu. With respect to the vasculature, Müller cells have been linked with the formation and maintenance of the blood-retinal barrier. Abnormalities in any of these vital Müller cell functions are associated with retinal compromise, including neuronal dysfunction and death.

The role of astrocytes in retinal function is less clear. Similar to Müller cells, retinal astrocytes are known to contain abundant glycogen and they may form a nutritive service in providing glucose to the neurons. However, they do not have processes that project to the synaptic layers of the retina and therefore, cannot regulate neuronal function in the same way as Müller cells. Retinal astrocytes probably do serve a role in ionic homeostasis in regulating extracellular potassium levels and metabolism of neurotransmitters like GABA. They also play an important role in controlling the development of the retinal vasculature given blood vessels that form the superficial plexus extend across an astrocyte template. Recently, regulation of vessel calibre and retinal blood flow has been attributed to macroglial cells in response to changing neural function.
Activation of retinal macroglia can lead to two opposite effects on ganglion cell processing. The first is inhibitory and is mediated by extracellular ATP, which is rapidly transformed into adenosine, when it is released from stimulated Müller cells.\textsuperscript{29} This may modulate a variety of neuronal classes from photoreceptors to ganglion cells given that they are known to express receptors to ATP.\textsuperscript{30} The second effect is stimulatory and is mediated by $d$-serine acting at the glycine-binding site of the NMDA receptor. In the retina, $d$-serine is present exclusively in Müller cells and astrocytes.\textsuperscript{31}

The ATP-mediated inhibitory effect on neurons is related to a large increase in intracellular calcium within both astrocytes and Müller cells.\textsuperscript{29} Mechanical, chemical and light stimulation can also evoke increases in intracellular calcium in both astrocytes and Müller cells that propagate to neighbouring glia.\textsuperscript{32} The source of the calcium elevations in retinal glia is thought to be primarily from intracellular stores, although there is also a range of other calcium permeable channels, pumps and exchangers that could mediate calcium influx into glia from the environment. As with other forms of stimulation, ischaemia leads to an increase in intracellular calcium response in astrocytes.\textsuperscript{33} It is thought that this may be an important protective mechanism because it is predicted that an elevated calcium response within glia might initiate vasoconstriction, limiting the region of ischaemia.

As with CNS astrocytes, retinal macroglia can release a number of gliotransmitters in response to injury as well as upregulate a number of growth factors and inflammatory mediators, in particular acute phase proteins, prostaglandin E2 (PGE2), cyclo-oxygenase and NO.\textsuperscript{34, 35}

\textbf{3.2.1.4 Astrogliosis}

Astrogliosis is a characteristic response of astrocytes in the CNS to trauma and a broad spectrum of disease processes.\textsuperscript{36} Gliosis refers to morphological changes observed in glial cells at the site of neuronal injury and can include hypertrophy of the cell body and nucleus, elongation of cytoplasmic processes and hyperplasia.\textsuperscript{37} Gliosis is thought to represent an attempt by the CNS to limit the site of injury. Glial cell hypertrophy and functional changes are thought to encapsulate an injured area,
thus protecting the neighbouring ‘normal healthy’ tissue. A key feature of retinal gliosis is the upregulation of GFAP, thought to be important structurally for supporting the new cellular shape of reactive glia. Gliosis may be either ‘conservative’, indicating that there is an increase in GFAP expression and functional changes, or ‘proliferative’, indicating that there is also glial proliferation and migration. With pathology, a spectrum of astrogliosis occurs, from functional anomalies within glia evident in early stages, followed by GFAP upregulation and finally proliferation and migration of glia. 26

Retinal gliosis is seen in both retinal and optic nerve pathology (the glial response to optic nerve injury is specifically outlined in following section). 26 Gliotic Müller cells are functionally altered. In particular, alteration of potassium channels may result in retinal dysfunction, with effects on neurotransmitter uptake systems and retinal fluid balance which are essential for normal neuronal function. 38

3.2.2 Retinal microglia

The origin of retinal microglia is still controversial, with reports of either a neuroepithelial and haemopoietic basis. Microglial precursors enter the developing retina from two main sources: the retinal margin and optic disc via the blood vessels of the ciliary body and iris, and the retinal vasculature. The first source of cells is thought to differentiate from optic nerve mesenchyme into ramified parenchymal microglia, whereas the second are probably blood-borne monocellular phagocyte series cells. Microglia are found in every layer of the retina, however are largely confined to the ganglion cell layer and inner plexiform layer. The roles of resting microglia in the normal retina are under investigated, but they are considered to play a role in host defence against invading microorganisms upon injury and breakdown of the retinal–blood barrier. 39
Neurodegeneration activates microglial cells and facilitates their phagocytic activity to clean up cell debris from the damaged retina. Under inflammatory conditions in the eye, microglia serve as antigen presenting cells and thus allow activation and proliferation of autoimmune T cells, which augment local inflammatory responses. T cells require the presence of microglia at the site of injury to facilitate a neuroprotective response, by mechanisms such as the production of growth factors from microglia. Enhancing the T cell immune response can improve the ability of microglia to take up excess amounts of extracellular glutamate.

However, an abnormal accumulation of activated microglia could also result in retinal damage. Microglial cells also produce neurotoxic compounds and overactivation of microglia may be associated with immune-mediated neurodegeneration.

3.3 Glial response following optic nerve injury

The macroglial and microglial response following optic nerve injury is a well-studied area both at the optic nerve lesion site and the retina.

3.3.1 Local Response

Crush lesions of the adult rat optic nerve result in an extended area of cell damage completely devoid of living cells, as well as a disruption of the blood-brain barrier. Repopulation of the lesion centre by reactive astrocytes that enclose axonal and myelin debris has been observed 1 week after injury. A massive infiltration of lysozyme-positive and ED1-positive microglia and macrophages into the lesion centre begins 2 days after the crush, peaking by 6 days and decreasing thereafter. In contrast, in the distal nerve stump, repopulation of ED 1-positive/lysozyme-negative
cells occurs 6 days after the crush and remained unchanged up to 3 weeks after the crush. These cells differed in shape and phagocytic ability from the macrophages in the lesion centre.\textsuperscript{43}

A closer examination of individual astrocytes following optic nerve injury showed that all astrocyte subtypes became reactive and led to glial scar formation.\textsuperscript{44} A prominent reaction was that astrocytes had withdrawn radial processes and extended a greater proportion of processes longitudinally, parallel to the long axis of the nerve and along the course of degenerated axons. This optic nerve astrogliosis response has been prevented by bcl-2 over-expression, an inhibitor of apoptosis.\textsuperscript{45}

The role of CNTF following optic nerve injury has been a particular area research interest. CNTF is thought to be important for survival and neurite extension by RGCs and it appears that the CNTF receptor is lost rapidly from RGC axons near the injury site, but appears in surrounding optic nerve macroglia.\textsuperscript{46} These findings could be interpreted to suggest that loss of the CNTF receptor from axons renders RGCs unresponsive to CNTF leading to cell death and that the appearance of the receptor on glia promotes scarring. Indeed in culture, astrocytes have been shown to secrete fibroblast growth factors in response to CNTF exposure.\textsuperscript{47}

\textbf{3.3.2 Retinal response}

Following interruption of the optic nerve or multiple penetrating wounds of the eye the radial fibres of Müller became intensely stained by GFAP, compared to just the inner retina surface in controls.\textsuperscript{48} A later study examined GFAP expression following optic nerve crush and showed levels were normal at 1 day post-crush, but increased 9-fold by day 3 and remained elevated over the 2 week period studied. They also showed that BDNF, a neuroprotectant that can prolong RGC survival, did not have a significant effect on the expression of GFAP. \textsuperscript{49}
Another study performed optic nerve crush in two species of rat.\textsuperscript{50} Despite a massive degeneration of RGCs in both species, only a minor increase in retinal GFAP-ir was observed in the inner retina of hooded rats compared to a significant increase in GFAP-ir in albino rats. Interestingly, a combination of optic nerve crush and administration of the proinflammatory agent LPS induced increased GFAP-ir in both species. While this data demonstrated that significant inter-species differences in retinal macroglial response following optic nerve injury exist, no microglia differences were observed with OX-42 labelling 1 week post-injury.

It appears that glial responses to optic nerve injury are not confined to the ipsilateral retina.\textsuperscript{51} Bromodeoxyuridine (BrdU) immunocytochemistry analysis of ipsilateral and contralateral retinas exhibited significantly higher densities of newborn cells in most retinal layers of injured animals compared to controls. Further double-labelling experiments confirmed the microglial and macroglial nature of these cells.

Crush injury to the adult rat optic nerve has been shown to activate intraretinal signalling cascades that can transform RGCs from a non-regenerative to a potentially regenerative state within 3–4 days. Explantation and culture of retina on glia from injured explants results in vigorous regeneration of RGC axons.\textsuperscript{52} This may be due to an orchestrated response of all retinal neurons and glia and further research has confirmed that marked pro-survival genomic changes occur in retinal neurons and glia following optic nerve injury.\textsuperscript{53} Genes that were regulated belonged to different functional categories such as antioxidants, anti-apoptotic molecules, transcription factors, secreted signalling molecules, and inflammation-related genes.

As in the optic nerve, retinal CNTF has been an area of intense interest. Upregulated expression of CNTF and GFAP in Müller cells in response to NMDA- and kainic acid (KA)- induced neuronal death has been demonstrated.\textsuperscript{54} CNTF release by retinal macroglia can be induced by intraocular inflammation from factors such as lens injury. This CNTF release from retinal Müller cells and astrocytes has been shown to bind to RGCs via receptor tyrosine kinases, improving RGC survival and
switching them to a regenerative state following axotomy. While activated macrophages and oncomodulin have also been suggested to be the mediators of this phenomenon, CNTF may play a more important neuroprotective role. In support, adenoviral-mediated transfer of CNTF to the retina rescued RGCs from axotomy-induced apoptosis. It was presumed this was due to the activation of the high affinity CNTF receptors on RGCs and modulation of low affinity inhibitory receptors on astrocytes. Seven days after intravitreal injections of CNTF, significant increases in astrocyte proteins GFAP, glutamate/aspartate transporter-1 (GLAST-1), glutamine synthetase (GS), and connexin 43 (Cx43) were observed. The authors therefore hypothesised that the neuroprotective effects of CNTF may result from a shift of retinal glial cells to a more neuroprotective phenotype.

As described above, the retinal microglial response to optic nerve injury is predominantly to facilitate the cleanup of degenerating neurons. It has been observed that retinal microglial cells displayed a strong territorial arrangement within the ganglion cell layer and inner plexiform layer after optic nerve transection, which probably aids the removal of cell debris and wound healing. While it is thought that this removal of debris has a positive effect, the same group showed that microglia-suppressing factors can retard axotomy-induced neuronal degradation and enhance axonal regeneration in vivo and in vitro.

3.4 Summary

The importance of glia in the CNS and retina was perhaps underestimated until more recent advances in our knowledge. Initially, astrocytes were seen simply as “brain glue” however it has become apparent that they form a highly communicative functional syncitium that is essential to normal neuronal function. CNS and retinal macroglia and microglia show specific responses to CNS injury and are thought to play a vital role in neuronal survival. In general, loss of astrocyte function appears to result in an increase in neuronal death following injury due to the release of various
gliotransmitters, antioxidants and upregulation of protective surface receptors. However, astrocyte-neuron interactions are clearly complex as astrocytes can also be neurodestructive via the release of free nitrogen and oxygen species. Similarly, CNS microglia also secrete a plethora of factors that can be both neuroprotective or neurotoxic. Microglia-astrocyte interactions are also critical in the modulation of inflammation following acute neuronal injury. The inflammatory process is a double-edged sword as it can cause secondary damage up to weeks after injury, yet the subsequent remodelling of brain tissue requires the efficient containment and removal of dead tissue. From the above evidence there can be little doubt that glial interactions in the CNS are critical to neuronal health, and it is the balance between various protective and destructive factors that are central to this.

3.5 References


55. Muller A, Hauk TG, Fischer D, Muller A, Hauk TG, Fischer D. Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation.[see comment]. *Brain*. Dec 2007;130(Pt 12):3308-3320.


Chapter 4: Objectives

The primary objective is to characterise the spatial and temporal relationship of Cx43 in the retina, optic nerve head and optic nerve in an *in vivo* rodent model of partial optic nerve injury.

The secondary objective is to relate any changes in Cx43 to changes in RGC numbers and CNS inflammatory markers, such as glia, to aide in determining the possible roles of Cx43 in optic nerve injury.

Given the current evidence in the literature, it is hopeful that this thesis will provide the foundation for Cx43 modulation as a neuroprotective mechanism following acute optic nerve injury.
SECTION 2: METHODS
Chapter 5: Materials and Methods

5.1 Animals

Wistar rats (250-350g) were obtained from the Vernon Jansen Unit (VJU), University of Auckland. All animal procedures in this study were approved by the Animal Ethics Committee at the University of Auckland and were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were housed under standard conditions and fed food and water ad libitum.

5.2 Surgical Procedures

5.2.1 Partial Optic Nerve Transection

The technique of partial optic nerve transection was adapted from methods outlined by Levkovitch-Verbin et al.¹ Prior to surgery, animals were anaesthetised with an intraperitoneal ketamine (80mg/kg) and xylazine (4mg/kg) mixture, and topical benoxinate 0.4% eye drops. Intraperitoneal anaesthesia was preferred to inhalational agents that have been shown to modulate Cx43 gap junctions.² To exclude intraocular pathology, pupil dilating eye drops (tropicamide 1.0%) were instilled and contact fundoscopy was performed with a glass slide.

A transverse incision was made close to the superior limbus through the conjunctiva and episclera that was large enough to admit two pairs of forceps. Minimal blunt dissection with straight forceps was required to visualise the optic nerve. With curved forceps, an assistant then gently retracted the globe and soft tissue back to fully expose the intraorbital segment of the optic nerve.

The methodology and parameters for partial optic nerve transection were finalised in a protocol development experiment (see Chapter 6.1). Briefly, the meninges (dura and arachnoid mater)
overlying the optic nerve were carefully divided superiorly, exposing the pia mater overlying the axon bundles. A 200µm incision was then made transversely across the optic nerve with diamond step knife and 45° blade (HUCO® VISION, Switzerland) at a point approximately 0.5mm behind the eye. Fundoscopy of the experimental eye was repeated to ensure patency of blood flow to the eye. The conjunctival incision was left to heal by secondary intention.

The same procedure was performed for sham surgery, although the diamond step knife was sheathed and placed on the exposed optic nerve.

5.2.2 Tissue Extraction and Timepoints

Animals were euthanatised by carbon dioxide (CO₂) inhalation at 4 hours, 8 hours, 24 hours, 3 days, 7 days, 14 days, 28 days and 56 days following partial optic nerve transection. A single conjunctival suture (6/0 silk, Ethicon®) was placed at the superior limbus for orientation.

To enucleate the eye, a 360° peritomy through tenon’s capsule (down to bare sclera) was performed at the equator of the globe. Extraocular muscles were detached and soft tissue was carefully dissected to free up the distal optic nerve. Enucleation was completed by severing the optic nerve approximately 3mm from the globe under gentle retraction, leaving an intact globe and intraorbital nerve specimen. Finally, the nerve segment was carefully stripped of any remaining meninges or soft tissue. The entire extraction process took less than 5 minutes per eye. To minimise physiological uncoupling of gap junctions post-mortem, tissue processing for analysis was performed immediately.

5.3 Tissue Processing

Tissue was either processed as cryosections, for histological, immunohistochemical and TUNEL analysis, or fresh frozen samples for Western blot and real-time PCR analysis.
5.3.1  Cryosections

Following extraction, tissue samples (globe with nerve segment) were immediately post-fixed by submersion in 1% paraformaldehyde for 30 minutes. Thorough rinsing with 1 x Phosphate Buffered Solution (PBS; Dulbecco A tablets from Oxoid Ltd, Hampshire, England) was followed by overnight cryoprotection with 15% sucrose. Samples were then embedded in Tissue-Tek Optical Cutting Temperature (OCT) medium (Sakura Finetek, Torrance, CA) before being snap frozen in liquid nitrogen. Frozen tissue blocks were stored in a -80°C freezer until required for cryosectioning.

Sagittal cryosections of 16µm thickness were cut at -20°C using a Microm HM550 Cryostat (Thermo-Scientific, Waltham, MA) and 3 sections were mounted on each Superfrost Plus electrostatic slide (Menzel-Gläser, Braunschweig, Germany). Cryosections eccentric to the optic nerve head were discarded. The middle sections of each slide were digitally photographed and, after the slides had dried for 30 minutes at room temperature, were stored at -20°C.

5.3.2  Fresh Frozen Samples

Following extraction, the optic nerve was divided from the globe. The optic nerve was divided into two segments, one including the injury site measuring approximately 1mm and a second segment distal to the injury of approximately 2mm. Each of these was stored in an RNA-free eppendorf (Ambion, Applied Biosystems) and snap frozen with liquid nitrogen.

The cornea was removed from then removed from globe and the lens was gently expressed. The remaining optic cup was divided into superior and inferior halves and the retina was gently peeled off the underlying choroid. Each retinal half was stored in an RNA-free eppendorf and snap frozen with liquid nitrogen.

5.4  Histological Analysis
Cryosections were stained using Mayer’s haematoxylin and eosin (1%) following standard histological procedures. In brief, slides were first warmed up to room temperature for 1-2 minutes, rinsed in tap water for 4 minutes to wash off the OCT medium. A dehydration (2 minutes of 70%, 95%, 100% ethanol; 4 minutes of pure xylene) and rehydration cycle (2 minutes of 100% 95%, 70% ethanol; 2 minutes of tap water) preceded staining in Mayer’s haematoxylin for 10 minutes. Excess stain was rinsed off in tap water and slides were then stained in eosin for 2 minutes. After rinsing in tap water, sections were further dehydrated through 95% ethanol and three times in 100% absolute ethanol, cleared in pure xylene three times, and mounted using a DPX mountant (BDH, Poole, UK).

5.5 Immunohistochemical (IHC) Analysis

Cryosections were warmed up to room temperature for 1-2 minutes and thoroughly rinsed in 1 x PBS to wash off the OCT medium. If either single or double labelling for Cx43, cryosections were further fixed in -20°C cold ethanol for 10 minutes, otherwise this step was missed. After a thorough rinse in phosphate buffered solution (PBS), sections were incubated for 1 hour at room temperature in the appropriate blocking buffer to prevent non-specific binding (10% normal goat serum (Invitrogen Corp., Carlsbad, CA) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for all except Brn3a labelling in which 10% horse serum (Invitrogen Corp., Carlsbad, CA) was substituted).

Primary antibodies were applied overnight at 4°C for Cx43 (rabbit polyclonal anti-Cx43, 1:2000, Sigma, catalogue number C-6219), astrocytes (mouse monoclonal anti-glial fibrillary acidic protein (GFAP)-Cy3 conjugate, 1:1000, Sigma, catalogue number C9205), microglia/macrophages (mouse monoclonal anti-OX-42 (CD11b), 1:100, Serotec, catalogue number MCA275B; mouse monoclonal anti-ED1 (CD68), 1:100, Serotec, catalogue number MCA341R), neutrophils (mouse monoclonal anti-myeloperoxidase (MPO), 1:10, abcam, catalogue number ab16886), retinal ganglion cells (anti-Brn-3a (C-20), 1:500, Santa Cruz Biotechnology Inc., catalogue number sc-31984) and blood vessel
endothelial cells (mouse monoclonal isolectin IB4 (IL-IB4)/Alexa 594 conjugate, 1:100, Molecular Probes, catalogue no. I21413). Where possible, mouse monoclonal primary antibodies were chosen to allow for double labelling with rabbit polyclonal anti-Cx43.

After rinsing 3 x 15 minutes in PBS, appropriate secondary antibodies were applied for 2 hours at room temperature including goat anti-rabbit Alexa 488 (1:1000, Invitrogen, catalogue number A-11034), goat anti-mouse Alexa488 (1:500, Invitrogen, catalogue number A-11029), goat anti-mouse Cy3 (1:500, Jackson ImmunoResearch Laboratories Inc., catalogue number 115-165-003) and donkey anti-goat Cy3 (1:1000, Jackson ImmunoResearch Laboratories Inc., catalogue number 705-165-147).

After rinsing 3 x 15 minutes in PBS and carefully drying each slide, the sections were mounted in ProLong® Gold Antifade reagent with DAPI (Invitrogen Corp, Carlsbad, CA) and #1 50mm coverslips were varnished in place.

Immunofluorescence was analysed by Leica DMRA microscope fitted with a Nikon Digital DS-U1 camera and an Olympus FV1000 confocal laser scanning microscope with Olympus FluoView software (version 1.7a). Single slice confocal images were captured at a speed of 4.0µs/pixel, with a resolution of 1024 x 1024 pixels, using a Kalman average of 4 and on sequential mode if using multiple lasers. Gain and offset were standardised using control tissue for all analyses. The software provided the ability to overlay retinal images from multiple lasers.

5.5.1 Optic nerve and nerve head IHC analysis

5.5.1.1 Optic nerve Cx43

Optic nerve and nerve head Cx43 was performed qualitatively on 3 sections of 6 animals per timepoint. Images of the optic nerve were taken at 10x and 20x magnification, centred on the injury site. A further 20x image was captured at the optic nerve head. Qualitative analysis was performed by comparing the intensity and distribution of Cx43-ir between images. For optic nerve head analysis, qualitative differences were compared between prelaminar, laminar and postlaminar
regions of the optic nerve head, as well as the peripapillary region of the retinal nerve fibre layer.

The defining boundaries of each of these regions were as per those used in similar studies by other authors. Specifically, the laminar region is said to be that region of the optic nerve that appears in line with the posterior sclera and contains the fibrous lamina cribrosa. The laminar region is also significant in that it contains the ‘transition zone’ in which the nerve fibres of the optic nerve undergo myelination. The prelaminar region is anterior to this and comprised of unmyelinated fibres, while the postlaminar region is posterior and comprised of myelinated nerve fibres.

5.5.1.2 Optic nerve astrocytes

The same sections and images used for optic nerve Cx43 analysis were used for optic nerve astrocyte analysis, since they were doubled labelled for GFAP and Cx43. Qualitative analysis was performed by comparing the intensity and distribution of GFAP-ir between images. Attention was also paid to any co-localisation between GFAP-ir and Cx43-ir using overlayed images.

