Microfluidic culture platform for studying neuronal response to mild to very mild axonal stretch injury^{a)}

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A new model for studying localised axonal stretch injury is presented, using a microfluidic device to selectively culture axons on a thin, flexible poly (dimethylsiloxane) membrane which can be deflected upward to stretch the axons. A very mild (0.5% strain) or mild stretch injury (5% strain) was applied to primary cortical neurons after 7 days growth *in vitro*. The extent of distal degeneration was quantified using the degenerative index (DI, the ratio of fragmented axon area to total axon area) of axons fixed at 24 h and 72 h post injury (PI), and immunolabelled for the axon specific, microtubule associated protein-tau. At 24 h PI following very mild injuries (0.5%), the majority of the axons remained intact and healthy with no significant difference in DI when compared to the control, but at 72 h PI, the DI increased significantly ($DI = 0.11 \pm 0.03$). Remarkably, dendritic beading in the somal compartment was observed at 24 h PI, indicative of dying back degeneration. When the injury level was increased (5% stretch, mild injury), microtubule fragmentation along the injured axons was observed, with a significant increase in DI at 24 h PI ($DI = 0.17 \pm 0.02$) and 72 h PI ($DI = 0.18 \pm 0.01$), relative to uninjured axons. The responses observed for both mild and very mild injuries are similar to those observed in the *in vivo* models of traumatic brain injury, suggesting that this model can be used to study neuronal trauma and will provide new insights into the cellular and molecular alterations characterizing the neuronal response to discrete axonal injury. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in children and young adults,¹ making TBI a significant public health problem. Widespread axonal damage throughout the brain, referred to as diffuse axonal injury (DAI), is one of the most common and important features of TBI.² DAI may be caused by rapid brain deformation, stretching, compression or shear forces occurring as a result of traumatic incidents such as motor vehicle accidents, falls and assaults.³ Primary damage to axons following TBI, progressively develops over a number of days into secondary processes, including axonal cytoskeletal disconnection

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and neuronal degeneration.^{4,5} The delayed response provides a “therapeutic window” for possible interventions to prevent or reverse these detrimental cellular changes. Critical to the development of such interventions is a complete understanding of the cellular mechanism that comprise the neuronal response to trauma.

To this end, a range of *in vitro* models of axonal injury have been developed. Such models include direct axonal transection,⁶ transient axonal stretch injury involving pressurized fluid deflection of axon bundles of primary cortical neurons,⁷ and graded axonal compression of squid giant axon.⁸ These models allow the study of axonal alterations in real-time and, facilitate investigations of the biochemical mechanisms involved in the progression of axonal pathology, however, the random spatial distribution of neurons limits the effectiveness of these platforms for studying and understanding TBI. There is only a finite distance over which the individual axonal response can be followed and traditional models do not allow for individualised control of the microenvironments of the distinct cellular compartments of the neuron (soma, axon, and dendrites) different, complicating the investigation of pathological changes within distinct cellular compartments of the neuron (soma, axon, and dendrites) in response to trauma and pharmacological treatment. Detection of these pathological changes may be particularly important in the investigations of mechanisms of secondary degeneration where the role of retrograde signaling to the soma is unclear.

Smith and co-workers⁵ developed a uniaxial *in vitro* model system in which neurons are grown on a flexible silicon membrane. They utilized a controlled air pulse to rapidly change the chamber pressure and deflect downward the portion of the substrate that contains the cultured axons, inducing tensile elongation. This model was recently adapted to include axon guiding structures.^{9,10} However, these guiding structures did not allow the fluidic isolation of the soma compartment, limiting the control of the microenvironment in this model, for example, it does not allow for the targeted exposure of the axon or soma to study potential therapeutic agents that may prevent axons from degenerating and/or may promote regeneration.

