Journal of Neuroscience Methods 245 (2015) 25–36

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Array tomography for the detection of non-dilated, injured axons in traumatic brain injury

Rachel E. Bennett[1], David L. Brody*

Department of Neurology, Washington University in St. Louis, 660 S. Euclid Avenue, Saint Louis, MO 63110, USA

HIGHLIGHTS

- Improved spatial resolution allows visualization of individual axons.
- Enhanced visualization of small non-dilated injured axons over conventional histology.
- Identified array-compatible antibodies for examining axon integrity and injury.

ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form 5 February 2015
Accepted 6 February 2015
Available online 14 February 2015

Keywords:
Array tomography
Axonal injury
Traumatic brain injury
Neurofilament
Tubulin
Electron microscopy

ABSTRACT

Background: Axonal injury is a key feature of several types of brain trauma and neurological disease. However, in mice and humans, many axons are less than 500 nm in diameter which is at or below the resolution of most conventional light microscopic imaging methods. In moderate to severe forms of axon injury, damaged axons become dilated and therefore readily detectible by light microscopy. However, in more subtle forms of injury, the damaged axons may remain undilated and therefore difficult to detect.
New method: Here we present a method for adapting array tomography for the identification and quantification of injured axons. In this technique, ultrathin (~70 nm) plastic sections of tissue are prepared, labeled with axon injury-relevant antibodies and imaged using conventional epifluorescence.
Results: To demonstrate the use of array-tomography-based methods, we determined that mice that received two closed-skull concussive traumatic brain injury impacts had significantly increased numbers of non-dilated axons that were immunoreactive for non-phosphorylated neurofilament (SMI-32; a marker of axonal injury), compared to sham mice (1682 ± 628 versus 339 ± 52 per mm^2, $p = 0.004$, one-tailed Mann–Whitney U test). Tubulin loss was not evident ($p = 0.2063$, one-tailed Mann–Whitney U test). Furthermore, mice that were subjected to more severe injury had a loss of tubulin in addition to both dilated and non-dilated SMI-32 immunoreactive axons indicating that this technique is suitable for the analysis of various injury conditions.
Comparison with existing method: With array tomography we could detect similar overall numbers of axons as electron microscopy, but accurate diameter measurements were limited to those with diameters >200 nm. Importantly, array tomography had greater sensitivity for detecting small non-dilated injured axons compared with conventional immunohistochemistry.
Conclusion: Imaging of individual axons and quantification of subtle axonal injury is possible using this array tomography method. This method may be most useful for the assessment of concussive injuries and other pathologies in which injured axons are not typically dilated. The ability to process moderately large volumes of tissue, label multiple proteins of interest, and automate analysis support array tomography as a useful alternative to electron microscopy.

Abbreviations: TBI, traumatic brain injury; rcTBI, repetitive closed-skull traumatic brain injury; APP, amyloid precursor protein; DTI, diffusion tensor imaging; MD, mean diffusivity; AD, axial diffusivity; NF, neurofilament; MBP, myelin basic protein; PBS, phosphate buffered saline; TBS, tris buffered saline; PFA, paraformaldehyde.

* Corresponding author. Tel.: +1 314 362 1381; fax: +1 314 362 3279.
E-mail address: brodyd@neuro.wustl.edu (D.L. Brody).
[1] Current address: Department of Neurology, Massachusetts General Hospital, Harvard Medical School, 114 16th Street, Charlestown, MA 02129, USA.

http://dx.doi.org/10.1016/j.jneumeth.2015.02.005
0165-0270/

1. Introduction

A barrier to analysis of axonal injury in mild traumatic brain injury (TBI) is the ability to resolve small, injured axons by light microscopy. This is due, in large part, to the low signal to noise of very small structures that are at or below the resolution of light microscopy. In humans and macaques, axon diameters within the corpus callosum range between 0.08 and >10 μm (Aboitiz et al., 1992; Lamantia and Rakic, 1990). In rodents, axonal diameter is smaller, typically in the 0.05–1 μm range (Kim et al., 1996; Olivares et al., 2001). Following moderately severe traumatic brain injury, swollen axons in corpus callosum can become very large (5 μm or more), and are clearly visible with standard immunohistochemistry using antibodies to amyloid precursor protein (APP) and neurofilaments (Blumbergs et al., 1994; Mac Donald et al., 2007a,b). However, several observations have led us to hypothesize that immunohistochemistry and light microscopy may not reflect the true amount of axonal injury present after TBI, particularly after mild concussive injuries.

