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PERSPECTIVES

Strategies to limit dysmyelination during secondary degeneration following neurotrauma

Following trauma to the central nervous system (CNS), cells in the lesion site die rapidly. In addition, neurons and glia beyond the initial lesion are vulnerable. These cells can undergo delayed death due to metabolic events that follow the initial trauma, *via* mechanisms thought to be triggered by glutamate-induced excitotoxicity and Ca^{2+} overload, leading to mitochondrial dysfunction, associated with increased oxidative stress (Camello-Almaraz et al., 2006; Peng and Jou, 2010). The resultant death of areas of grey and white matter adjacent to the lesion site is termed secondary degeneration, and is a feature of brain and spinal cord injury (Park et al., 2004; Giaume et al., 2007). Secondary degeneration contributes substantially to functional loss following neurotrauma (Profyris et al., 2004; Farkas and Povlishock, 2007) and rescuing this intact, but vulnerable, tissue is considered critical to minimising adverse sequelae and improving long term functional outcomes after CNS trauma (Fehlings et al., 2012). However, our understanding of many of the metabolic events thought to contribute to secondary degeneration is based largely on *in vitro* studies (Khodorov, 2004; Tretter et al., 2007; Peng and Jou, 2010) and there is a need to confirm the relevance of these mechanisms *in vivo*, as well as their structural and functional consequences.

To study and develop treatments for secondary degeneration, it is essential to have a reproducible *in vivo* model system that simulates the complex events that occur in humans and allows statistical verification of tissue rescue and functional improvements. Levkovitch-Verbin and colleagues developed an elegant partial optic nerve transection model in which only the dorsal optic nerve is injured, allowing spatial separation of the dorsal primary injury from ventral optic nerve white matter vulnerable to secondary degeneration (Levkovitch-Verbin et al., 2001; Levkovitch-Verbin et al., 2003; Blair et al., 2005). We and others have built upon these studies and this brief review describes some of this work further characterising metabolic and structural features of secondary degeneration following partial optic nerve transection, with particular reference to dysmyelination, and assessment of efficacy of treatment strategies to limit these changes.

Ca^{2+} changes and oxidative stress during secondary degeneration *in vivo*

Altered distribution of Ca^{2+} ions is thought to be a key early event in secondary degeneration, but these fine-scale changes are difficult to track *in vivo*. Using nanoscale secondary ion mass spectrometry (NanoSIMS) we have quantified changes in calcium (Ca) microdomains, which are localised areas of increased Ca^{2+} concentration (Rizzuto and Pozzan, 2006). We showed that the density of specific subsets of Ca microdomains selectively and significantly decreased after injury, in ventral optic nerve vulnerable to secondary degeneration (Wells et al., 2012; Lozic et al., 2014). Decreased density of Ca microdomains may be accompanied by an efflux of Ca^{2+} from these microdomains and future NanoSIMS assessments designed to quantify Ca^{2+} release are planned. We have also demonstrated increased immunoreactivity of the GluR1 subunit of the AMPA receptor in ventral optic nerve astrocytes in the first 24 hours after injury (Wells et al., 2012), perhaps contributing to changes in Ca^{2+} flux.

Increased Ca^{2+} flux has been associated with increased reactive oxygen and nitrogen species and oxidative stress *in vitro* (Camello-Almaraz et al., 2006; Peng and Jou, 2010). Excess influx of Ca^{2+} leads to perturbations in mitochondrial membrane potential, opening of the mitochondrial permeability transition and release of cytochrome c, which increases production of reactive oxygen species, overwhelms endogenous antioxidant responses and leads to oxidative stress (Kowaltowski et al., 2009; Peng and Jou, 2010). Oxidative stress has been demonstrated as a feature of traumatic brain and spinal cord injury (Park et al., 2004; Carrico et al., 2009). However, it is not yet clear if oxidative stress contributes to secondary degeneration *in vivo*. We have demonstrated increased immunoreactivity of the antioxidant enzyme manganese superoxide dismutase (MnSOD) in hypertrophic astrocytes, in the first minutes and days after injury (Fitzgerald et al., 2009a; Fitzgerald et al., 2010a), associated with increased reactive species (unpublished). However, antioxidant activity appears inadequate to limit these reactive species and prevent oxidative stress, as we observed structural changes in mitochondria of axons and glia (Cummins et al., 2013) and oxidative damage in optic nerve vulnerable to secondary degeneration, particularly in oligodendrocytes (Fitzgerald et al., 2010a; Szymanski et al., 2013). Protein carbonylation, indicated by increased carboxymethyl lysine (CML), was demonstrated from 1 day after injury (Wells et al., 2012; Szymanski et al., 2013), and we observed oxidative damage to DNA and lipids as well as protein nitration in the first week after injury (unpublished). Oxidative stress is likely exacerbated by inflammatory cell infiltration, which occurs in the first day after injury in the dorsal injury site and becomes apparent in ventral optic nerve vulnerable to secondary degeneration by day 3 (Fitzgerald et al., 2009a, 2010a). Taken together, our data indicate that spread of reactive species such as H_2O_2 *via* extracellular release and/or the astrocytic syncytium likely contributes to the spreading damage of secondary degeneration in neurons and glia.