In addition, studies show that axon degeneration can precede, and sometimes cause, neuronal death in several disorders,¹¹ creating a compelling need to understand the mechanisms of axon degeneration. Microfluidic devices that allow manipulation of fluids in channels with typical dimensions of tens to hundreds of micrometers have therefore emerged as a powerful platform for the study of neuron degeneration and regeneration.¹² The functionality and features such as microscale channels, pumps, and valves that can be incorporated into microfluidic devices allow physical and cellular manipulations which are not possible in conventional open cell culture systems.¹³ Specifically within the field of neuroscience, a microfluidic device developed by Jeon's group enabled the physical isolation of axons from their parent soma^{14,15} and the separation of the extracellular microenvironments of these cellular compartments through the incorporation of microgrooves (10 μm wide and 3 μm high) within microfluidic devices. Using such system, Hosie and colleagues¹⁶ demonstrated that glutamate receptors are present in axons and that they can respond directly to excitotoxic concentrations of glutamate independent of the soma. Kim and colleagues¹⁷ adapted this system into their “neuro-optical microfluidic platform” that also consists of a femtosecond laser to enable precise and reproducible axotomy of axons and mini incubator for continuous long term monitoring of post-injury events.

“Quake valves”¹⁸ are microvalves that were originally developed for pumping and valving in microfluidic systems. Within the field of neuroscience, Quake valves have been used to facilitate dynamic neuron and glia co-culture.¹⁹ They have also been utilised in neuronal trauma models to enable complete axotomy²⁰ and to apply graded compression to a single axon.²¹ Here, we have developed a new device that can apply mild to very mild stretch injuries to individual axons extending from primary cortical neurons by integrating Quake valves into the fluidically isolated microfluidic culturing devices. Previous studies¹⁵ used these microfluidic devices in combination with a glass coverslip, taking advantage of the well characterised culturing conditions on glass. Here, we used thin, elastic poly (dimethylsiloxane) (PDMS) membrane as a substrate to apply the stretch injury to the axons by pressurizing the Quake valve, requiring optimisation of the culturing conditions on PDMS. When the Quake valve is pressurized, the thin PDMS membrane is deflected upward and stretches the axons. Defined stretch injuries

applied using this device resulted in neuronal pathologies typical of those observed *in vivo*. This new device is unique owing to its ability to apply both mild (5%) and very mild stretch injury (0.5%) and to delineate alterations in response to discrete axonal injury in a fluidically isolated microenvironment. It also allows control over the length of the stretch, in this work set to 90 μm . The extent of the injury was quantified using immunocytochemical labelling and assessment of the axonal degenerative index.

II. MATERIALS AND METHODS

A. Characterization of PDMS/PDMS valve

A series of increasing gas pressures were applied to the Quake valve and once steady state was reached after 10 s, the membrane deflection was measured with an interference microscope Wyko NT9100 (Veeco Instrument Inc, USA) by dual Light Emitting Diode (LED) light optical profiling in the vertical scanning mode (VSI) through 20 $\times$ objective [Fig. 1].

B. Fabrication and assembly of axonal stretch injury platform

The axonal stretch injury device consisted of two independent PDMS structures separated by a thin PDMS membrane [Figs. 1(b) and 2]. Dissociated rat cortical neurons (harvested at embryonic day 18, E18) were grown in the upper PDMS microfluidic culturing device (Xona Microfluidic, CA), which has 450 μm long, 10 μm width, and 3 μm high microgrooves

FIG. 1. (a) Schematic showing experimental setup for PDMS membrane deflection characterization. Gas pressure was applied to the air channel by using an in-house built valve system which utilized a dynamic pressure regulator, USB-based controller box for the valve manifolds and Labview software. The computer sent the signal to the valve manifold through the controller box. The pneumatic valve opened and applied gas pressure to the embedded microchannel. The thin PDMS membrane deflected upward and deflection was measured with optical profiler system. (b) Photograph of assembled device comprising the Quake valve and culturing device. (c) Screen capture of optical profiler data used for quantifying deflection.

FIG. 2. Schematic drawing of microfluidic device used for simulating axonal stretch injury. (a) A thin PDMS membrane separates the Quake valves air-channel (bottom) from the overlying culturing chamber (Top). (b) Application of gas pressure to the air channel (positioned at 200–300 μm from microgrooves), causes upward deflection of the thin PDMS, which stretches the overlying axon. (c) Rat cortical neurons at 7 days in poly-L-lysine coated culturing device showing adequate axonal extension prior to axonal stretch injury. Scale bar = 100 μm .