5.5.1.3 Other Inflammatory markers

One animal per timepoint was analysed with inflammatory markers for activated microglia/macrophages (ED1), neutrophils (MPO) and blood vessel endothelial cells (IL-IB4). Cryosections were also doubled labelled for Cx43. Again, 10x and 20x images centred on the lesion site were captured. Qualitative comparison of ED1-ir, MPO-ir and IL-IB4 staining was made between timepoints.

5.5.2 Retinal IHC analysis

5.5.2.1 Retinal Cx43

Retinal Cx43 IHC analysis was performed qualitatively and quantitatively on the same 3 sections of 6 animals per timepoint used for optic nerve Cx43 analysis. Images of the retina containing the RGC, inner nuclear cell and outer nuclear cell layers, were captured at 60x magnification. Three retinal
images were acquired both superior and inferior to the optic nerve head, separated by a distance of one eyepiece field (60x eyepiece field = 300µm).

For qualitative analysis, the distribution and intensity of retinal Cx43-ir was compared between timepoints. Quantitative analysis was performed on the retinal nerve fibre and ganglion cell layers since these were the layers had the highest levels of Cx43-ir. Confocal images were exported as TIFF files to ImageJ software (version 1.41o). The image area was cropped to include the appropriate retinal layers and image type was converted to 8-bit black and white. A consistent threshold of 55 was applied to reduce background immunoreactivity, and an automated immunolabel spot count was then performed. (see Fig. 5-1)

5.5.2.2 Retinal Ganglion Cell Numbers

Brn3a has been validated as a reliable, efficient marker to quantify RGCs in optic nerve injured retinas. In contrast to other retinal images, those taken for RGC analysis were captured at 40x magnification as this was the best compromise between easy RGC discrimination and greater retinal area examined. Two retinal images were acquired both superior and inferior to the optic nerve head, separated by a distance of one eyepiece field (40x eyepiece field = 450µm).

For quantification of RGC numbers, Brn3a TIFF files were converted to 8-bit black and white and a threshold of 33 was applied. A “despeckle” process followed by two “erode” processes was applied to allow for easier discrimination of individual RGCs (see Fig. 5-1). A single, masked observer made manual cell counts on the final images.
Fig. 5-1. Post-imaging analysis for Cx43-ir (A-C) and RGC (D-F) quantification. (A) shows the original single-slice confocal image of Cx43-ir converted to (B) an 8-bit black and white image, with final image (C) after a threshold of 55 has been applied. (D) shows the original single-slice confocal image of BRN3a labelled RGCs converted to (E) an 8-bit black and white image, with final image (F) after a despeckle and 2 erode processes.

5.5.2.3 Retinal Macroglia

The same sections and images used for retinal Cx43 IHC analysis were used for retinal macroglial analysis, since they were double labelled for GFAP and Cx43. For qualitative analysis, the distribution and intensity of GFAP-ir was compared between retinal images. Attention was also paid to any co-localisation with Cx43-ir using overlayed GFAP/Cx43 images. For quantitative analysis, GFAP TIFF files were converted to 8-bit black and white, a threshold of 37 was applied, and an automated area count of immunolabel was performed.

5.5.2.4 Other Inflammatory markers

Qualitative assessment of immunolabelled activated microglia (OX-42 and ED1) and neutrophils (MPO) was performed on retinal sections. A basic quantitative analysis of OX-42 immunolabel was
also performed. Aggregated label, subjectively deemed to represent one cell, was manual counted in individual retinal layers.

5.6 TUNEL assay

Analysis of apoptotic cells by the terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) method was carried out using an ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon International, catalogue number s7111), following the prescribed methodology. Briefly, cryosections were rinsed in PBS before and after being post-fixed in a precooled ethanol/acetic acid (2:1) solution at -20°C for 5 minutes. An equilibration buffer was subsequently applied for a minimum of 10 seconds. Following this, sections were treated with a solution containing terminal deoxynucletidyl transferase and digoxigenin conjugated nucleotides for one hour at 37°C. This step was necessary to attach the digoxigenin-dUTPs to the terminal end of nucleic acids in DNA fragments caused by the apoptotic process. Sections were then incubated in a stop/wash buffer for 10 minutes at room temperature to halt the enzymatic dUTP addition. After washing in PBS, a fluorescein conjugated anti-digoxigenin antibody was applied to the sections for 30 minutes at room temperature, followed by further PBS washing. The assay was completed by mounting the slides with Prolong Gold anti-fade mounting medium on coverslips.

The distribution of TUNEL positive cells in the optic nerve and retina was compared between different timepoints.

5.7 Real-time polymerase chain reaction analysis

To determine the presence of Cx43 mRNA real-time quantitative polymerase chain reaction (RTqPCR) was performed. Three animals were selected from each of the control and 3 day post-
transection groups. Extracted retinal samples were frozen immediately in liquid nitrogen. Total RNA was isolated from the experimental and sham retinal samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method. Samples were homogenized with extraction RNA reagent (TRizol; Invitrogen, Carlsbad, CA) and purified using the PureLink™ RNA Micro Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. A Nanodrop Spectrophotometer (ND-1000; Thermo-Scientific, Wilmington, DE) was used to assess RNA quantity and quality. One hundred nanograms of total RNA was reverse transcribed into cDNA using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA) in a 20 µL reaction mixture under the following conditions: 25°C for 10 minutes; 42°C for 120 minutes; and 85°C for 5 minutes. The cDNA product was then amplified using PCR. PCR was performed using a Rotor-Gene™ 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). The reaction was initiated using a commercial system (FastStart Universal SYBR Green Master (Rox); Roche, Basel, Switzerland) according to the manufacturer’s specifications. The 50 µl reaction mixture consisted of 25 µl of green nucleic acid gel stain (FastStart Universal SYBR Green Master (Rox); Roche, Basel, Switzerland), 5 µl of cDNA, 0.5 µl of forward primer, 0.5 µl of reverse primer, and 19 µl of PCR-grade water. The primers used for connexin43 were 5’-GATTGAAGAGCACGGCAAGG-3’ (sense) and 5’-GTGTAGACCGGCTCAAG-3’ (anti-sense). The following conditions were used for 40 cycles: denaturation at 95°C for 10 minutes; annealing at 95°C for 15 seconds; and extension at 60°C for 1 minute.

The expression of connexin43 was normalised to β-actin, a house-keeping gene. The primers used for β-actin were 5’-GATTGGACCCACGCTTCTACA-3’ (sense) and 5’-ACTTTGGCTCATCTTTTCACGTTGG-3’ (anti-sense). Each PCR reaction was repeated three times for every eye at all time points. In addition, negative controls were performed without reverse transcriptase.

5.8 Western blot analysis
For Western blot analysis, eyes from three control and three animals 3 days post transection were enucleated and the retina dissected and immersed in liquid nitrogen. Samples were homogenized in ice-cold phosphate buffered saline containing 150 mM sucrose, 15 mM 4-(2-hydroxyethyl)-1-piperezineethanesulfonic acid (pH 7.9), 6 mM potassium chloride, 2 mM EDTA acid (pH 8.0), 1mM EGTA (pH 8.0), and a protease inhibitor (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland). Following the addition of 1% Triton-X100 (Sigma-Aldrich, St. Louis, MO), the homogenate was incubated for 1 hour on ice. The supernatant was collected after centrifugation at 10,000 rpm for 10 minutes and combined with a loading dye (125mM tris (pH 6.8), 0.8% sodium dodecyl sulfate (SDS), 2% glycerol, and 0.1% 8-mercaptoethanol).

Equal amounts of protein were loaded onto a 10% SDS polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. BenchMark™ Protein Ladder (Invitrogen, Carlsbad, CA) was included as a molecular weight standard. After incubation in blocking solution (5% non-fat milk powder and 0.1% Tween-20 in Tris-buffered saline (TBS)) for 1 hour at room temperature, the membrane was incubated sequentially with 1:6500 rabbit polyclonal anti-connexin43 antibody and 1:4000 rabbit anti-GAPDH antibody for 12 hours at 4°C. The membrane was then washed thoroughly with 0.1% Tween-20 in TBS and incubated with Alexa488-conjugated secondary antibodies at a dilution of 1:10000 for 1 hour at room temperature. Blots were washed with 0.1% Tween-20 in TBS before detection using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ). Chemiluminescence was detected using the LAS-3000 Imaging System (Fujifilm, Tokyo, Japan) and analysed using ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD).

5.9 Statistics
GraphPad Prism 5 for Windows was used to perform all statistical analyses. Statistical analysis on data employed Student’s t-test and one-way ANOVA when appropriate. P-values of <0.05 were considered to be significant.

5.10 References

SECTION 3:

RESULTS
Chapter 6: Optic Nerve Results

6.1 Injury model validation experiment

The importance of a consistent optic nerve injury was deemed central to inter-animal comparability for all experimental results. Any differences would be particularly masked in later timepoints, where progressive changes to injury morphology would mean that the depth of the original optic nerve lesion would be too difficult to estimate.

Optimisation of the partial transection injury model specific to the laboratory’s equipment was thus an essential first goal. During protocol development, it was noted that there was macroscopic variation in injury consistency between animals. Furthermore, the meninges covering the intraorbital optic nerve meant that it was necessary to dial up the diamond step-knife to a greater depth to achieve a transection injury involving the superior third of the optic nerve. As experience was gained from trial and error of various techniques and video footage from the model’s creators, it was apparent that a 300µm transection with meninges intact and a 200µm transection without prior dissection of superior meninges were the two leading injury protocols.

An experiment was therefore designed to determine which of the two techniques would prove most consistent. Eight animals were randomised between the two groups. A single masked surgeon (SSLC) performed all 8 injuries. Four hours later, the 8 animals were euthanised, tissue was extracted and processed into cryosections. Using the digital photographs of cryosections, the slide with the deepest injury and the two slides 100µm either side of this point were selected for H&E staining. Once stained for H&E, two masked observers qualitatively and quantitatively compared the injury depth and consistency between all 8 animals. Qualitatively, the injury depth was much more consistent in the group with meninges removed (see Fig. 6-1).
Fig. 6-1. Sagittal H&E images (10x) of the injury site (arrowhead) 4 hours after partial optic nerve transection. Large variation between injury depths and morphology is seen with the meninges intact technique used in (A) – (C). Smaller variation is observed with meninges off technique used in (D) to (F). RET = retina; ON = optic nerve; sup = superior surface; inf = inferior surface

6.2 Optic nerve H&E

The uninjured optic nerve has an orderly longitudinal arrangement of cell nuclei and the histological changes at due to optic nerve injury can be viewed in Fig. 6-2. Partial transection of the optic nerve caused bending of the nerve at the injury site that led to wound gaping at the cut edges, evident from 4 to 24 hours. From 3 days onwards this potential space created by the wound gape was slowly filled with disorganised cell nuclei and connective tissue, forming a cap-like scar over the superior aspect of the lesion by 14 days onwards. Disruption of the orderly nuclear arrangement was evident at the injury site and the adjacent tissue immediately proximal and distal to the cut ends, but inferior to the lesion site the linear architecture optic nerve nuclei remained preserved.
Fig. 6-2. Sagittal H&E images of injury site. Compared to (A) control nerve, obvious wound gaping is seen from (B) 4 hours onwards. By (E) 3 days to (F) 7 days, cellular material and disorganised material is beginning to fill the space left by the wound gape. At (G) 14 days and (H) 28 days, a “cap” of disorganised connective tissue now overlies the injury site. RET = retina; ON = optic nerve; sup = superior surface; inf = inferior surface

6.3 Optic nerve Cx43

Control tissue showed a relatively even distribution of punctate Cx43-ir along the length of the optic nerve, though at low magnification, it was evident that the immunolabel was organised into irregular columns along the long axis of the optic. There were also scattered areas of increased Cx43-ir that surrounded unlabelled areas. Double labelling with isolectin-IB4, an endothelial marker, showed that these scattered areas of increased Cx43-ir were orientated around blood vessels whose lumens were devoid of Cx43-ir. (See Fig 6-3.)

Fig. 6-3. Single-slice confocal images (20x) of Cx43-ir (green) and Isolectin IB4 label (red) in the control optic nerve. (A) depicts the distribution of Cx43 immunolabel in irregular columns that follow the long axis of the optic nerve. (B) is the same slice with isolectin IB4 label overlay (red), demonstrating the distribution of blood
vessels in relation to Cx43. The arrowheads show an example of an area of increased Cx43-ir surrounding a blood vessel cut in sagittal section.

As early as 4hrs after partial transection injury, a zone increased Cx43-ir was observed at cut edges of the optic nerve lesion site. This zone appeared to spread toward the centre of the nerve by 8 to 24hrs, but the increase in immunoreactivity was less marked. From 3 to 56 days, Cx43-ir was decreased within the centre of the lesion site. However from 14 to 56 days, increased Cx43-ir was observed in a band overlying the injury site superiorly that was very obvious by 56 days post-injury. No definite change in intensity or distribution of Cx43-ir was observed in areas of the optic nerve proximal and distal to the lesion site. (See Fig. 6-4)
Fig. 6-4. Single slice confocal images (20x) of the optic nerve lesion site labelled for Cx43 (green). The baseline distribution of Cx43-ir is shown in (A) control tissue. By (B) 4 hours post-injury, a clear zone of increased Cx43-ir surrounds the cut edges of the optic nerve. The Cx43-ir zone becomes less distinct by (C) 8 hours and (D) 24 hours, however looks to have extended around the lesion site (as depicted by white dotted lines). At (E) 3 days, there is a marked decrease in Cx43-ir at the centre of the lesion site and no clear areas of increased Cx43-ir. These observations remain consistent at (F) 7 days, (G) 14 days, (H) 28 days, despite the lesion site taking on a more bullous appearance from (G) 14 days. From (G) 14 days onwards, a new zone of increased Cx43-ir is
evident superficial to centre of the lesion that is particularly obvious by (I) 56 days. * denotes the centre of the lesion sites.

6.4 Optic nerve astrocytes

Astrocytes were immunolabelled with GFAP, an intermediate filament protein that is thought to be specific for astrocytes in the CNS (see Fig 6-5.). In comparison to the punctate nature of Cx43-ir, GFAP-ir was more confluent with areas of branching, consistent with the morphology of individual astrocyte cells. In control tissue, the distribution of GFAP-ir paralleled that of Cx43. While there was even distribution across the optic nerve, immunolabel was again orientated in longitudinal columns. Astrocytes are known to envelop blood vessels, thus the columns of absent GFAP-ir are likely to represent blood vessels cut in their long axis.

Following partial transection injury, the cut edges of the optic nerve were observed to have increased GFAP-ir by as early as 4 hours. This change was more obvious by 24 hrs to 3 days. GFAP-positive cells extended from the cut edges around the injury site from 7 to 56 days, and progressive disorganisation of GFAP-positive cell was evident with loss of longitudinal alignment and lengthening of astrocyte processes. Increased GFAP-ir in cells surrounding the lesion indicates that astrocytic activation was occurring both early at the cut edges, and late in a GFAP positive cap overlying the injury site.
Fig. 6-5. Single slice confocal images (20x) of the optic nerve lesion site labelled for GFAP (red). The baseline distribution of GFAP-ir is shown in (A) control tissue. By (B) 4 hours post-injury, a clear zone of increased GFAP-ir surrounds the cut edges of the optic nerve. The zone of GFAP-ir remains well confined to the cut edges at (C) 8 hours, (D) 24 hours and (E) 3 days. From (F) 7 days, the zone of increased GFAP-ir extends around the lesion site and by (G) 14 days, a cap of GFAP-positive cells now spans the gap left by the cut edges, obvious in earlier timepoints. This cap enlarges by (H) 28 days and (I) 56 days.
6.5 Colocalisation of Cx43/GFAP

In control tissue, significant colocalisation of Cx43-ir and GFAP-ir was noted representing that the majority of Cx43 was expressed on optic nerve astrocytes. (See Fig 6-6.)

Although an upregulation of both Cx43 and GFAP at the lesion site was observed in the hours after injury, the zones of increased immunoreactivity were not observed to completely colocalise. The zones of increased GFAP-ir were more toward the superior aspect of the injury site, overlying the more inferior zones of increased Cx43-ir. This raised the possibility that either other cell types were expressing Cx43 at the lesion, or that the astrocytes surrounding the GFAP upregulation were expressing increased levels of Cx43.

From 3 days till 28 days, although GFAP-ir remained elevated at and around the lesion site, Cx43-ir did not follow this distribution and was decreased in the centre of the lesion. The decrease in Cx43-ir in the centre of the lesion may represent GFAP-positive astrocytes that do not express Cx43. By 56 days, there was an increase in Cx43-ir in a band overlying the lesion which corresponded to GFAP-positive cells.
Fig. 6-6. Superimposed single-slice confocal images (20x) of the optic nerve lesion site labelled for Cx43 (green) and GFAP (red). Areas of yellow represent colocalisation. In (A) control tissue, significant colocalisation is seen, except for the Cx43 seen in endothelial cells (arrow). By (B) 4 hours, (C) 8 hours and (D) 24 hours post injury, increased areas of colocalisation are seen generally deep to cut edges, but the edges themselves remain absent of Cx43. By (E) 3 days to (F) 7 days post injury, a predominance of GFAP-ir at the lesion edges with absence of Cx43-ir is more obvious. By (G) 14 days and (H) 28 days, there is minimal colocalisation at the lesion centre. Finally at (I) 56 days, while there is still minimal colocalisation at the lesion centre itself, a band of colocalisation is evident superficial to this. * denotes centre of lesion sites.
6.6 Other inflammatory markers in optic nerve

The response of macrophages and neutrophils to partial transection injury was examined by immunolabelling with ED1 and MPO. Double-labelling was performed with Cx43 to determine if these cell types were expressing Cx43.

Control tissue showed minimal ED1-ir or MPO-ir throughout the optic nerve. After injury, MPO-positive cells were observed at the lesion site by 8hrs but not later. This indicates an early neutrophil response to optic nerve injury that is not prolonged. Cx43-ir did not colocalise with MPO-ir, but was generally abundant around MPO-positive cells and in fact some immunolabel was closely approximated to MPO-positive processes. Since MPO labels lysosomes of a neutrophil, it may be that some of the Cx43 was located on the neutrophil cell membranes. (see Fig. 6-7)

Fig. 6-7. Superimposed single-slice confocal images of the optic nerve, stained for MPO (red), Cx43 (green) and DAPI (blue). In (A) control nerve (20x), minimal MPO-ir is observed. By (B) 8 hours after injury (20x), MPO-positive cells are observed predominantly at the cut edges of the lesion. A “z-stacked” high magnification view (200x) of (C) an MPO-positive cell indicates Cx43 immunolabel is closely approximated but does not colocalise with MPO-positive processes.
ED1-positive cells were observed at the lesion site from 8 to 24 hours. By 3 to 7 days after injury, the majority of the ED1-positive cells were located distal to the lesion where axons are known to degenerate following injury. A more widespread distribution of ED1-positive cells was observed at 14 days and even by 28 days, ED1-positive cells remained scattered over the length of the optic nerve. (see Fig. 6-8) Colocalisation with Cx43 was not observed.

Fig. 6-8. Superimposed single-slice confocal images (10x) of the optic nerve, stained for ED1 (green) and DAPI (blue). In (A) control nerve, minimal ED1-ir is observed. By (B) 7 days after injury, ED1-ir is observed distal to the injury site (arrowhead). The increase in ED1-ir becomes more diffuse by (C) 14 days and (D) 28days post-injury.
To assess for any changes in vasculature around the lesion, sections were labelled for isolectin-IB4, an endothelial marker. Apart from the blood vasculature appearing a little more disorganised around the lesion site, not much qualitative change in vessel distribution or calibre was observed (images not shown). However at 3 days after injury, a dramatic influx of isolectin-IB4 positive cells surrounded the lesion, which were presumed to be activated microglia given that these are also labelled by isolectin-IB4. Their amoeboid morphology was starkly different to the ramified resting microglia (see Fig. 6-9)

![Fig. 6-9. Superimposed single-slice confocal images (20x) of the optic nerve, stained for isolectin-IB4 (red) and Cx43 (green). Compared to (A) control nerve, an influx of amoeboid isolectin-IB4 positive cells was evident at (B) 3 days after injury.](image)

### 6.7 Optic nerve TUNEL analysis

TUNEL analysis was used to assess the degree of apoptotic cell death occurring in the optic nerve following partial transection. Within control tissue, minimal TUNEL labelling was evident representing very little cell turnover by apoptosis.
After partial transection, a few in TUNEL-positive cells were observed by 8 hrs, steadily increasing to a maximum by 3 days. While most of the TUNEL-positive cells were located around the lesion site, there were a significant number spread along the length of the optic nerve both superior and inferior to the lesion. TUNEL-positive cells decreased in number from 7 days till 28 days, however interestingly did not return to control levels.

Fig. 6-10. Superimposed single-slice confocal images (10x) of the optic nerve, stained with TUNEL (green) and DAPI (blue). Very few TUNEL-positive cells are evident in (A) control nerve. An increase in TUNEL-positive cells is seen from (B) 24 hours to a maximum at (C) 3 days post-injury. TUNEL-positive cell numbers decreased from (D) 7 days, through till (E) 14 days and (F) 28 days post-injury.

6.8 Optic nerve head analysis

Similar to optic nerve tissue, the control optic nerve head (ONH) showed an even distribution of punctate Cx43-ir and GFAP-ir in the prelaminar, laminar and postlaminar regions. Following optic
nerve injury, changes in the pattern of Cx43 and GFAP immunolabelling were noted in the optic nerve head (ONH) and peripapillary retinal nerve fibre layer (PPNFL).

Cx43-ir was transiently increased throughout the laminar region of the ONH between 72 hours and 7 days following optic nerve injury, returning to normal levels by 28 days. Unlike the optic nerve injury site, no superior to inferior difference in Cx43-ir was observed within the area of Cx43 upregulation. In the prelaminar and postlaminar regions of the ONH, Cx43-ir did not differ from control images at any time point examined. No change in GFAP-ir was observed in any region of the ONH. (see Fig. 6-11)
Fig. 6-11. Single-slice confocal images (20x) of the optic nerve head, stained for Cx43 (green) and GFAP (red). An even distribution of Cx43-ir is observed in (A) control nerve head. An increase in Cx43-ir is evident in the laminar region (arrowheads) at (B) 3 days and (C) 7 days post-injury. No change in GFAP-ir is seen in (D) after injury.

In the PPNFL, an increase in Cx43-ir and GFAP-ir was observed 8 hours after optic nerve injury compared to controls. Again, the increase in Cx43-ir occurred in both the superior and inferior PPNFL despite the original injury being confined to the superior optic nerve. A secondary increase in GFAP-ir in both the superior and inferior PPNFL was observed from 72 hours to 28 days.

