Reactive species and oxidative stress in optic nerve vulnerable to secondary degeneration

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Abstract

Secondary degeneration contributes substantially to structural and functional deficits following traumatic injury to the CNS. While it has been proposed that oxidative stress is a feature of secondary degeneration, contributing reactive species and resultant oxidized products have not been clearly identified *in vivo*. The study is designed to identify contributors to, and consequences of, oxidative stress in a white matter tract vulnerable to secondary degeneration. Partial dorsal transection of the optic nerve (ON) was used to model secondary degeneration in ventral nerve unaffected by the primary injury. Reactive species were assessed using fluorescent labelling and liquid chromatography/tandem mass spectroscopy (LC/MS/MS). Antioxidant enzymes and oxidized products were semi-quantified immunohistochemically. Mitophagy was assessed by electron microscopy. Fluorescent indicators of reactive oxygen and/or nitrogen species increased at 1, 3 and 7 days after injury, in ventral ON. LC/MS/MS confirmed increases in reactive species linked to infiltrating microglia/macrophages in dorsal ON. Similarly, immunoreactivity for glutathione peroxidase and haem oxygenase-1 increased in ventral ON at 3 and 7 days after injury, respectively. Despite increased antioxidant immunoreactivity, DNA oxidation was evident from 1 day, lipid oxidation at 3 days, and protein nitration at 7 days after injury. Nitrosative and oxidative damage was particularly evident in CC1 positive oligodendrocytes, at times after injury at which structural abnormalities of the Node of Ranvier/ paranode complex have been reported. The incidence of mitochondrial autophagic profiles was also significantly increased from 3 days. Despite modest increases in antioxidant enzymes, increased reactive species are accompanied by oxidative and nitrosative damage to DNA, lipid and protein, associated with increasing abnormal mitochondria, which together may contribute to the deficits of secondary degeneration.

Keywords: neurotrauma; secondary degeneration; oxidative stress; reactive species; antioxidant enzymes; oxidative damage; mitophagy

Abbreviations: AGE, advanced glycation end-product; BSA, bovine serum albumin; CM-H_2DCF, chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate; CML, carboxymethyl lysine; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; GFAP, glial fibrillary acidic protein; GPx1, glutathione peroxidase 1; HNE, 4-hydroxynonenal; HO-1, heme oxygenase-1; LC/MS/MS, liquid chromatography/tandem mass spectroscopy; MPT, mitochondrial permeability transition; NADH/NADPH, nicotinamide adenine dinucleotide/ phosphate; 3-NT, 3-nitrotyrosine; 8OHDG, hydroxyguanosine; OMM/IMM, outer/ inner mitochondrial membrane; ON, optic nerve; PEG-SOD, polyethylene glycol superoxide dismutase; PFA, paraformaldehyde; ROS, reactive oxygen species; RNS, reactive nitrogen species; TPBN, phenyl-*N*-*tert*-butyl nitrone; VDAC, voltage dependent anion channel

Highlights: (max 85 ch each bullet, including spaces)

Contributors and consequences in oxidative stress after CNS injury are unknown in vivo

We demonstrate increased reactive species using *in vivo* labelling and LC/MS/MS

Anti-oxidant enzymes are also increased in nerve vulnerable to secondary degeneration

Nevertheless, there is oxidative and nitrosative damage to DNA, lipid and protein

We propose a working model of contributors and consequences in CNS oxidative stress

Introduction

The pathophysiology of injury to white matter tracts in the central nervous system (CNS) involves both primary and secondary mechanisms. Outside the primary injury area, neurons and glia remain vulnerable to secondary degeneration, with structural changes and delayed cell death resulting in progressive loss of function [1-4]. Excess Ca^{2+} flux and oxidative stress have been implicated in *in vitro* studies, as major contributors to damage following injury [5]. However, the biochemical processes and contributors leading to damage and death in nerve tissue vulnerable to secondary degeneration *in vivo* have not been characterized. Partial transection of the optic nerve (ON), involving a precise primary lesion only to dorsal axons, leaves those in ventral ON intact but vulnerable to secondary degeneration [6, 7]. This *in vivo* model allows spatial separation of primary from secondary injury, and the comprehensive assessment of biochemical pathways contributing specifically to secondary degeneration in a white matter tract of the CNS.

Oxidative stress is a hallmark of neurodegenerative diseases such as glaucoma [8-10] and CNS injury [1-3, 11-13] and occurs as a consequence of excess reactive species. Reactive species play a dual role, with both beneficial and harmful effects well documented, as reviewed by Valko et al., 2007 [14]. Enzymatic antioxidant defence systems such as glutathione peroxidase (GPx) and manganese superoxide dismutase (MnSOD), together with non-enzymatic antioxidants including ascorbic acid and glutathione, act to maintain redox balance [14]. However, when there is an abnormal increase in production of reactive oxygen or nitrogen species (ROS/RNS) and/or a decrease in antioxidant activity, antioxidant defences are overwhelmed, resulting in oxidative stress [15]. Excess ROS and RNS can oxidise DNA, lipids and proteins to toxic metabolites [16, 17]. Moreover, an altered redox environment can activate signalling pathways leading to cell damage and death [14]. Oxidative damage, particularly in the form of lipid

oxidation, has been demonstrated following traumatic brain injury [18]. Increases in a wide spectrum of indicators of oxidative stress provide circumstantial evidence that highly reactive oxidants mediate secondary degeneration following neurotrauma [19, 20], and damage in a range of neurological conditions [21, 22]. However, direct measures of the causes and consequences of oxidative stress in white matter exclusively vulnerable to secondary degeneration following neurotrauma are lacking.