connecting the soma and axon compartments. The bottom structure contained the Quake valve and was irreversibly sealed with the PDMS membrane using air plasma. In response to a pressure pulse, the Quake valve inflated and the PDMS membrane deflected upward, stretching the axons growing on top. The Quake valve microfluidic device was fabricated in PDMS (Sylgard 184, Dow corning, Michigan, USA) by soft lithography and replica molding procedure.²² The template to make the PDMS Quake valve device was fabricated by using an office laminator (Peach 3500, Laminator Systems, Australia), similar to the previously described protocol by Kazarian.²³ Briefly, the master template for the Quake valve was fabricated by laminating the poly (methymethacrylate) (PMMA) substrate (75 mm $\times$ 50 mm $\times$ 1 mm) with a 17 μm thick layer of dry film photoresist (Ordyl 317, Elga Europe, Italy) at 100 $^{\circ}\text{C}$ at a speed of 1350 mm/min. After lamination the substrate was exposed for 90 s through a transparency mask (Kodak Polychrome image setting film Pagi-Set, 4400 dpi, Pagination Design Services, Geelong, Australia) using a UV shark series high-flux LED array (OTLH-0480-UV, Opto Technology, Wheeling, IL, USA) as a light source.²⁴ The substrate was then baked for 20 min at 110 $^{\circ}\text{C}$ on a programmable hot plate (ECHOthermTM MODEL HS40, Torrey Pines Scientific, USA). The channels were developed and hard baked using the previously described procedure.²³ The template was ready for use as a master after being allowed to cool to room temperature. Curing agent and PDMS elastomer (1:10 ratio) were mixed and then poured over the template and degassed under vacuum. It was then cured for 60 min at 75 $^{\circ}\text{C}$. After curing, PDMS was detached and an air inlet was punched out with biopsy punch (1.5 mm diameter, Huat Instrument, USA). The resulting pieces were cleaned with compressed air and any remaining debris was removed by using a 3 M Scotch Brand 471 tape. A thin PDMS membrane was formed on a 1H, 1H, 2H, 2H perfluorooctyltrichol-silane (Fluorochem, UK) coated silicon wafer (100 mm diameter, 525 $\pm$ 25 μm thickness, one

side polished, test grade, SWI Semiconductor Wafer Inc, Taiwan) at rate of 1500 rpm and 2500 rpm at spinning time of 30 s by using a 8" Portable Precision Spin coater (Model P-6204, Cookson Electronics Equipment, IN, USA), which is a centrifuge-like device with a vacuum chuck and adjustable rotational speed to allow control of the uniformity and thickness of the film. To enable the inflation of the PDMS membrane, the Quake valve device was irreversibly sealed with the membrane through the activation of the surface using a handheld corona discharge unit (Electro Technic Product Inc., USA). Pressure was applied to the Quake valve by using an in-house assembly valve system developed by Quake and co-workers.¹⁸

C. Cell preparation

All experiments involving animals were conducted according to protocols approved by the Animal Ethics Committee of the University of Tasmania. Microfluidic devices used in this study were sterilized with 70% ethanol and UV light. Prior to cell seeding, the surface of the devices was hydrophilized using a handheld air plasma unit and then coated with 0.01% poly-L-lysine (PLL) (Sigma, USA) for three days. After that they were washed in milli-Q[®] water and placed in a standard humidified cell culture incubator set to 37 °C and 5% CO₂. The water was removed and the coated devices were filled with pre-warmed initial neuronal growth media consisting of Neurobasal[™], 10% heat inactivated foetal calf serum, 2% B27 supplement, 0.5 mM L-glutamine, 25 μ M glutamate, and 1% penicillin-streptomycin (Gibco/BRL, Life Technologies, USA) and incubated at 37 °C, 5% CO₂ for at least 3 h prior to plating the neurons. Primary cortical neurons were derived from E18 Sprague Dawley rat embryos as previously described.²⁵ Briefly, after dissection cells were chemically dissociated in 0.0125% trypsin (Life Technologies, USA) followed by washing and gentle manual dissociation. Primary neurons were then loaded into the somal compartment of the microfluidic devices at density of 9×10^6 cells/ml and incubated at 37 °C, 5% CO₂ for 5 min to enhance cell adhesion to the substrate. After incubation, both soma and axon compartments were filled slowly with pre-warmed initial neuronal growth media to minimise disturbing the cells. Cultures were grown at 37 °C, 5% CO₂. After 24 h, the initial neuronal growth media was replaced with subsequent growth media (initial growth media without the foetal calf serum and glutamate). The culture media was renewed three times a week to maintain neuronal viability.