Fig. 6-12. Superimposed single-slice confocal images (20x) of the parapapillary retinal nerve fibre layer (PPNFL) and optic nerve head, stained for Cx43 (green) and GFAP (red). A small amount of Cx43-ir and GFAP-ir is observed in (A) control PPNFL. An increase in Cx43-ir and GFAP-ir is evident in the superior and inferior PPNFL (arrowheads) at (B) 8 hours and (C) 28 days post-injury.
Chapter 7: Retinal Results

7.1 Retinal H&E

A decrease in cells of the GCL was qualitatively observed in the superior retina at 28 days following optic nerve partial transection (see Fig. 7-1). Pyknotic cells were also observed in this layer. Little histological change was seen in the other retinal layers.

![Fig. 7-1. 10x images of H&E labelled retinas. Cells in the GCL of (A) control retina are more abundant than those of (B) retina 28 days post injury.](image)

7.2 Retinal Cx43

In control tissue, Cx43-ir was primarily present in the retinal nerve fibre layer (RNFL) and ganglion cell layer (GCL), but was sparsely present in the superficial retinal layers (see Fig. 7-2). In the RNFL and GCL, Cx43-ir was found to predominantly co-localise with GFAP-ir, representative of retinal astrocytes or Müller cell endfeet (see Figs.7-3 and 7-4). However, minimal co-localisation of Cx43-ir was observed in the RNFL and GCL with GS-ir, a marker for Müller cells, and with IL-84-ir, a marker
for blood vessel endothelial cells (see Fig. 7-3). In the outer retinal layers, Cx43-ir primarily co-localised with IL-B4-ir, but not with GS-ir or GFAP-ir. (see Fig. 7-3)

Fig. 7-2. Superimposed single slice confocal images of retinal sections labelled for Cx43 (green) and DAPI (blue) showing a predominance of Cx43-ir in the RNFL and GCL, with minimal Cx43-ir in the superficial retinal layers. Scale bar = 50µm
Fig. 7-3. Superimposed single slice confocal images of retinal sections labelled for Cx43 (green), DAPI (blue) and various markers (red). In the RNFL and GCL, (A) shows co-localisation between Cx43-ir and GFAP-ir (red), (B) shows minimal co-localisation between Cx43-ir and IL-B4-ir (red), and (C) shows minimal co-localisation between Cx43-ir and GS-ir (red). In the superficial retinal layers, (D) shows no co-localisation between Cx43-ir and GFAP-ir, (E) shows some co-localisation between Cx43-ir and IL-B4-ir and (F) shows no co-localisation between Cx43-ir and GS-ir. All images are same magnification. Scale bar = 30 µm

Following injury, differences in superior retinal Cx43-ir were observed in RNFL and GCL, but not in the other retinal layers. A qualitative increase in retinal Cx43-ir was seen in the RNFL and GCL of the superior retina by 3 to 7 days following injury that disappeared by 14 days. A further rebound in superior retinal Cx43 was observed at 28 days, which appeared to normalise by 56 days (see Fig. 7-4). There was no apparent qualitative change in Cx43-ir in the inferior retina at any timepoint. No obvious change in Cx43-ir was observed in the superior or inferior outer retinal layers that would represent Müller cell Cx43 gap junctions.
Fig. 7-4. Superimposed single slice confocal images of superior retinal sections labelled for Cx43 (green), GFAP (red) and DAPI (blue). Baseline Cx43-ir and GFAP-ir displayed in (A) control retina, is predominantly seen in RNFL and GCL. No significant change in Cx43-ir or GFAP-ir is seen at (B) 8 hr following injury. Increased Cx43-ir is seen at (C) 24 hr, (D) 3 days, (E) 7 days, and maximally at (G) 28 days following injury. Decreased Cx43-ir is seen at (F) 14 days, and (H) 56 days following injury. A progressive increase in GFAP-ir is seen from (D) – (H), reaching significance from (D) 3 days. All images are same magnification. Scale bar = 50 µm

Quantitative analysis revealed a biphasic response of Cx43-ir in the retina peaking at days 3 and 28 post injury (see Fig. 7-5). The changes in Cx43 spot counts for the total, superior and inferior retina are outlined in Table 7-1. Superior retinal Cx43-ir was significantly increased compared to controls as early as 24 hours following injury (141.7% of controls, SD ± 53.1, p=0.0063) and continued to rise at 3
days (192.1% of controls, SD ± 88.7, p=0.0002). By 7 days, Cx43-ir had dropped to 136.6% (SD ± 41.3, 
p=0.0043) of controls, and at 14 days, levels were significantly lower than controls (73.8% of 
controls, SD ± 18.0, p=0.0028). A further maximal increase in Cx43-ir occurred at 28 days (212.1% of 
controls, SD ± 90.1, p<0.0001), followed by another decrease at 56 days (72.5% of controls, SD ± 
37.6, p=0.0232). No significant change in inferior retinal Cx43-ir was noted except at 28 days 
following injury (127.4% increase c.f. controls, SD ± 40.9, p=0.0481) (see Table 7-1).

![Graph showing quantification of Cx43-ir spot counts per retinal section.](image)

**Fig. 7-5.** Quantification of Cx43-ir spot counts per retinal section. Superior spot counts (light grey bars) and inferior spot counts (dark grey bars) stacked to show total retinal spot counts. Compared to controls, a significant increase in Cx43-ir spot counts is observed in the superior retina at 24hr, 3days, 7days, and 28 days post injury, and in the inferior retina at 28 days. A significant decrease is seen in the superior retina at 14 days and 56 days post injury when compared to control superior retina.
Table 7-1. Retinal Cx43 spot, RGC and GFAP area counts

<table>
<thead>
<tr>
<th>Time Point</th>
<th>N</th>
<th>Cx43 spot count/section</th>
<th>P vs Controls</th>
<th>RGC count/section</th>
<th>P vs Controls</th>
<th>GFAP area count/section (x 10^6)</th>
<th>P vs Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>Total Sup. 265.6 ± 98.3</td>
<td>Sup vs Inf 0.27</td>
<td>Total Sup. 84.5 ± 97</td>
<td>Sup vs Inf 0.83</td>
<td>Total Sup. 13.8 ± 3.9</td>
<td>Sup vs Inf 0.83</td>
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<td>143.4 ± 52.7</td>
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<td>109.4 ± 74.7</td>
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<tr>
<td></td>
<td>7</td>
<td>Total Sup. 265.1 ± 96.8</td>
<td>0.99 Sup vs Inf</td>
<td>Total Sup. 82.0 ± 11.2</td>
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<td>8hr</td>
<td>18</td>
<td>Total Sup. 299.0 ± 116.4</td>
<td>0.36 Sup vs Inf</td>
<td>Total Sup. 79.2 ± 13.5</td>
<td>0.29 Sup vs Inf</td>
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<td>0.25 Sup vs Inf</td>
<td>Total Sup. 81.9 ± 12.7</td>
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<td>Total Sup. 14.7 ± 4.1</td>
<td>0.52 Sup vs Inf</td>
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<td>0.0019 Sup vs Inf</td>
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<td>0.21 Sup vs Inf</td>
<td>Total Sup. 18.9 ± 5.2</td>
<td>0.0025 Sup vs Inf</td>
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<td>Total Sup. 67.8 ± 10.4</td>
<td>0.0005 Sup vs Inf</td>
<td>Total Sup. 19.5 ± 4.7</td>
<td>0.0004 Sup vs Inf</td>
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<td>Total Sup. 185.0 ± 56.1</td>
<td>0.0048 Sup vs Inf</td>
<td>Total Sup. 58.3 ± 14.9</td>
<td>&lt;0.0001 Sup vs Inf</td>
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<td>0.024 Sup vs Inf</td>
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<td>28day</td>
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<td>&lt;0.0001 Sup vs Inf</td>
<td>Total Sup. 39.8 ± 13.7</td>
<td>&lt;0.0001 Sup vs Inf</td>
<td>Total Sup. 28.5 ± 8.3</td>
<td>&lt;0.0001 Sup vs Inf</td>
</tr>
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<td>308.5 ± 131.0</td>
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<td>9.1 ± 4.7</td>
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<tr>
<td>56day</td>
<td>16</td>
<td>Total Sup. 224.6 ± 119.4</td>
<td>0.28 Sup vs Inf</td>
<td>Total Sup. 33.0 ± 10.9</td>
<td>&lt;0.0001 Sup vs Inf</td>
<td>Total Sup. 25.26 ± 14.1</td>
<td>0.0027 Sup vs Inf</td>
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<td>8.0 ± 3.7</td>
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<td></td>
<td>25.0 ± 7.7</td>
<td></td>
<td>12.7 ± 6.8</td>
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</tr>
</tbody>
</table>

*denotes statistical significance
The presence of Cx43 mRNA and protein in control and injured retinal samples was confirmed by RTqPCR and Western blot analysis respectively. It was not possible to accurately quantify relative levels of Cx43 mRNA or protein by either method. Extracted retinal samples were shown through immunohistochemical techniques to contain variable quantities of both neurosensory and pigmented retinal layers. The underlying retinal pigment epithelium, a structure expressing particularly high levels of connexin43, overwhelmed changes occurring in the inner retinal layers which were of a much smaller scale. The inability to separate retinal layers consistently and therefore distinguish the origin of the connexin43 mRNA or protein resulted in RTqPCR and Western blotting not being utilised as a quantitative tool.

7.3 Retinal Astrocytes

Evaluation of the retinal glial response following optic nerve injury was performed by analysis of GFAP-ir and GS-ir. Following injury, a qualitative increase in superior retinal GFAP-ir was observed in the RNFL, presumably in the retinal astrocytes (see Figs. 7-4 and 7-6). A qualitative difference in GFAP-positive Müller cells spanning the layers of the retina was not obvious (see Fig. 7-6). No significant qualitative change in GS-ir was observed in any of the retinal layers following injury (see Fig. 7-6).
Fig. 7-6. Superimposed single slice confocal images of superior retinal sections labelled for DAPI (blue) and glial markers (red). (A) shows GFAP-ir levelled to highlight Müller cell processes extending into superficial retinal layers. Using the same levelling parameters, minimal change is observed in the GFAP-positive Müller cell processes in (B) 28 days following injury. In contrast, although oversaturated, GFAP-ir is increased in the RNFL of (B) compared to (A). No significant change in GS-ir is seen throughout the retina in (C) control retina and (D) 28 days following injury. All images are same magnification. Scale bar = 50 µm

Quantitative analysis of GFAP-ir in the same 3 retinal sections per animal used for Cx43-ir analysis confirmed a progressive increase in area of GFAP-ir in the superior retina that became significant compared to controls at 3 days (153.7% increase, SD ± 55.6, p=0.0017), 7 days (158.2% increase, SD
\[107 \pm 46.1, p=0.0002\), 28 days (224.9\% increase, SD \pm 74.7, p<0.0001) and 56 days (178.9\% of controls, SD \pm 124.2, p=0.0149) after injury. The area of GFAP-ir in the inferior retina was significantly higher than controls at 28 days (186.7\% increase, SD \pm 97.7, p=0.0013) and 56 days (186.2\% of controls, SD \pm 100.0, p=0.0018) after injury. (Table 7-1)

![Graph showing area of GFAP immunolabel](image)

Fig. 7-7. Area of GFAP immunolabel. Compared to controls, a significant increase in area of GFAP-ir is observed in the superior retina at 3 days, 7 days, 28 days and 56 days post injury, and in the inferior retina at 28 days and 56 days. While the superior and inferior GFAP-ir at 14 days is not significantly different from controls, total GFAP-ir at 14 days is. Results are expressed as mean \pm SEM. * denotes statistical significance \(p<0.05\).

The increase GFAP-ir observed following partial transection was paralleled by increasing Cx43-ir except at 14 days, where total Cx43-ir was significantly less than controls \((p = 0.0048)\), and 56 days, where total Cx43-ir was not statistically different from controls \((p = 0.2813)\). Comparatively, total GFAP-ir was significantly higher than controls at these timepoints \((14 \text{ days}, p=0.0243; 56 \text{ days}, \ldots)\).
p=0.0027). This indicates that at these timepoints, GFAP-positive astrocytes were not expressing high levels of Cx43 protein. (see Fig. 7-8)

![Graph showing Cx43 spot counts and GFAP immunolabel areas](image)

Fig. 7-8. Cx43 spot counts and GFAP immunolabel areas parallel each other until day 14, where Cx43 levels drop but GFAP levels remain elevated. Elevation of both Cx43 and GFAP occurs by 28 days following injury, but at 56 days, Cx43 levels drop while GFAP-ir stays elevated. Results are expressed as mean ± SEM.

### 7.4 Retinal Ganglion Cell Numbers

RGC loss was determined by quantitative analysis of BRN3a-labelled RGCs (see Fig. 7-9) in 2 retinal sections per animal with six animals per time point (see Fig. 7-10). Superior RGC numbers following injury became significantly reduced compared to controls at 3 days (84.0% of controls, SD ± 16.2, p=0.0454) and continued to decline 7 days (66.9% of controls, SD ± 11.4, p<0.0001), 14 days (43.8% of controls, SD ± 15.6, p<0.0001), 28 days (21.3% of controls, SD ± 11.1, p<0.0001) and 56 days (18.8% of controls, SD ± 8.8, p<0.0001). A significant decrease in inferior RGC numbers was measured at 28 days (73.4% of controls, SD ± 22.8, p=0.0021) and 56 days (59.6% of controls, SD ± 18.4, p<0.0001) but not earlier (Table 7-1 and Fig. 7-10).
The onset of superior RGC loss at 3 days appeared to coincide with the early peak in superior Cx43-ir, which was initially elevated from 24 hours through till 7 days post injury. Similarly at 28 days, the significant loss of inferior RGCs compared to controls occurred in conjunction with a significant elevation of inferior retinal Cx43-ir. By 56 days, total RGC numbers were not significantly different from 28 days \( (p = 0.2347) \) and total Cx43 levels had returned to levels not statistically different from controls. (see Fig. 7-11)

Fig. 7-9. Superimposed single slice confocal images of retinal sections labelled for Brn3a (red) and DAPI (blue). Baseline Brn3a-positive RGCs displayed in (A) control retina. Arrowhead denotes a displaced amacrine cell in the GCL. A significant loss in superior RGCs is seen at (B) 3 days post-injury, through till (C) 56 days post injury. A significant loss in inferior RGCs is seen at (D) 28 days post-injury. All images are the same magnification. Scale bar = 50 µm
Fig. 7-10. Superior RGC loss becomes significant from 7 days post injury when compared to controls. Inferior RGC numbers are significantly lower than controls from 28 days post injury. Results are expressed as mean ± SEM. * denotes statistical significance p<0.05.
Fig. 7-11. Elevation in superior Cx43 spot counts reaches its first peak at 3 days in conjunction with significant superior RGC loss. At 28 days, a similar elevation in inferior Cx43 spot counts is paralleled by a significant loss of inferior RGCs. Results are expressed as mean ± SEM.

7.5 Retinal TUNEL analysis

TUNEL assay was used to confirm the extent of RGC apoptosis on 3 retinal sections per animal. In the superior retinal RGC layer, TUNEL-positive cells were detected as early as 24 hours (1 cell seen) following injury and reached 4-5 cells at 7, 14 and 28 days following injury. No TUNEL-positive cells in the RGC layer were detected in the inferior retina at any timepoint. (images not shown)

7.6 Other Inflammatory Markers in Retina

In addition to retinal astrocyte activation, OX-42 (microglia) and MPO (neutrophil) immunoreactivity was assessed following injury. A maximal increase in both OX-42-ir and MPO-ir was observed 14 days after injury in multiple retinal layers both in the superior and inferior retina (see Fig. 7-12). A basic quantitative analysis of OX-42-ir in the superior and inferior retina confirmed a consistent increase at 14 and 28 days following injury. (See Fig. 7-13)

No colocalisation was observed between OX-42-ir and Cx43-ir at high magnification (see Fig. 7-12). Because OX-42 is located in the plasma membrane, Cx43 immunolabel surrounding OX-42 positive cells in the GCL is likely to be expressed on other cell types. Similarly, no colocalisation was seen between MPO-ir and Cx43-ir at high magnification. However in contrast to OX-42, MPO is a cytoplasmic label, thus it could not be excluded that the abundance of Cx43 in close proximity was in fact expressed by these MPO-positive cells.
An analysis of retinal vasculature with isolectin-IB4 did not show any qualitative difference in distribution or calibre at any time point following injury (images not shown).

Fig. 7-12. Confocal images of retinal sections labelled for OX42 (red) and DAPI (blue) in (A) to (C), and MPO (red) and DAPI (blue) in (E) to (G). (A) control retina shows minimal OX42-ir. A multilayered increase in OX42-ir is observed at (B) 14 days after injury and (C) 28 days after injury. (D) is a high magnification (192x) “z-stacked” image of an OX42-positive cell (red) double labelled for Cx43 (green) showing no colocalisation. (E) Control retina shows minimal MPO-ir. An increase in MPO-ir is observed at (F) 14 days after injury that dissipates by (G) 28 days after injury. (H) is a high magnification (276x) “z-stacked” image of a MPO-positive cell (red) and Cx43 (green) showing no colocalisation.
Fig. 7-13. A consistent increase in OX42 positive cells was observed in the GCL, IPL, INL and OPL at 14 and 28 days post injury, compared to controls. Results are expressed as mean ± SEM.
SECTION 4:

DISCUSSION
Chapter 8: Discussion

8.1 General Discussion

Diseases of the optic nerve in humans are the second leading cause of blindness worldwide.\textsuperscript{12} Common to all types of optic nerve injury is the consequential loss of RGCs. Understanding the mechanisms behind this loss is thus of great importance scientifically to identify techniques to rescue RGCs from death.

Protection of RGC cell bodies from death is a prerequisite to achieving anatomical and functional restoration of the retinofugal pathway following axonal injury. Various substances through different routes of administration have been tested for their ability to rescue RGCs from death. Despite serial advances including neurotrophic and growth factors, apoptotic inhibitors and excitotoxic blockade, it appears difficult to permanently rescue RGCs that are committed to programmed cell death.

The focus of neuroprotection has thus moved towards prevention of secondary neuronal degeneration. This concept is based on increasing evidence that death of neurons can be associated with damage to other neurons that were not injured by the primary insult.\textsuperscript{3-5} Attempts have been made to identify the mediators of secondary degeneration, with the aim of neutralising them and/or their effects. Studies are increasingly aimed at the protection of neurons which, following acute nerve insult, do not sustain direct injury, but are adjacent to or surrounded by a damaged milieu and will consequently undergo secondary degeneration unless adequately treated.

Distinguishing RGCs that have degenerated as a result of the primary optic nerve injury and those that have degenerated as a result of a secondary process is not as simple as in other parts of the CNS. The first requirement would be a partial injury to the optic nerve that allows for a population of surviving RGCs and axons post-injury. The second requirement would be the ability to differentiate populations of RGC death from primary and secondary mechanisms. Logically, the temporal course of RGC loss following partial injury should help in this differentiation. As suggested
by Yoles et al\textsuperscript{6}, the secondary degeneration process lags in time from the primary RGC loss, both in the later and less severe RGC death in the inferior retina. While this is helpful, it does not provide complete differentiation because no clearly defined timepoint exists to separate RGC loss arising from primary or secondary degeneration. This is because following injury such as complete axotomy, there is a protracted phase of primary RGC loss that significantly overlaps with the beginning of secondary RGC loss seen in a partial injury.\textsuperscript{7} Levkovitch-Verbin et al have since developed a partial transection model that spatially segregates primary from secondary RGC degeneration by exploiting the topographic relationship between RGCs and their axons in the optic nerve. The model has been used successfully by other groups to determine the effects of glatiramer acetate and the calcium channel blocker, lomerizine, on primary and secondary degeneration of the optic nerve and RGCs.\textsuperscript{8-10}

The importance of glia in neuronal survival within the CNS and retina was perhaps underestimated until more recent advances in knowledge. Initially, astrocytes were seen simply as “brain glue” but it has become apparent that they form a highly communicative functional syncitium that is essential to normal neuronal function. Glial-neuronal interactions are complex and can result in neuroprotection via release of various gliotransmitters, antioxidants and upregulation of protective surface receptors\textsuperscript{11,12}, or neurodestruction via the release of free nitrogen and oxygen species.\textsuperscript{13} Glia are also critical in the modulation of inflammation following acute neuronal injury. The inflammatory process is a double-edged sword as it can cause secondary damage up to weeks after injury, yet the subsequent remodelling of brain tissue requires the efficient containment and removal of dead tissue.\textsuperscript{14,15}

Glial communication occurs via gap junctions, specialised cell-to-cell contacts that can transmit nutrients, metabolites, second messengers, cations and anions. A single gap junction consists of two hemichannels, or connexons, each of which is comprised of six connexin proteins. Cx43 is the most abundant glial connexin and is present in CNS astrocytes, including those of the optic nerve and
Following CNS injury, the response of Cx43 varies with severity of injury. Generally, mild to moderate injury appears to lead to increased Cx43-ir in the lesion site or in vulnerable CNS areas, whereas severe injury results in decreased Cx43-ir within the injury site probably due to cell death in that region, and a surrounding zone of increased Cx43-ir in what could be described as the penumbra of the injury in various models. There is little debate that Cx43 gap junction communication is an important mediator following CNS injury. In mild injury increased Cx43 expression may be involved in diffusion or spreading of substances such as potassium and glutamate that might otherwise reach toxic levels. For more extensive injuries, extensive Cx43 gap junction coupling may exacerbate the injury through ATP release, or by spreading toxins and death signals. In fact, the majority of evidence from studies using Cx43 gap junction blockade does indicate that neuroprotection is achieved. Exceptions to this include experiments that use global blockade, which should be interpreted with caution due to the nonspecific nature of agents such as carbenoloxone and octanol, and Cx43-knockout models, which result in permanent pre-injury Cx43 modulation that logically would lack gap junction recovery and thus impact negatively on a return to normal astrocytic homeostasis. Combined, the data suggests that permanent blockade of Cx43 may not be neuroprotective, but transient blockade targeting the window of initial Cx43 upregulation observed following injury is potentially therapeutic. Indeed, there is building in vitro and in vivo evidence for neuroprotection following transient Cx43 specific knockdown using specific Cx43 antisense oligodeoxynucleotides or Cx43 mimetic peptides, which then permit a return to normal Cx43 function.