ROS consist of radical and non-radical species that can be formed by the partial reduction of oxygen, and include superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the hydroxyl radical ($^{\bullet}OH$) [23]. RNS also include radical and non-radical species, such as nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$). There is evidence that in ON vulnerable to secondary degeneration, mitochondrial dysfunction and oxidative stress occur early after injury [24, 25]. However, the identities of the ROS and/or RNS that contribute, and the nature of oxidative damage that they cause, are not clearly understood. Most ROS are highly reactive molecules, rendering them unstable and difficult to detect in biological samples. Therefore, accurate detection of ROS/RNS requires labelling and/or imaging of live tissue, using particular compounds that react with reactive species and form fluorescent or other specific products. Here, we employ *in vivo* labelling of ROS/RNS, and immunohistochemical detection of antioxidant enzymes and indicators of oxidative damage to proteins, lipids and DNA following partial ON transection. The data generated allow us, for the first time, to develop a picture of the contributors to, and consequences of, oxidative stress in ON vulnerable to secondary degeneration *in vivo*.

Materials and Methods

Animals

Procedures were conducted in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Visual Research, and were approved and monitored by the University of Western Australia Animal Ethics Committee. Adult, female Piebald-Virol-Glaxo (PVG) hooded rats were procured from the Animal Resources Centre (Murdoch, WA), and housed under temperature controlled conditions on a 12 hour light/dark cycle, with access to standard rat chow and water *ad libitum*.

Partial ON transection

The partial ON transection procedure was conducted as described previously [12]. Briefly, PVG rats were anaesthetized intraperitoneally (i.p) (50 mg/kg ketamine hydrochloride and 10 mg/kg xylazil hydrochloride, Troy Laboratories, NSW, Australia). Right ONs were surgically exposed by incising the skin overlying the skull and deflecting lachrymal tissue. A diamond keratotomy knife (Geuder, Germany) was used to make a controlled dorsal incision in each ON, to a depth of 200 µm. Post-operative analgesia was administered subcutaneously (2.8 mg/kg carprofen, Norbrook Australia, Pty. Ltd., VIC, Australia). Controls were uninjured normal animals, as we have previously demonstrated no change following sham anaesthesia and surgery[7]. The contralateral ONs were not used as controls, due to demonstrated changes in the opposite eye following ON injury [26] and thus do not provide an appropriate or useful baseline for comparison of oxidative stress changes in our model. All animals were euthanased with Lethabarb© (800-1000 mg/kg i.p, Virbac, Australia Pty. Ltd., NSW, Australia) at 1 hour, 1, 3 or 7 days after injury (total n=24/time point).

Detection of reactive species in previously frozen sections

With animals under ketamine-xylazil anaesthesia as described above, right ONs (n=6/time-point) were dissected from the ocular cavity, collected onto a microscope slide maintained at -20^{o}C over a bed of dry ice, mounted in optical cutting temperature compound then snap-frozen in Eppendorf tubes in liquid nitrogen, and stored in airtight zip-lock bags at -80°C, to avoid desiccation. Tissue was cryosectioned longitudinally at -20^{o}C and free floating tissue sections (20 µm) from each experimental animal were collected into 30% sucrose in phosphate buffered saline (PBS) in one well of the top row of each of six 24-well trays, to facilitate subsequent labelling and avoid multiple freeze-thaw cycles. Trays containing sections were stored at -80^{o}C. Labelling and washing solutions were dispensed in rows of the trays and sections were transferred between solutions in droplets, using forceps that did not meet, to avoid damaging the tissue; incubations were timed to ensure equal durations. Sucrose was removed by washing sections in PBS followed by labelling at room temperature, in the dark, in either: 30 µM chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF) (Cell Biolabs, CA, USA) in PBS for 15 minutes; 100 µM Amplex UltraRed (Life Technologies, VIC, Australia) in 50 mM sodium citrate buffer (pH 6.5) containing 0.2 U/mL horse radish peroxidase (Life Technologies, VIC, Australia) for 30 minutes; or 10 µM dihydroethidium (DHE) (Life Technologies, VIC, Australia) in PBS for 10 minutes. Stock solutions of CM-H_2DCF and DHE were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) and stored at -20°C under argon; Amplex UltraRed was used according to manufacturer's instructions. Some sections were pretreated with 666 U/mL polyethylene glycol superoxide dismutase (PEG-SOD) in PBS for 15 minutes or 2 mM phenyl-*N*-*tert*-butyl nitrone (TPBN) for 60 minutes as described [27]. Tissue sections were washed for 10 minutes in PBS, and then fixed in 4% paraformaldehyde (PFA, ProSciTech, QLD, Australia) in PBS, for 10 minutes, washed in PBS, dried, and mounted on glass slides with Fluoromount-G (Southern Biotech, AL, USA).

Preliminary studies indicated that exposure of the sections to light (required for imaging) increased fluorescence dramatically within seconds, and that fixation reduced this problem.

