Published in final edited form as:

Dev Neurobiol. 2010 February ; 70(2): 126–133. doi:10.1002/dneu.20755.

Axoplasm Isolation from Peripheral Nerve

Ida Rishal¹, Izhak Michaelievski¹, Meir Rozenbaum¹, Vera Shinder², Katalin F. Medzihradszky³, Alma L. Burlingame³, and Mike Fainzilber^{1,*}

¹Dept. of Biological Chemistry, Weizmann Institute of Science, 76100 Rehovot, Israel

²Electron Microscopy Unit, Dept. of Chemical Research Support, Weizmann Institute of Science, 76100 Rehovot, Israel

³Mass Spectrometry Facility, Dept. of Pharmaceutical Chemistry, University of California San Francisco, 600 16th Street, MC 2240, San Francisco, CA 94158-2517, U.S.A.

Abstract

Localized changes in the composition of axonal cytoplasm (axoplasm) are critical for many biological processes, including axon guidance, responses to injury, neurite outgrowth, and axon-glia interactions. Biochemical and molecular studies of these mechanisms have been heavily focused on in vitro systems, due to the difficulty of obtaining subcellular extracts from mammalian tissues in vivo. Since in vitro systems might not replicate the in vivo situation, reliable methods of axoplasm extraction from whole nerve would be helpful for mechanistic studies on axons. Here we develop and evaluate a new procedure for preparation of axoplasm from rat peripheral nerve, based on incubation of separated short segments of nerve fascicles in hypotonic medium to separate myelin and lyse non-axonal structures, followed by extraction of the remaining axon-enriched material. We show that this new procedure reduces serum and glial cell contamination, and facilitates proteomic analyses of axonal contents.

Introduction

Localized changes in the composition of axonal cytoplasm (axoplasm) are critical for many biological processes, including axon guidance (Martin, 2004), responses to injury (Hanz and Fainzilber, 2006), neurite outgrowth (Wang et al., 2007), and axon-glia interactions (Twiss and Fainzilber, 2009). Such changes may include recruitment of new transcripts via calcium-regulated mechanisms (Yao et al., 2006) or via axonal transport (Willis et al., 2007; Vogelaar et al., 2009), localized protein translation following nerve lesion (Hanz et al., 2003; Perlson et al., 2005; Yudin et al., 2008) or during neurite outgrowth (Zheng et al., 2001; Willis et al., 2005), regulated post-translational modifications at distinct sites within an axon (van Niekerk et al., 2007), and organelle transfer from neighbouring cells (Court et al., 2008). Biochemical and molecular studies of these mechanisms have been heavily focused on in vitro systems such as compartmentalized cultures, due to the difficulty of obtaining subcellular extracts from mammalian tissues in vivo. This has limited much of the in vivo work on these issues to microscopy and imaging of fixed and sectioned tissue. Since in vitro systems might not replicate the in vivo situation, reliable methods of axoplasm extraction from whole nerve would be helpful for mechanistic studies on axons.

A mechanical extrusion procedure has been used for many years in studies of axoplasm from the squid giant axon (Brady et al., 1985), and similar methods have been used in other invertebrates (Marquis and Webb, 1974; Schmied et al., 1993; Perlson et al., 2004). The

*Corresponding author: mike.fainzilber@weizmann.ac.il.

structure of mammalian peripheral nerve (Figure 1A, B) complicates use of such straightforward extrusion procedures, hence in previous studies we have subjected cut nerve segments to a gentle homogenization procedure in isotonic solution to obtain axoplasm enriched preparations. Although Western blotting showed that extracts obtained by that procedure were largely free of glial cell contaminants (Hanz et al., 2003; Perlson et al., 2005; Yudin et al., 2008), initial attempts to carry out proteomic characterization of axoplasm failed due to the presence of a few highly abundant serum proteins. Serum contamination is a widespread problem in tissue proteomics, for example up to 80% of the proteins in human cerebrospinal fluid can originate from serum, and exclusion or depletion of these highly abundant proteins is necessary to study proteins of interest that are present at much lower concentrations (Shores and Knapp, 2007; Boschetti and Righetti, 2009; Ramstrom et al., 2009). Here we describe a new procedure that minimizes serum contamination in axoplasm preparations from rat sciatic nerve, and evaluate the uses and limitations of peripheral nerve axoplasm preparations.