8.2 Validation of partial transection model and Brn3a as a marker of RGC viability

The partial transection model allows for evaluation of primary and secondary RGC loss following axongenic injury. The model utilises the topographic distribution of RGC axons, in which axons of the upper optic nerve that are cut in a superior, partial transection belong only to RGCs residing in the
superior retina. This topographic relationship is known to be most preserved in the optic nerve closest to the globe, with the superior fibres occupying a more central location in the nerve as the brain is approached. A partial transection of the superior optic nerve close to the globe thus generates within the eye a superior zone of RGC death and an inferior retinal hemifield that acts as a zone affected by secondary degeneration. The model in itself is proof of the existence of secondary degeneration, which some researchers had previously doubted as a separate process from protracted primary RGC loss.

The data from this project confirmed the usefulness of the partial transection model to segregate and study the responses of primarily and secondarily injured RGC and the zones of retina in which they reside. A significant loss of superior RGCs was detected at 3 days following injury (16% loss, \( p=0.0454 \)), which compares relatively well to the original work by Levkovitch-Verbin et al \(^3\) of a 30.3% loss \((p=0.02)\) of superior RGCs at 4 days. By 7 days, superior RGC loss had increased to 33.1% \((p<0.0001)\) whereas Levkovitch-Verbin et al observed almost double the loss (62.8%, \( p=0.0018 \)) at 8 days. More superior RGC loss was observed at this project’s 28 day timepoint (80% loss, \( p<0.0001 \)) than their 4 week timepoint (46.3% loss, \( p=0.012 \)), which perhaps surprisingly showed less loss than at 8 days. In fact, the superior RGC observed by Levkovitch-Verbin et al did not exceed that seen at their 8 day timepoint, whereas in this project the percentage of superior RGC loss continued to decrease throughout all timepoints, a trend seen following complete axotomy injury that followed RGC loss out to 9 months post-injury.\(^7\) Perhaps of most interest was that inferior RGC loss was significant at the same 28 day timepoint with 26.6% loss \((p=0.0021)\) in this project compared to 39.2% \((p=0.0002)\) in Levkovitch-Verbin’s. In both this study as well as Levkovitch-Verbin’s, the inferior RGC loss had stabilised by 8-9 weeks to 40.4% and 34.5% losses respectively. The observation of inferior RGC loss in particular is critical as the model relies on this inferior RGC subset as an anatomical separation and the consequently delayed RGC death that occurs may be a result of spread of excitotoxic damage from RGCs that have died from transection of their axons in the
primary insult. The 40% loss of RGCs at 56 days thus becomes a therapeutic target for neuroprotective interventions.

There are certain inherent limitations to the partial transaction injury model. Injury consistency is a potential issue due to the mobility of the optic nerve in the orbit. A shallow lesion may result in less change in immunohistochemical markers, and a lesion that passes into the inferior optic nerve due to depth or inadvertent rotation of the optic nerve would damage inferior optic nerve axons and lead to inferior immunohistochemical changes unrelated to delayed death. However, in this project no significant inferior RGC loss was seen at 7 days, which is known to be the time when almost 50% of axotomised RGCs die.

In this project, immunohistochemical labelling with Brn3a was used to label RGCs. Brn3a is an RGC-specific marker that has previously been favourably compared with Fluorogold retrograde labelling following axotomy. Fluorogold retrograde labelling has known disadvantages that include the potential for infection or death due to the requirement for complete anaesthesia, drilling into the rodent skull, and precise injection of compounds into the brain. In addition, Fluorogold can relocate to other cells of the retina making counting more difficult and less accurate. Brn3a immunohistochemical labelling thus has the potential to provide a faster alternative method to evaluate RGC viability, whilst avoiding the risks of the surgical retrograde procedure.

Overall, the use of Brn3a as a marker of RGC loss compared relatively well with Fluorogold data by Levkovitch-Verbin et al, who used retrograde fluorogold labelling of retinal wholemounts. One should bear in mind a direct comparison is not possible due to differences in the depth of lesion. The depth used in this project was quoted at 200µm with meninges divided compared to 150µm with intact meninges in the Levkovitch-Verbin model.

Other alternative techniques for RGC labelling have been proposed. Thy1, Bex1/2, γ-synuclein, have been trialled as markers compatible for measuring RGC loss with limited success. The major
concerns with Thy1 is that the decrease in Thy1 does not correlate with RGC death, as there are no reports linking Thy1 to RGC survival, and that the Thy1 level decrease following optic nerve injury occurs before Fluorogold detected RGC loss. It is recognised that Brn3a levels also decrease earlier than Fluorogold detected RGC loss following axotomy and thus some have postulated that Brn3a may therefore suffer from similar limitations to Thy1. However, a clear link between Brn3a and RGC survival has been well-established. Brn3a antagonises p53 activation of pro-apoptotic proteins\textsuperscript{40}, Noxa and Bax, and through the activation of survival genes\textsuperscript{41}, Bcl-2 or Hsp27. Therefore, it is more likely that the loss of Brn3a expression happens earlier because it reflects a commitment to death preceding the disappearance of Fluorogold-labelled RGCs by phagocytic clearance.

Another limitation of the RGC labelling technique used was in tissue preparation. This project used retinal sections to label RGCs, which only gives a subset of the RGCs in the retina compared to retinal whole mounting which allows measurement of total RGC counts. Sagittal sections were taken only across the width of the optic nerve head, thus RGC numbers from the retina temporally and nasally eccentric to the optic disc are missing. While this project cannot comment on the total RGC population or any differences between the temporal and nasal retina, it does give an accurate representation of relative RGC numbers in the superior compared to inferior retina.

8.3 Cx43 changes following partial optic nerve transection

This project is the first to identify changes in Cx43 expression both in the optic nerve and retina following an \textit{in vivo} model of partial optic nerve injury.

In the optic nerve, an increase in Cx43 was observed around the transection site as early as 4 hours after injury that was associated with a similar increase in GFAP-ir. The increase in Cx43-ir was still evident at 8 and 24 hours following injury, although less pronounced by then. The early Cx43 response compares well to that seen following models of rodent spinal cord injury. Following acute
compression injury of the rat spinal cord, regions immediately adjacent to the lesion both rostrally and caudally exhibited intensified Cx43-ir at days 1 and 3, with associated reactive astrocytes displaying GFAP-ir. Similarly, following rodent spinal cord transection, an increase in Cx43-ir and Cx43 mRNA-positive cells in the gray matter adjacent to the lesion was observed from 4 hours post-injury that mainly colocalised with GFAP-ir. Another group looked closely at lesion site in a model of focal cerebral ischaemia induced by photothrombosis. In the cortex directly flanking the lesion, Cx43-ir was increased as early as 1 day after injury and was accompanied by an increase in reactive GFAP cells, thought to represent reactive astrocytes. A similar pattern was observed in post-mortem human brain samples with ischaemic damage. Cx43-ir was elevated in the lesion penumbra of both acute and chronic infarcted areas and was again mirrored by increased activated astrocyte numbers as measured by GFAP-ir. Given the above evidence, the increase in Cx43-ir and GFAP-ir observed around the transection site in this project suggest that early after the injury, astrocytes bordering the edge of the partial transection incision are becoming reactive and increasing their Cx43 gap junction communication. This response most likely represents an attempt at buffering of toxic substances released by cellular injury from the transection site to neighbouring healthy astrocytes. Certainly, the increase in Cx43 gap junctions also provides a route for the spread of axonal injury when the limits of toxic buffering are reached. Given that RGC loss was the primary focus, axonal counts in transverse sections of the optic nerve were out of the scope of this project but given these Cx43 findings, it would be of interest in the future to determine if indeed a spread of axonal loss occurs after this early increase in Cx43-ir around the lesion edges.

The increase in Cx43-ir seen in this project at any timepoint was, however, less marked than that seen in the afore-mentioned models of CNS injury and a laser-induced thermal optic nerve injury (unpublished results, T. Papchenko, Department of Ophthalmology, University of Auckland). There are two factors that may explain these differences. Firstly, the type of injury may influence the astrocytic response. A partial transection provides a small precise, clean, surgical incision injury compared to irradiation of a larger area of optic nerve with an argon laser creating a burn-like injury.
It is unsurprising that the local reaction to these different insults is less intense after the transection injury, given the likelihood that less necrotic cell death and inflammation occurs. Secondly, it is possible that the response of Cx43 in the CNS differs in grey matter as opposed to white matter. In the models of CNS injury described above, primary injury to the neuronal bodies of the grey matter and axons of the white matter occurred. By comparison, the lesion produced in this project was a purely axogenic injury with somatogenic neuronal damage only occurring as a consequence of the initial injury. In the studies above, most of the changes in Cx43-ir were observed in grey matter such as the cortex and striatum, or in the hippocampus, which contains many neuronal cell bodies. There is less information published on changes in Cx43 in white matter except in models of spinal cord injury, which involve both grey and white matter. These studies indicate that Cx43 upregulation occurs mostly in grey matter surrounding the lesion.

In this project, after the initial increase in Cx43-ir around the lesion site, a decrease in Cx43-ir was observed at the cut edges at 3 and 7 days. Some of this decrease is likely a result of cellular death and thus the inevitable loss of Cx43 protein. However, the presence of Cx43/GFAP co-localisation showed that these areas of decreased Cx43-ir actually corresponded to areas of increased GFAP-ir representative of reactive astrocytes. Thus it is likely that while some of the decrease in Cx43-ir may be due to cellular death, what was observed was predominantly the activation of reactive astrocytes that are not expressing Cx43 protein. This pattern continued from 14 to 28 days when a cap of GFAP-positive reactive astrocytes spanned the cut edges. At these timepoints, the centre of the lesion inferior to the GFAP cap and the cap itself remained low in terms of Cx43-ir. However by 56 days the mature reactive astrocytes, represented by the GFAP cap, co-localised with increased levels of Cx43-ir again while the centre of the lesion remains similar to previous timepoints. This cap of reactive astrocytes has also been termed a glial scar, which has been described following various CNS injuries and diseases. The glial scar is thought of as a physical and molecular barrier for axonal outgrowth, although more recently it has, conversely, been linked to the promotion of axonal sprouting. Haupt et al also described the upregulation of Cx43 in a glial scar following
photothrombotic ischaemic injury to rodent cortex. They observed a band of GFAP-positive reactive astrocytes that expressed high amounts of Cx43 mRNA by day 14 and was visible through till day 60 following injury. They supposed a role for Cx43 in mediating proliferation of the glial scar.

In the optic nerve head, the laminar region showed a transient increase in Cx43-ir at 3 days and 7 days post-injury. However, this increase in Cx43 ir was uniform throughout the laminar region with no predilection for the superior zone that would correspond to injured superior axons. No change in Cx43-ir was detected in the pre and post laminar regions of the optic nerve head post-injury. In addition, there was no visible astrocytosis or increase in GFAP-ir. The predilection for the laminar region of the nerve head points to the possibility that the lower compliance of the fibrous lamina cribrosa may result in additional mechanical damage to axons which swell after anterograde injury due to axoplastic stasis. Another concept is that the axons in the laminar region, which are undergoing myelination, are somehow more susceptible to damage than the other regions.

In the retina, changes in Cx43-ir were different in the superior and inferior halves. More specifically, following partial transection of the superior optic nerve, the corresponding superior retina showed a significant increase in Cx43-ir from as early as 24 hours post injury, at which time TUNEL-positive RGC apoptosis had also begun. Cx43-ir peaked at almost 200% of controls at 3 days following injury, dropping down to subnormal levels at 14 days and increasing to maximal levels by 28 days following injury. The initial increase in superior retinal Cx43-ir preceded significant superior RGC loss, with superior RGC counts 66.9% of controls at 7 days following injury. By comparison, inferior retinal Cx43-ir only significantly changed at 28 days post-injury, which were elevated to 127% of control levels. This was paralleled by significant inferior RGC loss at 28 days, with inferior RGC counts 73% of controls.

A potential limitation of this data was the range of physiologic variability seen with various immunohistochemical markers such as Cx43, as seen with the high standard deviations even in control retinas. To work around this issue, the experimental numbers were increased to 6 per
timepoint for immunohistochemistry and 3 retinal sections per animal were thoroughly examined to create a large numerical database. In addition, attempts were made to quantify the total amount of retinal Cx43 mRNA using RTqPCR and Cx43 protein via western blotting to further elucidate whether the changes in Cx43-ir seen were due to de novo synthesis, or changes in the cellular distribution of Cx43 protein. While both techniques confirmed the presence of Cx43 mRNA and Cx43 protein in both control and injured tissue, quantification results were misleading secondary to contamination of the tissue specimens by retinal pigment epithelium, a structure whose abundance in Cx43 protein masked changes in the neurosensory retina. Current evidence points toward de novo synthesis being the most likely mechanism behind the increase in Cx43-ir observed. Cameron et al\(^44\) showed upregulation of Cx43 transcripts using a DNA microarray and real time PCR analysis following mechanical injury to zebrafish retina. Following focal cerebral ischemia, reactive astrocytes have been shown to envelope the lesion and upregulate Cx43 mRNA protein expression.\(^19\) Complete transection of the adult rat spinal cord led to an upregulation of Cx43 mRNA and protein on GFAP-positive astrocytes within hours, remaining significantly elevated up to 28 days after the injury.\(^20\) However, the possibility remains that the increase in Cx43-ir observed may result from the cellular redistribution of astrocytic gap junction protein from a pool of Cx43 not normally detectable by immunohistochemistry, as proposed by Hossain et al\(^16\) following a global cerebral ischemia model in which Cx43-ir increased but Cx43 protein levels by Western blot did not.

To identify the retinal cell types expressing Cx43, double labelling immunohistochemical techniques were used. Cx43-ir predominantly co-localised with GFAP-ir in the RNFL and GCL, which could represent Cx43 expression by either retinal astrocytes or the endfeet of Müller cells as has been previously studied by Zahs et al\(^45\). However, the minimal co-localisation observed between Cx43-ir and GS-ir, a Müller cell-specific marker, throughout the retina suggests the former is most likely. This is in agreement with Ball et al\(^46\), who demonstrated Müller cell Cx43 staining in the goldfish and mudpuppy retina but not the rat retina, and with the vast amount of literature reporting astrocytic Cx43 (see Chapter 2). In the more superficial layers, Cx43-ir showed some co-localisation with IL-IB4
labelling of blood endothelial cells, which did not change following transection injury. This is in contrast to the upregulation in Cx43 observed in small vessel walls following spinal cord injury \textsuperscript{32} and skin injury \textsuperscript{47,48}. An increase in Cx43 gap junction communication has also been observed in the microglia of primary glial cell cultures \textsuperscript{49} and brain stab wounds \textsuperscript{50}, but no definite Cx43-ir co-localisation with OX-42-ir was noted in the retinal sections of this project.

The inflammatory response observed following partial transection is also of interest. GFAP positive glial activation was seen to predominantly occur in the retinal astrocytes of the RNFL and GCL in the superior retina until 28 days when the inferior retina also became significantly higher. Surprisingly, there was no increase in GFAP-ir or GS-ir in the other retinal layers as has previously been described in rodent Müller cell fibres following optic nerve crush injury. \textsuperscript{51,52} Perhaps an explanation is the difference in injury model as a crush would affect a greater volume of axons than a partial transection. Astrocyte activation paralleled Cx43 levels well except at 14 days and 56 days post-injury where superior Cx43-ir was decreased despite increased GFAP-ir. At these times, decreased gap junction communication was occurring despite a persisting astrocytosis. The microglial and neutrophil response to injury was more widespread with an increase in OX-42-ir and MPO-ir observed throughout the layers of the superior and inferior retinas. This could represent either direct proliferation of the microglial population, recruitment and differentiation of circulating monocytes into microglia, or activation and hypertrophy of resting microglia. \textsuperscript{53}

### 8.4 RGC Loss and Cx43

There is abundant evidence that RGC loss after injury to their axons at the optic nerve head or optic nerve is signalled from the injury site through retrograde axonal transport, leading to decreased neurotrophic support from the central target and/or the arrival at the RGC cell body of molecules signalling injury. \textsuperscript{54,55} This results in programmed death of the RGC, or apoptosis. In this project, a
A low number of TUNEL-positive RGCs was observed which compared well with data following complete axotomy by Isenmann et al. They assessed TUNEL-positive RGCs and detected the occasional positive RGC from 2 to 24 hours post axotomy. Their data showed a peak of TUNEL-positive RGC apoptosis at 6 days (3 cells/section), which dropped sharply to occasional cells by 28 days. By comparison, in this project there was a more constant number of TUNEL-positive RGCs in the superior retina at 7, 14 and 28 days (1-2 cells/section) and no cells in the inferior retina. This difference may be due to a difference in injury models. It is unsurprising that no TUNEL-positive RGCs were detected in the inferior retina in this project as inferior retinal RGC loss was less severe than superiorly. The ongoing apoptosis seen in the superior retina of this project compared to the drop off with complete axotomy may well be because of the existence of a subpopulation of unaxotomised RGCs that is suffering from secondary death from mechanisms described below.

One mechanism behind apoptotic RGC death is through excitotoxic damage. Following optic nerve injury, an increase in extracellular glutamate and calcium ions have been implicated in the excessive stimulation of excitatory amino acid receptors such as the NMDA receptor. It is thus intuitive than the surrounding retinal glia, which are critical for the buffering of such excitotoxic substances plays a significant role in regulation of RGC death following injury. The retinal Cx43 changes observed following distal optic nerve injury also suggest that the RGC-glia interplay is highly significant in the retinal response to RGC axon injury. The data in this project supports the participation of astrocytic upregulation of Cx43 in the early phenomena of this injury process. The initial increase in superior retinal Cx43 detected coincided with, or even preceded, significant superior RGC death, suggesting that the Cx43 changes are not a reactive or secondary event, but instead occurs early in the injury pathway. The retinal astrocytes may well be reacting to local signals and events generated by RGC body injury. One could speculate that cell-to-cell interactions with the RGC in the initial stages of injury are leading to astrocytic and microglial activation. Retinal glia abundantly express Cx43, and are known to play a role in regulation of neurotransmitters such as glutamate, growth factors, and inflammatory processes after damage. It is likely that the Cx43
localised in retinal macroglia in close proximity to the RGC cell layer may regulate excitotoxic RGC apoptosis. In brain slices subjected to acute injury, reactive gliosis and upregulation in astrocytic Cx43 has been observed. Lesion spread has been documented to occur in the slices via gap junction-mediated calcium signalling between astrocytes. Mechanical damage on the cut surface of the slices may induce hemichannel opening, allowing release of ATP into the extracellular space. Increase in ATP then activates a G-protein-coupled receptor signalling cascade, which results in elevation of intracellular calcium levels, and further release of ATP and glutamate. Toxic signals such as mobilised calcium and inositol triphosphate may also passively diffuse into cells coupled by gap junctions. This amplification of toxic signals causes irreversible cellular damage, extending the area of the lesion. These processes are likely to be occurring in the retina as well.

A further possibility is the fact that astrocytes are linked via their Cx43 gap junctions in a functional syncytium. Injury at the level of the optic nerve may be signalled through this astrocytic network back into the retinal astrocytes. However this is less likely given that if this were the case, we would expect that retrograde spread through the astrocytic syncytium would result in a much more global retinal astrocytic Cx43 response than that observed, which was limited to the superior retina alone until later in the process.

As discussed above, this project used the partial transection model to segregate and study the responses of primarily and secondarily injured RGC and the zones of retina in which they reside. As previously reported by Levkovitch-Verbin et al, and indirectly suggested in prior studies by Yoles et al, the secondary degeneration process lags in time after primary RGC loss. This project confirms that later and less severe RGC loss occurs in the inferior retina following partial superior optic nerve transection. It adds the important new finding that there is a parallel dissociation in time between Cx43 upregulation in upper and lower retina in the model, with Cx43 increase delayed in the zone of putatively secondary degeneration inferiorly, just as RGC cell body loss is delayed there. This could be interpreted to mean that the inter-astrocytic communication via gap junctions, while potentially
permitting broad geographic effects, is in fact limited to the zone of active injury. It is not certain at this time as to whether the Cx43 changes are beneficial or detrimental to RGC survival, although it is tempting to speculate the latter given the above-mentioned literature and that the rise in superior retinal Cx43-ir precedes RGC loss. The spacing between later time points may well be masking the exact relationship between Cx43 increase and RGC loss at this time. In addition, the Cx43 upregulation may simply reflect spreading inflammation, which is causing RGC loss, rather than a direct causal relationship between Cx43 expression and RGC loss per se. Studies to inhibit or to stimulate Cx43 presence and function are needed to investigate these issues.

8.5 Final conclusion, future directions and potential therapeutic implications

Cx43 gap junction communication has been implicated in the spread of neuronal death in other parts of the CNS. There is an abundance of Cx43 in both the mammalian optic nerve and retina, but a paucity of data on changes in Cx43 expression following optic nerve injury. This project was therefore primarily designed to evaluate the spatial and temporal response of optic nerve and retinal Cx43 following an in vivo model of optic nerve injury.

Results demonstrate that in the first few hours after partial superior optic nerve transection, an early upregulation in Cx43-ir occurs around the optic nerve injury site. In the ensuing days, this is followed by a delayed increase in Cx43-ir throughout the laminar region of the optic nerve head. Within the retina, Cx43 protein is shown to be upregulated in the superior retinal zone corresponding to optic nerve injury beginning at 24 hours and peaking at 3 days, with a second peak in both the superior and inferior retinal zones at 28 days. An associated inflammatory response was observed including activation of astrocytes, microglia, and an influx of macrophages and neutrophils.

The biphasic peaks of Cx43-ir in the retina coincided with significant RGC loss, but perhaps most important was the subset of inferior RGC loss, representative of secondary degeneration, that began
at 28 days and reached a 40% loss by 56 days post-injury. This 40% loss thus becomes a therapeutic target for neuroprotective interventions.

The temporal and spatial relationship between Cx43 upregulation, astrocytic activation and RGC loss indicates that retinal glial gap junction communication may have a role in mediating RGC damage. This is consistent with the concept that RGC loss and related inflammatory response can be prevented by modulation of Cx43, either at the optic nerve or retina. Future studies will therefore be directed at modulating Cx43 expression to determine if this impacts on RGC survival.

8.6 References


SECTION 4:

APPENDICES
Response of Retinal Connexin43 to Optic Nerve Injury

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PURPOSE. To characterize the spatial and temporal expression of Connexin43 (Cx43) after partial optic nerve transection and evaluate its relationship to retinal ganglion cell (RGC) loss and retinal glial response.

METHODS. Partial, unilateral, superior optic nerve transection was performed in 150 Wistar rats. The retinas were evaluated at 8 and 24 hours and 3, 7, 14, 28, and 56 days after injury. Immunohistochemical analysis identified changes in several markers including Cx43 immunoreactivity (ir), RGC counts (Bn3a), and retinal astrocytes (GFAP).