D. Axonal stretch

A controlled gas pressure was applied to the Quake valve to deflect the PDMS membrane, thereby stretching the individual axons located above the air channel. The pressure was applied to using an in-house built valve system that utilized a dynamic pressure transducer (Pneumadyne, Inc., Plymouth, MN), USB-based controller for the 24 solenoid pneumatic valves and Labview software (National Instrument, Texas, USA). The introduction of gas into the valve was gated by a solenoid. A controller box, connected with the computer sent out a signal to a relay circuit. The relay circuit opened a pneumatic valve through the solenoid, and the pneumatic valve then applied gas pressure pulse to the Quake valve on the device. Uniaxial strains were applied 7 days after plating of primary cortical neurons in the somal compartment of the microfluidic device.

E. Immunocytochemistry

At 24 and 72 h after injury cells were fixed with 4% paraformaldehyde (Sigma, USA) for 1 h at room temperature. After removal of the fixative, the cells were washed with 0.01 M phosphate buffered saline (PBS). The PDMS substrates were then incubated with primary antibodies diluted in diluent (0.01 M PBS containing 0.3% Triton X-100) for 1 h at room temperature and at 4 °C overnight. Primary antibody to the axon specific microtubule associated protein tau (1:5000, rabbit polyclonal, Dako, Denmark), NFM (neurofilament M; 1:1000, Serotec, USA), and MAP2 (microtubule associated protein 2; 1:1000, Millipore, USA) were used. Following incubation with the primary antibody, cells were rinsed with 0.01 M PBS and incubated in the

dark with secondary antibody for 2 h at room temperature. Secondary antibodies (Mouse IgG Alexa Fluor 488 and Rabbit IgG Alexa Fluor 594, Molecular Probes, USA) were diluted 1:1000 in 0.01 M PBS. The cells were then washed in 0.01 M PBS followed by milli-Q water and mounted on glass slides with Permafluor mounting medium (Immunotech, France). The slides were then allowed to air dry in the dark at room temperature.

F. Quantification of axon degeneration

Fixed, immunofluorescent labelled samples were visualized with a Leica DMLB2 fluorescent microscope (Leika, Germany) and images were acquired with a CCD camera (ORCA, Japan) and recorded in NIH elements software (Nikon, Japan). To quantify axonal degeneration, we used the method described by Sasaki and colleagues.²⁶ Here, we analysed axonal degeneration by comparing axonal tau labelling among experimental groups at distal region of axons. Tau is a microtubule associated protein localised specifically to axons and routinely used to visualise these processes.²⁷ Briefly, each tau labelled image was binarized based on pixel intensity using NIH imageJ software. The total number of detected black pixels was defined as the total axon area. Degenerated axons that were fragmented and beaded were detected as particulate structure. Using a particle analyser algorithm of ImageJ, axonal regions with circularity more than 0.2 were determined and designated as fragmented. A degeneration index (DI) was calculated as the ratio of fragmented axon area over total axon area. To facilitate the comparison between injuries, we measured the DI relative to control at 24 h and 72 h post injury (PI). For each culture, two randomly selected images (20 $\times$) of fluorescently labelled axons were captured. Images from three different devices from three separate cultures were analysed, and the data arising from processing these 18 images are expressed with means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using Student's t-test. p-values < 0.05 were considered significant.

III. RESULTS

A. Axonal injury device design and operation

To apply the stretch injury, flexible elastic PDMS was chosen as the substrate for culturing. The isolated axonal stretch injury device contains two independent PDMS structures separated by a thin PDMS membrane [Figs. 1(b) and 2]. The upper structure will be referred to as the culturing device and the bottom structure will be referred to as the Quake valve device. The culturing device was a commercially available device used to culture neurons in a fluidically isolated microenvironment.¹⁴ It was positioned 200–300 μ m from the microgrooves and sealed with a thin PDMS membrane on top of which the neurons grow. This flexible elastic membrane is deflected upward when the Quake valve below is connected to gas, stretching the axons growing on top.