In vivo labelling with dihydroethidium (DHE) and detection of reactive species by LC/MS/MS quantification of DHE and its oxidation products.

As DHE is only sparingly soluble in aqueous buffers, DHE was bound to bovine serum albumin (BSA, Fraction V-essentially fatty acid free, Sigma-Aldrich, MO, USA) on the day of use as follows. Twenty five millimolar DHE stock was prepared in argon purged DMSO with vigorous vortexing and minimal exposure to light. The DHE stock was added drop-wise to 7.5 mM BSA in sodium phosphate buffer (50 mM, pH 7.4) such that the final concentrations were DHE 2.5 mM, DMSO 10% and BSA 6.8 mM. Following incubation at 37^{o}C for 15 minutes, diethylene triamine pentaacetic acid (Sigma-Aldrich) was added to a final concentration of 0.1 mM and the DHE-BSA preparation was stored on ice, in the dark until use.

Under ketamine-xylazil anaesthesia as described above, the ON partial transection injury sites of animals that had undergone partial ON transection 1 or 7 days previously were re-exposed and the dural sheath around the injury site (both dorsal and ventral) removed. Fifty µL of DHE-BSA was pipetted directly onto the ON at the injury site, ensuring full immersion of the nerve in labelling solution. ONs were incubated *in situ* in the presence of DHE-BSA for 1 hr, with overlying tissue restored to its normal position to minimise light exposure; additional anaesthesia was administered if required. At the conclusion of DHE-BSA labelling, prior to euthanasia, right ONs were dissected from the ocular cavity, briefly rinsed in PBS to remove excess DHE-BSA, and collected onto a microscope slide maintained at -20°C over a bed of dry ice. Frozen ONs were cut into dorsal and ventral segments using a scalpel, and then pooled

dorsal or ventral ON segments from 3 animals were snap-frozen in liquid nitrogen as described above. Three sets of three ON segments from animals at each time point were collected (n=3, total animals=9/time-point): similarly located and labelled ON segments from completely normal animals were used as controls.

The pooled ON segments were then subjected to quantification of DHE and its major oxidation products, ethidium (E^+), 2-hydroxyethidium (2-OH-E^+, the superoxide-specific product of DHE), and 2-chloroethidium (2-Cl-E^+, the HOCl-specific product of DHE) as described previously[28]. For the detection of RNS, we used the putative nitrated HE (2-nitroethidium, 2-NO_2-E^+) as the specific product of the reaction of ONOO- with DHE. 2-NO_2-E^+ was detected using the general LC/MS/MS parameters described previously[28] and the following precursor to product ion transition, m/z 359→312, with collision energy set at 35 V. Briefly, ON segments were cut into small pieces, 100 µL of 80% N_2-bubbled ethanol added and subjected to three freeze/thaw cycles in liquid N_2. After centrifugation at 14,000 g (4°C, 15 min), DHE, E^+, 2-OH-E^+, and 2-Cl-E^+ were then detected and quantified in the supernate against authentic standards by LC/MS/MS analysis.

Immunohistochemical assessments

Following euthanasia, animals were transcardially perfused with 0.9% saline followed by 4% PFA (0.1 M phosphate buffer; pH 7.2). ONs (n=5/time point) were cryosectioned and immunohistochemical analyses conducted according to established procedures [12], using primary antibodies recognising: GPx1 (1:250; Abcam, Cambridge, UK); haem oxygenase-1 (HO-1) (1:200; Abcam, Cambridge, UK); 3-nitrotyrosine (3NT) (1:500; Abcam, Cambridge, UK); 4-hydroxynonenal (HNE) (1:200; Jomar Bioscience); 8-hydroxyguanosine (8OHDG)

(1:500; Abcam, Cambridge, UK); co-labelled with primary antibodies to identify specific cell types: activated microglia/macrophages, ED1 (1:500; Millipore, MA, USA)[29]; resident microglia/macrophages, Iba1 (1:400; Novachem, VIC, AUS)[30]; mature oligodendrocytes, CC1 (1:500; Millipore, MA, USA)[31]; astrocytes, GFAP (1:1000; Abcam, Cambridge, UK) and Hoechst nuclear stain (1:1000; Invitrogen, VIC, Australia). Secondary antibodies were species specific AlexaFluor® 555 and 488 (1:500; Invitrogen, VIC, Australia).

Microscopy and image analysis

Reactive species and immunohistochemical labelling were visualised in a single section of ventral ON directly below the primary injury site for each animal, and photographed using either a Leitz Diaplan fluorescence microscope (Leica, Germany) or, where colocalisation of markers with specific cell types was required, a Nikon Eclipse Ti inverted microscope (Nikon Corporation, Japan). A series of optical images at 0.5 µm increments along the z-axis were acquired from the middle 6 µm of each 14 µm section imaged using the Nikon microscope: images were collected and deconvoluted using autoquant blind deconvolution with Nikon Elements AR software. All images for each outcome measure were captured at constant exposures and in a single session. Image analysis was conducted on a single image using Image J/Fiji analysis software, setting constant arbitrary threshold intensities for all images in an analysis and semi-quantifying mean intensities of the whole ventral ON images and mean intensities and areas above the set threshold. Transmission electron microscopy (TEM) and quantification of mitochondrial autophagic profiles was conducted as described [24] (n=4 animals/time point), using a using a Philips CM-10 TEM (Eindhoven, The Netherlands) attached to an Olympus Megaview III camera (1376x1032 pixels), at an accelerating voltage of 80 kV at 25000x magnification, corresponding to a field of view size of 10.4 μm^2.