RESULTS. After injury, superior retinal Cx43-ir peaked at 3 days (192.1% of control; \( P = 0.0002 \)) and 28 days (212.1% of control; \( P = 0.0027 \)) and troughed at 14 days (73.8% of control; \( P = 0.0028 \)) and 56 days (72.5% of control; \( P = 0.0232 \)). Inferior retinal Cx43-ir was elevated at only 28 days (127.4% increase; \( P = 0.0481 \)). Superior RGC loss began at 3 days (84.0% of control; \( P = 0.0454 \)) and continued to decline by 56 days (18.8% of control; \( P < 0.0001 \)). Inferior RGC loss began at 28 days (73.4% of control; \( P = 0.0021 \)). An increase in GFAP-ir occurred in the superior retina from day 3 (153.7% of control; \( P = 0.0017 \)) and from day 28 (186.7% of control; \( P = 0.0013 \)) in the inferior retina, persisting in both the superior and inferior retina to 56 days (\( P = 0.0027 \)).

CONCLUSIONS. A biphasic upregulation of retinal Cx43 protein occurs in the superior retina with peaks at 3 and 28 days after injury, but at only 28 days in the inferior retina. There is an associated loss of RGCs and a retinal astrocytic inflammatory response. (Invest Ophthalmol Vis Sci. 2011;52:3620–3629) DOI:10.1167 iovs.10-6318

Connexin43 (Cx43) is the most ubiquitous gap junction protein in the central nervous system (CNS) and is predominantly found in brain astrocytes.1 Gap junctions are specialized cell-to-cell contacts that provide direct intercellular communication of molecules less than 1200 Da in size, which includes nutrients, metabolites, second messengers, cations, and anions.2 In the retina, Cx43 is principally found in macroglia, forming gap junctions between various combinations of astrocytes and Müller cells.3 There is accruing evidence that changes in the spatial and temporal expression of Cx43 occur in various models of CNS injury and that the severity of the injury governs the specific response of astrocytic Cx43. Generally speaking, mild to moderate injury leads to increased Cx43 immunoreactivity (ir) at the lesion site or in vulnerable CNS areas.4,5 Severe injury results in decreased Cx43-ir at the lesion site, which is surrounded by a zone of increased Cx43-ir in what could be described as the penumbra of the injury.4,6–14 These observations indicate that astrocytic gap junction communication is an important mediator after CNS injury, and the preponderance of present evidence suggests that preventing Cx43 upregulation that occurs after CNS injury increases neuronal survival.15–18 The most likely mechanisms by which Cx43 modulation leads to neuroprotection is by blocking Cx43 gap junctions, which would prevent the spread of cell death from areas of injured neurons to healthy bystanders,19 or by gap junction hemichannel events.20

Optic nerve injury models are useful in study of CNS disorders due to the accessibility of the optic nerve axons and their distant retinal ganglion cell (RGC) bodies in the retina.21 After complete transection of the optic nerve in rats, there is an initial 1-week period of RGC body survival, followed by substantial RGC loss during the next 2 weeks.22 RGC axons in the optic nerve distal to the injury site degenerate more rapidly. There is consistent evidence that RGCs die by apoptosis.23–24 A body of evidence now shows that the cellular presence of Cx43 and gap junction communication strongly influences apoptotic activity though there is debate as to whether it is facilitatory or inhibitory,20,25,26 A model in which the superior optic nerve axons are transected was developed to exploit the topographic distribution of RGC axons, generating within the eye a superior zone in which there is direct RGC axonal injury and an opposite inferior hemifield that might serve as a zone without direct injury that is exposed to a toxic environment, similar to that of the penumbra zone in CNS lesions.27 This paradigm has demonstrated that secondary injury in the inferior retina exhibits slower and less significant RGC loss than in the primarily injured superior retina.28 This approach allows investigation of the relevance of spatial or temporal changes in Cx43 in the retina after partial optic nerve transection. The present research details the relationship of changes in Cx43 in both superior and inferior retina in this model, relating them to apoptotic RGC neuronal loss and the response of retinal glia.

MATERIALS AND METHODS

Animals

Wistar rats (250–350 g) were obtained from the Vernon Jansen Unit (VJU), University of Auckland. All animal procedures in this study were approved by the Animal Ethics Committee at the University of Auckland and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were housed under standard conditions and fed food and water ad libitum.

Partial Optic Nerve Transection Model

The technique of partial optic nerve transection was adapted from methods outlined by Levkovitch-Verbin et al.27 Briefly, unilateral partial optic nerve transection was performed in animals that were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (4 mg/kg) and topical benoxinate 0.4% eye drops. Intracocular disease was ex-
cluded by examination after instillation of mydriatic eye drops (tropiccamide 1%). A superior conjunctival incision was made, and the eye was gently retracted outward with forceps, exposing the nerve behind the eye. The meninges overlying the optic nerve was carefully divided superiorly, exposing the optic nerve. A 200-μm incision was then made across the optic nerve with diamond step knife and 45° blade (Huco Vision, St. Blaise, Switzerland) at a point 0.5 to 1.0 mm behind the eye. The conjunctival incision was self-closing. Each surgically injured eye was inspected ophthalmoscopically to ensure patency of blood flow to the eye. The same procedure was performed for sham injured eye was placed on the exposed optic nerve. The meninges overlying the optic nerve was carefully divided superiorly, exposing the optic nerve. A 200-μm incision was then made across the optic nerve with diamond step knife and 45° blade (Huco Vision, St. Blaise, Switzerland) at a point 0.5 to 1.0 mm behind the eye. The conjunctival incision was self-closing. Each surgically injured eye was inspected ophthalmoscopically to ensure patency of blood flow to the eye. The same procedure was performed for sham surgery, but the diamond step knife was sheathed and placed on the exposed optic nerve.

Animals were euthanatized by carbon dioxide (CO2) inhalation at 8 and 24 hours; 3, 7, and 14 days; and 1 and 2 months after partial optic nerve transection. A single conjunctival suture was placed superiorly and 24 hours; 3, 7, and 14 days; and 1 and 2 months after partial optic nerve transection. A single conjunctival suture was placed superiorly and

**Immunohistochemistry**

Six animals per time point were used for immunohistochemical analysis. Samples were immediately postfixed after extraction by submergence in 1% paraformaldehyde for 30 minutes, followed by overnight cryoprotection with 15% sucrose. Sagittal cryosections of 16-μm thickness were mounted on slides (Superfrost Plus; Menzel-Gläser, Braunschweig, Germany), dried for 30 minutes at room temperature, and stored at −20°C. Sections including the optic nerve head as a landmark were used for immunolabeling.

The sections were further fixed in −20°C ethanol for 10 minutes if they were for Cx43 and astrocyte labeling; otherwise this step was omitted. After a thorough rinsing in phosphate-buffered solution (PBS), the sections were preincubated in the appropriate blocking buffer (10% normal goat serum and 0.1% Triton X-100 in PBS for all except RGC labeling in which 10% horse serum was substituted) for 1 hour at room temperature.

Primary antibodies were applied overnight at 4°C for Cx43 (rabbit polyclonal anti-Cx43, 1:2000; Sigma-Aldrich, St. Louis, MO), retinal ganglion cells (anti-Brn-3a, 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA), astrocytes (mouse monoclonal anti-glial fibrillary acidic protein [GFAP]-Cy3 conjugate, 1:1000; Sigma-Aldrich), Müller cells (mouse monoclonal anti-glutamine synthetase [GS], 1:200; Abcam, Cambridge, UK), activated microglia (mouse monoclonal anti-IOX-42 [CD11b], 1:100; Serotec, Oxford, UK), and blood vessel endothelial cells (mouse monoclonal anti-isocitrate B4; IL-B4)/Alexa 594 conjugate, 1:100; Invitrogen-Molecular Probes, Eugene, OR).

After rinsing in PBS, appropriate secondary antibodies were applied for 2 hours at room temperature, including goat anti-rabbit Alexa 488 (1:1000; Invitrogen, Carlsbad, CA), goat anti-mouse Alexa488 (1:500; Invitrogen), goat anti-mouse Cy3 (1:500, Jackson ImmunoResearch Laboratories Inc.), and donkey anti-goat Cy3 (1:1000, Jackson ImmunoResearch Laboratories Inc.).

After further rinsing in PBS, the sections were mounted in antifade reagent with DAPI (ProLong Gold; Molecular Probes) on coverslips, which also provided precise identification of retinal and nerve morphology.

**Imaging**

Immunofluorescence was analyzed using a microscope (DMRA; Leica, Wetzlar, Germany) fitted with a confocal scanning microscope (model FV1000 with Fluoview software; ver. 1.7a; Olympus, Tokyo, Japan). Single-slice confocal images were captured at a speed of 4.0 μm/pixel, with a resolution of 1024 × 1024 pixels, using a Kalman average of 4 and on sequential mode if using multiple lasers. Gain and offset were standardized using control tissue for all analyses. Images of the retina containing the RGC layer and the inner nuclear (INL) and outer nuclear (ONL) cell layers, were captured at 60× magnification, except for RGC analysis (40×). Three retinal images were acquired both superior and inferior to the optic nerve head, separated by a distance of one eyepiece field (60× eyepiece field, 300 μm; 40× eyepiece field, 450 μm).

**Cx43 Immunohistochemical Analysis**

Retinal Cx43 immunohistochemical analysis was performed qualitatively and quantitatively on three sections of each animal. For quantitative analysis, confocal images were exported as TIFF files into ImageJ software (version 1.41o; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Image area was cropped to include the retinal glia only, and image type was converted to 8-bit black and white. A consistent threshold of 55 was applied to reduce background immunoreactivity, and an automated immunolabel spot count was then performed (Fig. 1).

**RGC Immunohistochemical Analysis**

Bn3a has been validated as a reliable, efficient marker to quantify RGCs in optic nerve-injured retina.29 For quantification of RGCs, Bn3a TIFF files from two sections of each animal were converted to 8-bit black and white and a threshold of 35 was applied. A "despeckle" process followed by two "erode" processes were applied, to allow for easier discrimination of individual RGCs. A single, masked observer made manual cell counts on the final images (Fig. 1).
Astrocyte Immunohistochemical Analysis

Retinal GFAP immunohistochemical analysis was performed qualitatively and quantitatively on the same sections used for Cx43 immunohistochemical analysis (three sections per animal). For quantitative analysis, GFAP TIFF files were converted to 8-bit black and white and a threshold of 37 was applied, and an automated area count of immunolabeled cells was performed.

Inflammatory Response Immunohistochemical Analysis

Qualitative assessment of immunolabeled activated microglia (OX-42) and blood vessel endothelial cells (isoechin-IB4 [IL-B4]) were performed on retinal sections. A basic quantitative analysis of OX-42 immunolabeling was also performed. Aggregated label, subjectively deemed to represent one cell, was manually counted in individual retinal layers.

TUNEL Assay

Analysis of apoptotic cells by the terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) method was performed with an in situ fluorescein apoptosis detection kit (ApopTag Plus; Chemicon International, Temecula, CA) the prescribed methodology. Briefly, sections were rinsed in PBS before and after being postfixed in a precooled ethanol/acetic acid (2:1) solution at −20°C for 5 minutes. An equilibration buffer was subsequently applied for a minimum of 10 seconds. After this, sections were treated with a solution containing terminal deoxynucleotidyl transferase and digoxigenin-conjugated nucleotides for 1 hour at 37°C. This step was necessary to attach the digoxigenin-dUTPs to the terminal end of nucleic acids in DNA fragments caused by the apoptotic process. Sections were then incubated in a stop/wash buffer for 10 minutes at room temperature to halt the enzymatic dUTP addition. After washing in PBS, a fluorescein conjugated anti-digoxigenin antibody was applied to the sections for 30 minutes at room temperature, followed by further PBS washing. The assay was completed by mounting the slides with a DAPI mounting medium on coverslips.

Polymerase Chain Reaction of Cx43 mRNA and Western Blot Analysis

To determine the presence of Cx43 mRNA real-time quantitative polymerase chain reaction (RTqPCR) was performed. Three animals were selected from each of the control and 3-day posttransection groups. Extracted retinal samples were frozen immediately in liquid nitrogen. Total RNA was isolated from the experimental and sham retinal samples by the acid guanidinium thiocyanate-phenol-chloroform extraction method. The samples were homogenized with RNA extraction reagent (TRIzol; Invitrogen, Carlsbad, CA) and purified (PureLink RNA Micro Kit; Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. A spectrophotometer (NanoDrop ND-1000; Thermo-Scientific, Wilmington, DE) was used to assess RNA quantity and quality. One hundred nanograms of total RNA was reverse transcribed into cDNA (SuperScript VILO cDNA synthesis kit; Invitrogen) in a 20-μL reaction mixture under the following conditions: 25°C for 10 minutes; 42°C for 120 minutes; and 85°C for 5 minutes. The cDNA product was then amplified by PCR. PCR was performed on a real-time rotary analyzer (RotorGene 6000; Corbett Life Science, Sydney, Australia). The reaction was initiated with a commercial system (FastStart Universal SYBR Green Master [Roxy]; Roche, Basel, Switzerland) according to the manufacturer’s specifications. The 50-μL reaction mixture consisted of 25 μL of green nucleic acid gel stain (FastStart SYBR Green [Roxy]; Roche), 5 μL of cDNA, 0.5 μL of forward primer, 0.5 μL of reverse primer, and 19 μL of PCR-grade water. The primers used for connexin43 were 5’-GATTGAGACCGCAGCAAGG-3’ (sense) and 5’-GTTGAGACCGGCTCAAGG-3’ (antisense). The following conditions were used for 40 cycles: denaturation at 95°C for 10 minutes, annealing at 95°C for 15 seconds, and extension at 60°C for 1 minute.

The expression of connexin43 was normalized to β-actin, a housekeeping gene. The primers used for β-actin were 5’-GATTGGCCACCACTCTTCTACA-3’ (sense) and 5’-ACTTGTGGCATCTTTTCACGGTG-3’ (antisense). Each PCR reaction was repeated three times for every eye at all time points. In addition, negative controls were performed without reverse transcriptase.

For Western blot analysis, eyes were enucleated from three control and three animals 3 days after transection, and the retina dissected and immersed in liquid nitrogen. The samples were homogenized in ice-cold phosphate-buffered saline containing 150 mM sucrose, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9), 6 mM potassium chloride, 2 mM EDTA acid (pH 8.0), 1 mM EGTA (pH 8.0), and a protease inhibitor (Complete Protease Inhibitor Cocktail, Roche). After the addition of 1% Triton X-100 (Sigma-Aldrich), the homogenate was incubated for 1 hour on ice. The supernatant was collected after centrifugation at 10,000 rpm for 10 minutes and combined with a loading dye (125 mM Tris [pH 6.8], 0.8% sodium dodecyl sulfate [SDS], 2% glycerol, and 0.1% B-mercaptoethanol).

Equal amounts of protein were loaded onto a 10% SDS polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. A molecular weight standard (Benchmark Protein Ladder; Invitrogen) was included. After incubation in blocking solution (5% nonfat milk powder and 0.1% Tween-20 in Tris-buffered saline [TBS]) for 1 hour at room temperature, the membrane was incubated sequentially with 1:6500 rabbit polyclonal anti-connexin43 antibody and 1:4000 rabbit anti-GAPDH antibody for 12 hours at 4°C. The membrane was then washed thoroughly with 0.1% Tween-20 in TBS and incubated with Alexa488-conjugated secondary antibodies at a dilution of 1:10,000 for 1 hour at room temperature. Blots were washed with 0.1% Tween-20 in TBS before detection with Western blot detection reagents (Amersham ECL Plus; GE Health care, Piscataway, NJ). Chemiluminescence was detected with an imaging system (LAS-3000; Fujifilm, Tokyo, Japan) and analyzed with ImageJ software.

Statistics

Data were analyzed with used Student’s t test or one-way ANOVA, whichever was appropriate (Prism 5 for Windows; GraphPad, San Diego, CA). P < 0.05 was considered to be significant.

RESULTS

Biphasic Retinal Cx43 Response after Partial Optic Nerve Transection

Cx43-ir was primarily present in the retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL), but was sparsely present in the superficial retinal layers (Fig. 2). In the RNFL and GCL, Cx43-ir was found to predominantly co-localize with GFAP-ir, representative of retinal astrocytes or Müller cell end feet (Figs. 3, 4). However, minimal co-localization of Cx43-ir was observed in the RNFL and GCL with GS-ir, a marker for Müller cells, and with IL-B4-ir, a marker for blood vessel endothelial cells (Fig. 3). In the more superficial retinal layers, Cx43-ir primarily co-localized with IL-B4-ir, but not with GS-ir or GFAP-ir, representative of Müller cell processes (Fig. 3).

After injury, differences in superior retinal Cx43-ir were observed in RNFL and GCL (Fig. 4), but not in the other retinal layers. Quantitative analysis revealed a biphasic response of Cx43-ir in the retina, peaking at days 3 and 28 after injury (Fig. 5). The changes in Cx43 spot counts for the total, superior, and inferior retina are outlined in Table 1. Superior retinal Cx43-ir was significantly increased compared with controls, as early as 24 hours after injury (141.7% of control, SD ± 53.1%; P = 0.0065) and continued to increase at 3 days (192.1% of control, SD ± 88.7%; P = 0.0002). By 7 days, it had dropped to 136.6% (SD ± 41.3%; P = 0.0043) of control, and at 14 days, the levels were significantly lower than the control (73.8% of control, SD ± 18.0%; P = 0.0028). A further maximum increase in
Cx43-ir occurred at 28 days (212.1% of control, SD/H11006 90.1%; P/H11021 0.0001), followed by another decrease at 56 days (72.5% of control, SD/H11006 37.6%; P/H11005 0.0232). No significant change in inferior retinal Cx43-ir was noted except at 28 days after injury (127.4% increase cf. controls, SD/H11006 40.9%; P/H11005 0.0481; Table 1).

The presence of Cx43 mRNA and protein in control and injured retinal samples was confirmed by RT-qPCR and Western blot analysis, respectively. It was not possible to accurately quantify relative levels of Cx43 mRNA or protein by either method. Extracted retinal samples were shown through immunohistochemical techniques, to contain variable quantities of both neurosensory and pigmented retinal layers. The underlying retinal pigment epithelium, a structure expressing particularly high levels of connexin43, overwhelmed changes occurring in the inner retinal layers which were of a much smaller scale. The inability to separate retinal layers consistently and therefore distinguish the origin of the connexin43 mRNA or protein resulted in the decision not to use RT-qPCR or Western blot analysis as a quantitative tool.

**Cx43 Upregulation Coincides with Significant RGC Loss**

RGC loss was determined by quantitative analysis of Brn3a-labeled RGCs in two retinal sections per animal with six animals per time point (Fig. 6). The number of superior RGCs after injury was significantly reduced compared with the control number at 3 days (84.0% of control, SD/H11006 16.2%; P/H11005 0.0454) and continued to decline at 7 (66.9% of control, SD/H11006 11.4%; P < 0.0001), 14 (43.8% of control, SD/H11006 15.6%; P < 0.0001), 28 (21.3% of control, SD/H11006 11.1%; P < 0.0001), and 56 (18.8% of control, SD/H11006 8.8%; P < 0.0001) days. A significant decrease in the number of inferior RGCs was measured at 28 (73.4% of control, SD/H11006 22.8%; P = 0.0021) and 56 (59.6% of control, SD/H11006 18.4%; P < 0.0001) days but not earlier (Table 1, Fig. 5).

The onset of superior RGC loss at 3 days appeared to coincide with the early peak in superior Cx43-ir, which was initially elevated from 24 hours through to 7 days after injury. Similarly, at 28 days, the significant loss of inferior RGCs compared with controls occurred in conjunction with a significant elevation of inferior retinal Cx43-ir. By 56 days, the total number of RGCs was not significantly different from that at 28 days (P = 0.2347), and total Cx43 had returned to levels not significantly different from the control. The TUNEL assay confirmed the presence of a low number of TUNEL-positive cells in the RGC layer of multiple retinal sections.

**Retinal Astrocyte Activation Occurs after Partial Transection**

Evaluation of the retinal glial response after optic nerve injury was performed by analysis of GFAP-ir and GS-ir. After injury, a
The present study is the first to evaluate the spatial and temporal response of retinal Cx43 after an in vivo model of optic nerve injury. Optic nerve injury evoked a biphasic response of retinal Cx43 protein expression in the retinal area corresponding to axonal damage (superior), and a delayed elevation in the retinal area unaffected by the primary injury (inferior). In both areas, the peak in Cx43-ir coincided with significant RGC loss. There was also an associated inflammatory response including activation of astrocytes and microglia.

More specifically, after partial transection of the superior optic nerve, the superior retina showed a significant increase in Cx43-ir as early as 24 hours after injury, peaking to almost 200% of control at day 3, and remaining elevated 7 days after injury. This increase in superior retinal Cx43-ir coincided with, or even preceded, significant superior RGC loss which began at 3 days after injury, with RGC counts declining to 66.9% of control at 7 days after injury. Superior retinal Cx43-ir dropped to subnormal levels at 14 days but increased again to maximum levels by 28 days after injury. By comparison, inferior retinal Cx43-ir significantly changed at only 28 days after injury, when it was elevated to 127% of control levels. This was again paralleled by significant inferior RGC loss at 28 days, with inferior RGC counts down to 73% of control.

RGC loss by Brn3a analysis of retinal sections compared relatively well with data from Levkovitch-Verbin et al., who used retrograde Fluorogold labeling of retinal wholemounts, bearing in mind that our incision was 50 μm deeper than theirs. With respect to superior RGC loss, our data showed less loss at 7 days (−33%) than their 8-day time point (−63%), but more loss at 28 days (−80%) than their 4 week time point (−46%). Of interest, both sets of data do not show significant differences in inferior RGC counts until 4 weeks (−27% cf. −39%). TUNEL analysis confirmed the presence of a low number of TUNEL-positive cells likely to represent RGC apoptosis.

**Discussion**

The present study is the first to evaluate the spatial and temporal response of retinal Cx43 after an in vivo model of optic nerve injury. Optic nerve injury evoked a biphasic response of retinal Cx43 protein expression in the retinal area corresponding to axonal damage (superior), and a delayed elevation in the retinal area unaffected by the primary injury (inferior). In both areas, the peak in Cx43-ir coincided with significant RGC loss. There was also an associated inflammatory response including activation of astrocytes and microglia.

