Neuroscience 177 (2011) 269–282

AXOTOMY-INDUCED CYTOSKELETON CHANGES IN UNMYELINATED MAMMALIAN CENTRAL NERVOUS SYSTEM AXONS

C. BALARATNASINGAM,[a,b] W. H. MORGAN,[a] L. BASS,[c] M. KANG,[a] S. J. CRINGLE[a,b] AND D.-Y. YU[a,b]*

[a]*Centre for Ophthalmology and Visual Science, The University of Western Australia, 2 Verdun Street, Nedlands, Perth, Australia*

[b]*The Australian Research Council Centre of Excellence in Vision Science, The University of Western Australia, 2 Verdun Street, Nedlands, Perth, Australia*

[c]*Department of Veterinary Anaesthesia, Murdoch Veterinary School, Murdoch University, 90 South Street, Murdoch, Perth, Australia*

Abstract—Oligodendrocyte-derived myelin retards the ability of CNS axons to regenerate following transection. The intrinsic response of CNS axons to an axotomy insult may be vastly different in the absence of myelin. However, the paucity of adequate experimental models has limited detailed investigation of cellular behaviour following axon transection in an unmyelinated CNS environment. In this study we perform laser-induced axotomy of the porcine retinal ganglion cell axon, a physiologically unmyelinated, mature CNS axon that is structurally similar to humans to infer knowledge about axonal behaviour in the absence of myelin. Axotomy-induced changes to the neuronal cytoskeleton and supporting astrocytes during the early stages after transection are delineated by examining the sequence of neurofilament subunit, microtubule (TUB), microtubule associated protein (MAP), glial fibrillary acidic protein (GFAP) and terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) modification. Axonal transection induced an increase in the expression of neurofilament light at regions within, and immediately adjacent to, sites of axotomy. Other neurofilament subunits were not altered at sites of transection. Unlike myelinated axons where an increase in GFAP staining within hypertrophic glial scars have been shown to inhibit axonal repair we demonstrate a decrease in GFAP staining within regions of increased or preserved neurofilament expression. The behaviour of TUB and MAP proteins following transection of unmyelinated CNS axons are similar to what has previously been described in myelinated CNS axons. This study provides fundamental insights into astrocyte and axonal behaviour acutely after axotomy and demonstrates a series of degenerative events in unmyelinated CNS axons, which in comparison to prior reports are different to myelinated CNS axons. The findings of this report have relevance to understanding pathogenic mechanisms underlying neuro-degeneration in the CNS.

*Correspondence to: D.-Y. Yu, Centre for Ophthalmology and Visual Science and the Australian Research Council Centre of Excellence in Vision Science, The University of Western Australia, Perth, Australia. Tel: +61-8-9381-0716; fax: +61-8-9381-0700.
E-mail address: dyyu@cyllene.uwa.edu.au (D.-Y. Yu).

Abbreviations: GFAP, glial fibrillary acid protein; MAP, microtubule associated protein; NF-H, neurofilament heavy; NF-L, neurofilament light; NF-M, neurofilament medium; RGC, retinal ganglion cell; ROI, region of interest; TUB, microtubules.

Key words: axotomy, cytoskeleton, neurofilament, astrocyte, central nervous system, myelin.

Interruption of the axonal conduit provokes a complex array of structural and metabolic changes which can culminate in neuronal death (Koliatsos and Price, 1996). Glial cells and myelin membrane proteins are inherently related to pathogenic mechanisms that underlie myelinated axonal degeneration and modify axonal behaviour post-injury through inhibitory, permissive and repulsive guidance cues (Caroni et al., 1988; Yiu and He, 2006; Ng et al., 1996). *In vitro* and *in vivo* studies have revealed that in the absence of oligodendrocyte-derived myelin the cascade of cellular events that precede neuronal demise are remarkably different (Schnell and Schwab, 1990; Kim et al., 2003; Keirstead et al., 1995). These previous studies have demonstrated delayed-onset neuronal degeneration and, in distinct cellular environments, neuronal recovery post-transection. Although these investigations have greatly improved our understanding of myelin-mediated mechanisms of axonal degeneration, the results of these experiments to some extent have been confounded by the influence of genetic deletions inducing unknown variations in the genetic background of experimental models and the iatrogenic effects of immunological techniques utilised for receptor antagonism and demyelination. Studies utilising physiologically unmyelinated CNS axons to examine the neuronal response post-axotomy may remove some of these confounding factors thereby improving our understanding of biological processes involved in axonal degeneration in the central nervous system.