Statistical analyses

All data were expressed as mean ± SEM: statistical analyses were conducted using SPSS statistical software (IBM). Analyses of DHE *in vivo* labelling data employed two-way ANOVA comparing dorsal to ventral ON, and control to injured outcomes. All other data were analysed using one-way ANOVA (F value, degrees of freedom (dF)) and Bonferroni Dunn or Dunnett's post-hoc tests as appropriate (p value); or Student's t-test where comparisons were between two values ($p<0.05$ as significant).

Results

Increased reactive species in ON vulnerable to secondary degeneration

Dyes that fluoresce upon oxidation by a range of reactive species were used first to assess reactive species in fresh frozen tissue sections. In ventral ON vulnerable to secondary degeneration, there was a small but significant increase in mean CM-H_2DCF derived fluorescence intensity above an arbitrary but constant threshold, at 1 day post-injury ($F=4.25$, $dF=4$, $p=0.002$; Figure 1A, 127% of control), but no significant changes in mean area of fluorescence above that threshold ($F=0.28$, $dF=4$, $p=0.88$; Figure 1B). Similarly, there was a significant increase in the mean Amplex UltraRed derived fluorescence intensity above threshold at 1 and 3 days post-injury in ventral ON ($F=32.23$, $dF=4$, both $p<0.0001$; Figure 1D, 165% and 143% of control respectively), but no significant changes in the mean fluorescence area above the threshold ($F=1.91$, $dF=4$, $p=0.14$; Figure 1E). There were no changes in the mean fluorescence intensities of the whole ventral ON image for CM-H_2DCF or Amplex Ultrared (not shown). In the primary injury, the particularly bright staining patterns obtained with CM-H_2DCF and Amplex UltraRed were reminiscent of infiltrating microglia/macrophages (Figures 1C and F) [12], although definitive immunohistochemical identification of these cells was precluded by the need to use non-fixed tissue. Additionally, although there were no significant changes in the mean DHE derived fluorescence intensity above threshold in the ventral ON at any day post-injury, ($F=1.14$, $p= 0.37$; Figure 1G), we detected a substantial and significant increase in the mean DHE derived fluorescence area above the threshold in ventral ON 7 days following injury ($F=10.76$, $dF=4$, $p<0.0001$; Figure 1H, representative images in J, 383% of control) and the mean intensity of the whole ventral ON image ($F=8.15$, $dF=4$, $p=0.0002$, not shown, 165% of control), indicating greater spread of reactive species recognised by this dye. Pre-treatment of sections with PEG-SOD resulted in a significant decrease in mean DHE derived fluorescence intensity in ventral ON at 3 days following injury ($p\leq0.05$, Figure

1I): note that absolute values of fluorescence intensity are not comparable between experiments conducted on different days (e.g. Figure 1G and I). Pre-treatment of sections with the reactive species scavenger TPBN resulted in no difference in mean CM-H_2DCF derived fluorescence intensity ($p>0.05$, not shown).

In vivo labelling of ON with DHE-BSA, followed by LC/MS/MS analysis of tissue, allowed detection and quantification of: 2-hydroxy-ethidium (2-OH-E^+), a $O_2^{-\bullet}$-specific product [32]; ethidium (E^+), a non-specific oxidation product arising from a number of species reacting with DHE [33]; and 2-chlorethidium (2-Cl-E^+), a specific product formed from DHE by chlorinating species such as hypochlorous acid (HOCl) [28] (Figure 2). However, we were not able to detect any significant levels of putative 2-NO_2-E^+, a specific product of the reaction of HE with $ONOO^-$ (data not shown). Binding of DHE to BSA (used to prepare a form of DHE soluble in an aqueous buffer) did not apparently change its suitability as a redox dye, as there was no difference in the intensity or appearance of labelling of fresh frozen sections with DHE-BSA, compared to DHE (e.g. mean ± SEM fluorescence intensity above set threshold: DHE = 30.9±3.1; DHE-BSA = 29.3±1.4; n=3/group). We did not detect any significant differences in the amounts of 2-OH-E^+ or E^+ in normal compared to injured ON segments ($F=3.03$, $dF=2$, $p=0.09$; $F=1.03$, $dF=2$, $p=0.39$ respectively), or between dorsal and ventral ON ($F=3.32$, $dF=1$, $p=0.10$; $F=4.09$, $dF=1$, $p=0.07$ respectively; Figures 2 A, B). Similarly, when the data were expressed as a ratio of oxidized to total probe detected (to account for any variation in DHE uptake), despite a trend towards an increased ratio of E^+/DHE following injury, there were no significant differences in the ratios of 2-OH-E^+/DHE or E^+/DHE in normal compared to injured ON segments ($F=1.47$, $dF=2$, $p=0.27$; $F=3.30$, $dF=2$, $p=0.07$ respectively), or between dorsal and ventral ON ($F=1.23$, $dF=1$, $p=0.29$; $F=0.91$, $dF=1$, $p=0.36$ respectively; Figures 2 D, E). However, there was significantly more 2-Cl-E^+ in dorsal ON than ventral ON at days 1 and 7

following partial ON transection (F=6.62, dF=1, p=0.05, p=0.03 respectively; Figure 2C, 344% and 764% of ventral 2-Cl-E^+ respectively). Furthermore, the ratio of 2-Cl-E^+/DHE in dorsal ON was significantly increased at 1 and 7 days after injury compared to normal (F=17.18, dF=2, p<0.0001, p=0.01 respectively, 297% and 216% of control respectively), and was significantly higher than the ratio of 2-Cl-E^+/DHE in ventral ON at these times (F=12.28, dF=1, p=0.01, p=0.02 respectively, 181% and 187% of ventral 2-Cl-E^+/DHE respectively: Figure 2F).

