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Neurofilament dot blot assays: Novel means of assessing axon viability in culture

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ABSTRACT

Axonal structure and integrity are vital to overall neuronal maintenance and action potential propagation. Neurofilaments (NFs) are one of the main cytoskeletal components of axons and phosphorylation of NF subunits regulates speed of NF transport through axons and determines optimal axonal calibre required for signal propagation. Many previous studies of neuroprotective agents have focussed on neuronal viability in models of neurodegenerative disease, without specifically considering axon function as an indicator of neuronal damage. In this study, we have focused on developing novel assays for determining axon viability by measuring levels of neurofilament phosphorylation in cultured cortical neurons. The nitric oxide donor DETANONOate (NO) was used as an inflammatory insult and glial cell line-derived neurotrophic factor (GDNF) and superoxide dismutase (SOD) were tested as potential axonal protective agents. Using 'dot blot' methodologies, we show a decrease in NF phosphorylation in cortical neurons exposed to NO-mediated cell toxicity and an attenuation of NO-mediated changes in NF phosphorylation associated with GDNF and SOD treatment. These results correlated well with immunocytochemical counts. We propose therefore that the dot blot assay is a novel method for assessing axonal integrity *in vitro* and may play a useful role in the future for testing the effects of agents on axonal viability, providing a reliable and reproducible screening method for potential therapeutics for neurodegenerative diseases.

1. Introduction

Axons are composed of three main cytoskeletal components; actin microfilaments, microtubules and intermediate filaments (Black and Lasek, 1980). Type IV intermediate filaments are composed of neurofilament (NF) subunits, which are the most extensively phosphorylated proteins found in neurons (Lee and Cleveland, 1996). The main NF subunits, NF-L (68 kDa), NF-M (150 kDa) and NF-H (190–210 kDa), have a head domain (N-terminus) and hyper-variable tail domain (C-terminus) (Petzold, 2005). Post-translational phosphorylation, mediated through activation of proline-directed kinases, occurs extensively at the C-terminal (Perrot et al., 2008). Both NF-L and NF-M are expressed before NF-H in development and have roles in establishing neuronal phenotype and initiating/maintaining neurite outgrowth, whereas NF-H is expressed after synaptogenesis and functions in stabilising axons (Carden et al., 1987). The rate of NF transport is inversely related to its phosphorylation state, and phosphorylation of C-terminal sidearms slows NF axonal transport and increases inter-filament spacing, causing an overall increase in axonal calibre, which, in turn, is important in optimising axonal saltatory conduction (Petzold, 2005). Conversely, loss of NF phosphorylation within axons is associated with increased susceptibility to proteolysis and is a hallmark of progressive white matter central nervous system diseases (Goldstein et al., 1987; Trapp et al., 1998).

Dysfunction of NF subunits plays a part in many neurodegenerative diseases, usually resulting in NF accumulation, causing disruption of axonal transport (Perrot et al., 2008). For example, NF-H over-expression has been found in sporadic amyotrophic lateral sclerosis (ALS) which leads to intra-neuronal NF aggregation, known as axonal spheroids (Murayama et al., 1992; Cote et al., 1993). Also, in Charcot-Marie-tooth disease (CMT) several mutations in the NF-L subunit have been found, again causing NF aggregation (Brownlees et al., 2002; Perez-Olle et al., 2005).

Specifically, oxidative damage has been associated with damage to axons in a variety of diseases of the central nervous system (Mattson and Liu, 2002). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced during inflammatory processes and are toxic to the cell at high concentrations. Previous studies have shown that ROS/RNS are neurotoxic *in vitro* and compounds such as nitric oxide (NO) mediate specific axonal injury (Wilkins and Compston, 2005). ROS/RNS can be broken

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; Min, minimal serum free media; NF, neurofilament; NO, nitric oxide; SOD, superoxide dismutase; ROS, reactive oxygen species.

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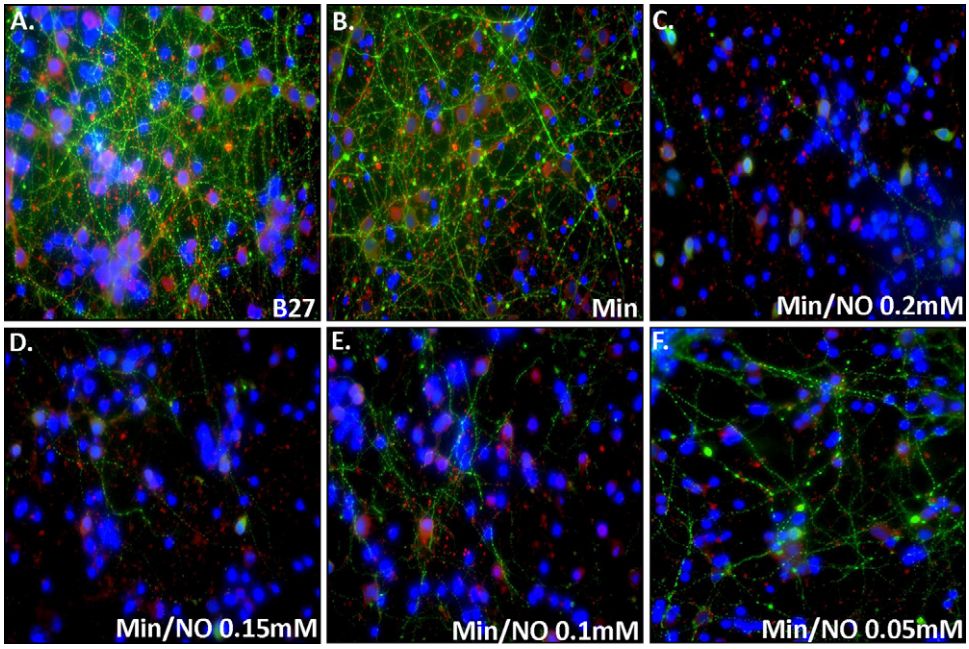