B. Device characterization

To determine the degree of axonal stretch, the physical extent of membrane deflection was measured by using an optical profiler system [Fig. 1(c)]. Membrane deflection, and therefore the degree of axonal stretch, depends on the thickness of the PDMS membrane and the applied pressure. In these experiments, PDMS membrane with thickness of $\sim$ 60 μ m (spin speed 1500 rpm), and $\sim$ 15 μ m (spin speed 2500 rpm) were used, as determined by an optical profiler system (data not shown). For the experiments presented here, a 35 psi pulse was applied to a 60 μ m PDMS membrane, resulting in a 4.3 μ m upward deflection [Fig. 3]. The Quake valve in this study was 90 μ m wide, therefore a mild, 0.5% strain was obtained (estimated by calculating the length of the membrane based on the channel width and deflected height using Pythagoras theorem). It is important to note this stretch is significantly smaller than strains previously applied in the literature, for example, Dollé *et al.* reported a deflection of up to 1 mm resulting in a 11% strain.²⁸ We also applied a 25 psi pressure to a 15 μ m PDMS membrane and thereby obtained 14.1 μ m upward deflection which resulted in a 5% strain to axons [Fig. 3].

FIG. 3. Relationship between applied gas pressure and membrane deflection at steady state for $\sim 60 \mu\text{m}$ PDMS membranes (1500 rpm) and $\sim 15 \mu\text{m}$ PDMS membranes (2500 rpm). A constant valve opening of 10 s was applied to ensure deflection measurements at steady state.

C. Response to axonal stretch injury

Primary cortical neurons from E18 embryos were seeded inside the poly-L-lysine coated culturing devices and allowed to grow for 7 days *in vitro* (DIV) for adequate axonal extension prior to stretch injury [Fig. 2(c)]. After 7 DIV, a pressure pulse (10 s) was applied to the Quake valve to induce a very mild (0.5%) axonal stretch injury to the overlying axon. Double fluorescent immunolabelling for the dendritic marker protein MAP2 and the axonal proteins tau and NFM demonstrated smooth continuous expression of these proteins in control, uninjured cultures [Figs. 4(a) and 4(b)]. Tau and NFM beading were observed along the length of very mild injured axons 24 h after a very mild (0.5%) injury [Fig. 4(d)]. In order to examine if axonal

FIG. 4. Immunocytochemistry images of uninjured and injured neurons 24 h following 0.5% injury. Cell bodies and dendrites ((a) MAP2 labelling), and axons ((b) tau and NFM labelling), in the control chambers were smooth and uniformly labelled for cytoskeletal markers. At 24 h post injury cortical cultures exposed to axonal stretch injury showed dendritic blebbing, and irregular MAP2 expression ((c) MAP2 labelling). The injured axons ((d) tau and NFM labelling) underwent characteristic beading and degeneration, showing punctate accumulation of tau and NFM within the swollen portions of the axon. Scale bar = $50 \mu\text{m}$.

injury resulted in retrograde signs of degeneration in the neuronal soma, we examined the somal compartment. At 24 h PI stretched neurons demonstrated dendritic beading and irregular MAP2 expression in the somal compartment [Fig. 4(c)].

To quantify the extent of distal axonal degeneration at different post-injury time points, we analyzed tau immunolabelled images of fixed axons using a particle analyzer algorithm of ImageJ software [Fig. 5] at distal region of axons. After very mild injuries (0.5%), the majority

FIG. 5. Axonal stretch injury to cultured cortical neurons resulted in axonal degeneration. (a) Tau-labeled (microtubule marker) axons following 0.5% injury (very mild) and 5% injury (mild) at 24 h and 72 h time point. Binarized images show fragmented axons defined by Analyze Particle function in ImageJ software. Stretch injury induced progressive distal degeneration leading to axonal beading and microtubule fragmentation. Scale bar = 50 μ m. (b) The degeneration index increased significantly at 72 h following 5% injury, compared to the degeneration index following 0.5% injury. However, there was no significant difference between control and 24 h following 0.5% injury. The degeneration index increased significantly at 72 h following 0.5% injury if compared to the control. Axonal degeneration index is significantly higher than controls at both 24 h and 72 h following 5% injury. The * symbol represents a statistical difference ($p < 0.05$) versus control at each time point. The † symbol represents a statistical difference ($p < 0.05$) versus very mild stretch (0.5% injury) at post 72 h.

