Experimental Neurology 224 (2010) 415–423

Contents lists available at ScienceDirect

Experimental Neurology

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

Dendritic alterations after dynamic axonal stretch injury *in vitro*

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ARTICLE INFO

Article history:
Received 3 February 2010
Revised 29 April 2010
Accepted 3 May 2010
Available online 18 May 2010

Keywords:
Dendrite
Traumatic brain injury
Diffuse axonal injury
Cerebral cortex
In vitro

ABSTRACT

Traumatic axonal injury (TAI) is the most common and important pathology of traumatic brain injury (TBI). However, little is known about potential indirect effects of TAI on dendrites. In this study, we used a well-established *in vitro* model of axonal stretch injury to investigate TAI-induced changes in dendrite morphology. Axons bridging two separated rat cortical neuron populations plated on a deformable substrate were used to create a zone of isolated stretch injury to axons. Following injury, we observed the formation of dendritic alterations or beading along the dendrite shaft. Dendritic beading formed within minutes after stretch then subsided over time. Pharmacological experiments revealed a sodium-dependent mechanism, while removing extracellular calcium exacerbated TAI's effect on dendrites. In addition, blocking ionotropic glutamate receptors with the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 prevented dendritic beading. These results demonstrate that axon mechanical injury directly affects dendrite morphology, highlighting an important bystander effect of TAI. The data also imply that TAI may alter dendrite structure and plasticity *in vivo*. An understanding of TAI's effect on dendrites is important since proper dendrite function is crucial for normal brain function and recovery after injury.

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability among people less than 45 years old. It afflicts up to two million people each year in the United States (US), and it is estimated that 2% of the US population lives with long-term TBI-related cognitive, behavioral and motor disability. TBI also may initiate insidious neurodegeneration that is associated with a greater risk of Alzheimer's disease (Rasmusson et al., 1995; Van Den Heuvel et al., 2007). Traumatic axonal injury (TAI), which results from damaged or dysfunctional axons throughout the white matter, is the most common pathology in TBI (Smith and Meaney, 2000), and is thought to contribute to the neurologic deficits found in most TBI survivors (Scheid et al., 2006; Kumar et al., 2009). However, how TAI affects cognitive outcome in humans is uncertain, particularly after mild or moderate TBI (Wallesch et al., 2001; Felmingham et al., 2004; Scheid et al., 2006), the most prevalent forms of TBI.

Dendrite diversity is a striking characteristic of the brain. In addition, dendrites can undergo rapid structural changes in response to a variety of stimuli (Cline, 2001; Jan and Jan, 2001), and this plasticity is essential to the cellular response of learning and memory (Comery et al., 1996; Lamprecht and Ledoux, 2004; Kozorovitskiy et al., 2005; Sjostrom et al., 2008). By contrast, any alteration in dendrite growth and/or structure can result in impaired brain function, e.g. cognitive dysfunction. Interestingly, impaired learning and memory are observed in many TBI survivors regardless of injury severity, and similar deficits are observed in animal models of TBI (Dixon et al., 1987; Lyeth et al., 1990; Winocur, 1990; Smith et al., 1991; Squire and Zola-Morgan, 1991; Smith et al., 1993). Additional evidence suggests that altered dendrite structure may underlie the cognitive deficits observed after TBI (Fineman et al., 2000; Zhu et al., 2000; Hoskison et al., 2009). For example, hippocampal long-term potentiation (LTP), that may account for many types of learning and is associated with dendritic plasticity, is suppressed for days to weeks following experimental TBI (Miyazaki et al., 1992). At the cellular level, changes in dendrite structural proteins such as microtubule-associated protein 2 (MAP2) and neurofilament proteins are prominent in animal models of TBI (Taft et al., 1992; Posmantur et al., 1994, 1996; Kanayama et al., 1996; Lewen et al., 1996; Folkerts et al., 1998; Saatman et al., 1998), and are also observed in the human brain of autopsy specimens after TBI (Castejon et al., 1997, 2004; Castejon and Arismendi, 2003). In addition, cerebrospinal fluid (CSF) MAP2 levels are increased in patients with severe TBI (Hayes et al., 2008). Together these data underline the importance of understanding how TBI affects

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0014-4886/$ – see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.expneurol.2010.05.001

dendrite structure and/or function, which may lead to novel insights about recovery after TBI.

There has been extensive research into the pathology and mechanisms of TAI (Povlishock, 1992; Smith and Meaney, 2000, Tang-Schomer et al., 2010). However, the effect of TAI on dendrite structure is less well understood. Therefore, we examined immediate and evolving dendritic changes using a well-established *in vitro* model of isolated axonal stretch injury (Smith et al., 1999; Wolf et al., 2001; Iwata et al., 2004), in which mechanical loading conditions replicate those occurring in head trauma (Meaney et al., 1995). We observed that stretch-induced axonal injury caused transient dendritic beading. This beading was sodium-dependent, exacerbated by extracellular calcium removal and blocked by N-methyl-D-aspartate receptor (NMDAR) antagonists. Our findings raise the possibility of a direct mechanical link between TAI and dendrite injury, and in particular that TAI may have a bystander effect on dendrites. Since dendrite structure and cognition are interconnected, understanding the mechanism(s) of dendrite injury after TBI may increase insight into TBI's pathobiology and lead to novel strategies to improve cognitive outcome.