Along with others, we have developed a model of concussive traumatic brain injury in mouse that results in pronounced behavioral impairments (Creed et al., 2011; Creeley et al., 2004; DeFord et al., 2002; Kane et al., 2012; Laurer et al., 2001; Longhi et al., 2005; Meehan et al., 2012; Mouzon et al., 2012; Shitaka et al., 2011; Uryu et al., 2002; Zohar et al., 2003). However, the underlying pathology is quite subtle. For example, in our repetitive closed skull traumatic brain injury (rcTBI) model, standard immunohistochemistry techniques including amyloid precursor protein labeling (APP) reveal only occasional axonal varicosities that appear to be largely resolved by 7 days. However, widespread abnormalities are apparent on silver staining and electron microscopy indicating that injured axons are present throughout the ipsilateral corpus callosum and external capsule at 7 days post-injury (Shitaka et al., 2011). These axons display compaction of cytoskeletal elements, organelle accumulation, and axolemma collapse. A key observation is that few of these injured axons appear to have diameters greater than 1 micron, and most are less than 500 nm—at or below the resolution of standard light microscopy techniques. Indeed other investigators have documented cases of axonal injury without axonal swelling, and it may be possible for axon degeneration to proceed without the classic "beads-on-a-string" morphology (Stone et al., 2001). Additionally, in this concussive injury model, white matter abnormalities are apparent by diffusion tensor imaging (DTI), where mean (MD) and axial diffusivity (AD) are both significantly reduced at 7 days post-injury (Bennett et al., 2012). Neither MD nor AD correlates with the amount of silver staining or Iba-1 labeling for microglia (Bennett et al., 2012). The inability to explain DTI abnormalities by standard histological techniques further supports the idea that we are underestimating the amount of axonal injury in rcTBI by these methods.

To test this hypothesis, we adapted array tomography for measuring axonal injury. Array tomography was developed in the lab of Stephen Smith to quantitatively measure synapses in the cortex (Micheva and Smith, 2007). In this method, improved spatial resolution is achieved along the z-axis through physical sectioning on an ultramicrotome, which greatly improves the signal to noise ratio and allows identification of individual synapses (Kay et al., 2013; Micheva and Smith, 2007). While this technique has not been rigorously validated by quantitative EM, the use of several antibodies to label pre- and post-synaptic densities and the careful co-registration of fluorescent labels with scanning electron micrographs has confirmed the spatial correlation of immunofluorescence with ultrastructural details (Micheva and Smith, 2007). Further, the advantages of this technique over traditional electron microscopy are the ability to assay larger volumes of tissue in a relatively high-throughput fashion, to label multiple proteins of interest, and to perform these experiments with a standard epifluorescent microscope.

Here, we outline a method for using array tomography to examine axon injury. We show preliminary data using this technique to resolve injured and uninjured axons at a level of resolution not previously possible except with electron microscopy. Altogether, this is a promising new method for quantitative analysis of axons that could be applied to many fields in addition to traumatic brain injury.

2. Materials and methods

2.1. Animals

Male C57Bl/6j mice were purchased from Jackson Laboratory between 6 and 8 weeks of age (stock# 000664). Two male APP knockout mice were obtained from Jackson Laboratory (stock# 004133) at 2 months of age (Zheng et al., 1995). Two 12-month-old male TauP301S mice and two 3-month-old tau knockout mice (gift from Marc Diamond) were also used for these experiments and were bred in house (Tucker et al., 2001). All animals were housed in accordance with the Animal Studies Committee at Washington University in Saint Louis. Mice were provided with food and water ad libitum and were maintained under a 12-h light/dark cycle.

2.2. Surgical procedures

Mice were subjected to closed-skull sham injury or rcTBI injury ($n = 5$ per group) as previously described (Shitaka et al., 2011). Briefly, mice are anesthetized, placed in a stereotaxic frame, and a midline incision is made. An electromagnetic impactor fitted with a rubber tip is centered over the intact skull 1.5 mm lateral to midline and 1.8 mm posterior to bregma. A 3.3 mm depth impact is delivered at 5 m/s with a dwell time of 100 ms. The incision is then sutured closed and mice recover on a heating pad.

A second group of mice underwent controlled cortical impact injuries at a depth of 1.0 mm ($n = 2$) or 1.5 mm ($n = 4$), which has been described elsewhere (Brody et al., 2007). Briefly, a 5 mm craniotomy is performed over the left parietal cortex. An electromagnetic impactor fitted with a 3 mm metal tip is positioned 1.2 mm left of midline and 1.5 mm anterior to lambda. Impacts are delivered at 5 m/s with a dwell time or 100 ms. After irrigation with phosphate buffered saline (PBS) a plastic skull cap is glued in place, the midline incision is sutured closed, and the mice are allowed to recover on a heat pad before being returned to their cage. In all experiments, sham mice were subject to the same surgical procedures as rcTBI mice, but no impact was delivered.

For conventional immunohistochemistry, additional mice underwent either sham ($n = 1$), rcTBI ($n = 2$), or a 2.0 mm CCI ($n = 1$) procedure.

2.3. Tissue embedding and sectioning for array tomography and electron microscopy

Tissue embedding and sectioning was performed as described by others with minor modifications (Kay et al., 2013). Briefly, animals were sacrificed by deep anesthesia with isofluorane followed by cardiac perfusion with 10 ml room temperature 0.3% heparin in 0.01 M PBS. This was immediately followed by perfusion with 10 ml 4% paraformaldehyde (PFA, cat# 15710 E.M.S), 0.025% sucrose in 0.01 M PBS. Brains were removed and placed in fixative for 20–30 min and then were sectioned into 1 mm thick coronal slabs using a razor blade and a brain slicing matrix. Corpus callosum