Increases in antioxidant enzyme immunoreactivity in ON vulnerable to secondary degeneration

There was a small but significant increase in the mean intensity of immunoreactivity of the antioxidant enzyme GPx1, when assessing the whole of ventral ON images at 3 days following injury (F=3.00, dF=4, p=0.05; Figure 3A, 119% of control) compared to normal ON. However there were no significant changes in the mean intensity above a set threshold (F=1.26, dF=4, p=0.32, not shown) or the area above that set threshold (F=2.78, dF=4, p=0.06; Figure 3B), implying a diffuse increase, as observed in Figure 3G. GPx1 immunoreactivity co-localised with cell somata of CC1+ cells, although some CC1+ cells were not GPx1 immunoreactive (arrowheads). Occasional co-localisation with ED1+ cells and MBP was much less pronounced (arrows, Figures 4C-E). There was also a significant increase in intensity of HO-1 immunoreactivity when assessing the whole of ventral ON images at 7 days following injury (F=3.50, dF=4, p=0.002; Figure 3F, 123% of control). While there were no significant changes in the mean intensity above a set threshold (F=2.58, dF=4, p=0.07, not shown), the area above that set threshold was substantially and significantly increased at 7 days (F=3.03, dF=4, p=0.003; Figure 3G, 219% of control). HO-1 immunoreactivity was also present in a somewhat diffuse pattern across the ventral ON, that co-localised with Iba1+ cells at 3 days after injury

(note that the particular image chosen to demonstrate co-localisation had higher than average numbers of Iba1+ cells, arrow, Figure 3H).

Increases in protein nitration, lipid oxidation and oxidative damage to DNA in ON vulnerable to secondary degeneration

We then investigated whether markers of oxidative damage were increased in ON vulnerable to secondary degeneration. We detected a significant increase in both the mean intensity of immunoreactivity of 3NT (a marker of protein nitration) in the whole ventral ON image ($F=6.51$, $dF=4$, $p=0.0003$; Figure 4A, 160% of control), and a large increase in the area above a set threshold intensity ($F=9.96$, $dF=4$, $p<0.0001$; Figure 4B, 430% of control) at 7 days following injury, compared to normal ON. While there appeared to be a significant change in the mean intensity above that threshold ($F=2.98$, $dF=4$, $p=0.04$, not shown), *post hoc* tests did not reveal a significant difference from normal, at any time-point. 3NT immunoreactivity was not co-localised with MBP at 7 days (Figure 4C), but was observed increasingly in CC1+ somata as time passed after injury (arrows, Figure 4I).

There was no significant change in the mean intensity of HNE immunoreactivity in the whole ventral ON image after injury ($F=1.51$, $dF=4$, $p=0.25$; Figure 4D), however the mean intensity above the set threshold was slightly increased at 3 days following injury ($F=4.24$, $dF=4$, $p=0.003$; not shown, 129% of control), and the area above that threshold increased substantially ($F=3.94$, $dF=4$, $p=0.002$; Figure 4E, 231% of control). Representative images demonstrate that HNE immunoreactivity occasionally co-localised with MBP (Figure 4F) and was co-localised to most CC1+ myelinating oligodendrocyte somata (green, arrows, Figure 4J). HNE

immunoreactivity also occasionally colocalised with GFAP (blue) immunoreactivity (purple, Figure 4J).

There was also a significant increase in immunoreactivity of 8OHDG (a marker of DNA oxidation) in ventral ON from 1 day after injury, when assessing the mean intensity of the whole image ($F=12.00$, $dF=4$, $p<0.0001$; Figure 4G, 135% of control at day 1 to 164% at day 7), the mean intensity of immunoreactivity above the set threshold ($F=20.00$, $dF=4$, $p<0.0001$, not shown, 110% of control at day 1 to 124% at day 7) and the mean area above that threshold ($F=11.60$, $dF=4$, $p<0.0001$; Figure 4H, 198% of control at day 1 to 252% at day 7). Cellular distribution of 8OHDG immunoreactivity was widespread, with many cell somata demonstrating 8OHDG immunoreactivity by 1 day after injury (Figure 4K).

Increases in mitochondrial autophagic profiles in ON vulnerable to secondary degeneration

Ultrastructural analyses of mitochondria revealed a small subset which featured second double membranes, very high electro-density and/or vacuole-like features, clearly indicative of autophagic processes [24]. Given the complexities associated with definitive identification of mitophagy, these structures were classified as mitochondrial autophagic profiles and their incidence quantified (40 fields of view/ animal, total area assessed = 416μm^2). We observed a large, significant increase in the incidence of mitochondrial autophagic profiles in ventral ON from 3 days after injury ($F=73.01$, $dF=4$, $p\leq0.0001$, Figure 5A). Representative images illustrate mitochondrial autophagic profiles with a second double membrane (Figure 5B, arrow), compared to normal mitochondria (Figure 5C). Furthermore, the incidence of abnormal mitochondria was also increased during secondary degeneration: representative images at day 1

illustrate mitochondria with swollen regions, that were highly electron dense or that featured a lysosomal sac (arrows, Figures 5D-F respectively).

