Neurobiology of Disease

Oligodendroglia Are Particularly Vulnerable to Oxidative Damage after Neurotrauma In Vivo


1Experimental and Regenerative Neurosciences, School of Biological Sciences, 2School of Molecular Sciences, 3Centre for Microscopy, Characterisation, and Analysis, University of Western Australia, Perth, 6009 Western Australia, Australia, 4Curtin Health Innovation Research Institute, Curtin University, Bentley, Perth, 6102 Western Australia, Australia, and 5Perron Institute for Neurological and Translational Science, Sarich Neuroscience Research Institute, Nedlands, 6009 Western Australia, Australia

Loss of function following injury to the CNS is worsened by secondary degeneration of neurons and glia surrounding the injury and is initiated by oxidative damage. However, it is not yet known which cellular populations and structures are most vulnerable to oxidative damage in vivo. Using Nanoscale secondary ion mass spectrometry (NanoSIMS), oxidative damage was semi-quantified within cellular subpopulations and structures of optic nerve vulnerable to secondary degeneration, following a partial transection of the optic nerve in adult female PVG rats. Simultaneous assessment of cellular subpopulations and structures revealed oligodendroglia as the most vulnerable to DNA oxidation following injury. 5-Ethynyl-2'-deoxyuridine (EdU) was used to label cells that proliferated in the first 3 d after injury. Injury led to increases in DNA, protein, and lipid damage in oligodendrocyte progenitor cells and mature oligodendrocytes at 3 d, regardless of proliferative state, associated with a decline in the numbers of oligodendrocyte progenitor cells at 7 d. O4+ preoligodendrocytes also exhibited increased lipid peroxidation. Interestingly, EdU+/mature oligodendrocytes derived after injury demonstrated increased early susceptibility to DNA damage and lipid peroxidation. However, EdU-/mature oligodendrocytes with high 8-hydroxyguanosine immunoreactivity were more likely to be caspase3+. By day 28, newly derived mature oligodendrocytes had significantly reduced myelin regulatory factor gene mRNA, indicating that the myelination potential of these cells may be reduced. The proportion of caspase3+ oligodendrocytes remained higher in EdU− cells. Innovative use of NanoSIMS together with traditional immunohistochemistry and in situ hybridization have enabled the first demonstration of subpopulation specific oligodendroglial vulnerability to oxidative damage, due to secondary degeneration in vivo.

Key words: myelin regulatory factor gene; NanoSIMS; oligodendrocyte precursor cell; oligodendroglia; oxidative stress; proliferation and differentiation

Significance Statement

Injury to the CNS is characterized by oxidative damage in areas adjacent to the injury. However, the cellular subpopulations and structures most vulnerable to this damage remain to be elucidated. Here we use powerful NanoSIMS techniques to show increased oxidative damage in oligodendroglia and axons and to demonstrate that cells early in the oligodendroglial lineage are the most vulnerable to DNA oxidation. Further immunohistochemical and in situ hybridization investigation reveals that mature oligodendrocytes derived after injury are more vulnerable to oxidative damage than their counterparts existing at the time of injury and have reduced myelin regulatory factor gene mRNA, yet preexisting oligodendrocytes are more likely to die.

Introduction

Following injury to the CNS, secondary degeneration results in structural changes to axons and myelin, as well as death of neurons and glia in areas surrounding the initial injury site, leading to further loss of function (Lu et al., 2000; Park et al., 2004). However, it is not known which cellular populations and structures...
are most vulnerable to secondary degeneration in vivo. Secondary degeneration can be effectively modeled by a partial optic nerve transection, where dorsal axons are transected by the primary injury leaving initially unaffected ventral axons vulnerable to secondary degeneration (Fitzgerald et al., 2009a, 2010). As such, findings using the model are highly applicable to the development of therapeutic strategies to limit damage and functional loss following neurotrauma. Using this and other models, we and others have demonstrated that progression of secondary degeneration features oxidative stress, occurring as a consequence of the overproduction of reactive oxygen and nitrogen species, ultimately resulting in the oxidation of DNA, lipids, and proteins (Park et al., 2004; Xiong et al., 2007; O’Hare Doig et al., 2014). Reactive species and oxidative stress are observed early in the partial optic nerve transection model, with increased immunoreactivity and manganese superoxide dismutase activity in astrocytes as early as 5 min after injury in optic nerve vulnerable to secondary degeneration (Fitzgerald et al., 2010), and increased hypochlorous acid indicative of myeloperoxidase activity from macrophages in the injury site soon thereafter (O’Hare Doig et al., 2014). It has been postulated that selective vulnerability of oligodendroglia, particularly oligodendrocyte progenitor cells (OPCs) to oxidative damage (Back et al., 2002; Fünfschilling et al., 2012), may underlie much of the pathology of secondary degeneration (Szymanski et al., 2013). Previous studies have assessed oxidative damage to neurons (Coyle and Puttfarken, 1993), glia and oligodendrocytes (Thorburn and Juurlink, 1996; Juurlink et al., 1998; Zibreva et al., 2010). However, many of these studies have used immortalized cell lines or primary OPC cultures and compared relative expression of ion channels as a surrogate indicator of vulnerability. Comparative in vivo studies indicate that OPCs are more vulnerable than mature oligodendrocytes in neonatal hypoxic-ischemic injury (Back et al., 2002), whereas studies using inhibitors of complex IV indicate greater vulnerability of differentiated oligodendrocytes (Zibreva et al., 2010). In vivo comparative measures of damage specific to neurons, myelin, OPCs, and oligodendrocytes following neurotrauma are lacking.

OPCs have an increased susceptibility to oxidative damage, attributed to their high iron content, low reduced glutathione levels (Thorburn and Juurlink, 1996), and low antioxidant defenses (French et al., 2009; Volpe, 2011). When considering neurotrauma, a loss in OPC numbers over time, with a concomitant increase in newly derived mature oligodendrocytes has been demonstrated following spinal cord injury (Watanabe et al., 2002). However, the influence of differentiation and proliferative state on cellular vulnerability following neurotrauma in vivo are yet to be explored. Selective vulnerability of OPCs is thought to impact upon function through both lack of availability of OPCs to generate new myelinating oligodendrocytes as well as compromised neuroglial signaling (Fields, 2015; Gautier et al., 2015). Importantly, the mechanisms driving depletion of OPCs are currently unknown, and it is not known whether proliferating cells are more susceptible to oxidative damage following neurotrauma.