Cytoskeleton proteins are highly concentrated within the intracellular compartment of CNS axons and maintain an intimate relationship with glia and myelin membrane proteins (Bray and Gilbert, 1981; Liuzzi and Tedeschi, 1992; Brady et al., 1999). Neurofilaments, microtubules (TUB) and microtubule associated proteins (MAPs) constitute a major portion of the axonal cytoskeleton and collectively play an important role in conferring structural integrity to axonal arborisations (Fuchs and Cleveland, 1998). Cytoskeleton proteins also regulate axonal function through their involvement in vital homeostatic processes such as cell signalling and synaptic plasticity (Lariviere and Julien, 2004). Neurofilaments are obligate hetero-polymers that are composed of three individual subunits: neurofilament heavy (NF-H), neurofilament medium (NF-M) and neurofilament light (NF-L) in order of decreasing mass (Julien and Mushynski, 1998). Each neurofilament subunit is capable of autonomously regulating neuronal function through distinct molecular pathways, however, both NF-H

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2010.12.053

and NF-M are dependent on subunit NF-L for neurofilament assembly (Julien, 1997). Microtubules and MAPs function as the cytosolic scaffolding along which motor proteins involved in axonal transport mobilise (Sato-Harada et al., 1996) and in this regard play a different homeostatic role to neurofilament subunits. Despite these functional differences there is some co-dependency between the two families of proteins with recent studies revealing that microtubule assembly and polymerisation is influenced by the behaviour of neurofilament subunits (Bocquet et al., 2009).

Cytoskeleton modification is an early and important pathogenic event in the process of neuronal degeneration (Balaratnasingam et al., 2008, 2010). Although transection of myelinated axons induces an immediate decline in many cytoskeleton proteins (Wong and Oblinger, 1990) there is increasing experimental evidence to suggest that neuronal demise is predominantly modulated by distinct neurofilament subunits (Balaratnasingam et al., 2007; Toth et al., 2008). In particular, there has been renewed interest concerning the relationship between NF-L behaviour and the temporal characteristics of neuronal degeneration. Sciatic nerve crush experiments have revealed that axonal degenerative and regenerative mechanisms are vastly different in the absence, or presence, of NF-L (Toth et al., 2008; Zhu et al., 1997). NF-L in the peripheral nervous system interacts with Schwann cells via Mtmr2 proteins (Bolis et al., 2009; Previtali et al., 2003) and it is possible that previously reported NF-L behaviour in myelinated axons may to some extent reflect activity that is modulated by structural proteins found in myelin. It is possible that the response of NF-L protein in the absence of myelin may be different.

Few studies have examined the response of CNS cytoskeleton proteins under conditions where the tonic inhibitory effects of myelin and myelin-producing-glia have been removed. This has made it difficult to determine if cytoskeleton protein changes reported in previous studies were the intrinsic neuronal response to sub-compartmental disruption or secondary to the axon-modulated effects of myelin membrane proteins. The intra-retinal portion of the retinal ganglion cell (RGC) axon, in many animal species, is physiologically unmyelinated. Consequently, experimental models of retinal axonal injury are a reliable means of examining the early intrinsic response of neuronal elements to injury in the absence of CNS myelin inhibition. This study reports the temporal sequence of cytoskeleton protein change 1 and 6 h after axotomy of physiologically unmyelinated CNS axons. The intra-retinal portion of the porcine retinal ganglion cell axon—an unmyelinated, unbranched, mature CNS axon that is structurally similar to human (Bristow et al., 2002)—is axotomised with precision using argon laser and the behaviour of cytoskeleton protein subunits are documented. Axon-glial relationships are also investigated by correlating cytoskeleton behaviour with glial fibrillary acid protein (GFAP) changes. The purpose of this report is to delineate the sequence of cytoskeleton change when the extrinsic inhibitory effects of myelin are not present. By doing so, we aim to make inferences about axogenic mechanisms that contribute to neuronal degeneration. This report also presents a novel experimental model that can be used to reliably address issues concerning axonal degeneration in an unmyelinated CNS environment.