Discussion

Increased reactive species and resultant oxidative damage to DNA, lipid and protein have been implicated in secondary degeneration, largely based on *in vitro* studies [5], but *in vivo* characterisation of contributors to, together with consequences of, oxidative stress are lacking. We have used an *in vivo* ON partial transection model, enabling analysis of ON exclusively vulnerable to secondary degeneration, to characterise increases in reactive species in white matter vulnerable to secondary degeneration. Despite relatively modest increases in antioxidant enzyme immunoreactivity, increases in oxidative and nitrosative damage were detected in DNA, lipid and protein, particularly evident in oligodendrocytes. This oxidative damage may contribute to the structural and functional deficits of secondary degeneration following traumatic injury to the CNS.

Changes in reactive species during secondary degeneration

In conditions of excess Ca^{2+} and altered mitochondrial membrane potential, electron leakage to oxygen at complexes I and III of the electron transport chain can increase, resulting in increased formation of $O_2^{-\bullet}$ [15, 34]. Superoxide dismutases reduce $O_2^{-\bullet}$ to H_2O_2 [35], and their enzyme activity can increase in response to $O_2^{-\bullet}$ production [36]. We have previously shown increases in MnSOD immunoreactivity in hypertrophic astrocytes from 5 minutes to 3 days in ON vulnerable to secondary degeneration [12]. This increase in MnSOD is associated with decreased catalase activity [13], consistent with the presence of oxidative stress and our observed increases in CM-H_2DCF and Amplex Ultrared fluorescence in ventral ON. However, it is important to bear in mind the limitations in conclusions one can draw from results derived from CM-H_2DCF and Amplex Ultrared [37]. H_2DCF reacts with a wide range of one-electron-oxidising species, including heme proteins such as cytochrome *c* and different ROS and RNS.

As such, the probe cannot be used to specifically quantify H_2O_2 or other ROS [37]. Similarly, Amplex Ultrared-derived fluorescence requires the presence of H_2O_2, plus a peroxidase, so that any change in signal is not a direct measure of H_2O_2. These limitations may help explain why CM-H_2DCF staining was not more pronounced than Amplex Ultrared fluorescence in ventral ON. Moreover, a range of sources of fluorescence on tissue sections may further preclude comparison of staining intensities or definitive identification of individual reactive species present. By comparison, *in vivo* labelling with DHE-BSA, followed by LC/MS/MS analysis of tissue, allows more definitive identification of some individual reactive species. For example, the increased 2-Cl-E^+ that we observed in the primary injury following *in vivo* labelling is likely due to exposure of DHE to HOCl derived from myeloperoxidase, characteristic of infiltrating inflammatory cells [28], and coinciding with the marked fluorescence in dorsal ON, in tissue sections stained with CM-H_2DCF and Amplex Ultrared.

The substantial increase in DHE derived fluorescence in ventral ON tissue sections could be interpreted as increased $O_2^{-\bullet}$ [38]. However lack of increase in the $O_2^{-\bullet}$-specific product 2-OH-E^+ [33, 28] in ventral ON, labelled *in vivo* with DHE, indicates that increased DHE staining was more likely due to formation of ethidium, a non-specific oxidation product that can arise from a number of species reacting with DHE, as well as cytochrome *c* [39]. Our observed increases in Tunel+ oligodendrocyte precursor cells in ventral ON in the first week after injury are consistent with increased cytochrome *c* derived ethidium [39, 40]. There was a trend towards increased ethidium detected using the LC/MS/MS approach, particularly in the primary injury site, but this did not reach significance, and caution should be exercised when interpreting these findings. Indeed, we have previously observed decreases in DHE staining in fixed tissue vulnerable to secondary degeneration[25], indicating significant variability depending upon the timing of fixation and analysis technique employed. Furthermore, while PEG-SOD pre-

treatment of tissue sections reduced DHE derived fluorescence, presumably due to reductions in primarily extracellular $O_2^{-\bullet}$- and possibly intracellular ROS in sectioned cells, we did not observe a decrease in TPBN scavenged reactive species detected using CM-H_2DCF. It has been demonstrated that effects of scavengers in cell free systems do not necessarily correspond to effects in dissociated retinae [41], and therefore perhaps in live tissue sections. Taken together, and bearing in mind the caveats already mentioned, our results indicate that infiltrating inflammatory cells at the primary injury are a significant source of reactive species, primarily HOCl. Additional reactive species including H_2O_2, $^{\bullet}OH$ and ONOO may also contribute to the damage of secondary degeneration, but definitive identification of these species remains to be established *in vivo*.