Studies comparing the degree of damage in cellular sub-populations and structures, such as oligodendroglia, myelin, and paranodes, have not been possible using conventional immunohistochemical techniques, due to the inherent limitations of fluorescence microscopy. Using Nanoscale secondary ion mass spectrometry (NanoSIMS) to image metal isotope-conjugated antibodies, it is theoretically possible for simultaneous analysis of up to 100 antigens of interest, with the same level of reliability as immunohistochemistry (Angelo et al., 2014) and without secondary antibody emission overlap (Bandura et al., 2009). NanoSIMS images can be interpreted using immunointensity analysis techniques (Angelo et al., 2014; Lozic et al., 2016) that reveal comparative, semiquantitative information regarding the intensity of labeling in different cells and cellular components in the tissue. Here, oxidative damage to oligodendrocyte subpopulations and cell structures was compared in areas of white matter vulnerable to secondary degeneration following partial optic nerve transection, using NanoSIMS analysis. Complementary immunohistochemical and in situ hybridization analyses, identifying cells that had proliferated and/or differentiated using 5-ethyl-2-deoxyuridine (EdU), were used to illuminate functional significance of oxidative damage in specific oligodendroglial subpopulations, dependent upon DNA damage and proliferative status.

Materials and Methods

Animal procedures

All procedures involving animals were approved by the University of Western Australia Animal Ethics Committee (approval number RA3/100/673 and RA3/100/1485) and adhered to the National and Medical Research Council Australian Code of Practice for the care and use of animals for scientific purposes. Adult female PVG rats were procured from the Animal Resources Centre (Murdoch, Western Australia) and housed under temperature-controlled conditions on a 12 h light/dark cycle, with access to rat chow and water ad libitum. Female rats were used to complement our existing body of published work examining the pathology of secondary degeneration following partial optic nerve transection (Fitzgerald et al., 2009a; Payne et al., 2012; O’Hare Doig et al., 2014); the surgery for this valuable model is not practical in mice due to the size of the knife relative to the orbit and the presence of large blood vessels. The partial optic nerve transection procedure was conducted as described previously (Fitzgerald et al., 2010). In brief, rats were anesthetized with xylazine (10 mg/kg i.p. i.lium Xylazil; Troy Laboratories), in combination with ketamine (50 mg/kg Ketamil, Troy Laboratories), the dorsal aspect of the right optic nerve was incised to a depth of 200 μm, 1 mm behind the eye. Postoperative analgesia was provided once (2.8 mg/kg s.c. carprofen, Norbrook). EdU (Invitrogen) was administered via intraperitoneal injection at 20 mg/kg to rats to be used for immunohistochemical outcomes twice a day at 12 h intervals for 3 d, commencing during postoperative analgesia. All animals were killed with Lethabarb (sodium pentobarbitone 850 mg/kg plus sodium phenytoin 125 mg/kg; Provet). Animals were transcardially perfused with saline followed by 4% PFA (Sigma-Aldrich) in 0.1 M phosphate buffer, pH 7.2–7.4. Optic nerves were harvested and postfixed in 4% PFA (Sigma-Aldrich) for 1 h and then cryoprotected in 15% sucrose until cryosectioning. Optic nerves were cryosectioned transversely at 14 μm thickness for immunohistochemistry and 10 μm thickness for NanoSIMS.

Experimental design and statistical analysis

In total, 8 groups of animals were used for immunohistochemical and/or in situ hybridization outcomes; uninjured 3 d (n = 10), injured 3 d (n = 10 for immunohistochemistry and n = 8 for in situ hybridization and caspase3 outcomes), uninjured 7 d (n = 10), injured 7 d (n = 10), uninjured 28 d (n = 10), and injured 28 d (n = 10 for immunohistochemistry and n = 8 for in situ hybridization and caspase3 outcomes), with EdU administered to all animals. There were no significant differences recorded between the uninjured groups and therefore controls were combined for statistical comparisons. There were 2 groups used for NanoSIMS outcomes; uninjured 3 d (n = 3) and injured 3 d (n = 3). The numbers of animals per group for NanoSIMS analyses were appropriate given the fine-scale nature of the ultrastructural analysis and were similar to those described in published electron microscopy (Fitzgerald et al., 2009b; Xing et al., 2014) and NanoSIMS (Lozic et al., 2016) studies. Power analyses indicated that the numbers of animals per group would be sufficient to detect differences, based upon Type I/II errors of α = 0.05 and β = 0.2 for both NanoSIMS and immunohistochemistry measures. The study design assessed between-subject factors, with no repeated
measures, analyzing effects of injury, and time after injury, on a range of NanoSIMS and immunohistochemistry-based outcomes.

For NanoSIMS data sampling, 20× magnification light-microscopy images of H&E-stained-optic nerve were used to select at least one FOV (50 μm × 50 μm) in the ventral aspect of each optic nerve for NanoSIMS imaging. Additional FOV were used when available. Regions encompassing linear nuclei immediately ventral to the injury site, or equivalent in normal nerve, were selected for analysis. Choice of FOV was hypothesis driven; regions that would encompass a range of cell types and structural features in nerve exclusively vulnerable to secondary degeneration were actively selected. For immunohistochemistry and in situ hybridization sampling, a single FOV was selected per section, and there was one section per animal, located in ventral nerve at the injury site.

Data were expressed as mean ± SEM. Statistical analyses were conducted using SPSS statistical software (IBM), comparing day 3, 7, and 28 injured tissue to grouped control groups using either one-way or two-way ANOVA with Bonferroni or Sidak post hoc tests as appropriate. Normality of data was assumed and homogeneity of variances was tested using Levene’s test. p values alone were presented for ANOVA tests; p values alone were presented for post hoc comparisons. Student’s paired t tests were used for comparisons between in situ hybridization outcomes or proportions of caspases3,7 cells, for 8-hydroxyguanosine (8OHdG) low or high immunoreactivity subpopulations or for EDU− or EDU+ cells. For NanoSIMS analyses, variances were not homogeneous; therefore, a significance value of p ≤ 0.01 was used. For immunohistochemistry and in situ hybridization analyses, where variances were equal, significance was set at p ≤ 0.05. Significances less than p = 0.001 are denoted as p < 0.001. Multiple comparisons were conducted in a hypothesis driven fashion, comparing the effects of injury and time after injury on the selected outcomes, in regions of the optic nerve established as vulnerable to secondary degeneration (Fitzgerald et al., 2009a).