Materials and Methods

Animals and housing

This study was conducted in accordance with care guidelines issued by the Weizmann's Institutional Animal Care and Use Committee (IACUC). Adult male Wistar rats 8-10 weeks of age were housed in institutional standard cages (4 rats per cage) on a 12-h light/12-h dark cycle with free access to water and food before experimental procedures. Animals were sacrificed by CO₂ inhalation and cervical dislocation.

Electron microscopy

Sciatic nerves were processed for electron microscopy at different stages of method development as described below. Nerves or fascicles were fixed with 2.5% glutaraldehyde in 0.1M NaCaCo buffer (pH 7.4) for 1 hour in room temperature followed by at least 24 hours at 4°C. Nerves were then cut into 1 mm blocks, and post-fixed in 1% osmium tetroxide in 0.1M NaCaCo with 0.5% potassium dichromate and 0.5% potassium hexacyanoferrate for 2 hours, followed by incubation in 2% UrAc dissolved in double distilled water (DDW) for 2 hours at room temperature in the dark. Subsequent dehydration in a series of ethanols and propylene oxide preceded embedding of the blocks in EMBed 812 (Electron Microscopy Sciences). Blocks were sectioned on an ultramicrotome at 70-80 nm and collected on copper grids. Carbon coated copper grids were used for negative staining. Grids were stained in uranyl acetate and lead citrate and analyzed under 120kV on a Tecnai 12 (FEI) Transmission Electron Microscope with a Megaview III CCD camera using AnalySIS software.

Antibodies and Western blots

The antibody against Importin $\alpha 3$ was a kind gift from Dr. Matthias Koehler (MDC, Berlin). Mouse anti-Dynein intermediate chain clone 74.1 was from Chemicon (MAB1618), rabbit anti-NFH was from Chemicon (AB1989); mouse anti-NFH clone N52 was from Sigma; mouse anti-Importin β clone 31H4 was from Sigma (I2534); mouse anti-CNPase was from Chemicon (MAB326); mouse anti-GFAP clone G-A-5 was from Sigma (G6171); rabbit anti-albumin and rabbit anti-transferrin were from Cedarlane (CLAG5140 and GLAG5240 respectively); mouse anti-tubulin $\beta 3$ was from Sigma (T2200); and rabbit anti-gERK was from Sigma (M5670). For Westerns, axoplasm samples were resolved on 10% SDS-PAGE and transferred to nitrocellulose, and after reaction with the desired antibodies were visualized using horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:10,000) with chemiluminescent detection (Pierce Biochemical).

Mass Spectrometry

Both axoplasm preparations were denatured with 6 M Guanidine chloride and reduced with 2.1 mM TCEP for 1 hour at 57°C. Iodacetamin (IAA) was used for alkylation for 45 min at room temperature under darkness, before overnight digest of the protein mixture at 37°C with side-chain protected trypsin (Promega, Madison, WI) at a molar ratio of 1:50 trypsin:protein. The peptide mixture was desalted using C18 ziptip and seppac cartridges (Waters, Milford, MA) and dried in speedvac. Desalted digests were fractionated by strong cation exchange chromatography over a PolyLC column (Polysulfoethyl A, 200×2.1 mm 5µm bead, 200 Å pores) on an Akta HPLC (GE Healthcare). Peptides were eluted in a gradient of 0-350 mM KCl in 5 mM KH₂PO₄, 30 % acetonitrile; concentrated by speedvac and desalted over a C18 macrotrap peptide reverse phase column (Michrom Bioresources, Auburn, CA) using a gradient of acetonitrile in 0.1 % TFA (trifluoroacetic acid).

Samples were dissolved in 0.1 % HCOOH and samples of approximately 100 fmole peptides were injected onto the reverse phased nano-column. Liquid chromatography flow rate was set to 425 nl/min, with a protocol duration of 63 min, and a gradient from 4.9 % to 49.882 % of acetonitrile in 0.1 % TFA. Minimal peak resolution was set to 8000. The HPLC (Agilent 1100, Agilent Technologies, Palo Alto, CA) was connected online to a quadrupole-orthogonal acceleration TOF (QSTAR pulsar or Elite, Applied Biosystems, Foster City, CA) mass-spectrometer. Data was acquired in an information dependent manner: MS acquisitions (1 sec) were followed by CID experiments. The two most abundant multiply charged ions were selected for subsequent collision induced dissociation (CID). First a regular CID acquisition was performed, where the collision energy was automatically adjusted according to the peptide charge and m/z value. Then with a high resolution precursor ion selection and preset collision energy only the reporter ion region was monitored. Precursor ions already analyzed were excluded from the selection process for 1 minute. Peaklists were generated with Analyst software using Mascot.dll version 1.6b20.