Fig. 1. The nitric oxide donor DETANONOate causes reductions in axonal and whole neuronal viability *in vitro*: cortical neurons (5DIV) were exposed to (A) base media (B27); (B) serum free minimal media (MIN); or (C–F) varying concentrations of DETANONOate (NO) for 24 h. Cultures stained with axonal marker SMI312 (green), neuronal marker βIII tubulin (red) and nuclear stain DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

down into harmless by-products through the actions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). We have previously shown that extracellular-SOD (SOD-3) has neuroprotective properties *in vitro* (Kemp et al., 2010b). Additionally, glial cell line-derived neurotrophic factor (GDNF), which plays an important role in neurite outgrowth and axonal maintenance, is a specific axonoprotective agent in cortical neuronal cultures (Wilkins et al., 2003).

Current research into neurodegenerative disorders focuses on attempts to define neuroprotective agents. Many studies use neuronal viability and 'whole cell' function as a read out for neuroprotective effects. We are interested in determining the influence of putative neuroprotective agents on axons specifically, since pathological and physiological processes occurring in the axon may differ from those occurring in the cell body or dendrites (Finn et al., 2000). Furthermore, certain neurological diseases are characterised by extensive axonal structural and functional change (Ferguson et al., 1997; Trapp et al., 1998). For these diseases, protection of neurofilaments within axons and specifically preservation or restoration of neurofilament phosphorylation may be an important therapeutic approach (Wilkins and Scolding, 2008). The aims of this study are to explore new methods for assaying axon viability, specifically focussing on levels of NF phosphorylation within the axon. We have shown that the 'dot blot' assay using a number of phosphorylated NF specific antibodies is a reliable readout of axonal integrity. Furthermore, we have shown that NO causes dose dependent reductions in phosphorylated NF within cultured axons and that both GDNF and SOD attenuate these changes.

2. Materials and methods

2.1. Neuronal cell culture

Neuronal cultures were prepared from cortices of E18 rat embryos, as previously described (Wilkins et al., 2001). In brief, pregnant females (time mated), were euthanised and embryos removed. Cortices were isolated and meninges removed. Following enzymatic and mechanical dissociation, cells were counted and plated onto poly-L-lysine coated 13 mm coverslips at 3×10^5/coverslip in 24-well plates. Cortical cells were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 2% B27 (Gibco, Paisley, UK) and 1% penicillin and streptomycin (B27 medium). 5 days after plating, neurons (identified by βIII tubulin staining) represented $97.9 \pm 1.2\%$ ($n = 3$) of the total cells present. The remaining cells were predominantly GFAP expressing astrocytes and GalC expressing oligodendrocytes. After 5 days *in vitro*, cortical neurons were exposed to experimental conditions. The basal medium for all experiments (aside from the control B27 media condition) was 'minimal' media (Min), which consisted of Dulbecco's modified Eagles medium supplemented with: 1% insulin-free Sato, -penicillin/streptomycin, -holo-transferrin and 0.5% L-glutamine. The insulin-free Sato contained 100 μg/mL of bovine serum albumin and transferrin, 16 μg/mL putrescine, 0.06 μg/mL progesterone and 0.04 μg/mL of selenite, thyroxine and triiodothryonine. A stock solution (50 mM in 10 mM NaOH) of (Z)-1-[2-(aminoethyl)-N-(2-ammonioethyl)amino]daizen-1-ium-1,2-diolate (DETANONOate; Alexis Biochemicals, Nottingham, UK) was prepared immediately before use. Recombinant rat GDNF (R&D Systems) was used at concentrations of 500ng/mL and 1 μg/mL. SOD derived from human erythrocytes (Sigma–Aldrich) was used at concentrations of 100 U/mL and 400 U/mL.

2.2. Immunocytochemistry

Evaluation of cortical neuronal cell survival was carried out using immunocytochemistry and examination of cellular morphology. Neuronal cultures were stained after fixation with 4% paraformaldehyde and permeabilization with 100% methanol at −20 °C for 10 min. Primary antibodies used for all counting data were phosphorylated axon marker SMI312 (1:600) (Sternberger – Cambridge Biosciences, UK) and neuronal marker βIII tubulin (1:300) (Sigma–Aldrich). Species specific Alexa Fluor® 488 and 555 conjugated secondary antibodies (1:500) (Invitrogen,