NanoSIMS

Metal-antibody conjugation. Metal-conjugated primary antibodies were prepared using the MaxPAR antibody conjugation kit (DVS Sciences) according to the manufacturer’s protocol (Table 1). Following the conjugation procedure, antibodies were diluted in PBS stabilization buffer: supplemented with 1% (w/v) BSA (Thermo Fisher Scientific) and 0.05% (w/v) sodium azide (Sigma-Aldrich) at concentrations between 0.25 and 0.4 mg/ml and stored at 4°C. Metal conjugation to the Click-iT EdU AlexaFluor-647 (Invitrogen) reagent was performed at a ratio of 4:1 of 144Nd:AlexaFluor-647, the ratio selected based on optimization using fluorescence spectrometry. Conjugation was demonstrated by spectral shift in the fluorescence emission. However, as excess unbound metal could not be sufficiently removed by extensive dialysis and interfered with sampling of other metals, the Click-iT reaction was not performed on this tissue.

Substrate preparation. TPO glass wafers (Corning) were precoated with 10 μg/ml poly-L-lysine (Sigma-Aldrich) for 1 h at room temperature, followed by 10 μg/ml laminin (Sigma-Aldrich) at 37°C overnight. Optic nerves were transversely cryosectioned at 10 μm thickness. Sections to be used for NanoSIMS analysis were stained with H&E free floating, then placed onto the precoated TPO glass wafer and allowed to adhere for 10 min at room temperature. The sections were then subjected to immunohistochemical procedures as previously described (Fitzgerald et al., 2010). In brief, sections were incubated for 24 h at 4°C with metal-conjugated primary antibodies recognizing 8OHdG (RRID:AB_297482), CC1 (RRID:AB_443473), myelin basic protein (MBP) (RRID:AB_1141521), 3-nitrotyrosine (3NT) (RRID:AB_310089), Caspr (RRID:AB_809934), βIII-tubulin (βIII), NG2 (RRID:AB_443419), and olig2 (RRID:AB_2157554), all at concentrations of 1 μg/ml. Sections of each configuration: (1) 41K (H&E), 144Nd, 151Eu (Caspr), 158Gd (CC1), 162Dy (8OHdG), 170Er (3NT), and 175Lu (MBP); and (2) 27Al (H&E), 144Nd (for preliminary EDU analysis), 152Sm (olig2), 158Gd (CC1), 162Dy (8OHdG), 172Yb (NG2) and 176Yb (βIII).

NanoSIMS quantification. NanoSIMS analyses result in secondary ion maps, with individual pixels representing the counts of secondary ions acquired within the acquisition time. The use of pseudo-coloring allows assignment of color to the lanthanide metal signals. White represents overlap of multiple colors, such as red, cyan, and purple. Analysis of these maps was conducted using the OpenMIMS plugin for Fiji (version 2.0; National Institutes of Health). Semi-quantitative analyses of 8OHdG and 3NT (antibodies conjugated to 162Dy and 170Er, respectively) were performed by determining the mean secondary ion count, defined as counts per pixel within specified ROI. The ROI were defined by manually tracing around areas with high secondary ion counts, present as a result of labeling with metal-conjugated antibodies recognizing βIII, MBP, Caspr, NG2, olig2, or CC1 (antibodies conjugated to 176Yb, 175Lu, 151Eu, 152Sm, or 158Gd, respectively). Area and mean counts were reported as pixel intensity. Visualization of structural information was achieved using overlays of secondary ion images in Photoshop CC 2017 (Adobe), each assigned to a color channel as indicated on the respective images.

Immunohistochemistry, microscopy, and analysis

Immunohistochemical analyses were conducted according to previously described procedures (Fitzgerald et al., 2010). Primary antibodies used for immunohistochemical assessments were as follows: 8OHdG (1:400 or 1:1000, goat, Abcam, ab10802, RRID:AB_297482) (1:400, mouse, Abcam, ab62623, RRID:AB_940049), hydroxynonanoyl (HNE) (1:400, rabbit, Abcam, ab46545, RRID:AB_722490), 3NT (1:500, rabbit, Abcam, ab61392, RRID:AB_942087), NG2 (1:400, mouse, Abcam, ab50009, RRID:AB_881569), olig2 (1:500, goat, R&D Systems, AF4218, RRID:AB_2157554), CC1 (1:500 or 1:750, mouse, Calbiochem, ab16794, RRID:AB_443473), O4 (1:50, mouse, EMD Millipore, MAB345, RRID:AB_94872), MBP (1:300, rabbit, Abcam, RRID:AB_1141521), and
activated-caspase3 (1:500, rabbit, Abcam, ab15847, RRID:AB_443014). Secondary antibodies were species-specific AlexaFluor-350, -488, and -555-conjugated antibodies (1:500; Invitrogen). EdU+ cells were visualized using a Click-IT Edu AlexaFluor-647 Imaging Kit (Invitrogen) according to the manufacturer’s instructions. Similarly, TUNEL+ cells were visualized using a Click-IT TUNEL AlexaFluor-488 Imaging Kit (Invitrogen) according to the manufacturer’s instructions.

In situ hybridization analyses were conducted using the RNAsecope Multiplex Reagent Kit version 2 system, using custom-made probes to myelin regulatory factor gene (MYRF) and ID2 (both C1 hybridized), Sox10 and Nkx2.2 (both C2 hybridized); Nkx2.2 outcomes were somewhat variable and are not described further. The Multiplex Fluorescent Reagent Kit version 2 and TSA amplification (PerkinElmer) was used largely according to the manufacturer’s instructions (document 323100-USM) with the following optimizations: pretreatment was with 5 min boiling in target retrieval reagent, followed by 20 min 33% protease incubation at room temperature. Immunohistochemistry was conducted after completion of the in situ hybridization protocol and was followed by Edu detection in accordance with recommendations in technical note 323100-TN, using primary antibody dilutions of 1:300 for CC1 (RRID:AB_443473), 1:250 for 8OHdG (RRID:AB_297482) and 1:300 for MBP (RRID:AB_1141521), incubating overnight at room temperature. Secondary antibodies were donkey anti-goat AlexaFluor-350 (1:250; Invitrogen) for 8OHdG and CC1 and TSA amplification (PerkinElmer) with biotinylated secondary antibodies and streptavidin-HRP for NG2 (RRID:AB_10672215) and CC1 (RRID:AB_443473) as appropriate. TSA fluorescein and Cy3 and Cy5 fluorophores (PerkinElmer) were used to amplify each of the fluorescent signals for the three or four color analyses, in accordance with the instructions in the Multiplex Fluorescent Reagent Kit version 2.