Dysmyelination during secondary degeneration

When we looked more closely at the cell types exhibiting signs of oxidative stress, we observed that increased CML immunoreactivity was particularly prominent in oligodendrocytes vulnerable to secondary degeneration ((Szymanski et al., 2013) and unpublished), despite reports of resistance of mature oligodendrocytes to oxidative damage (Back et al., 2005). Concurrently, paranodes significantly lengthened, as did Nodes of Ranvier, and there was greater incidence of abnormal node/paranode structures (Szymanski et al., 2013). Similar changes have been reported in models of inflammatory demyelinating disease, including multiple sclerosis (Lonigro and Devaux, 2009; Oluich et al., 2012). Later after injury myelin became increasingly decompacted, with increased thickness of myelin, due to loosening of myelin lamaellae, and increased numbers of intraperiodic lines (Payne et al., 2011, 2012). These kinds of abnormalities and perturbations in myelin, referred to as dysmyelination, have been reported following spinal cord injury and in demyelinating diseases including multiple sclerosis (Krsulovic et al., 1999; Rosenbluth and Schiff, 2009; Nomura et al., 2013). Visuomotor function, which is signifi-

cantly compromised from one day after injury (unpublished), progressively worsened in the 6 months following partial transection (Payne et al., 2012). Oligodendrocyte precursor cells (OPCs) proliferated and appeared to differentiate and possibly remyelinate axons, at least to some extent. However, there was significant death of OPCs and their total numbers remained chronically lower in ventral optic nerve vulnerable to secondary degeneration (Payne et al., 2013). Depletion of OPCs may have compromised normal adult myelinogenesis (Young et al., 2013), neuromodulation (Polito and Reynolds, 2005) and myelin repair. We consider it likely that oxidative stress in oligodendrocytes and their precursors contributes to the dysmyelination we observe, as well as associated chronic functional loss. It is interesting to note that following partial optic nerve transection, multifocal electroretinogram responses are reduced in inferior retina vulnerable to secondary degeneration (Chu et al., 2013), perhaps further contributing to loss of visual function.

Strategies to limit dysmyelination during secondary degeneration

A key goal of increasing understanding of the metabolic and structural features of secondary degeneration is to enable rational design of treatment strategies to limit these changes. We have used the relatively CNS specific voltage gated calcium channel inhibitor lomerizine (Hara et al., 1999) as a strategy to limit excess Ca^{2+} flux, and potentially oxidative stress, during secondary degeneration. While lomerizine reduced necrotic and to a lesser extent apoptotic death of retinal ganglion cells vulnerable to secondary degeneration (Fitzgerald et al., 2009a; Fitzgerald et al., 2009b), it did not fully restore visual function (Fitzgerald et al., 2009a; Selt et al., 2010). More recently we have combined lomerizine with additional Ca^{2+} channel inhibitors: the highly soluble AMPA receptor antagonist YM872 (also known as zonampanel or INQ) (Atsumi et al., 2003; Furukawa et al., 2003); and the $P2X_7$ receptor antagonist oxATP (Wang et al., 2004; Matute et al., 2007). Only treatment with all three of the Ca^{2+} channel inhibitors in combination reduced myelin decompaction, lengthening of Nodes of Ranvier and CML immunoreactivity (indicative of reduced oxidative stress) in oligodendroglia of ventral optic nerve (Savigni et al., 2013). The combination of three Ca^{2+} channel inhibitors also preserved visual function following partial optic nerve transection (Savigni et al., 2013). From this work we can conclude that inhibiting multiple Ca^{2+} permeable receptors is beneficial for preventing dysmyelination and preserving function in white matter vulnerable to secondary degeneration. However, beneficial effects may not be due solely to reduced Ca^{2+} influx from the extracellular space *per se*. Inhibition of downstream signalling pathways of individual receptors, such as those leading to calpain mediated axonal degeneration (Thompson et al., 2010), and/or resultant reductions in release from intracellular Ca^{2+} stores (Stirling et al., 2014) may also contribute to beneficial effects. Furthermore, we have not yet ascertained whether it is inhibition of Ca^{2+} permeable receptors in oligodendrocytes, OPCs and/or other cell types, such as astrocytes, neuronal somata and/ or axons, or even photoreceptors, which is beneficial.