More specifically, after partial transection of the superior optic nerve, the superior retina showed a significant increase in Cx43-ir as early as 24 hours after injury, peaking to almost 200% of control at day 3, and remaining elevated 7 days after injury. This increase in superior retinal Cx43-ir coincided with, or even preceded, significant superior RGC loss which began at 3 days after injury, with RGC counts declining to 66.9% of control at 7 days after injury. Superior retinal Cx43-ir dropped to subnormal levels at 14 days but increased again to maximum levels by 28 days after injury. By comparison, inferior retinal Cx43-ir significantly changed at only 28 days after injury, when it was elevated to 127% of control levels. This was again paralleled by significant inferior RGC loss at 28 days, with inferior RGC counts down to 73% of control.
lesion and upregulate Cx43 mRNA protein expression. Complete transection of the adult rat spinal cord led to an upregulation of Cx43 mRNA and protein on GFAP-positive astrocytes within hours, remaining significantly elevated up to 28 days after the injury. However, the possibility remains that the increase in Cx43-ir that we observed resulted from the cellular redistribution of astrocytic gap junction protein from a pool of Cx43 not normally detectable by immunohistochemistry, as proposed by Hossain et al. \(^9\) after a global cerebral ischemia model in which Cx43-ir increased but Cx43 protein levels by Western blot did not.

To identify the retinal cell types expressing Cx43, double-labeling immunohistochemical techniques were used. Cx43-ir predominantly co-localized with GFAP-ir in the RNFL and GCL, which could represent Cx43 expression by either retinal astrocytes or the end feet of Müller cells as has been previously studied by Zalis et al. \(^9\) However, the minimal co-localization we observed between Cx43-ir and GS-ir, a Müller cell-specific marker, throughout the retina suggests that the former is most likely. This is in agreement with Ball et al. \(^32\) who demonstrated Müller cell Cx43 staining in goldfish and mudpuppy retinas but not the rat retina and with the vast amount of literature studying astrocytic Cx43 (for review, see Chew et al. \(^33\)). In the more superficial layers, Cx43-ir showed some co-localization with IL-4-ir, a marker for blood endothelial cells, which did not change after transection injury. This is in contrast to the up-regulation in Cx43 observed in small vessel walls after spinal cord injury \(^15\) and skin injury. \(^34,35\) An increase in Cx43 gap junction communication has also been observed in the microglia of primary glial cell cultures \(^36\) and brain stab wounds, \(^37\) but no definite Cx43-ir co-localization with OX-42-ir was noted in this study.

The inflammatory response observed after partial transection is also of interest. GFAP-positive glial activation was seen to predominantly occur in the retinal astrocytes of the RNFL and GCL in the superior retina until 28 days when the inferior also became significantly higher. Surprisingly, there was no increase in GFAP-ir or GS-ir in the other retinal layers, as has been described in rodent Müller cell fibers after optic nerve crush injury. \(^38,39\) Perhaps an explanation is the difference in injury model, as a crush would affect a greater volume of axons than a partial transection. Astrocyte activation paralleled Cx43 levels well except at 14 and 56 days after injury where superior Cx43-ir was decreased despite increased GFAP-ir. At these times, decreased gap junction communication was occurring despite a persisting astrocytosis. The microglial response to injury was more widespread with an increase in OX-42-ir observed throughout the layers of the superior and inferior retinas. This could represent either direct proliferation of the microglial population, recruitment and differentiation of circulating monocytes into microglia, or activation and hypertrophy of resting microglia. \(^40\)

The retinal Cx43 changes observed after distal optic nerve injury suggest that the RGC–glia interplay is highly significant in the retinal response to RGC axon injury. There is abundant evidence that RGC loss after injury to their axons at the optic nerve head or optic nerve is signaled from the injury site through retrograde axonal transport, leading to decreased neurotrophic support from the central target and/or the arrival at the RGC cell body of molecules signaling injury. \(^41,42\) Our data support the participation of astrocytic upregulation of Cx43 in the early phenomena of this injury process. The initial increase in the level of Cx43 in the superior retina that we detected coincided with, or even preceded, significant superior retinal RGC death, suggesting that the Cx43 changes are not a reactive or secondary event, but instead occurs early in the injury pathway. The retinal astrocytes may well be reacting to local signals and events generated by RGC body injury. We speculate

![Figure 5.](image-url)
that cell-to-cell interactions with the RGCs in the initial stages of injury lead to astrocytic and microglial activation. Retinal glia abundantly express Cx43 and are known to play a role in the regulation of neurotransmitters, such as glutamate, growth factors, and inflammatory processes after damage. It is possible that the Cx43 localized in retinal macroglia in close proximity to the RGC cell layer may regulate excitotoxic RGC apoptosis. In brain slices subjected to acute injury, reactive astrocytes are known to upregulate Cx43 in astrocytic gap junctions and are linked via their Cx43 gap junctions in a functional syncytium.

mobilized calcium and inositol triphosphate may also passively diffuse into cells coupled by gap junctions. This amplification of toxic signals causes irreversible cellular damage, extending the area of the lesion. This process may well occur in the retina, as well. A further possibility is that astrocytes are linked via the Cx43 gap junctions in a functional syncytium. It is conceivable that injury at the level of the optic nerve is signaled through this astrocytic network back into the retinal astrocytes. However, this is unlikely, given that the astrocytic Cx43 response is limited to the superior retina alone until later in the process. We used the partial transaction model to segregate and study the responses of primarily and secondarily injured RGC and the zones of retina in which they reside. As reported by Levkovich-Verbin et al. and indirectly suggested by Yoles and Schwartz, the secondary degeneration process lags in time from primary RGC loss, both in the later and less severe

<table>
<thead>
<tr>
<th>Time Point</th>
<th>n</th>
<th>Cx43 Spot Count/Section</th>
<th>P vs. Controls</th>
<th>RGC Count/Section</th>
<th>P vs. Controls</th>
<th>GFAP Area Count/Section (× 10^−4)</th>
<th>P vs. Controls</th>
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</thead>
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<tr>
<td>Control</td>
<td>18</td>
<td>Total 265.6 ± 98.3</td>
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<td>Total 84.5 ± 9.7</td>
<td>—</td>
<td>Total 13.8 ± 3.9</td>
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<tr>
<td></td>
<td>Sup. 143.4 ± 52.7</td>
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<td>Sup. 42.6 ± 8.8</td>
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<td>Sup. 7.0 ± 2.6</td>
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<td>Inf. 109.4 ± 74.7</td>
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<td>Inf. 41.9 ± 5.0</td>
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<td>Inf. 121.7 ± 54.2</td>
<td>0.70 Inf. 40.7 ± 6.4</td>
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<td>8 h</td>
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<td>Total 299.0 ± 116.4</td>
<td>0.36 Total 79.2 ± 13.5</td>
<td>0.29 Total 14.8 ± 4.6</td>
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<td>Sup. 183.2 ± 80.1</td>
<td>0.087 Sup. 39.3 ± 8.8</td>
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<td>Inf. 115.8 ± 74.0</td>
<td>0.80 Inf. 39.9 ± 10.1</td>
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<td>24 h</td>
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<td>0.25 Total 81.9 ± 12.7</td>
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<td>Sup. 206.1 ± 77.3</td>
<td>0.0063* Sup. 37.3 ± 7.1</td>
<td>0.14 Sup. 8.4 ± 2.7</td>
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<td>Inf. 97.2 ± 48.6</td>
<td>0.57 Inf. 44.3 ± 8.1</td>
<td>0.40 Inf. 6.3 ± 2.7</td>
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<td>3 d</td>
<td>18</td>
<td>Total 421.4 ± 170.3</td>
<td>0.0019* Total 78.3 ± 13.5</td>
<td>0.21 Total 18.9 ± 5.2</td>
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<td>Sup. 279.4 ± 129.0</td>
<td>0.0002* Sup. 35.8 ± 6.9</td>
<td>0.045* Sup. 10.8 ± 3.9</td>
<td>0.0017*</td>
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<td>Inf. 142.0 ± 68.6</td>
<td>0.19 Inf. 42.6 ± 9.2</td>
<td>0.85 Inf. 8.1 ± 2.0</td>
<td>0.11</td>
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<tr>
<td>7 d</td>
<td>18</td>
<td>Total 330.6 ± 87.6</td>
<td>0.044* Total 67.8 ± 10.4</td>
<td>0.0005* Total 19.5 ± 4.7</td>
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<td>0.0043* Sup. 28.5 ± 4.8</td>
<td>&lt;0.0001* Sup. 11.1 ± 3.2</td>
<td>0.0002*</td>
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<tr>
<td></td>
<td>Inf. 131.8 ± 51.7</td>
<td>0.31 Inf. 39.3 ± 7.7</td>
<td>0.34 Inf. 8.4 ± 2.5</td>
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<tr>
<td>14 d</td>
<td>18</td>
<td>Total 185.0 ± 56.1</td>
<td>0.0048* Total 58.3 ± 14.9</td>
<td>&lt;0.0001* Total 18.2 ± 6.7</td>
<td>0.024*</td>
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<td>Sup. 107.3 ± 26.2</td>
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<td>&lt;0.0001* Sup. 7.8 ± 3.2</td>
<td>0.078</td>
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<tr>
<td></td>
<td>Inf. 77.7 ± 47.7</td>
<td>0.14 Inf. 39.6 ± 10.4</td>
<td>0.50 Inf. 9.4 ± 5.2</td>
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<tr>
<td>28 d</td>
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<td>Total 461.5 ± 150.8</td>
<td>&lt;0.0001* Total 39.8 ± 13.7</td>
<td>&lt;0.0001* Total 28.5 ± 8.3</td>
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<tr>
<td></td>
<td>Sup. 308.5 ± 131.0</td>
<td>&lt;0.0001* Sup. 9.1 ± 4.7</td>
<td>&lt;0.0001* Sup. 15.8 ± 5.2</td>
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<tr>
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<td>Inf. 153.0 ± 49.1</td>
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<td>0.0021* Inf. 12.7 ± 6.7</td>
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<td>56 d</td>
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<td>Total 224.6 ± 119.4</td>
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<td>Sup. 105.5 ± 54.6</td>
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<td>Inf. 119.1 ± 82.4</td>
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* Statistically significant.

![Figure 6](image-url)

**Figure 6.** Superimposed single-slice confocal images of retinal sections labeled with Brn3a (red) and DAPI (blue). Baseline Brn3a-positive RGCs displayed in (A) control retina. **Arrowhead:** a displaced amacrine cell in the IPL. A significant loss in superior RGCs was seen at (B) 3 days through to (C) 56 days after injury. A significant loss in inferior RGCs was seen at (D) 28 days after injury. All images are the same magnification. IPL, inner plexiform layer. Scale bar, 50 μm.
RGC death in the inferior retina. The model has been used successfully by other groups to determine the effects of glatiramer acetate and the calcium channel blocker lomerizine on primary and secondary degeneration of the optic nerve and RGCs.\textsuperscript{28,59,60} Our work adds the important new finding that there was a parallel dissociation in time between Cx43 upregulation in upper and lower retina in our model, with Cx43 increase delayed in the zone of putatively secondary degeneration inferiorly, just as RGC cell body loss was delayed there. This result could be interpreted to mean that the interastrocytic communication via gap junctions, while potentially permitting broad geographic effects, is in fact limited to the zone of active injury. We cannot be certain at this time as to whether the Cx43 changes are beneficial or detrimental to RGC survival, although it is tempting to speculate the latter, given the above-mentioned literature and that the increase in superior retinal Cx43-ir precedes RGC loss. The spacing between later time points may well be masking the exact relationship between Cx43 increase and RGC loss at this time. Studies to inhibit or to stimulate Cx43 presence and function are needed to investigate these issues.

The authors recognize that there are certain limitations to the injury model. Injury consistency was a problem because of the mobility of the optic nerve in the orbit. A shallow lesion may result in less changes in immunohistochemical markers and a lesion passing into the inferior optic nerve due to inadvertent rotation of the optic nerve would be even more detrimental by leading to inferior immunohistochemical changes unrelated to delayed death. However, no significant inferior RGC loss was seen at 7 days, which is known to be the time when almost 50% of axotomized RGCs die. Another limitation was the range of physiologic variability seen with various immunohistochemical markers such as Cx43, as seen with the high standard deviations even in the control retinas. To work around this issue, we used six animals per time point for immunohistochemistry and thoroughly examined three retinal sections per animal to create a large numerical database.

In summary, Cx43 protein is shown to be upregulated in retinal zones corresponding to optic nerve injury beginning at 24 hours and peaking at 3 days, with a second peak throughout the retina at 28 days. This study is the first to demonstrate the biphasic response of Cx43 in the superior retina after superior optic nerve transection. The temporal and spatial relationship between Cx43 upregulation, astrocytic activation and RGC loss indicates that retinal glial gap junction communication may...
have a role in mediating RGC damage. Future studies will be directed at modulating Cx43 expression to determine whether it has an impact on RGC survival.

References


Review

Role of connexin43 in central nervous system injury

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A B S T R A C T

Gap junctions are specialized cell-to-cell contacts that provide direct intercellular communication. In the central nervous system (CNS), gap junction coupling occurs between both neurons and glial cells. One of the most abundant gap junction proteins in the CNS is connexin43 (Cx43). The functional syncytium formed by astrocytes via Cx43 gap junction intercellular communication has, for example, been implicated in maintaining the homeostasis of the extracellular milieu of neurons. In particular, astrocytes are involved in the spatial buffering of many ions, signalling molecules and energy sources. In this review, the role of Cx43 following CNS injury is examined by combining evidence surrounding the response of Cx43 to CNS injury and the effects of Cx43 gap junction blockade on neuronal survival in various models of injury. Combined evidence suggests that transient blockade targeting the window of initial Cx43 upregulation observed following injury is potentially therapeutic.

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Introduction

Gap junctions are specialized cell-to-cell contacts that provide direct intercellular communication of small molecules (less than 1200 Daltons), which can include nutrients, metabolites, second messengers, cations and anions (Sohl et al., 2005; Kumar and Gilula, 1996). A
single gap junction channel consists of two hemichannels, or connexons, each of which is composed of six connexin proteins. The connexin protein itself consists of four plasma membrane spanning sections, two extracellular loops, one intracellular loop and an intracellular N- and C-terminal (Fig. 1). At least 20 connexin genes have been described in mammals and are most commonly named by their respective proteins’ molecular mass in kiloDaltons (Sohl and Willecke, 2003).

In the central nervous system (CNS), gap junction coupling occurs between neurons, astrocytes, oligodendrocytes, microglia and ependymal cells, as well as between different cell types. Unopposed hemichannels have the potential to provide a direct link between the intracellular components and the extracellular environment. Under normal physiological conditions gap junctions function in an open state, allowing intercellular communication, whereas hemichannels are closed to the passage of substances (Decrock et al., 2009). Furthermore, recent evidence suggests a functional role for connexin proteins irrespective of their channel forming abilities (Stout et al., 2004).

Eleven connexins have been identified in various CNS cell types and during different stages of development. Of these, connxin 43 (Cx43) is the most ubiquitously expressed and is predominantly found in astrocytes, activated microglia, developing neurons, and the smooth muscle and endothelial cells of blood vessels (Contreras et al., 2004; Nagy and Rash, 2000; Naus et al., 1991). In astrocytes, they are abundant at end-foot processes along blood vessels thus impacting on the blood–brain barrier, as well as within astrocytic processes that surround chemical synapses (Danesh-Meyer and Green, 2008). Activated microglia, responding to CNS injury by proliferation and migration to the injury site, may establish physical contact and gap junction formation while in close proximity following insult (Eugenin et al., 2001). Due to the low sensitivity and/or resolution of many analytical techniques it is often difficult to assign the expression of Cx43 to a specific cell class. Thus multi-technique approaches are commonly employed when identifying the composition and origin of gap junctions.

The functional syncytium formed by astrocytes via Cx43 gap junctional intercellular communication has been implicated in maintaining the homeostasis of the extracellular milieu of neurons (Cronin et al., 2008). In particular, astrocytes are involved in the spatial buffering of extracellular potassium ions (Wallraff et al., 2006), glutamate and other signalling molecules (Hansson et al., 2000), energy sources (Dienel and Cruz, 2003) and mediation of intercellular calcium ion signalling (Charles et al., 1992).

The purpose of this review is to determine the role of Cx43 following CNS injury by combining evidence surrounding the response of Cx43 to CNS injury and effects of Cx43 gap junction blockade on neuronal survival in various models of CNS injury.

**Response of Cx43 to CNS injury**

It is the response of Cx43 to CNS injury that first highlighted Cx43 as an important mediator of CNS injury. In vitro work confirmed that astrocytic Cx43 gap junctions and hemichannels may remain functionally open following injury and in vivo work has shown significant changes in both spatial and temporal Cx43 protein expression observed following various models of CNS injury.

**In vitro**

Models of in vitro ischemia have been used to study Cx43 gap junctional communication and hemichannel activity in astrocyte cultures. Cotrina et al. (1998) observed that while ischemia led to a decrease in Cx43 gap junction coupling, the gap junctions did remain functional meaning intercellular communication could still occur under ischemic conditions. Specifically, astrocyte death evoked by either ionophore exposure or metabolic inhibition resulted in increased cytosolic calcium and a subsequent decrease in gap junctional coupling. This reduction in astrocytic Cx43 gap junction coupling was confirmed following iodoacetate-induced hypoxia by Li and Nagy (2000; 2000), who also observed an associated dephosphorylation of Cx43, thought to contribute to the uncoupling process. The idea that decreased but functional Cx43 gap junction communication can occur in ischemic conditions is significant as it means the intercellular transfer of ions and metabolites from dying astrocytes to healthy ones can occur.

A further noteworthy change following chemical ischemia by inhibition of glycolytic and oxidative metabolism was the permeabilization of astrocytes to external lucifer yellow and ethidium bromide. This was thought to be due to opening of Cx43 hemichannels since astrocytes were impermeable if gap junction blockade with 18α-glycyrrhetinic acid (AGA) was performed or Cx43-knockout was used (Contreras et al., 2002). In support of these findings was work performed by Retamal et al. (2006) using

**Fig. 1.** A connexin protein consists of four plasma membrane (PM) spanning domains, two extracellular (EC) loops, one intracellular (IC) loop and an intracellular N- and C-terminal. Six connexin proteins form a connexon, or hemichannel, which interacts with a hemichannel on a neighbouring cell to produce a gap junction.
cortical astrocyte cultures and inducing metabolic inhibition with antimycin A and iodoacetic acid. Following injury a marked increase in ethidium bromide dye uptake was observed comparable to the increased abundance of Cx43 cell surface hemichannels. Dephosphorylation and/or oxidation of the hemichannels were implicated as potential mechanisms for the enhanced cell permeability along with nitrosylation of intracellular Cx43 cysteine residues increasing the hemichannel surface expression (Retamal et al., 2006). These experiments raised the concept of Cx43 hemichannels as another theoretical route of entry for extracellular ions and metabolites that may be released by dying cells.

In vivo

Ischemia

Models of CNS ischemia affect the expression of Cx43 in many neural areas, particularly in the hippocampus where increases in Cx43 immunoreactivity (Cx43-ir) have been observed in the CA1/2 pyramidal subfields following transient forebrain ischemia (Rami et al., 2001). Consequently, many of the in vitro and in vivo models used to assess modulation of Cx43 utilize hippocampal tissue.

A similar increase in Cx43-ir in hippocampal and striatal areas was observed 2 days after mild to moderate global cerebral ischemia induced by bilateral carotid artery occlusion in rats (Hossain et al., 1994a). However following severe ischemia, there was an area of reduced Cx43-ir surrounded by a zone of increased Cx43-ir. Immunoelectron microscopy confirmed a greater preponderance of gap junctions among astrocytic processes in the vicinity of degenerating neurons and increased intracellular Cx43 in astrocytic processes and cell bodies. However, no change in Cx43 protein levels was detected by Western blot. From these results the authors inferred that a process of gap junction reorganization from a pool of Cx43, normally undetectable by immunohistochemistry, was involved following ischemia, and that the qualitative nature of the response was dependent on the severity of neuronal damage or loss.

The same group employed a rodent model of focal cerebral ischemia by transient occlusion of the middle cerebral artery to demonstrate that astrocytic Cx43 epitope masking, dephosphorylation and cellular redistribution occurs after a focal ischemic brain injury (Li et al., 1998). After 1 h of ischemia and 24 h of reperfusion, unphosphorylated Cx43-ir was absent in the ischemic core within the hypothalamus, but persisted in a thin corridor at the ischemic penumbra which contained presumptive apoptotic cell profiles. Similar results were obtained in ischemic striatum and cerebral cortex, though with a delayed time course that depended on severity of the ischemic insult.

Another rodent model of focal cerebral ischemia utilized photothrombosis to induce small and defined ischemic lesions restricted to the cortex (Haupt et al., 2007a). In addition to assessing Cx43-ir, in-situ hybridization was applied to study Cx43 mRNA expression. In the cortex of the injured hemisphere, there was a transient downregulation of Cx43 mRNA on day 1 post-ischemia, followed by an increase of Cx43 mRNA positive cells by days 3 and 7 that subsequently normalised. This was accompanied by a generalised reduction in Cx43-ir at all time points. The difference in Cx43 mRNA and protein expression was explained by the possible internalization and degradation of Cx43 protein molecules following ischemia. The underlying hippocampus followed a similar Cx43 mRNA and protein pattern to that displayed in the cortex, except in the stratum oriens subjacent to the injury, which showed elevated Cx43-ir.

In a separate experiment (Haupt et al., 2007b), the group looked more closely at the lesion site, surrounding cortex and developing glial scar following photothrombosis. In the cortex directly flanking the lesion, Cx43-ir was increased as early as 1 day after injury, becoming denser by 7 days and remaining elevated through until day 60. This was accompanied by an increase in glial fibrillar acidic protein (GFAP), a marker for astrocytes, over the same time course. Of note was the appearance of a band of highly GFAP reactive cells and increased Cx43-ir, likely to represent reactive astrocytes at the bottom of the lesion by day 14.

The observations from these experiments raise the concept of a penumbra of increased Cx43-ir surrounding a focal lesion following two rodent models of cerebral ischemia. Nakase et al. (2006) confirmed a similar pattern in post-mortem human brain samples with ischemic damage. Those brains that had suffered embolic strokes were considered as an acute ischemic model, while multiple infarction brains were considered as a chronic ischemic model. Cx43-ir was elevated in the lesion penumbra of both acute and chronic models, as well as in the normal white matter of the chronic model. The distribution of increased Cx43-ir was mirrored by a similar pattern of increase in astrocyte numbers, which also showed expansion of their processes indicative of activation, as measured by GFAP immunoreactivity. In fact, Cx43-ir was predominantly observed in GFAP positive cells in both models; however a subset of Cx43 positive but GFAP negative areas existed in the acute model. Double immunolabelling for microglia, with CD68 and Cx43, revealed that microglia expressed Cx43 more abundantly in the acute model than in the chronic model.