Fluorescence imaging was performed using an Si1 inverted microscope (Nikon). Images were captured in a stack of 13 visual slices at 0.5 μm increments along the z-axis, obtained from the middle 6 μm of the optic nerve section. All images for assessment of each outcome measure were captured at a constant exposure, using identical microscope settings. The selected FOVs were located in ventral optic nerve directly below the dorsal primary injury site. A single FOV was selected per section, and there was one section per animal. Images were deconvolved using autoquant blind deconvolution with Nikon Elements AT software. OPCs were identified as NG2+/olig2+ cells, premyelinating O4 cells were identified as O4+ and mature OLs were identified as CC1+ cells (Payne et al., 2013). O4 immunoreactivity was present diffusely on cell processes, so O4+ cells were defined as cells with >50% O4 immunoreactivity surrounding a Hoechst nucleus. EdU+ cells were defined as newly derived cells. Numbers of cells within oligodendroglial subpopulations were quantified. This nonstereological technique does not allow for an estimate of total cell numbers within the nerve but does allow for comparative assessment between time points after injury. 8OHdG, 3NT, and HNE immunointensities were assessed within ROI defined by the oligodendroglial identification markers for OPCs, premyelinating oligodendrocytes, and mature oligodendrocytes on a single, central visual slice. The presence of the analyzed trans-scription factors further confirmed cellular identity within the oligodendroglial lineage (Wang et al., 2001; Fancy et al., 2004; Liu et al., 2007; Emery et al., 2009). Subpopulations of cells were analyzed for mRNA expression or the proportion of TUNEL+ or caspase3+ cells, based upon whether 8OHdG immunoreactivity in these cells was above or below an arbitrarily defined and constant threshold, or whether they were EdU+ or EdU−. The mean intensity of MBP immunoreactivity in an area immediately surrounding CC1+ oligodendrocytes was also quantified, separately for EdU+ and EdU− cells. Analysis of immunointensity and cell numbers were performed using Fiji analysis software. Normalization to background is not possible for oxidative stress immunointensity data, as the oxidized proteins, lipid, and DNA are diffusely distributed throughout the tissue, and there is nowhere that can be conclusively described as background. Data shown are mean arbitrary fluorescence intensities for 8OHdG, 3NT, HNE, and MBP as well as for ID2, Sox10, and MYRF mRNA. Mean counts of OPCs, O4s, and mature oligodendrocyte cells were expressed at number of cells/mm2. Numbers of TUNEL+ or caspase3+ cells were expressed as a proportion of the total number of CC1+ mature oligodendrocytes.

Results

The NanoSIMS was used to compare the vulnerability of cellular subpopulations and structures within regions of secondary degeneration following neurotrauma in vivo. Imaging of metal isotope-conjugated antibodies using NanoSIMS has been demonstrated to have the same level of reliability as immunohistochemistry (Angelo et al., 2014), without secondary antibody emission overlap (Bandura et al., 2009).

Identification of oxidative damage in oligodendroglia, myelin structures, and axons

To capitalize on the semiquantitative information inherent in the secondary ion images produced by NanoSIMS, regions of lanthanide metal/antibody labeling indicating cell subtypes or structural features were analyzed. FOV for analysis were selected using longitudinally oriented optic nerve sections viewed by light microscopy (Fig. 1a). The dark streaks (arrow) visualized via the
H&E staining are nuclei and likely represent oligodendroglia arranged in linear arrays, as described in studies of oligodendroglial development (Walsh et al., 2016). FOV were chosen to encompass these features. Intensities of metal/antibody labeling indicating oxidative damage within these defined regions were semiquantified, making it possible to compare oxidative damage between these cellular subpopulations and structures, for a specific indicator of oxidative damage. We did not attempt to compare intensity of labeling between oxidative damage indicators, as while the degree of metal labeling is consistent for any individual outcome measure, intensity may be different for different antibodies. ROIs were identified based on elevated pixel intensities of the metals bound to antibodies recognizing CC1 for mature but not necessarily myelinating oligodendrocyte somata, MBP for myelin ensheathing axons, Caspr for paranodes, βIIIT for axons, and NG2 and olig2 for oligodendroglia (Fig. 1). The pixel intensity of 8OHdG and 3NT labeling in these ROI were then determined to semiquantify DNA oxidation and protein nitration, respectively. Two sets of comparisons were conducted: the first assessing the effects of injury on pixel intensities within each cellular subpopulation or structure, and the second comparing pixel intensities in the various subpopulations or structures to each other. The FOV captured by the NanoSIMS and used in these first analyses were too small to identify sufficient numbers of NG2+/olig2+ cells for valid statistical analysis of oxidative damage within OPCs. Therefore NG2+ and olig2+ ROI were analyzed separately. However, it is important to note that the olig2+ transcription factor is expressed in all oligodendroglia (Payne et al., 2013) and NG2 profiles include pericytes (Ozerdem et al., 2002), as well as neuronal stem cells (Richardson et al., 2011), astrocytes (Zhu et al., 2008), and areas of extracellular matrix (Busch and Silver, 2007). The use of pseudo-coloring allowed assignment of color to the lanthanide metal signals, and overlay of these colors is as expected (e.g., blue overlaid with red appears purple). Tissue sections labeled with lanthanide-conjugated antibodies displayed expected labeling patterns. For example, olig2 was present in nuclei identified on41K images of the same FOV, and NG2 and CC1 ROI corresponded to expected cellular distributions (Fig. 2a,b, arrowheads). The NanoSIMSs images are generated as a reflection of ablation of a very small depth of tissue. As such, not all features expected in tradi-