We are also pursuing anti-oxidant strategies in an effort to reduce dysmyelination due to secondary degeneration. Irradiation with red/near-infrared light (R/NIR-IT, 630–1,000 nm) was developed as a therapeutic strategy for the treatment of a range of injuries and diseases, following observations of beneficial effects on minor wound healing in space (Whelan et al., 2001). Specific to the nervous system, beneficial effects have been reported following retinal degeneration (Natoli et al., 2010; Albarracin et al., 2011), CNS injury (Byrnes et al., 2005), stroke (Lapchak et al., 2007) and peripheral nerve damage (Rochkind et al., 2009; Ishiguro et al., 2010), as summarized in our recent review (Fitzgerald et al., 2013). While there is controversy regarding the mechanism of action of R/NIR-IT, one hypothesis is that it acts by improving oxidative metabolism and reducing oxidative stress. The enzyme cytochrome c oxidase, complex IV of the electron transport chain, is proposed to act as a photoacceptor for irradiation at these wavelengths, with absorption spectra matching efficacious wavelengths (Moody, 2005; Wong-Riley et al., 2005). Specifically, irradiation is thought to lead to activation *via* changes in the oxidation-reduction state of this enzyme (Karu et al., 2008).

We have demonstrated that 670 nm R/NIR-IT delivered by light emitting diode (LED) array increased cytochrome c oxidase activity in optic nerve vulnerable to secondary degeneration (Szymanski et al., 2013). This was accompanied by reduced MnSOD immunoreactivity in astrocytes (Fitzgerald et al., 2010b), reduced incidence of mitochondrial autophagic profiles (Cummins et al., 2013), rescue of node/paranode abnormalities and preservation of visual function (Fitzgerald et al., 2010b; Szymanski et al., 2013). Nevertheless scepticism regarding efficacy of R/NIR-IT as a treatment for CNS injury remains, largely due to uncertainty regarding penetrance of the irradiation and lack of consensus on optimal treatment parameters, even within a single type of CNS injury (Fitzgerald et al., 2013). Our current efforts are focussed on developing an optimal R/NIR-IT treatment protocol for prevention of dysmyelination during secondary degeneration following partial optic nerve transection *in vivo* and conducting multi-centre comparative assessments of efficacy of a single R/NIR-IT treatment paradigm across multiple CNS injury types.

Additional strategies we are pursuing to limit dysmyelination and functional loss due to secondary degeneration following neurotrauma include use of nanotechnologies to deliver rationally designed inhibitors and anti-oxidants to areas of nerve specifically vulnerable to secondary degeneration. We have demonstrated anti-oxidant capacity of phospholipid calix[4]arene formulations *in vitro* (James et al., 2013) and developed multimodal polymeric nanoparticles, functionalised with magnetite nanoparticles and fluorescent dyes for tracking by magnetic resonance imaging and fluorescence microscopy respectively, for delivery of therapeutics (Evans et al., 2011). We have shown effective release of lomerizine from these multimodal nanoparticles (Evans et al., 2012) and demonstrated lack of toxicity following injection of our nanoparticles into a partial optic nerve injury site (Harrison et al., 2012). Polymeric nanoparticles have the potential to safely deliver effective anti-oxidant treatment strategies to specific cell types vulnerable to secondary degeneration, overcoming solubility and delivery limitations, and we are currently undertaking studies to assess their efficacy in this regard.

Summary and conclusions

The progression of secondary degeneration following partial optic nerve transection is characterised by initial, rapid onset

alterations to Ca^{2+} distributions and increases in indicators of oxidative stress, particularly in astrocytes. Reactive species and altered Ca^{2+} flux may spread to ventral optic nerve vulnerable to secondary degeneration *via* the astrocytic syncytium. Oxidative stress in oligodendrocytes and alterations to node/paranode structure are evident by 24 hours after injury in ventral optic nerve vulnerable to secondary degeneration, before detection of inflammatory cell infiltration at 3 days. OPC numbers are also reduced from 3 days, despite proliferation of these cells. While retinal ganglion cell axonal loss is evident in ventral optic nerve by 7 days, secondary death of retinal ganglion cell somata is not detected until 2 weeks after injury and is followed by continued axonal swelling and decompaction of myelin surrounding remaining vulnerable axons. Chronic functional loss persists until at least 6 months following injury. Treatment strategies including combinations of Ca^{2+} channel inhibitors and R/NIR-IT have been shown to limit oxidative stress, dysmyelination and functional losses of secondary degeneration. However, it is likely that multi-faceted combinatorial treatment strategies will be required to limit the many aspects of damage during secondary degeneration, especially in more complex models and in patients suffering from neurotrauma.

Melinda Fitzgerald
Experimental and Regenerative Neurosciences, School of Animal Biology, The University of Western Australia, Hackett Drive, Crawley, WA 6009, Australia

Corresponding author: *Melinda Fitzgerald, Ph.D., Experimental and Regenerative Neurosciences, School of Animal Biology, The University of Western Australia, Hackett Drive, Crawley, WA 6009, Australia, lindy.fitzgerald@uwa.edu.au.*

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