Samples spectra were automatically analyzed using ProteinProspector ver 5.0, against the UniProt.2008.03.18.random.concat database (147182/11606446 entries searched). Only tryptic peptides were considered, up to two mis-cleavages were allowed. Fixed modifications were: carbamidomethylation of Cys residues, iTRAQ4plex-modification of Lys-side chains and N-termini. Variable modifications considered in the analysis included N-terminal acetylation of proteins; cyclization of N-terminal Gln residues, methionine oxidation and serine/threonine/tyrosine phosphorylation. 3 variable modifications per peptide were permitted. Mass tolerance in the QSTAR experiments was set to 0.2 Da and 100 ppm for the fragment ions and precursor ions, respectively. E-value threshold was set to 0.01, and peptides with scores under 30 were excluded.

Statistical analyses

Data were expressed as mean ± standard error of mean (SEM). All statistical tests were carried out with SigmaPlot 11.0 software. Statistical significance was evaluated using one-sample t-test with hypothesized population value 100. Values of P<0.05 were considered as significant.

Results and Discussion

Our previous method for axoplasm isolation was based on manual squeezing of dissected sciatic nerve segments in eppendorf tubes with a plastic pestle, under phosphate-buffered saline (PBS) or nuclear transport buffer (NTB) (see Supplementary Methods). Western blot quality control of such axoplasm preparations did not reveal glial contamination (Hanz et al., 2003; Perlson et al., 2005), but mass spectrometry showed significant levels of serum protein contaminants (Table S1). Electron microscopy of the residual material remaining

after isotonic axoplasm extraction from rat sciatic nerve revealed extensive destruction of cellular and subcellular structures, including blood vessels and nuclei (Figure 1C,D). We therefore attempted to modify the extraction procedure to develop a method that would allow purer axoplasm preparations. Initial attempts to dissociate the nerve by enzymatic digestion with collagenase and dispase in isotonic buffers as commonly used for in vitro dissociation of neurons (Yudin et al., 2008) caused widespread tissue destruction (Figure S1A). Manual removal of the epineurium in isotonic PBS helped to separate axon bundles (Figure S1B). Previous studies have shown that it is possible to isolate axons from the central nervous system by osmotic manipulations (DeVries et al., 1972). Moreover, myelin can be separated from nervous tissue in hypotonic solutions that cause the myelin sheath to peel off from the axon (DeVries et al., 1972; Larocca and Norton, 2007). We therefore assessed the effect of incubating complete sciatic nerve or dissociated fascicles in solutions of different osmolarity. The first attempt entailed usage of a hypertonic solution of 10 x PBS supplemented with protease inhibitors. The expectation was that a hypertonic environment should not damage non-neuronal cells, while simultaneously encouraging axoplasm extrusion from the cut open ends of the axons. Manual removal of the epineurium damaged myelin and Schwann cells, causing increased contamination (Figure S1C,D). Although the procedure seemed to work for whole nerve, there was still significant contamination with serum proteins such as albumin, transferrin and hemoglobin, most likely due to concomitant leakage of material from disrupted vasculature (Figure 3 C,D).