The reduction in the concentration of extracellular Ca2+ associated with global ischemia of the CNS is also likely to result in an increased probability of Cx43 hemichannel opening and interaction between the cellular contents and extracellular matrix (Retamal et al., 2007; Ye et al., 2003; Rana and Dringen, 2007). It seems likely that other factors, such as reactive oxygen species, may cause alterations in the functional state of hemichannels during ischemia (Spray et al., 2006).

Excitotoxins

In support of their cerebral ischemia work, Hossain et al. (1994b) showed that following stereotactic injection of both kainic acid and N-methyl-D-aspartate (NMDA) into rat brain striatum, there was decreased Cx43-ir at the lesion site where there was a depletion of neurons surrounded by a zone of increased Cx43-ir. They demonstrated altered immunohistochemical recognition of Cx43 epitopes and purported that Cx43 molecular modification occurs in excitotoxin-lesioned tissue (Hossain et al., 1994b; Sawchuk et al., 1995; Vukelic et al., 1991).

Trauma

Following acute compression injury of the rat spinal cord, alteration in Cx43-ir was observed in the white and gray matter area of spinal cord for up to 7 days (Theriault et al., 1997). At 1 and 3 days post injury, Cx43-ir was decreased in the lesion epicenter, whereas immediately adjacent regions both rostrally and caudally exhibited intensified staining. By 7 days, all Cx43-ir had returned to normal/resting levels. Reactive astrocytes displaying GFAP appeared by day 1 and were prominent by day 3. Their distribution in white and gray matter corresponded closely to that of Cx43 staining at day 1 and 7, but less so at 3 days when GFAP-positive profiles were present at sites where Cx43 labelling was absent. Ultrastructural observations of the areas of intensified Cx43-ir on nonjunctional astrocytic membranes led the authors to believe these areas represented injury-induced gap junction disassembly and Cx43 dispersal in plasma membrane leading presumably to an uncoupling of astrocytes immediately adjacent to the site of injury.

Using a model of rodent spinal cord transection injury, Lee et al. (2005) examined Cx43, GFAP and OX-42 (microglia marker) immunoreactivity. An increase in Cx43-ir and Cx43 mRNA-positive cells in the gray matter adjacent to the lesion was observed from 4 h post-injury and remained higher than controls at 4 weeks. Cx43-ir mostly colocalized with GFAP rather than with OX-42, indicating that such upregulation was mainly in astrocytes rather
than microglia, though the distribution of OX-42 staining was not described.

Utilizing a brain stab wound injury model, activated microglia were shown to induce Cx43 expression by double labelling sections with isolectin-B4 (microglia marker) and Cx43 (Eugenin et al., 2001). Four days following CNS injury approximately 60% of microglia expressed Cx43 compared to 5% under normal conditions. This corresponds to the period of maximal microglia proliferation after injury with induction of Cx43 presumably triggered by inflammatory mediators.

Another rodent model of traumatic brain injury utilizes lateral fluid percussion, which is thought to be selective for the vulnerable hippocampal CA3 subfield, dentate hilar neurons and cortical neurons. (Ohsumi et al. 2006) observed an initial reduction in Cx43-ir within the hippocampus up to 6 h after injury, followed by a subsequent increase in Cx43-ir from 24 to 72 h. The injured cortex showed a similar increase in Cx43-ir by 72 h compared to controls.

Peripheral nervous system

Due to the intimate relationship between the peripheral nervous system (PNS) and the CNS the response of Cx43 in injury to the former affords mention. It has been established that damage to regions of the CNS can result in an increased expression of Cx43 mRNA and protein in the injury zone, with severe insult causing a site-specific loss of expression surrounded by an upregulated penumbra. Likewise, injury to the PNS can produce responses in Cx43 expression of a similar nature.

A crush injury to the sciatic nerve resulted in dramatic increases to Cx43 mRNA and protein expression in endoneurial fibroblasts within the crush affected region and distal areas at 3 days post-trauma which returned to control levels by 12 days (Chandross et al., 1996). Immediately following the injury Cx43 expression was localized in the cytoplasm before being distributed to the plasma membrane, an indication of de novo synthesis. The rapid return of Cx43 expression to control levels subsequent to the injury suggested the function of the gap junction is limited to early regenerative and degenerative events. This study is consistent with previous observations involving sciatic nerve transection and prolonged capsaicin induced C- fiber discharge resulting in an upregulation of Cx43 (Lin et al., 2002).

Chang et al. (2000) investigated the effects of peripheral axotomy of motor neurons located in the lumbar spinal cord. Following peripheral nerve injury a recoupling of gap junctions, detectable by dye coupling, was observed between the motor neurons. The unchanged expression of Cx43 in the motor neurons led to the hypothesis of gap junction reestablishment through reorganization of the existing Cx43 gap junction proteins. Similarly, peripheral axotomy of facial sensory ganglion cells resulted in increased Cx43-ir in the injured motor neurons and surrounding glial cells 4 days post-trauma (Rohlmann et al., 1993). These findings illustrate functional effects on the CNS as a result of PNS injury.

Role of Cx43 in CNS injury

Neuroprotective or neurodestructive?

While there is little controversy surrounding the great body of evidence outlining changes in Cx43 expression following CNS injury, the role of Cx43 gap junctions and hemichannels in these disease states is of some debate. Adding fuel to the discussions are the actions of gap junction proteins independent of channel formation. Two seemingly disparate views of the role of gap junctional communication in response to CNS injury have been established.

The “good samaritan effect” put forward by Farahani et al. (2005), proposed a number of possible mechanisms by which gap junction communication may be beneficial following injury (see Fig. 2). Cellular injury results in accumulation of deleterious metabolites in the cytoplasm, such as calcium ions and glutamate, key players in excitotoxicity (Perez Velazquez et al., 2003). Gap junction communication would allow the passage of these factors into neighbouring healthy cells. By allowing the buffering of toxic metabolites by healthy cells, injured cells may be prevented from dying. In addition to the idea that astrocytes may buffer neurons from excitotoxicity and hypoxic depolarisation by taking up extracellular glutamate and potassium, they also secrete various neurotrophic factors and cytokines that may stimulate the survival of neighbouring neurons and protect neurons from excitotoxic, metabolic and oxidative insults. Ischemia-induced elevation of endothelin-1 and norepinephrine may lead to enhanced astroglial glycogenesis and glycolysis producing pyruvate and lactate that can pass via gap junction channels to neighbouring neurons to be used as an energy substrate. Reduced glutathione, synthesized in astrocytes, may pass via gap junctions to neighbouring neurons to protect them from oxidative stress.

The opposing view of the role of gap junction communication following CNS injury is that by allowing the spread of death messengers from injured cells to otherwise healthy neighbouring cells, the zone of damage extends. This “gap junction-mediated bystander effect” has been demonstrated by the ability of herpes simplex virus type 1 thymidine kinase (HSV-tk)-expressing cells incubated with ganciclovir to induce cytotoxicity in neighbouring HSV-tk-negative (bystander) human glioblastoma cells in primary culture (Asklund et al., 2003; Freeman et al., 1996; Moolten, 1986). Asklund et al. (2003) further demonstrated that ganciclovir treatment led to the death of 90% of cells in culture within 1 week, but that the loss could be inhibited by the addition of AGA, a gap junction inhibitor that was shown to inhibit intercellular fluorescent dye transfer and immunodetection of Cx43 by Western blot.

Another concept specific to damage spread in the CNS is that of spreading depression, a slowly propagating depression of cerebral neuronal activity and transmembrane ionic gradients, that arises in response to a variety of noxious stimuli (Nedergaard et al., 1995). It is experimentally evoked by applying potassium chloride or glutamate to exposed cortical tissue, or by electrical stimulation. Spreading depression bears a strong resemblance to gap junction-mediated calcium waves triggered by glutamate among cultured astrocytes. Experimental evidence now supports the notion that astrocytic calcium waves constitute the leading edge of a propagating, spreading depression wave. It has been demonstrated that astrocytic calcium increments precede the depolarizing wave of spreading depression by several seconds in acutely prepared hippocampal slices (Rawanduzi et al., 1997).

There are a number of mechanisms proposed for the action of hemichannels in the outcome of injured neurons also. Similar to gap junction channels the bidirectional movement of molecules through the open hemichannel may contribute to the loss of essential substances from the cell to the extracellular environment or to the intake of toxic factors. Kang et al. (2008) demonstrated the permeability of Cx43 hemichannels to ATP using patch-clamp recordings and bioluminescence imaging. The loss of intracellular ATP may have direct affects on the cell itself or act to protect or harm nearby cells through the interactions of its breakdown product adenosine with target receptors (Dunwiddie and Masino, 2001; Lin et al., 2008). Glutathione release from astrocyte cultures in response to a lack of extracellular divalent cations, a condition known to increase the probability of open hemichannels, was demonstrated by Rana and colleagues (2007). It is unclear whether this would harm the cell by compromising the integrity of its antioxidant defence or...
act extracellularly to intercept free radicals before they could threaten the cells. It is also possible that opening of hemichannels could collapse Na⁺/K⁺ gradients or cause an influx of toxic levels of Ca²⁺ (Contreras et al., 2002). As mentioned with adenosine, the released substances may adversely or benefitically act on nearby cells' receptors. An additional mechanism determining the fate of a cell in response to injury involves cell volume regulation, a parameter intricately linked to the functional state of hemichannels (Rodriguez-Sinovas et al., 2007; Quist et al., 2000).

The fact that Cx43 mediated injury resistance or amplification does not necessarily require functional gap junctions or hemichannels provides an intriguing alternative to current neuroprotective and neurodestructive theories. Dang et al. (2003) illustrated using immunofluorescence and Western blotting that expression of only the C-terminal region of Cx43, a non channel forming domain, was enough to elicit a negative response in cell growth. Location of the COOH-terminal portion of Cx43 predominantly in the nucleus suggests the effects may be exerted at a genetic level. Under circumstances of increased protein degradation, for example during ischemia, the action of Cx43 may play a significant role in the fate of injured and healthy cells in a mechanism independent of gap junction activity.

In order to determine the role of Cx43 in the CNS response to injury, investigators have studied the effect of altering the expression of Cx43 and thus astrocytic gap junctional communication in the CNS. A complicating factor in this is the differential regulation and functions of gap junctions and unopposed hemichannels, two structures assembled from the same protein.

Methods of altering Cx43 expression

A wide range of neurotransmitters, growth factors, and cytokines regulate or alter Cx43 expression and gap junction permeability in astrocytes (De Maio et al., 2002; Salameh and Dhein, 2005). For example, treatment of cultured astrocytes with interleukin-1 beta (IL-1) for several hours reduced Cx43 levels and cell to cell communication (John et al., 1999a; John et al., 1999b). Reactive astrocytes, as well as activated microglia, have been shown to produce IL-1 and activated microglia in cultures have been shown to reduce astrocytic gap junctional communication (Faustmann et al., 2003; Rouach et al., 2002). Chemical regulation of Cx43 by insulin-like growth factor and insulin uncoupling has been demonstrated to occur via a particle-receptor mechanism (Homma et al., 1998). Endothelins have been shown to decrease the expression of phosphorylated Cx43 forms and are potent inhibitors of gap junction communication in astrocytes (Blomstrand et al., 2004). Even human marrow stromal cells have been shown to increase Cx43 expression in co-culture with astrocytes (Gao et al., 2005). Of particular note is that halothane, used in numerous animal studies as inhalational anaesthesia, has also been shown to influence gap junctional communication in cultured cells (Li et al., 1998).

While many factors have been shown to influence Cx43 expression, experimental blockade of Cx43 expression is the key to understanding its role in CNS injury. This has been performed using three methods: knockout mice models, global gap junction blockade, and specific transient blockade.

Connexin knockouts permanently delete a gene, but permit compensatory changes in gene expression. Inducible knockouts allow for greater time and tissue specificity. Homozygous Cx43-knockout transgenic mice, with permanent deletion of the Cx43 gene, do not survive beyond the perinatal period due to malformation of the conus region overlying the pulmonary outflow tract. Failure at the pulmonary gas exchange, heart embryonic alteration and delayed ossification and osteoblast dysfunction has been noted (De Maio et al., 2002). While homozygous Cx43-knockout mouse tissue has been used for in vitro studies, it is the heterozygous Cx43-knockout mice, which have normal survival, that are used for in vivo studies. Knockout models cannot however differentiate between the involvement of gap junctions and hemichannels in in vivo studies as both are suppressed through reduced Cx43 expression. Sushansian et al. (2001) confirmed that astrocytic cultures from homozygous and heterozygous Cx43-knockout mice had lower levels of Cx43, a more dispersed Cx43 distribution, and decreased dye-coupling compared to wild-type animals. A key issue
with Cx43-knockout models is their permanent effect on Cx43-gap junction communication, which is known to be necessary for normal astrocytic homeostasis (Bennett et al., 2003; Nagy and Rash, 2000). Although reduction in Cx43 expression is achieved through use of knockout models concerns arise over the possible compensatory effects from other connexin proteins. Connexin30 (Cx30) gap junction proteins have been clearly demonstrated accompanying, and even colocalizing with, Cx43 on astrocytes (Nagy et al., 1997, 1999). Any effects observed using Cx43-knockout animals should be interpreted with caution as Cx30 may, at least partially, compensate for the loss in Cx43 gap junction activity.

Global gap junction blockade is usually induced by global gap junction blockers such as carbenoxolone, a derivative of glycyrrhetic acid, which is extracted from the licorice root (Blanc et al., 1998). Carbenoxolone is thought to uncouple gap junctions by disrupting connexon particle arrangements. It is relatively specific for gap junctions and nontoxic compared with more harmful agents such as octanol and heptanol (Contreras et al., 2004). The major criticism of these global gap junction blockers are that while they appear relatively specific for gap junctions, they are not specific for different connexins and that some can reduce transmission at chemical synapses by a direct effect (Puil and el-Beheiry, 1990; Rouach et al., 2003). In addition, hemichannel effects need to be considered.

Specific transient connexin blockade can be induced by agents such as antisense oligodeoxynucleotides (AS-ODNs) or mimetic peptides (Contreras et al., 2004; Evans and Boitano, 2001; Green et al., 2001; O’Carroll et al., 2008). The advantage of these agents is not only their specificity for Cx43, but their transient knockdown of gap junction communication allows for quick recovery and return of normal astrocytic homeostasis (Green et al., 2001). AS-ODNs are short chain nucleotides that temporarily block the expression of a target gene by binding to its mRNA, preventing translation and thus production of a specific protein (Moore and Burt, 1994). Because unmodified AS-ODNs have a short half-life of about 20 min, after which they are degraded by cell nucleases, incorporation of a Pluronic gel delivery system aids penetration of AS-ODNs into cells and acts as a reservoir to provide sustained release (Green et al., 2001). Knockdown of Cx43 expression can be achieved for 24 to 48 h using this gel delivery system. Connexin mimetic peptides are small peptide sequences designed against the extracellular regions of the connexin molecule. They may impair the interactions of the extracellular loops by binding to recognition sites on the hemichannel. Mimetic peptides have been shown to regulate both hemichannels and gap junctions independently of each other dependent on administration concentration (O’Carroll et al., 2008).

While each of these methods results in decreased Cx43 expression, contradictory results obtained must be interpreted with caution due to key differences in specificity and duration of action.

In vitro/ex vivo studies

Knockout

Hippocampal organotypic slice cultures were used by Frantseva et al. (2002a) in a traumatic model of weight-drop injury. Hippocampal slices were taken from newborn homozygous, heterozygous and wild-type Cx43-knockout mice. Cell death was evaluated in the neocortical region, and was found to be significantly lower in slices from Cx43-knockout animals 24 h after the impact when compared to wild-type and heterozygote littermates.

Global blockade

Several lines of evidence using global blockade of gap junctions suggest that gap junction coupling between cells play a role in bystander killing. Lin et al. (1998) generated two different glial lines to evaluate the role of gap junction coupling following metabolic injury. The first line was transfected with cDNA for Cx43 (Cx+) and demonstrated functional gap junction coupling, but remained susceptible to metabolic injury, oxidative stress and calcium ionophore. The second line underwent double transfection with Cx43 and bcl2 (bcl + Cx+), an anti-apoptotic gene allowing increased resistance to injury. As expected, when bcl + Cx+ cultures were exposed to the calcium ionophore, less than 5% of cells died. However when mixed cultures were exposed to the calcium ionophore, death of the vulnerable Cx+ cell line occurred but more importantly, 95% of the bcl + Cx+ line also died. The death of the injury resistant cells indicated that gap junction coupling between the Cx+ cells and the bcl + Cx+ cells contributed to the increased death of the previously resistant cells. This spread of injury from the Cx+ cells to the bcl + Cx+ cells was confirmed by blockade with a global gap junction blocker, AGA, which significantly reduced the incidence of bcl + Cx+ cell death in co-culture. Death of bcl+ cells could also be modified by varying the degree of Cx43 coupling, with intermediate levels inducing less death than their high-expression clones. The potency of bystander killing was also a direct function of the coupling index between resistant and non-resistant cells, with lowering of the concentration of non-resistant Cx+ cells resulting in a reduced amount of bystander death. Increase in death of bcl + Cx+ cells was also demonstrated by substituting the Cx+ cells for primary astrocytes. Annexin V staining confirmed that death of bcl + Cx+ cells was not occurring by the primary exposure, but by delayed secondary mechanisms. Gap junction coupling was shown to raise calcium levels in resistant cells, which usually had reduced calcium levels, to levels of surrounding non-resistant cells.

Other investigators have also evaluated the role of gap junctions using global gap junction blockers with various in vitro hippocampal tissue models. Frantseva et al. (2002a) incubated wildtype organotypic hippocampal slices with global gap junction blockers, carbenoxolone or octanol, following traumatic weight drop injury. This resulted in significant neuroprotection, measured over 72 h, with improved synaptic function and decreased cell death in the CA1-3 pyramidal layers if the blocker was added up to 2 h after the traumatic insult. However, preincubating and removing carbenoxolone 5–10 min after the impact resulted in no neuroprotection. While gap junction blockade was observed to offer neuroprotection, promotion of gap junction communication with intracellular alkalinisation by bicarbonate incubation enhanced the cell loss.

The same group (Frantseva et al., 2002b) repeated the experiment with a different injury model of in vitro ischemia-reperfusion with deoxygenated glucose-free culture medium. Incubating the organotypic cultures with carbenoxolone again resulted in significant neuroprotection with cell loss in the pyramidal cell body layers 36% of that displayed in untreated slices subjected to identical insults. Significant neuroprotection was observed if the blocker was added 2 h after the ischemic episode, though in this case cell death was higher than that found when the drug was present throughout the experiment. Addition of carbenoxolone 24 h after the ischemic injury did not result in appreciable neuroprotection in any hippocampal area. From these results, the global blockade of gap junction communication was seen to have a neuroprotective role. These results suggest that there is a critical time, between 2 and 24 h after injury, in which gap junction coupling results in the spread of injury. Blockade of gap junction communication during this time may be neuroprotective.

In agreement with these findings is work by de Pina-Benabou et al. (2005) who used a different oxygen-glucose deprivation (OGD) regime on organotypic hippocampal slice cultures. When a global gap junction blocker, carbenoxolone, was added to the culture medium 30 min before, during or 60 min after OGD, markedly delayed death of CA1 pyramidal neurons was observed by propidium iodide labelling 24 h following the insult.
Nodin et al. (2005) induced chemical ischemia on primary rodent hippocampal astrocyte cultures and studied the effects of carbeneoxide, glycyrhrizic acid, heptanol and octanol. Their model utilized iodoacetate to induce ATP depression and subsequent apoptosis. Initiation of apoptosis was defined by the number of Annexin V positive cell groups. The spread of apoptosis by how much these Annexin V cell groups enlarged. Following gap junction blockade, the number of apoptotic processes remained similar to controls, but did not enlarge indicating that gap junction blockade inhibited the spreading but not initiation of apoptosis. In separate experiments employing calcium chelation to prevent the rise in intracellular calcium from iodoacetate, they observed an earlier apoptosis initiation by slower progression of ATP decline and no decrease in Annexin V positive cells. Similarly, broad spectrum caspase inhibition also did not alter the Annexin V positive cells.

The work of Ye and colleagues (2003) demonstrated the influence of functional hemichannels in cultured hippocampal astrocytes and their potential role in CNS injury. Passage of extracellular lucifer yellow dye into astrocytes exposed to a divalent-cation free solution, conditions known to induce opening of hemichannels, was observed as well as the release of large amounts of glutamate and other amino acids from the astrocytes. These observations were effectively blocked with the gap junction blocking agents carbeneoxide, heptanol, heptanol, flufenamic acid and AGA. It was suggested that the amino acid efflux, produced under conditions similar to those experienced during ischemia, may contribute to the injury cascade. Ex vivo experiments on isolated optic nerves corroborated the in vitro results and strengthened the argument for functional hemichannel involvement in response to injury.

Other investigators have demonstrated that gap junction inhibition with octanol and carbeneoxide significantly reduces bystander killing in other in vitro CNS models. Cusato et al. (2003) induced retinal cell death with cytochrome c (Cc) and examined the induced cells and their neighbours for apoptotic morphology or caspase-3 cleavage. Specifically they used a scrape-loading technique to introduce Cc into the cytoplasm of retinal cells in situ. Rhodamine dextran (RD) served as a marker for these cells. While the majority of RD-labelled, Cc-scrape loaded cells were pyknotic, bystander killing was observed in the form of neighbouring, unlabelled pyknotic nuclei. Similarly, caspase-3 cleavage was detected in both RD-labelled and unlabelled bystander cells, confirming the presence of bystander cell apoptosis.

C6 glioma cells are widely used as models for astrocytes in gap junction investigations due to conservation of receptor types, ion channels and transport systems accompanied with a decreased degree of gap junction coupling. Despite the expression of non-functional mutant Cx43 significant resistance to injury by a variety of apoptotic stimuli was observed in C6 cell cultures in a mechanism deemed independent of gap junction coupling or hemichannel activation (Lin et al., 2003). In support of this notion was the continued protective effect despite pharmacological inhibition using AGA as well as physical isolation of cells through low density plating. The effects were attributed to Cx43 mediated cytoskeletal reorganization and rapid normalization of elevated calcium levels. Direct interaction between Cx43 and cytoskeletal proteins has been well established (Giepmans, 2004).