of the axons remained intact and healthy at 24 h PI with no significant difference in degenerative index when compared to the control. However, the degenerative index increased significantly at 72 h following the very mild injury ($DI = 0.11 \pm 0.03$). When the injury level was increased (5% injury, mild injury), we observed increased signs of degeneration including beading and fragmentation at both time points with a significant increase in the degenerative index at 24 h PI ($DI = 0.17 \pm 0.02$) and 72 h PI ($DI = 0.18 \pm 0.01$) compared to the uninjured control. Additionally, the degenerative index was increased significantly at 72 h following mild injury (5%), compared to the degeneration index following very mild injury (0.5%).

IV. DISCUSSION

TBI is a major public-health problem and the majority of the cases are considered to be mild TBI.²⁹ Currently, there is no effective therapeutic intervention that can preserve or restore the cognitive, sensory, or motor dysfunction that can result from TBI.³⁰ In addition, there is growing awareness that repetitive mild TBIs, such as concussion, can produce persistent cognitive, behavioral, and psychiatric problems as well as lead to the development of a particular type of neurodegeneration, now known as chronic traumatic encephalopathy (CTE).³¹ Therefore, new models that mimic specific and defined aspects of mild TBI are required in order to fully elucidate the mechanisms underpinning the neuronal response to injury.

However, to the best of our knowledge, no *in vitro* system is able to study precise and reproducible mild axonal stretch injury in a fluidically isolated microenvironment. Recent studies demonstrated that microfabrication technologies enable the development of powerful platforms to culture and manipulate neurons and able to study nerve injuries.³² Therefore, a novel microfluidic device to simulate mild (5%) and very mild stretch injury (0.5%) of axons by incorporating microfluidic valve technology into our devices has been developed in the current investigation, capable of applying mild to very mild injuries by stretching only a 90 μm long section of the axons. The microfluidic culture chamber design developed by Taylor and co-workers¹⁴ for fluidic isolation of the axon and soma was used, together with a flexible thin PDMS membrane that can be locally deflected upward using the valve technology. This allows axons growing on top of the PDMS membrane to be discretely stretched, providing a new platform to study stretch injuries and, in later stages, has the potential to test the effects of therapeutic agents in isolated axonal or somal compartments following stretch injury. More recently, Dolle and colleagues²⁸ developed a new device where uniaxial strains were applied to the elastic PDMS substrate on which axons extend between two organotypic hippocampal slices. This device is similar to our device, where upward deflection is applied to the axons growing on PDMS; however, this device applied stretch injury range from 11% to 42%, in a non-fluidically isolated microenvironment over a length of approximately a millimeter, whereas our device applied stretch injury in fluidically isolated microenvironment at a relatively small strain (0.5% or 5%). These significant differences in stretching regimes each provide access to unique scenarios to simulate clinical manifestations.

Here, flexible elastic PDMS was chosen as the substrate for culturing because PDMS has advantages of being inexpensive, permeable to gas, optically clear and can be simply sterilized by ethanol or UV light exposure.³³ In addition, PDMS has also been widely used in a range of biological application, providing evidence of its biocompatibility.³⁴ For example, Dolle *et al.*²⁸ proved that organotypic hippocampal slices cultured on elastic PDMS substrate remain healthy over three weeks in culture. In addition, we do not observe any detachment of axons after the application of stretch injury, suggesting that the axons are firmly attached to the surface of thin PDMS substrate and the axons are stretching in the same strain as the PDMS substrate. The Quake valve was positioned 200–300 μm from the microgrooves. In the same culturing device, Taylor and colleagues¹⁴ observed axons began to extend into the axonal chamber after 4 DIV with the extensive growth of axons in axon compartment levelling off at 7 DIV. At 7 DIV, we confirmed all axons that had extended into the axon compartment, past the Quake valve, and ensured these were long, relatively unbranched axons. Based on this information, all the axons in the axon compartment would be subjected to stretch injury when applying the stretch injury

at 200–300 μm from the axon grooves at 7 DIV, selecting these conditions for the stretch injury studies.