Figure 2. Pseudofluorescence composite images confirming appropriate pixel distribution of metal-labeled antibodies of interest and negative controls. The pseudofluorescent composite images show distinctly different pixel distributions. a, b. The same FOV from normal optic nerve, captured using the two NanoSIMS scan settings described in Figure 1, to detect specific lanthanide-metal-conjugated antibodies of interest. a, 41K (gray) shows the location of nucleic and cytoplasmic regions. Olig2 (blue) and NG2 (green) indicate OPCs when colocalized (arrowheads). Olig2 and βIIIT (red) are present in nuclei and cytoplasm, respectively. b, 8OHdG (blue) staining pattern colocalizes with nuclei (arrowhead), within CC1+ (green) mature oligodendrocytes. CC1 and Caspr (red) labeling, both in the cytoplasm, with distinct staining patterns in line with their known cell structure distributions. *Center of the image from the first imaging scan. The NanoSIMS images of tissue not incubated with lanthanide-conjugated antibody. c, d. Distinctly different pixel distribution from the sections labeled with lanthanide-conjugated antibodies shown in Figure 1 (and a, b). c, d. The same FOV from normal optic nerve, captured using the NanoSIMS scan settings described in Figure 1 to detect the specific lanthanide metals of interest. c. Images acquired using the NanoSIMS calibrated to detect the following: 144Nd, 151Eu, 158Gd, 162Dy, 170Er, and 175Lu. d. Images acquired using the NanoSIMS calibrated to detect the following: 144Nd, 152Sm, 158Gd, 162Dy, 172Yb, and 176Yb within the same FOV. The NanoSIMS scan setting used to detect specific metal lanthanides are indicated on each image (c, d).
Comparative analysis of DNA oxidation and protein nitration in cellular subpopulations and structures vulnerable to secondary degeneration in vivo. A, Mean ± SEM pixel intensity indicating increased DNA oxidation in CC1⁺, Caspr⁺, NG2⁺, and olig2⁺ areas following injury relative to control (*), as well as significant increases in DNA oxidation in olig2⁻ areas compared with other cellular subpopulations and structures following injury (* over bar). B, Mean ± SEM pixel intensity indicating increased protein nitration in CC1⁺, MBP⁺, Caspr⁺, βIIIIT⁺, NG2⁺, and olig2⁻ areas following injury (*). Representative pseudofluorescent composite images show (c) DNA oxidation (red) in CC1⁺ (green), Caspr⁺ (purple), NG2⁺ (green), and olig2⁻ (blue) areas in control and injured optic nerve and (d) protein nitration (red) within CC1⁺ (green), MBP⁺ (blue), Caspr⁺ (purple), NG2⁺ (green), olig2⁻ (blue), and βIIIIT⁺ (purple) areas. 8OHdG and 3NT pixel intensity images for control and injured nerve were each taken of the same FOV. For example, some of the red 3NT signal in the first image of injured tissue, colocalizing with white olig2/NG2/3NT in the second image. *p ≤ 0.01, n = 3/group.

Oligodendroglial DNA to oxidative stress in regions remote from the initial injury site. Assessments of 3NT pixel intensity revealed that protein nitration increased at 3 d after injury in mature oligodendrocytes (p = 0.002), NG2⁺ cells (p = 0.002), olig2⁻ cells (p = 0.001), βIIIIT⁺ axons (p = 0.004), and paranodes (p = 0.001) (F = 0.752, df = 10, two-way ANOVA) (Fig. 3b,d). However, in contrast to the higher DNA oxidation observed in Olig2⁻ cells, comparisons between ROI in terms of 3NT pixel intensity at 3 d after injury showed no significant differences between the cell populations and structures (Fig. 3b,d). It is important to view the NanoSIMS images in the context of the multiple images of the same FOV. For example, some of the red 3NT signal in the first image of Figure 3d (injured, blue arrow) appears not to be colocalized with a cellular element. However, if compared with the adjacent image of the same FOV, the red signal can be seen to colocalize with the white olig2/NG2/3NT signal. Comparisons in basal levels of DNA oxidation and protein nitration between
oxidative stress (It is widely acknowledged that oligodendroglia are vulnerable to secondary degeneration of oligodendroglia along the differentiation lineage; cells that had proliferated in the 3 d after injury were labeled with EdU in vivo. Following injury, there was a significant increase in the number of newly derived OPCs at days 3 (p ≤ 0.001) and 7 (p ≤ 0.001) following injury compared with uninjured controls (F = 3.78, df = 117, two-way ANOVA). Despite the increase in the number and proportion of newly derived OPCs, the total numbers of OPCs (newly derived and previously existing populations together) decreased in areas of secondary degeneration 7 d after injury (p = 0.029), and remained depleted at 28 d (p ≤ 0.001) (Fig. 4a). Dying cells were quantified using TUNEL staining; however, the numbers of these cells were low, precluding useful statistical comparisons (data not shown).

There was a significant increase in the numbers of newly derived EdU+ premyelinating oligodendrocytes (O4+) at 3 (p ≤ 0.001), 7 (p ≤ 0.001) and 28 (p = 0.004) days after injury (F = 2.85, df = 79, two-way ANOVA) (Fig. 4b). The total numbers of O4+ cells increased at 3 (p = 0.037) and 7 (p = 0.001) days after injury compared with controls (Fig. 4b). It has been previously documented that, in the uninjured, mature CNS, there is a relatively slow turnover of mature OLs (Young et al., 2013). Similarly, we observed very low numbers of newly derived EdU+ mature OLs in control uninjured animals, and only in 2 animals. Following injury, there was a significant increase in the numbers of newly derived mature oligodendrocytes at 28 d following injury (p ≤ 0.001) (F = 6.28, df = 119, two-way ANOVA) (Fig. 4c). It is worthwhile noting that, while these data are a snapshot in time and cannot be taken as cumulative, the total number of newly derived mature oligodendrocytes (Fig. 4c) was lower than the numbers of newly derived OPCs or of premyelinating oligodendrocytes at 3, 7, and 28 d (Fig. 4a,b). Oxidative damage to oligodendroglia following neurotrauma

It is widely acknowledged that oligodendroglia are vulnerable to oxidative stress (Roth and Núñez, 2016). However, direct comparisons between oxidative damage in newly derived oligodendroglial subpopulations and subpopulations existing at the time of injury, within regions of secondary degeneration in vivo have not yet been conducted. Accordingly, the immunointensities of markers indicating oxidative damage to DNA, proteins, and lipids were compared in EdU+ and EdU- OPCs, premyelinating, and mature oligodendrocyte subpopulations.