We then tried axoplasm extraction under hypotonic conditions, to separate myelin and lyse non-axonal structures. The epineurium was removed from freshly dissected sciatic nerve and fascicles were separated by mechanical dissociation with fine forceps. Fascicles were then incubated in 0.2X PBS with protease inhibitors. Duration of the hypotonic incubation was assessed by EM imaging to determine efficiency of Schwann cell destruction versus possible damage of axons. The EM survey showed that complete separation of myelin layers was achieved within 30 minutes, while Schwann cell lysis required between 1-2 hours, without deleterious effects on axons (Figure 2). Longer incubations of up to 24 hours destroyed axons. We therefore limited the incubation time in hypotonic solution to two hours, followed by extensive washing. This procedure resulted in a preparation of axon processes with lengths up to 15 mm, devoid of Schwann, blood and other non-neuronal cells. Axonal ultrastructure was retained, with intact axolemma, neurofilaments, tubules, and endosomal structures, with slight signs of swelling in mitochondria (Figure 2D). Extraction of axoplasm was then carried out in 1x PBS, which at this stage should actually be hypertonic in relation to axon contents. A detailed protocol for this procedure is provided in Supplementary Methods. Electron microscopy of negatively stained axoplasm showed more homogeneity in axoplasm from hypotonic extraction, as compared to the previously used isotonic squeeze method (Figure 3A, B). Axoplasm from the hypotonic extraction procedure contained an even spread of small size particles and vesicles in the 2-10 nm range, while axoplasm from isotonic squeeze contained a heterogeneous mix of clumps of vesicles and particles in a wider size range of 10-70 nm.

We assessed serum and non-neuronal contaminations in the axoplasm preparations by Western blot and mass spectrometry analyses. Western blots revealed lower levels of serum markers (albumin, transferrin) in axoplasm from the hypotonic extraction procedure in comparison to axoplasm from isotonic squeeze or hypertonic extraction procedures (Figure 3 C,D). Levels of the glial markers CNPase and GFAP were also reduced in hypotonic extracted axoplasm (Figure 3C, F). Pellets from hypotonic and hypertonic extraction procedures were extracted again in isotonic solution and analyzed by Western blot. The glial marker CNPase was enriched in the pellet fraction, while axonal proteins were depleted, further supporting efficacy of the procedures (Figure 3C-E). Ultracentrifugation of the axoplasm did not markedly change contaminant levels (Figure 3D). Importins are found in

axoplasm as core components of the dynein-linked retrograde injury signaling complex (Hanz et al., 2003) and importin $\alpha 3$ was indeed found to increase in correlation with dynein in axoplasm from the hypotonic extraction procedure (Figure 3E), while levels of importin $\beta 1$ were somewhat decreased (Figure 3C). Axonal markers like neurofilament heavy chain (NFH) and tubulin $\beta 3$ were enriched in axoplasm from the hypotonic procedure (Figure S2). Overall quantification of these different markers is shown in Figure 3G and in Figure S2B, demonstrating that the new procedure suffers from less serum and non-neuronal cell contamination, while maintaining levels of axonal proteins. Moreover, batch to batch variability of the new method is much improved over the isotonic squeeze procedure (Figure S2).

Axoplasm samples were further compared by mass spectrometry, which is highly sensitive to the presence of serum proteins or other abundant contaminants. We first carried out MS/MS analyses of peptides from tryptic digests of crude axoplasm extracts from both methods. Spectra were included on the analyses only if they had extensive y- and b-ion coverage and E-value < 0.01. The results are summarized in Figure 4A-C, and show a striking reduction in the number of peptides identified from serum proteins. Although the mass spectrometric method we used is not strictly quantitative, the number of identified peptides per protein roughly correlates with abundance of that protein in the sample. Hence, the marked decrease in number of peptides identified for albumin, transferrin, and globins, indicate a significant reduction in serum contamination in the hypotonic extraction method. In addition to these highly abundant proteins, other blood-born contaminants such as hemopexin precursor, creatine kinase (muscle), Vitamin D-binding protein precursor, and Apolipoprotein E precursor were shown to be reduced. Reduction of other proteins of non-axonal origin, e.g. periaxin and cofilin, was also observed. Peptides for ubiquitous housekeeping proteins, e.g. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Fructose-bisphosphate aldolase A, and axonal proteins such as dihydropyrimidinase-related protein 2, were found at similar levels in axoplasm from both methods (Figure 4C). On the other hand, the hypotonic extraction method seemed to introduce higher levels of very basic proteins, such as myelin basic protein and histones. These might arise from a different type of contamination introduced by the procedure, or in part reflect unexpected occurrence of such proteins in cytoplasm (Xu et al., 2009).