EXPERIMENTAL PROCEDURES

Animals

Eight female White Landrace pigs were used. Pigs used for these experiments were between 8 and 11 weeks of age, weighing 18–31 kg. Animals used for this series of experiments were also used for a previously published report (Balaratnasingam et al., 2010). All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The study was approved by the University of Western Australia Animal Ethics Committee. Four female pig eyes were also obtained from the local abattoir and used for studying normal astrocyte and axonal morphology in flat mount porcine retina.

Anaesthesia

Anaesthesia was induced with an i.m. injection of tiletamine/zolazepam 4.4 mg/kg (Zoletil 100 mg/ml, Virbac, Peakhurst, NSW, Australia) and Xylazine 2.2 mg/kg (Xylazil 100 mg/ml, Troy Laboratories, Smithfield, NSW, Australia). Anaesthesia was maintained with a constant rate infusion of propofol 12 mg/kg/h (Fresol 1%, Fresenius Kabi Austria GmbH, A-8055 Graz, Austria) and a constant rate infusion of fentanyl citrate 30 μg/kg/h (Fentanyl Injection 50 μg/ml, Mayne Pharma Pty Ltd, Mulgrave, VIC, Australia). Pancuronium (Pancuronium Injection BP 2 mg/ml, AstraZeneca, North Ryde, NSW, Australia) was given at 0.2 mg/kg, i.v. followed by a constant rate infusion of 0.3 mg/kg/h, i.v. to induce muscle relaxation.

Following induction, pigs were placed in sternal recumbency, intubated and ventilated (Ohmeda 7000, BOC Health Care, Madison, WI, USA). Ventilator settings were adjusted to maintain end-tidal carbon dioxide ($PaCO_2$) tensions between 30 and 45 mmHg. Oxygen and nitrogen flow rates were adjusted to maintain arterial oxygen tensions between 80 and 115 mmHg. Inspired oxygen concentrations and end-tidal carbon dioxide tensions were monitored continuously (Capnomac Ultima, Datex, Helsinki, Finland). The auricular artery was catheterised for continuous blood pressure measurement (Gould P23 ID pressure transducers connected to conditioning module Analog Devices, Norwood, MA, USA) and to allow collection of arterial blood samples. Arterial blood gas samples were collected every 60 min to permit measurement of pH, carbon dioxide and oxygen tensions using a model 238 pH/blood gas analyser (CIBA Corning Diagnostics, Halsted, England). Heart rate was monitored via electrocardiogram (Cardiocap, Datex, Helsinki, Finland).

To maintain diastolic pressure above 60 mmHg, a balanced isotonic fluid solution (Hartmann's, Baxter Healthcare Pty Ltd, Toongabbie, NSW, Australia) was administered at 10 ml/kg/h, supplemented when necessary with dobutamine 1–5 μg/kg/h (Dobutamine Injection 12.5 mg/ml, Mayne Pharma Pty Ltd, Mulgrave VIC, Australia) and/or Dextrans 70 at 5–10 ml/kg/h (Gentran 70 6% w/v, Baxter Healthcare Ltd, Thetford, Norfolk, England). Intravenous fluids and drugs were administered via a 22G catheter (BD Insyte, Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT, USA) in the auricular vein.

Rectal temperature was maintained between 37 and 39.5 °C with a thermal blanket (Homeothermic Blanket Control Unit, Harvard Apparatus Limited, Edenbridge, Kent, England) beneath and above the pig. Plastic bubble wrap was used at extremities to reduce heat loss. Euthanasia on completion of the experiment