Our results are consistent with a working model of changes in reactive species following partial ON injury, in which increased Ca^{2+} [42] and SOD, together with phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase lead to increased H_2O_2 produced from $O_2^{-\bullet}$, in mitochondria, the cytosol and extracellularly (Figure 6). HOCl derived from myeloperoxidase-containing inflammatory cells will also be present intra- and extracellularly at the primary injury site in the first day after injury [7], but is not likely to diffuse to other parts of the tissue due to its high reactivity. However $O_2^{-\bullet}$ and particularly the more stable H_2O_2, together with excess Ca^{2+}, may spread to ventral ON vulnerable to secondary degeneration through membranes, in the case of H_2O_2, and *via* interconnecting gap junctions of the astrocytic syncytium, as has been demonstrated in endothelial cells [43]. Increased MnSOD in ventral astrocytes [12] may exacerbate this phenomena by rapidly converting mitochondrial $O_2^{-\bullet}$ to H_2O_2. Astrocytes contact myelinating oligodendrocytes and axons at the Node of Ranvier and may also disseminate ROS through hemi-channels into the extracellular space. Rapid spread of ROS from astrocytes to myelinating oligodendrocytes is consistent with our demonstrations of

increased oxidative stress in oligodendrocytes and node/paranode abnormalities in secondary degeneration in the first day after injury [25]. Inflammatory cells spread into ventral ON by 3 days after injury [7], likely furthering the spread of ROS/RNS and associated oxidative stress. Direct reaction of DNA with reactive species such as HOCl, $^{\bullet}OH$ and $ONOO^{-}$ [44, 45] are likely to have led to our observed increases in oxidized DNA at this time, and may in part account for the early and sustained death of oligodendrocyte precursor cells in ON vulnerable to secondary degeneration [40] and later death of neurons by necrosis and to a lesser extent, apoptosis [46]. It is likely that the proposed model is of relevance to neurotrauma and neurodegenerative conditions where glutamate excitotoxicity, associated with changes in Ca^{2+} flux, initiate focal increases in reactive species. Such changes have been reported in models of optic nerve injury [42], Parkinson's disease [47], spinal cord injury[48] and neurodegenerative diseases [19].

Antioxidant responses and oxidative/nitrosative damage during secondary degeneration

GPx1 is one of the enzymes that reduces free H_2O_2 to H_2O; increased immunoreactivity in ventral ON at 3 days may reflect increased synthesis of the enzyme as a compensatory response to high levels of H_2O_2[49] present in a range of cell types in ventral ON. Specifically, the co-localisation of GPx1 immunoreactivity with CC1+ oligodendrocytes and MBP within these cells, although modest, may indicate a response to limit H_2O_2 in oligodendrocytes. Oligodendrocytes form CNS myelin which is rich in lipid and iron and highly vulnerable to selective oxidative damage [50, 51]. GPx1 has been reported to reduce some lipid hydroperoxides [52] and consequent lipid oxidation leading to HNE [53, 54]. However, GPx1 increases were small, not sustained and were associated with increased HNE, followed by protein tyrosine nitration (Figure 6), in oligodendrocytes as well as other cells. Although we have not directly measured antioxidant enzyme activity, from these data we can infer that antioxidant responses in oligodendrocytes vulnerable to secondary degeneration are inadequate to prevent lipid

oxidation, perhaps contributing to the structural abnormalities in myelin and functional loss we observe at this time [25, 7], as well as myelin decompaction despite sustained oligodendrocyte numbers later after injury [4, 40]. Our findings are in accordance with other reports of oxidative damage in white matter and oligodendrocytes, associated with glutamate excitotoxicity, following injury to the CNS [55-57], although vulnerability is thought to be maturation dependent in these cells [58, 59]. It is interesting to note that the increased HNE immunoreactivity was due to an increased area of positive cells and a greater intensity above a set threshold, indicating more or swollen cells that were immunopositive for lipid oxidation, as well as increased lipid oxidation within these cells. Oxidized lipid is associated with structural changes to mitochondria, including progression to mitophagy in both axons and glia at this time. The substantial increase in incidence of abnormal mitochondria is likely to reflect profoundly altered oxidative metabolism and possibly increased reactive species, at least in this mitochondrial subset.

Protein tyrosine nitration is a major cytotoxic pathway in the CNS, potentially contributing to a range of CNS insults and neurodegenerative disorders [60-62].The increased co-localisation of3NT immunoreactivity with CC1+ myelinating oligodendrocytes indicates nitrosative damage in these cells, which may further contribute to abnormal myelin in secondary degeneration[4]. A possible source of the implied reactive nitrogen species is myeloperoxidase [63], consistent with our observed increase in 2-Cl-E^{+}. Nitration of tyrosine residues of MnSOD has been shown to inactivate the enzyme [64]. Our observed greater than 4 fold increase in 3NT at 7 days after injury may explain the reduced immunoreactivity of MnSOD at this time [12], resulting in a feed forward loop of increased $O_2^{-\bullet}$ and other reactive species, including $ONOO^{-}$ (Figure 6). Under oxidative stress conditions, free haem released from haem proteins also becomes highly cytotoxic [65], accelerating the production of ROS. HO-1 catalyses the degradation of haem [66],

and the greater than 2 fold increase in immunoreactivity co-localised with resident activated microglia in ventral ON indicates a protective role for these cells, as has been previously reported [67].