In order to assess the possibility of carrying out detailed proteomic studies after these procedures, we carried out an additional mass-spectrometry analysis after multidimensional separation of the axoplasm over a strong cation exchange column followed by reverse phase chromatography linked to nano-electrospray ionization MS/MS. 540 proteins were identified from hypotonic extract axoplasm by this method, with 187 of these represented by at least three peptides (Table S2, Figure 4 D, E). DAVID bioinformatics annotation analysis of the identified proteins assigned 462 proteins to different tissues and organs, approximately 55% of which were categorized as neural specific (insets in Figure 4 D, E). Hence, the hypotonic axoplasm extraction procedure reduces serum and non-neuronal contamination of the preparations, and facilitates downstream proteomic studies on these samples. We hope that the methods characterized above and detailed in the protocols provided in Supplementary On-line Methods will be of use in characterizing biochemical events in nerve axoplasm, thus allowing cytoplasmic events in axons to be studied in vivo as well as in vitro.

Supplementary Figure Legends

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge funding support from the Israel Science Foundation, the Adelson Medical Research Foundation (AMRF), the International Institute for Research in Paraplegia (IFP), and the National Institutes of Health (NCRR P41RR001614 and NCRR RR012961). Electron microscopy was conducted at the Irving and Cherna Moskowitz Center for Nano and Bio-Nano Imaging at the Weizmann Institute of Science. M.F. is the incumbent of the Chaya Professorial Chair in Molecular Neuroscience at the Weizmann Institute of Science.

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Figure 1. Structural characteristics of peripheral nerve before and after axoplasm extraction by isotonic squeeze

(A) Schematic of tissue organization in peripheral nerve. Vascularization shown in blue. (B) Electron micrograph of a longitudinal section through adult rat sciatic nerve. NMYAx - nonmyelinated axons; SW - Schwann cell; MY - myelin; NF - neurofilament. Scale bar 5 μm . (C, D) Electron micrographs of sections through residual material from sciatic nerve after axoplasm extraction by the isotonic squeeze method reveal extensive structural disruption. CO - collagen, NU - nucleus of a Schwann cell. Scale bars 1 μm .

Figure 2. Effects of hypotonic incubation on sciatic nerve

(A) Electron micrograph of a longitudinal section through sciatic nerve after manual separation of fascicles and 15 minutes incubation in 0.2X PBS. Scale bar 2 μ m. Note the beginning of myelin layer separation. (B) 30 minutes incubation improves separation of myelin layers. Schwann cell lysis is observed, without apparent damage to axons. Scale bar 2 μ m. (C) 1 hour incubation. Note formation of large myelin vesicles which extrude axons out of the myelin sheath. Scale bar 500 nm. (D) 2 hours incubation. Note that the myelin sheath has detached from axons, which remain largely intact. Scale bar 2 μ m. (E) 24 hours incubation in hypotonic solution disrupts axonal structure. Scale bar 1 μ m. (F) Cross section through sciatic nerve after 24 hours incubation in 0.2X PBS without prior removal of connective tissue and manual separation of fascicles. Note that the intact nerve is remarkably resistant to such an extensive hypotonic incubation. Scale bar 1 μ m. MY - myelin; NF - neurofilament, SW - Schwann cell, Ax - Axon.

Figure 3. Axoplasm characterization and quality control

(A) Electron micrograph of negative staining of axoplasm from the isotonic squeeze method shows a nonhomogeneous preparation containing vesicular structures of different sizes. (B) Negative staining of axoplasm from the hypotonic extraction procedure shows a more homogenous preparation containing numerous small size vesicle-like structures. Scale bar in both images 200 nm. (C-F) Western blot comparison of soluble and pellet fractions of axoplasms extracted by different methods and cleared by regular centrifugation or ultracentrifuge (UC). Albumin and transferrin were used to monitor serum contamination, CNPase and GFAP for Schwann cells and other glia, importins and dynein intermediate chain (IC) for cell body components and retrograde signaling complexes, and general Erk1 and Erk2 MAP kinases (gERK) as a loading control. (G) Levels of different proteins in the soluble fraction from hypotonic extraction axoplasm as a percentage of their level in isotonic squeeze axoplasm. All data was normalized to the average of the control group (isotonic squeeze axoplasm), taken as 100% in each blot. Statistics were by one-sample t-test with hypothesized population mean 100, p-value < 0.05. Note markedly reduced levels of serum and glia contaminants, versus enrichment of dynein associated proteins. 80 µg protein per lane.