DNA oxidation was significantly increased in both newly derived (p ≤ 0.001) and previously existing (p ≤ 0.001) OPCs 3 d after injury, compared with control animals (F = 1.164, df = 92); newly derived and preexisting OPC DNA oxidation returned to control levels at 7 and 28 d after injury (Fig. 5a). Both populations of OPCs displayed increased protein nitration, indicated by 3NT immunointensity 3 d after injury (newly derived p = 0.002, previously existing p = 0.050, F = 2.306, df = 84, two-way ANOVA); this also returned to control levels at 7 and 28 d after injury (Fig. 5b). Similarly, both newly derived and preexisting OPCs showed increased immunofluorescence of HNE in OPCs at 3 d after injury (p = 0.017 and p ≤ 0.001, respectively, F = 1.780, df = 92, two-way ANOVA), which returned to control levels at 7 and 28 d after injury (Fig. 5c). There were no differences between EdU+ and EdU- OPCs in terms of the degree of oxidative damage after injury, regardless of the oxidative damage indicator (Fig. 5d–f; EdU- cells indicated by >, EdU+ cells indicated by >>). The data indicate that OPCs have transient increases in oxidative damage to DNA, lipid, and protein at 3 d after injury.

Analysis of O4+ cells showed no increases in 8OHdG (p > 0.05, F = 0.406, df = 71, two-way ANOVA) (Fig. 6a) or 3NT (p > 0.05, F = 0.391, df = 68, two-way ANOVA) (Fig. 6b) immunoreactivity at any time point after injury, in either the newly derived or previously existing subpopulations. There was, however, a significant increase in HNE immunointensity in the nonproliferating O4+ subpopulation at 3 d after injury (p = 0.001, F = 1.750, df = 47, two-way ANOVA) (Fig. 6c). The lack of newly derived EdU+ O4+ cells in the control group rendered comparisons of EdU+ cells derived after injury to control cells impossible; therefore, no statistical comparisons were made between newly derived O4+ subpopulations (Fig. 6c). Representative images show EdU- cells indicated by >, EdU+ cells indicated by >> (Fig. 6d–f).

Both newly derived (p ≤ 0.001) and preexisting (p ≤ 0.001) mature oligodendrocyte populations showed increased DNA oxidation 3 d after injury (F = 1.189, df = 87, two-way ANOVA),...
Figure 5. 8OHdG-, 3NT-, and HNE-derived immunofluorescence in OPCs vulnerable to secondary degeneration in vivo. Mean ± SEM fluorescence intensity within OPCs (NG2+/olig2+ cells) is shown as follows: (a) 8OHdG, (b) 3NT, and (c): HNE labeling in uninjured optic nerve and at 3, 7, and 28 d following partial transection. Arbitrary values for immunofluorescence were categorized as EdU− (black bars) or EdU+ (gray bars) to discriminate any differences due to proliferative state. Representative images of (d) 8OHdG, (e) 3NT, and (f) HNE labeling are of OPCs located within the ventral optic nerve vulnerable to secondary degeneration. d–f, Cells indicated are NG2+/olig2+/EdU− or NG2+/olig2+/EdU+ (>). Statistical comparisons were made across and between groups. *p < 0.05, differences in immunointensity compared with controls. Scale bar: d–f, 10 μm.

Figure 6. 8OHdG-, 3NT-, and HNE-derived fluorescence in premyelinating oligodendrocytes vulnerable to secondary degeneration in vivo. Mean ± SEM fluorescence intensity within premyelinating oligodendrocytes (O4− cells) is shown as follows: (a) 8OHdG, (b) 3NT, and (c): HNE labeling in uninjured optic nerve and at 3, 7, and 28 d following partial transection. Arbitrary values for immunofluorescence were categorized as EdU− (black bars) or EdU+ (gray bars) to discriminate any differences due to proliferative state. Representative images of (d) 8OHdG, (e) 3NT, and (f) HNE labeling are of premyelinating oligodendrocytes located within the ventral optic nerve vulnerable to secondary degeneration. d–f, Cells indicated are O4−/EdU− (>) or O4−/EdU+ (>>). Statistical comparisons were made across and between groups. *p < 0.05, differences in immunointensity compared with controls. Scale bar: d–f, 10 μm.
returning to control levels at 7 and 28 d (Fig. 7a). Interestingly, newly derived EdU+ mature oligodendrocytes displayed significantly higher DNA oxidation than their previously existing counterparts 3 d after injury (p \leq 0.001), although, as shown in Figure 4c, this represents a very small proportion of the total numbers of mature oligodendrocytes (Fig. 7a; EdU− cells indicated by >), EdU+ cells indicated by >>). Similarly, both newly derived (p = 0.001) and previously existing (p \leq 0.001) populations of mature oligodendrocytes displayed increased protein nitration immunoreactivity 3 d after injury (F = 2.044, df = 90, two-way ANOVA), returning to control levels at 7 and 28 d after injury. There were no statistically significant differences in 3NT immunoreactivity between previously existing and newly derived mature oligodendrocytes at any time point (Fig. 7b). Both EdU+ (p \leq 0.001) and EdU− (p \leq 0.001) mature oligodendrocytes demonstrated increases in lipid peroxidation 3 d after injury (F = 4.08, df = 89, two-way ANOVA). Newly derived mature oligodendrocytes maintained higher immunointensity of lipid peroxidation at 7 d after injury compared with their previously existing counterparts (p = 0.003), and relative to the immunointensity of control HNE (p = 0.010). Previously existing mature oligodendrocytes returned to control levels at this time. Representative images show EdU− cells indicated by >, EdU+ cells indicated by >> (Fig. 7d−f).

**Functional significance of oxidative damage to oligodendroglia following neurotrauma relative to proliferative state**

The effects of increased oxidative damage to DNA on expression of mRNA for transcription factors associated with OPC differentiation and oligodendrocyte myelination were assessed using fluorescent in situ hybridization together with fluorescence immunohistochemistry. Comparisons were made between cells that were at baseline or had elevated 8OHdG immunoreactivity at 3 d, as this was the time point at which most significant increases in 8OHdG were observed (Figs. 5−7). At day 28, populations were distinguished based upon whether they were EdU+ and therefore newly derived soon after the injury; or EdU−, and were therefore either derived later than 3 d after injury, or were preexisting at the time of injury.