Materials and methods

Animal welfare

All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, U.S. Department of Health and Human Services, Pub No 85-23, 1985, and approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). The minimum number of animals necessary for scientific validity was used and efforts were made to minimize animal suffering.

Dissociated cerebral cortex neuronal cultures

Primary neuron cultures from E18 Sprague–Dawley rat embryos (Charles River Laboratories, Wilmington, MA) were prepared as previously described (Iwata et al., 2004). Briefly, neocortices were freed of meninges, dissociated and maintained in culture with Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen) containing 0.5 mM glutamine and 5% fetal bovine serum. Cells were plated at a density of 3.75×10^5 cells/cm^2 on a deformable membrane (Specialty Manufacturing, Saginaw, MI) coated with 1 mg/ml poly-L-lysine in custom-designed stainless steel wells (Smith et al., 1999). Two 2×10 mm silicone barriers (constructed from SYLGARD, Dow Corning Corporation, Midland, MI) with 0.4 mm wide micro-channels were placed on the deformable membrane at equal distance from the center of the well to create two cell-free gaps. These micro-channels were fabricated onto the surface of a stamp by casting polydimethylsiloxane (PDMS, Dow Corning) from patterned lithographic masters, as described previously (Tang et al., 2003). Using this system, we established 2 mm long axon tracks that span two neuronal populations. This micro-channel design offers several advantages over one-gap cultures (Iwata et al., 2004): 1) it provides directional cues for length-wise growth of axons, 2) parallel axon tracks are well separated and 3) as an essential requirement for anisotropic materials' elastic responses (Meaney et al., 1995), the longitudinal tracks insure a uniaxial stretch of most axons. We also used a novel method of water in the micro-channels to prevent cells from entering the gap (Tang-Schomer et al., 2010), instead of using pre-fabricated multi-layered channels (Taylor et al., 2005), so avoiding sophisticated lithography. Cells were grown for 11 days *in vitro* (DIV) when axon growth between the two cell populations separated by the gap is well-established. The barriers then were removed before axonal stretch injury was performed.

Axonal stretch injury

A specially designed axon stretch injury apparatus was used (Smith et al., 1999). To induce the injury, the culture well was placed in a device consisting of an aluminum cover block, a stainless steel plate with a machined 2×10 mm slit, and an air pulse-generating system. The culture well was inserted into the cover block and then placed on the slit plate so that the region containing the axon tracks coincided with the slit. The top plate was attached to the microscope stage, creating a sealed chamber. The top plate had a quartz viewing window in the center, an air inlet for compressed air and a dynamic pressure transducer (model EPX-V01-25P/16F-RF, Entran, Fairfield, NJ) to monitor internal chamber pressure. The introduction of compressed air into the chamber was gated by a solenoid (Parker General Valve, Elyria, OH). The solenoid and the pressure transducer were controlled and monitored by an analog-to-digital board (Metrabyte, Keithley Instruments, Cleveland, OH) integrated with a computer data acquisition system (Capital Equipment Corporation, Bellerica, MA). The device was mounted on the stage of a Nikon inverted microscope (Optical Apparatus, Ardmore, PA), to allow observation of the axon tracks throughout the experiment. To induce axonal stretch, a controlled air pulse was used to rapidly change the chamber pressure and deflect downward only the membrane containing the axon tracks within the gap. A strain rate of 20–50 s^{-1} was applied to the axons, well within the range for traumatic injury experienced by the human brain during rotational acceleration (Denny-Brown and Russell, 1940; Meaney et al., 1995). The nominal uniaxial strain (ε) was calculated by determining the centerline membrane deflection (δ) relative to the slit width (w) and substituting into the geometric relationship:

$$\varepsilon = \frac{w^2 + 4\delta^2}{4\delta w}\sin^{-1}\left(\frac{4\delta w}{w^2 + 4\delta^2}\right) - 1.0.$$

Experiments were conducted with a peak internal chamber pressure of 13–15 ψ to induce a transient uniaxial axonal strain calculated at 1.8–1.9 or 80–90% beyond axons' initial length.

Drug treatment

Before axonal stretch, cells were washed once and incubated in control salt solution (CSS, 15 min at +37 °C) consisting of 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, and 25 mM HEPES, pH 7.4. In some experiments, cells were pre-incubated (15 min) with tetrodotoxin (1 μM) or MK-801 (20 μM) by diluting drug stock solutions directly into CSS-containing cultures. Then, axon stretch was performed and cells were either fixed after 5 min and processed for immunocytochemistry or subjected to cell viability analysis. For time-course experiments, CSS was exchanged for culture medium immediately after stretch, and the cells returned to the incubator for 3 h and 5 h, until immunocytochemical or cell viability analyses were performed.

Calcium-free experiments were conducted in calcium-free CSS containing the calcium-chelating agent EGTA (1 mM). Cells were washed 3 times in calcium-free CSS, and incubated for 15 min at +37 °C before axonal stretch was performed. Sodium-free experiments were conducted by replacing sodium with N-methyl-D-glucamine and followed the same procedure described for calcium experiments.

Immunocytochemistry

Cells were fixed in cold 4% paraformaldehyde/4% sucrose in PBS for 20 min, thoroughly washed in PBS and permeabilized with 0.5% Triton X-100 for 5 min. Cells were pre-incubated in 5% goat serum (GS) in PBS for 30 min at room temperature, and then double-stained