Conclusions

While ROS are generated as natural by-products of oxidative metabolism, important for cell signaling and homeostasis, when over-produced during secondary degeneration *in vivo*, they are associated with spread of reactive species and oxidative damage despite modest increases in antioxidant enzyme immunoreactivity. Myeloperoxidase-derived chlorinating species and increased lipid oxidation and protein nitration likely contribute to the structural and functional abnormalities in myelin that are a feature of secondary degeneration *in vivo*. As such, oligodendrocytes and/ or their precursors may present an attractive target for antioxidant therapeutic intervention to limit secondary degeneration following neurotrauma. This study provides an insight into therapeutic windows of opportunity in the treatment of secondary degeneration in the ON.

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Figure Legends

Figure 1. CM-H_2DCF, Amplex UltraRed, and DHE derived fluorescence in ventral ON vulnerable to secondary degeneration. Mean ± SEM fluorescence intensity and area, above an arbitrary constant threshold, are shown following CM-H_2DCF (A, B), Amplex UltraRed (D, E) or DHE labelling (G-I) in normal ON and at 1 hour, 1, 3 and 7 days following partial ON transection, in ventral ON vulnerable to secondary degeneration: *indicates significantly different from normal ($p \leq 0.05$), n=6/time point. Representative images of the entire ON at the injury site following CM-H_2DCF (C) or Amplex UltraRed (F) labelling are shown: note prominent immunoreactivity in the dorsal (uppermost) primary injury necessitates reduced image exposure such that ventral immunoreactivity is faint. Representative images of DHE labelling are of ventral ON immediately below the injury site (J).

Figure 2. Quantification of DHE products detected using LC/MS/MS after in vivo labelling of ON with DHE, following partial transection. Mean ± SEM amounts (fmol) of hydroethidine (2-OH-E^+; A), ethidium (E^+; B) and 2-chloroethidium (2-Cl-E^+; C) in homogenates of pooled dorsal or ventral ON segments after *in vivo* labelling with DHE of normal ON or 1 or 7 days following partial ON transection. Mean ± SEM ratios of 2-OH-E^+/DHE (D), E^+/DHE (E) and 2-Cl-E^+/DHE (F) are also shown: *indicates significant differences ($p \leq 0.05$), n=3, total animals=9/time-point.

Figure 3. GPx1 and HO-1 immunoreactivity in ventral ON vulnerable to secondary degeneration Mean ± SEM immunoreactivity of the entire image or the area above a set threshold intensity are shown for GPx1 (A, B) and HO-1 (F, G), from normal ON and ON 1 hour, 1, 3 or 7 days following partial ON transection, in ventral ON vulnerable to secondary

degeneration: *indicates significantly different from normal ($p \leq 0.05$), n=6/time point. Representative images demonstrate immunoreactivity of: GPx1 (red), co-localised with green CC1 (C), ED1 (D) or MBP (E), arrows and Hoechst nuclear dye; and HO-1 co-localised with Iba1 and Hoechst (H); arrowheads indicate lack of co-localisation. Note the HO-1/Iba1 image at 3 days has higher than average numbers of Iba1+ cells and was chosen to demonstrate observed co-localisation, arrow: scale bars=20µm.

Figure 4. 3NT, HNE and 8OHDG immunoreactivity in ventral ON vulnerable to secondary degeneration. Mean ± SEM immunoreactivity of the entire image or the area above a set threshold intensity are shown for 3NT (A, B), HNE (D, E) and 8OHDG (G, H), from normal ON and ON 1 hour, 1, 3 or 7 days following partial ON transection, in ventral ON vulnerable to secondary degeneration: *indicates significantly different from normal ($p \leq 0.05$), n=6/time point. Representative images demonstrate immunoreactivity of: 3NT (red), co-labelled to detect green MBP at 7 days (C), or CC1 and Hoechst (I); HNE (red) co-localised with MBP in normal ON (F), or CC1 (green) and GFAP (blue) (J) and; 8OHDG (green) (K). Examples of co-localisation are indicated with arrows, scale bars=20µm.

Figure 5. Abnormal mitochondria in ventral ON vulnerable to secondary degeneration. Mean ± SEM incidence of mitochondrial autophagic profiles in a 416µm^2 region from normal ON and ON 1 hour, 1, 3 or 7 days following partial ON transection, in ventral ON vulnerable to secondary degeneration: *indicates significantly different from normal ($p \leq 0.05$), n=4/time point. Arrows in representative images indicate mitochondria with a swollen region (D), that were highly electron dense (E) or that featured a lysosomal sac (F), scale bars=0.5µm.

Figure 6. Schematic diagram illustrating changes in reactive species, antioxidant enzymes and oxidized products during secondary degeneration, based upon reactions described in *in vitro* studies [23, 68, 15, 69, 54, 70] and including findings of the present and previous studies in ON vulnerable to secondary degeneration [12, 25]. Note: reactions shown are indicative of processes rather than balanced equations and not all possible reactions are shown; GPx is also present in the cytoplasm; myeloperoxidase is secreted, although it can re-enter cells; some reactions shown are specific or more abundant in particular cell types e.g. NADPH oxidase and myeloperoxidase in inflammatory cells. Abbreviations are: AGE, advanced glycation end-product; CML, carboxymethyl lysine; MPT, mitochondrial permeability transition; NADH/NADPH, nicotinamide adenine dinucleotide/ phosphate; OMM/IMM, outer/ inner mitochondrial membrane and VDAC, voltage dependent anion channel

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