OPC differentiation factors ID2 (Wang et al., 2001; Havrda et al., 2014) and Sox10 and MYRF (Stolt et al., 2002; Liu et al., 2007) were compared in NG2+ or CC1+ cells that were high or low in 8OHdG immunoreactivity, relying upon oligodendroglial transcription factors to confirm OPC identification. There were no changes in intensity of ID2 (Fig. 8a; p = 0.65, df = 7, t test) or Sox10 expression (data not shown; p = 0.17, df = 7, t test) relative to 8OHdG immunoreactivity at day 3, and there were also no differences relative to EdU defined proliferation status at day 28 (Fig. 8b, ID2; p = 0.09, df = 6, t test), although Levene’s test indicated significantly greater variability in EdU+ OPCs than in EdU− OPCs (p = 0.03). Similarly, at day 3 after injury, increased 8OHdG immunoreactivity did not lead to altered Sox10 (p = 0.17, df = 7, t test) or MYRF (p = 0.90, df = 7, t test) mRNA expression in CC1+ mature oligodendrocytes (Fig. 8c,e). However, at day 28, while there was still no change in Sox10 mRNA (p = 0.28, df = 6, t test), newly derived CC1+ oligodendrocytes had significantly less MYRF mRNA (p = 0.00, df = 6, t test) than their preexisting counterparts (Fig. 8d; for representative image, see Fig. 8g). MYRF is a transcription factor, turned on as OPCs differentiate into oligodendrocytes, and critical for oligodendrocyte maturation (Koenning et al., 2012). Throughout life, MYRF
is also critical for oligodendrocyte myelin maintenance, and the loss of this factor can directly lead to significant demyelination (Koenning et al., 2012). As such, reduced MYRF may be indicative of reduced myelination capacity in newly derived oligodendrocytes. The immunoreactivity of MBP in the areas immediately surrounding CC1$^+$ oligodendrocyte somata was also quantified relative to proliferative status, at day 28. MBP immunoreactivity immediately surrounding EdU$^+$ newly derived oligodendrocytes was not significantly less than that surrounding preexisting oligodendrocytes ($p = 0.33$, df = 6, t test; data not shown). This is perhaps not surprising, given that myelin internodes are frequently $>$100 $\mu$m in length, depending upon the proliferative history of the cell, and the myelin measured close to a particular oligodendrocyte in a 2D plane may be formed by a different oligodendrocyte with its soma 40–100 $\mu$m distant in any direction (Young et al., 2013).

The proportion of CC1$^+$ mature oligodendrocytes that were TUNEL$^+$ and caspase3$^+$ was compared for cells that were high or low in 8OHdG immunoreactivity at day 3. Mature oligodendrocytes with low 8OHdG immunoreactivity were generally not either TUNEL$^+$ or caspase3$^+$, and there was a significantly increased proportion of TUNEL$^+$ oligodendrocytes in the elevated 8OHdG subpopulation (Fig. 9a; $p = 0.04$, df = 7, t test). Similar findings were observed when caspase3 was used as an indicator of apoptotic cell death (Fig. 9c; $p = 0.00$, df = 7, t test).

For oligodendrocytes with elevated 8OHdG, the proportion of TUNEL$^+$ cells was not different for EdU$^-$ and EdU$^+$ cells (Fig. 9b; $p = 0.53$, df = 7, t test). However, when apoptotic death was considered more specifically (Degterev and Yuan, 2008), the percentage of caspase3$^+$ cells was higher for EdU$^-$ than for EdU$^+$ cells (Fig. 9d; $p = 0.00$, df = 7, t test). Similarly, at day 28, the proportion of caspase3$^+$ cells was significantly higher in EdU$^-$ than EdU$^+$ mature oligodendrocytes (Fig. 9e; $p = 0.00$, df = 7, t test).

**Discussion**

Multiplex NanoSIMS immunohistochemistry has enabled demonstration of selective vulnerability of oligodendroglia to DNA oxidation following neurotrauma in vivo. Detailed in vivo subpopulation and fate mapping analyses of oligodendroglial subpopulations along the differentiation lineage revealed susceptibility of OPCs and mature oligodendrocytes to DNA oxidation, protein nitration, and lipid peroxidation, with mature oligodendrocytes newly derived directly after injury more vulnerable than preexisting or later derived cells to DNA and protein damage. Reduced MYRF mRNA in the newly derived mature oligodendrocytes indicates that these cells may be compromised in their ability to make myelin. However, it is the EdU$^-$ cells that were preexisting at the time of injury that are more likely to die at day 3. EdU$^-$ oligodendrocyte vulnerability to death at day 28 may also include cells derived later after injury, and further studies will be required to assess effects of proliferation along a substantially more extended time course to differentiate vulnerability of later derived oligodendrocytes from their preexisting counterparts. The findings complement and extend previous studies suggesting vulnerability of oligodendroglia in vitro (Back et al., 1998; Fern and Möller, 2000) and in a range of pathologies, such as periventricular white matter damage (Back et al., 2005), hypoxia-ischemia and spinal cord injury (Watanabe et al., 2002) in vivo. Here we demonstrate higher levels of DNA oxidation in OPCs than axons and that OPC numbers decrease by 7 d after injury. We have previously demonstrated that retinal ganglion cells vulnerable to secondary degeneration do not begin to die until 2–3 weeks after injury (Fitzgerald et al., 2009a, b).

Together, with the oxidative damage in mature oligodendrocytes at 3 d, it is clear that changes to OPCs and oligodendrocytes precede loss of neurons in secondary degeneration and are likely to be a contributing factor to functional loss.

Using NanoSIMS, the cellular subpopulations and structures most vulnerable to oxidative damage within the CNS following...
neurotrauma were identified. NanoSIMS imaging eliminates many of the limitations of multiplex imaging, as there are no autofluorescent background signals. Importantly, because the NanoSIMS has the capacity to resolve a fraction of a Dalton, there is no spectral overlap between mass-adjacent lanthanide metals. The quantitative accuracy between immunohistochemistry fluorescence intensity and NanoSIMS pixel intensity has been extensively tested, and linear regression analysis has revealed robust similarities between the two imaging methods (Angelo et al., 2014). NanoSIMS revealed DNA oxidation increased in oligodendroglial cell bodies following injury, whereas there were no such increases in myelin. 8OHdG is mainly expressed in the nuclei of cells but has also been identified within cytoplasmic regions via incorporation in mitochondrial DNA (Tsai et al., 2011). The significant increase in DNA oxidation in olig2+ oligodendrocytes comparisons are confined to EdU+/− as 8OHdG immunoreactivity is not elevated. Each symbol represents the average for an animal. Error bars indicate mean values for the group. Statistical comparisons were made between groups. *p ≤ 0.05, differences in percentages of TUNEL− or caspase3− cells. f, Representative image of caspase3, EdU, CC1, caspase1, EdU and CC1 immunoreactivity at 28 d. CC1+/caspase3+/EdU− (>) and CC1+/caspase3+/EdU+/−(>) cells are indicated; caspase3+/EdU+ oligodendrocytes are seldom observed. Scale bar, 20 μm.