While the above studies demonstrate the neuroprotective effects of global gap junction blockade, other investigators have observed an increase in neuronal damage and extent of injury after blocking gap junctions. Blanc et al. (1998) investigated the effect of high gap junction blockade, by AGA or halothane, on neuronal vulnerability to oxidative injury in embryonic rat hippocampal cell cultures. Following oxidative insults with iron sulphate (FeSO\(_4\)) and 4-hydroxynonenal, gap junction blockade markedly enhanced the generation of intracellular peroxides, the impairment of mitochondrial function and the amount of cell death (as measure by intracellular lactate dehydrogenase release) in neurons but not astrocytes. Antioxidants (propyl gallate and glutathione) blocked the death of neurons exposed to FeSO\(_4\) and AGA. Neuronal intracellular calcium levels also increased with gap junction blockade following FeSO\(_4\) exposure, and the calcium channel blocker nimodipine prevented impairment of mitochondrial function and cell death, whereas glutamate receptor antagonists were ineffective. In an attempt to best replicate in vivo conditions, they exposed organotypic hippocampal slice cultures to FeSO\(_4\) and kainate oxidative insults, and showed that AGA also exacerbated these injuries. Finally, they excluded nitric oxide and impaired glutamate transport as a mediator of the effects of AGA.

Exacerbation of injury following gap junction blockade was also shown by Ozog et al. (2002) and Naus et al. (2001) in a model of glutamate cytotoxicity in co-cultures of mice cortical astrocytes and neurons. Treatment with carbeneoxide prior to glutamate exposure led to a 20–25% increase in cell death as assessed by terminal dUTP nick end labelling (TUNEL) and propidium iodide staining, with associated increases in lactate dehydrogenase release.

Specific transient blockade

Cx43-specific blockade has been shown to have a neuroprotective effect following several in vitro studies. Frantseva et al. (2002a; 2002b) used Cx43 AS-ODNs to confirm their neuroprotective knockout and global blockade results in both trauma and ischemia models with organotypic hippocampal slices. Partial knockdown of Cx43, confirmed functionally and biochemically by Western blot, resulted in significantly less pyramidal and dentate gyrus cell death at 48 h compared to controls or sense oligodeoxynucleotides.

Further work with Cx43 AS-ODNs was performed by Danesh-Meyer et al. (2008) in an in vitro ischemic optic nerve model. Results demonstrated that in addition to limiting lesion spread at 24 h, Cx43 AS-ODNs modulated several facets of the inflammatory process with reduced tissue swelling, improved vascular integrity and slowed differentiation of inflammatory cells (astrocytes and microglial cells). In control tissue, Cx43 upregulation was generalised and persisted for 2 to 3 days post-ischemia. Cx43 AS-ODNs knocked down this upregulation and any increases in expression were confined to the cut edges of the optic nerve. These findings supported the concept that ischemic injury to the optic nerve is associated with an upregulation in the local inflammatory response that is at least partially exacerbated by a Cx43 gap junction-mediated bystander effect.

O’Carroll et al. (2008) used Cx43 mimetic peptides in an ex vivo model of spinal cord injury where cut spinal cord segments were placed in culture. The amount of tissue swelling and extrusion from both dural ends was compared following co-culture with Cx43 mimetic peptides for 24 h. An almost 50% reduction in swelling compared with controls was observed. A similar reduction in astrocyte activation and neuronal loss with mimetic peptide treatment was demonstrated by both Western blotting and immunohistochemistry. Cx43 analysis showed that the increase seen at 4 h and beyond in response to injury was markedly reduced by mimetic peptide treatment. Gap junction blockade with Cx43 mimetic peptide was confirmed with dye spread studies and the neuroprotective effect was both concentration and time dependent suggesting the possibility of a dual Cx43 role in response to the CNS injury. Initially following the injury hemichannels may have facilitated the cell swelling process and movement of toxic materials from injured cells, an effect disrupted by low concentrations of mimetic peptide within the first 24 h post-injury. Subsequent to this astrocytic gap junctions were likely to be involved in long term survival through spatial buffering of signals and were effectively blocked by high concentration mimetic peptide application.
Knockouts

Nakase et al. (2003b) performed various experiments using a rodent stroke model by middle cerebral artery occlusion in heterozygous Cx43-knockout mice. Firstly the investigators demonstrated that the area of infarction was more extensive in the knockout mice than their wildtype counterparts four days after injury (Siushansian et al., 2001).

Their second experiment (Nakase et al., 2003a) measured markers of apoptosis as well as stroke volume using the same injury model. They observed increased apoptosis by TUNEL labelling in the penumbra, increased caspase-3 levels in the stroke lesion, and again an increased infarct volume in Cx43-knockout mice when compared to wildtype. Interestingly, the number of TUNEL positive cells in the knockout mice was still increased at 4 days despite apoptosis usually reaching a maximum at 24 h or 48 h after ischemia. The authors therefore speculated that Cx43 gap junctions allow removal of pro-apoptotic and cytotoxic agents from the ischemic lesion. It was also observed that the average length of astrocytic processes was significantly increased in the penumbra of knockout mice. While there was no significant difference between the number of GFAP-positive astrocytes in knockout and wildtype mice, they found a reduced area of astrogliosis by immunohistochemistry but an increased ratio of GFAP by western blot in the knockout mice compared to controls.

A potential limitation of these experiments is the unwanted effects of global Cx43-knockout on other cell types. The investigators addressed this in a third set of experiments (Nakase et al., 2004) by breeding transgenic mice lacking Cx43 in astrocytes (Cre+) but with normal Cx43 levels in other cell types. In this way, they could specifically analyze the neuroprotective role of astrocytic gap junctions in focal ischemia. Animals with astrocyte-directed ablation of Cx43 exhibited a significantly increased infarct volume compared to wildtypes, as well as significantly enhanced apoptosis as detected by TUNEL labelling and cleaved caspase-3 immunolabelling. These results additionally supported the notion that a decrease or loss of astrocytic Cx43 increases the vulnerability to ischemic stroke. An increase in CD11b immunolabelling, an inflammatory marker for microglia and macrophages, was also observed in the lesion of Cre+ mice and it was therefore postulated that an increase in cytokine production from activated astrocytes might cause enhanced inflammation after ischemic insult.

It must be reiterated that these Cx43-knockout experiments remain limited by the lack of gap junction recovery and thus return of normal astrocytic homeostasis that features in the transient gap junction blockade models.

Global blockade

There is substantial evidence that global gap junction blockade has a neuroprotective effect following in vivo CNS injury. Using the rodent model of focal brain ischemia by middle cerebral artery occlusion, Rawanduzy et al. (1997) intraperitoneally pretreated rats with the global gap junction blocker, octanol and compared the sizes of the ischemic lesions to those that received vehicle dimethyl sulfoxide. Histopathological analysis, performed 24 h later, showed a significantly decreased mean infarction volume in the octanol group when compared to wildtype. Interestingly, the number of TUNEL positive cells in the knockout mice was still increased at 4 days despite apoptosis usually reaching a maximum at 24 h or 48 h after ischemia. The authors therefore speculated that Cx43 gap junctions allow removal of pro-apoptotic and cytotoxic agents from the ischemic lesion. It was also observed that the average length of astrocytic processes was significantly increased in the penumbra of knockout mice. While there was no significant difference between the number of GFAP-positive astrocytes in knockout and wildtype mice, they found a reduced area of astrogliosis by immunohistochemistry but an increased ratio of GFAP by western blot in the knockout mice compared to controls.

Specific transient blockade

Cronin et al. (2008) used transient Cx43 specific blockade in an in vivo model of CNS injury. They manipulated the expression of Cx43 protein in the hours following two rodent models of spinal cord injury with Cx43 AS-ODNs. Within 24 h following a partial transection injury, spinal cords were less swollen and inflamed in external appearance, and showed less tissue disruption, than controls. Cx43 AS-ODNs was confirmed to reduce the elevation of Cx43 and GFAP levels normally seen in response to transection or compression injuries. The functional consequences of Cx43 AS-ODNs application were also assessed by observation of the locomotor ability following injury. Treated rats showed clear improvements in locomotion compared to controls. Assessment of tissue inflammation was conducted with markers for neutrophils (myeloperoxidase), activated microglia (OX42 in injury site) and leakage from damaged vasculature (intravenous FITC-Bovine Serum Albumin). Fewer neutrophils and microglia, along with reduced leakage from blood vessels, were observed at the lesion site in Cx43 AS-ODNs treated animals. Furthermore, the morphology of the microglia in treated animals was more stellate than typical activated, rounded, phagocytic forms seen in controls.

Finally, Li et al. (2005) looked at the effects of Ginko biloba extract and nimodipine on Cx43 mRNA and protein expression, and neurological function after rodent cerebral ischemia. They showed improved neurological function and lower Cx43 mRNA and protein levels (by Western blot) following intraperitoneal nimodipine 1 h prior to injury, or Ginko biloba extract administered orally for 7 days prior to injury.

Discussion

Cx43 remains the most widely studied connexin protein in the literature. The preceding observations of Cx43 immunohistochemistry following CNS injury, outlined in Table 1, indicate that the response of Cx43 varies with severity of injury. Generally, mild to moderate injury appears to lead to increased Cx43-ir in the lesion site...
Table 1
Response of Cx43 following CNS injury.

<table>
<thead>
<tr>
<th>Model and injury</th>
<th>Changes in Cx43/gap junction coupling</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td></td>
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<tr>
<td>Astrocytic cultures</td>
<td>Decreased but functional gap junction coupling</td>
<td>Cotrina et al. (1998)</td>
</tr>
<tr>
<td>Iontophore exposure or metabolic inhibition</td>
<td>Dephosphorylation of Cx43, epitope masking, gap junction internalization</td>
<td>Li and Nagy, 2000; Nagy and Li, 2000</td>
</tr>
<tr>
<td>Astrocytic cultures</td>
<td>Permeabilization of astrocytes thought to be due to opening of Cx43 hemichannels</td>
<td>Contreas et al. (2002)</td>
</tr>
<tr>
<td>Iodoacetate hypoxia</td>
<td>Increased Cx43 hemichannel surface expression possibly due to S-nitrosylation</td>
<td>Retamal et al. (2006)</td>
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<tr>
<td>Astrocytic diseases</td>
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<td>Chemical ischemia by metabolic inhibition</td>
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<td>Chemical ischemia by metabolic inhibition</td>
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<tr>
<td>In vivo</td>
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<tr>
<td>Global cerebral ischemia</td>
<td>In hippocampus and striatum, increased Cx43 levels following mild-moderate insult, but decreased Cx43 levels with surrounding increased Cx43 following severe insult</td>
<td>Hossain et al. (1994a)</td>
</tr>
<tr>
<td>Focal cerebral ischemia</td>
<td>Decreased Cx43 levels in ischemic core, but increased in a thin corridor at the ischemic penumbra</td>
<td>Li et al. (1998)</td>
</tr>
<tr>
<td>Focal cerebral ischemia</td>
<td>Increased Cx43-ir in vulnerable CA1/2 hippocampal subfields but not resistant CA3 subfield</td>
<td>Rami et al. (2001)</td>
</tr>
<tr>
<td>Focal cerebral ischemia</td>
<td>In cortex and hippocampus, transient downregulation of Cx43 mRNA followed by upregulation; reduced Cx43-ir except in hippocampus subjacent to injury</td>
<td>Haupt et al. (2007a,b)</td>
</tr>
<tr>
<td>Photothrombosis of cortex</td>
<td>Increased Cx43-ir in cortex directly bordering lesion</td>
<td>Haupt et al. (2007a,b)</td>
</tr>
<tr>
<td>Human cerebral ischemia</td>
<td>Increased Cx43-ir in injury penumbra</td>
<td>Nakase et al. (2006)</td>
</tr>
<tr>
<td>Post-mortem stroke</td>
<td>Decreased Cx43 levels in lesion surrounded by increased Cx43-ir in lesion border</td>
<td>Sawchuk et al., 1995; Hossain et al., 1994a,b; Vukelic et al., 1991</td>
</tr>
<tr>
<td>Excitotoxic cerebral damage</td>
<td>Decreased Cx43-ir at lesion epicenter with increased Cx43-ir in adjacent regions</td>
<td>Theriault et al. (1997)</td>
</tr>
<tr>
<td>NMDA injection</td>
<td>Decreased Cx43-ir at lesion epicenter with increased Cx43-ir in adjacent regions</td>
<td>Lee et al. (2005)</td>
</tr>
<tr>
<td>Spinal cord trauma</td>
<td>Decreased Cx43-ir by 24–72 h</td>
<td>Obushima et al. (2006)</td>
</tr>
</tbody>
</table>

or in vulnerable CNS areas as displayed by Rami et al. (2001) and Haupt et al. (2007a,b) utilizing brain ischemia models, Obushima et al. (2006) with a traumatic brain injury model and corroborated through PNS investigations. Severe injury on the other hand seems to result in decreased Cx43-ir within the injury site probably due to cell death in that region, and a surrounding zone of increased Cx43-ir in what is described as the penumbra of the injury in various models. This has been well documented in the brain (Hossain et al., 1994a,b; Li et al., 1998; Haupt et al., 2007a,b; Nakase et al., 2006; Sawchuk et al., 1995; Vukelic et al., 1991) and spinal cord (Theriault et al., 1997; Lee et al., 2005). Increased cell surface expression (Retamal et al., 2006) and open probability of Cx43 hemichannels (Contreras et al., 2002) has also been implicated in the response to CNS injury (see Table 1). Furthermore, there is evidence to suggest the action of Cx43 at a genetic level, independent of any channel activity (Dang et al., 2003).

There can be little debate that Cx43 gap junction communication and hemichannel functional state are important mediators following CNS injury. In mild injury increased Cx43 expression may be involved in diffusion or spreading of substances such as potassium and glutamate, either buffered through gap junctions or released into the extracellular matrix via hemichannels (Ye et al., 2003), that might otherwise reach toxic levels. For more extensive injury there is, however, evidence in support of both neuroprotection and neurodestruction following Cx43 blockade (outlined in Tables 2 and 3). In these injuries extensive coupling may exacerbate the injury through ATP release, or by spreading toxins and death signals. The majority of this evidence comes from studies using global gap junction blockade, with most of these results indicating that neuroprotection is achieved. A reduction in neuronal cell death is commonly observed when blocking agents are applied within 2 h following an injury (Frantseva et al., 2002a; Frantseva et al., 2002b; de Pina-Benabou et al., 2005; Perez Velazquez et al., 2006). Pretreatment, up to 60 min prior to injury, has shown promising results in terms of cell survival too (de Pina-Benabou et al., 2005; Rawanduzi et al., 1997; Rami et al., 2001; Perez Velazquez et al., 2006; Cusato et al., 2003). Accompanying the decrease in cell death following gap junction blockade is improved synaptic functioning and vascular integrity and reduced tissue swelling and astrocyte activation (Frantseva et al., 2002a; Danesh-Meyer et al., 2008; O’Carroll et al., 2008; Cronin et al., 2008). There is limited evidence of increased neurodestruction following global gap junction blockade in vivo. However, results from experiments using global blockade should be interpreted with caution due to the nonspecific nature of agents such as carbenoxolone and octanol. Cx43-knockout models are another popular choice and interestingly most of the in vivo evidence in support of continued neurodestruction or lesion spread comes from Cx43 knockout mice (Siushansian et al., 2001; Nakase et al., 2003a,b; Nakase et al., 2004). Again caution should be used when interpreting results from Cx43 knockout models as other connexin proteins may compensate for the reduced expression of Cx43. While the above data is seemingly disparate, it may not necessarily be mutually exclusive. Cx43-knockout models result in permanent Cx43 modulation and thus it would make logical sense that the lack of gap junction recovery would impact negatively on a return to normal astrocytic homeostasis.

Results suggest a critical time in which gap junction coupling and hemichannel activation results in the mediation of injury. Indeed the role of Cx43 appears to vary with time following an injury. Initially after a CNS insult Cx43 hemichannels may be involved in the swelling of cells and spread of neurotoxic molecules from injured cells into the extracellular milieu. At later stages spatial buffering through the action of astrocytic gap junctions appears to be required for long term neuroprotection. Combined, the data suggest that permanent blockade of Cx43 may not be neuroprotective, but transient blockade targeting the window of initial Cx43 upregulation observed following injury is potentially therapeutic. Transient Cx43 specific knockdown, by
Cx43 antisense oligodeoxynucleotide or Cx43 mimetic peptides, leads to a return to normal Cx43 function and there is building evidence of neuroprotection following their application both in vitro and in vivo. These exciting neuroprotective interventions may well be the way forward in this field and future work should be directed at determining the efficacy and optimum dosing regimens for transient Cx43 blockade following various models of CNS injury.

### Table 2
Role of Cx43 blockade following CNS injury—neuroprotective.

<table>
<thead>
<tr>
<th>Model and injury</th>
<th>Gap junction blockade</th>
<th>Effects vs. controls</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampal organotypic slices Weight-drop trauma</td>
<td>Cx43 knockout mice</td>
<td>Decreased neocortical cell death at 24 h</td>
<td>Frantseva et al. (2002a,b)</td>
</tr>
<tr>
<td>Hippocampal organotypic slices Weight-drop trauma</td>
<td>Carbenoxolone or octanol (post-injury)</td>
<td>Decreased pyramidal cell death and improved synaptical function</td>
<td>Frantseva et al. (2002a,b)</td>
</tr>
<tr>
<td>Hippocampal organotypic slices Weight-drop trauma</td>
<td>Cx43 AS-ODNs</td>
<td>Decreased cell death at 48 and 72 h</td>
<td>Frantseva et al. (2002a,b)</td>
</tr>
<tr>
<td>Hippocampal organotypic slices Hypoxia-hypoglycemia</td>
<td>Carbenoxolone (pre-injury)</td>
<td>Decreased pyramidal cell death</td>
<td>Frantseva et al. (2002a,b)</td>
</tr>
<tr>
<td>Hippocampal organotypic slices Hypoxia-hypoglycemia</td>
<td>Cx43 AS-ODNs</td>
<td>Decreased pyramidal and dentate gyrus cell death</td>
<td>Frantseva et al. (2002a,b)</td>
</tr>
<tr>
<td>Hippocampal astrocyte cultures</td>
<td>Carbenoxolone</td>
<td>Reduction in death of pyramidal neurons</td>
<td>Frantseva et al. (2002a,b)</td>
</tr>
<tr>
<td>Hippocampal astrocyte cultures Chemical ischemia</td>
<td>Carbenoxolone, heptanol, octanol</td>
<td>Inhibited the spreading of astrocytic apoptosis</td>
<td>Nodin et al. (2005)</td>
</tr>
<tr>
<td>Transfected gial culture</td>
<td>Carbenoxolone, heptanol, octanol, AGA, flufenamic acid</td>
<td>Reduced glutamate/aspartate release through hemichannels</td>
<td>Ye et al. (2003)</td>
</tr>
<tr>
<td>Retinal whole-mounts</td>
<td>Carbenoxolone or octanol</td>
<td>Reduction in bystander killing/apoptosis</td>
<td>Cusato et al. (2003)</td>
</tr>
<tr>
<td>C6 glioma cell culture</td>
<td>AGA</td>
<td>No significant change in cell death due to mechanism of injury resistance independent of gap junction coupling and hemichannel activation</td>
<td>Lin et al. (2003)</td>
</tr>
<tr>
<td>Optic nerve segments</td>
<td>Cx43 AS-ODNs</td>
<td>Reduced tissue swelling, inflammation and lesion spread. Improved vascular integrity</td>
<td>Danesh-Meyer et al. (2008)</td>
</tr>
<tr>
<td>Spinal cord segments Transaction trauma</td>
<td>Cx43 mimetic peptide</td>
<td>Reduction in tissue swelling, astrocyte activation and neuronal loss</td>
<td>O’Carroll et al. (2008)</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
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<tr>
<td>Rodent stroke</td>
<td>Octanol (Intraperitoneal)</td>
<td>Decreased infarct volume and inhibition of spreading depression</td>
<td>Rawanduzu et al. (1997)</td>
</tr>
<tr>
<td>Transient forebrain ischemia</td>
<td>Octanol (Intraperitoneal)</td>
<td>Reduced neuronal death in pyramidal hippocampal subfields</td>
<td>Rami et al. (2001)</td>
</tr>
<tr>
<td>Bilateral carotid artery occlusion</td>
<td>Carbenoxolone, AGA, endothelin</td>
<td>Decreased cell death and lipid peroxidases</td>
<td>Perez Velazquez et al. (2006)</td>
</tr>
<tr>
<td>Perinatal ischemia</td>
<td>Carbenoxolone</td>
<td>Decreased neuronal death, apoptosis, histopathologic damage and developmental impact</td>
<td>de Pina-Benabou et al. (2005)</td>
</tr>
<tr>
<td>Developing retina Normal apoptosis</td>
<td>Carbenoxolone</td>
<td>Decreased clustering of dying cells</td>
<td>Cusato et al. (2003)</td>
</tr>
<tr>
<td>Spinal cord trauma Partial transection Compression injury</td>
<td>Cx43 ASODN</td>
<td>Decreased tissue swelling, inflammation and improved locomotor ability</td>
<td>Cronin et al. (2008)</td>
</tr>
<tr>
<td>Rodent stroke Middle cerebral artery occlusion</td>
<td>Ginko biloba or nimodipine</td>
<td>Improved neurological function and lower Cx43 mRNA/protein levels</td>
<td>Li et al. (2005)</td>
</tr>
</tbody>
</table>

### Table 3
Role of Cx43 blockade following CNS injury—neurodestructive.

<table>
<thead>
<tr>
<th>Model and injury</th>
<th>Gap junction blockade</th>
<th>Effects vs. controls</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampal cell cultures and organotypic slices Oxidative injury with FeSO4</td>
<td>AGA or halothane</td>
<td>Increased cell death, generation of intracellular peroxidises and impaired mitochondrial function</td>
<td>Blanc et al. (1998)</td>
</tr>
<tr>
<td>Astrocyte-neuron co-cultures Glutamate cytotoxicity</td>
<td>Carbenoxolone</td>
<td>Increased cell death, increased LDH release</td>
<td>Ozog et al., 2002; Naus et al., 2001</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rodent stroke</td>
<td>Cx43-knockout heterozygotes</td>
<td>Increased area of infarction at 4 days</td>
<td>Siushansian et al. (2001)</td>
</tr>
<tr>
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