Figure 9. TUNEL labeling and caspase3 immunoreactivity relative to 8OHdG immunoreactivity or EdU defined proliferation status in oligodendrocytes. Percentages of CC1+ oligodendrocytes that are TUNEL− at 3 d after injury; comparisons are made between all CC1+ cells based on 8OHdG immunoreactivity (a), then between EdU− and EdU+ cells, both with elevated 8OHdG (b). Similarly, percentages of CC1+ oligodendrocytes that are caspase3+ at 3 d after injury based upon 8OHdG immunoreactivity (c), then between EdU− and EdU+ cells, both with elevated 8OHdG (d). e, At 28 d, caspase3+ oligodendrocyte comparisons are confined to EdU+/− as 8OHdG immunoreactivity is not elevated. Each symbol represents the average for an animal. Error bars indicate mean values for the group. Statistical comparisons were made between groups. *p ≤ 0.05, differences in percentages of TUNEL− or caspase3− cells. f, Representative image of caspase3, EdU, CC1, caspase1, EdU and CC1 immunoreactivity at 28 d. CC1+/caspase3+/EdU− (>) and CC1+/caspase3+/EdU+/−(>) cells are indicated; caspase3+/EdU+ oligodendrocytes are seldom observed. Scale bar, 20 μm.
injury in this model, indicating that differentiation does not ac-
count for all of the OPC depletion (Payne et al., 2013).

CCI 1− mature oligodendrocytes display increased DNA ox-
idation 3 d after injury, with a small subpopulation of newly de-
rived oligodendrocytes exhibiting even higher DNA oxidation
than their previously existing counterparts. There was no dis-
cernable decrease in the numbers of these mature preexisting cells
until 28 d after injury. It has been demonstrated that senescent
fibroblasts exhibit significantly higher levels of DNA oxidation
than young cells (Chen et al., 1995), with foci of DNA damage
particularly evident in newly senescent (i.e., recently divided
cells) and associated with shortening of telomeres (von Zglinicki,
2002). Newly differentiated oligodendrocytes are susceptible to
oxidative challenge (Ziabreva et al., 2010); however, differentia-
tion does not necessary lead to increased oxidative damage to
DNA (Covacci et al., 2001). We see that not all oligodendrocytes
with elevated BOHdG die, and newly derived oligodendrocytes
with elevated BOHdG have a lower percentage caspase3 . Sus-
ceptibility of an individual cell likely depends on the interplay of
multiple factors, including its proliferative history, antioxidant
defenses, and the microenvironment to which it is exposed. Fate
mapping data indicate that the newly derived mature oligoden-
drocytes are also vulnerable to lipid peroxidation but, neverthe-
less, go on to replace existing oligodendrocytes. However, these
newly differentiated oligodendrocytes may not be able to gener-
ate myelin due to their substantially reduced levels of MYRF
(Emery et al., 2009; Koenning et al., 2012; Bujalka et al., 2013;
Hornig et al., 2013). Although it is not clear how much MYRF is
required for myelination, these newly derived cells appear to be
compromised and are likely to have reduced ability to generate
new myelin to repair the damage of secondary degeneration.
While we were unable to demonstrate reduced MBP adjacent
to newly derived oligodendrocytes, the lack of a marker that iden-
tifies myelin associated with a particular oligodendrocyte soma in
this rat model precludes definitive conclusions regarding
whether myelin is produced by these cells. Nevertheless, the dys-
function of newly derived and increased early death of existing
and also potentially later generated oligodendrocytes observed in
the current study likely contributes to the dysmyelination and
chronic loss of function observed during secondary degeneration
at 28 d and 3 and 6 months in this model (Payne et al., 2012;
Szymanski et al., 2013), OPC differentiation into mature myeli-
nating oligodendrocytes ensheathing axons is a metabolically
demanding process that increases the susceptibility of these my-
elinating oligodendrocytes to apoptosis (Garbern, 2007; Butts et
al., 2008). This, in conjunction with the hostile environment
present following neurotrauma, potentially culminates in the in-
creased susceptibility to oxidative damage of myelinating oligo-
dendroglia to death. Although some remyelination is indicated by
the shorter internodes and increased number of intraperiodic
lines observed at 3 months in this model (Payne et al., 2012,
2013), functional deficits persist, emphasizing the lack of effective
myelin protection and/or repair.

It has generally been accepted that mature oligodendrocytes
are postmitotic and are incapable of proliferating in response to
injury (Mori and Leblond, 1970). Early reports of proliferation of
oligodendrocytes in demyelinating lesions (Herndon et al., 1977)
and following trauma (Ludwin, 1979) did not clearly differentiate
between label incorporated into precursor cells that had newly
differentiated, or that was incorporated directly into oligoden-
drocyte DNA, perhaps as a result of DNA repair. It is likely that
the small number of EdU + mature oligodendrocytes seen in con-
trols and at 3 and 7 d after injury in the current study were labeled
as a result of DNA repair involving baseline levels of differentia-
tion of OPCs. The larger increase in the numbers of newly derived
oligodendrocytes at 28 d after injury are likely derived from ma-
turing OPC populations, differentiating as a consequence of in-
jury. It has been suggested that OPC differentiation into mature
oligodendrocytes is compromised in models of multiple sclerosis
(Mi et al., 2009; Fancy et al., 2010). However, the scenario follow-
ning physical CNS injury may be different, as indicated in spinal
cord injury (Zai and Wrathall, 2005) and by the significant num-
bers of newly differentiated oligodendrocytes observed in the
current study. It is important to note that the current study as-
essed proliferation occurring immediately after injury; and al-
though out of the scope of this study, it would be interesting to
assess oxidative damage and differentiation in cellular subpopu-
lations that arise within the multiple later phases of injury.

In conclusion, this work provides in vivo experimental evidence
for the important role of oxidative damage in oligodendroglia in
the pathology of secondary degeneration following neurotrauma.
The semiquantification of oxidative damage to DNA, proteins,
and lipids in oligodendroglia shows, for the first time, acute dam-
age to specific subpopulations of these cells at different stages
following injury. Future fate mapping of proliferating OPCs and
their progeny beyond 28 d after injury will enable investigation of
whether early proliferative responses result in the derivation of
new, healthy oligodendrocytes and compact myelin. Protecting
against early oxidative damage due to secondary degeneration will
be worth considering in the design of therapeutic strategies aiming to
preserve structure and function following neurotrauma.

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