Traditionally, only strains over 10% were thought to cause injury.³⁵ There are a few previous studies that have reported different experimental models for stretching axons in uniaxial field, ranging from 15% to 65% in a cultured N-Tera2 human neuron cell line⁵ from 30% to 75% in a model of primary cortical neurons.³⁶ Dendritic alterations following axonal stretch injury were examined as part of this study. Dendritic beading was observed along the dendrite shaft, a finding consistent with previous studies performing *in vitro* axonal stretch injury.⁹ Changes in dendritic structural proteins, such as MAP2 and neurofilaments, are also prominent in animal models of TBI.^{37–39} Since dendrites are essential to the processes of learning and memory,^{40,41} changes in dendritic structure can result in cognitive dysfunction. Evidence from previous studies demonstrated that altered dendritic structure may contribute to the cognitive deficit observed following TBI.⁴² Understanding the mechanisms by which axonal stretch injury affects dendrite structure and function may provide additional insight into TBI pathophysiology and lead to novel strategies to improve learning, memory and behavioral dysfunction after TBI.

Most research into axonal stretch injuries focused on axonal effects, reporting widespread axon swelling and degeneration within 24 h post injury. A 1%–6% stretch injury model developed by Staal and Chung⁷ showed neurofilament alterations characteristic of DAI at 48 h post injury, but no distinguishable difference in axonal morphology at 24 h PI. In the current study, after mild axonal stretch injury (5%), microtubule fragmentation along the injured axons was observed both at 24 h and 72 h PI. Similarly, axonal degeneration increased at 24 h post 0.5% injury (very mild injury), although there was no significant difference between control and very mild stretch at this time point. However, 72 h post 0.5% injury, microtubule fragmentation was significantly greater in stretched axons compared to the unstretched axons. These findings are considered hallmark pathological features of DAI and have not previously been studied or reported for these small and very mild injuries.

The current study has the ability to reproducibly perform a very small level of strain (0.5% increase in original axon length) as compared to the bundle axonal stretch injury (1%–6%) demonstrated by Staal and Chung.⁷ For comparison, the strain rate applied by Staal and Chung was in the range of 0.8–4.2 s^{-1} while we have applied strain rates of significantly lower magnitude, from $5 \times 10^{-4} \text{ s}^{-1}$ (0.5% increase in original axon length) to $5 \times 10^{-3} \text{ s}^{-1}$ (5% strain). Significant changes in both dendritic and axonal structure were observed in our study even after this relatively mild injury. This suggests that even very small stretch injury to axons will induce degenerative changes. The current model is also designed to address single axons responses, therefore, circumventing the added variables of investigating bundles of axons of unknown density and varying length that are not attached to the substrate.

Previous studies⁵ observed delayed elastic effects where undulation and waves were observed immediately after injury and then returned to the original axonal length after a period of time. We have not observed this effect on our device, possibly due to the fact that the delayed elastic effect are only observed at high strain rates such as 19 s^{-1} applied by Smith *et al.*⁵ in comparison with the relatively small strain rate magnitudes such as $5 \times 10^{-4} \text{ s}^{-1}$ (0.5% strain) and $5 \times 10^{-3} \text{ s}^{-1}$ (5% strain) reported here. Our small strain rate also explains why we do not observe primary axotomy after application of stretch injury although the time duration of 10 s.

In addition to the capacity to apply mild axon transient injury, our device also has the capability to investigate the injury-induced sub-cellular alterations because of the fluidic isolation of soma and axon in the microfluidic culturing devices in combination with the discrete and transient stretch of individual axons. Therefore, several aspects of DAI including changes in intracellular calcium levels,⁴³ cytoskeletal structure,⁶ membrane permeability,⁴⁴ and axonal swelling formation⁴⁵ can be studied through this model. In addition, the fluidic isolation of this device allows independent chemical treatments on the axonal side or somal side and will enable insights into signalling between the axon and soma by investigating the site specific neuronal response to blockers of protein synthesis, proteasome degradation, apoptosis, or cytoskeletal stabilization after direct axonal stretch injury. This approach has the capability to investigate a

range of different stretch injuries from mild to severe, at different distances from the soma and over different lengths and at different intervals due to the ability to precisely control the location and dimensions of the valve, the thickness of the PDMS membrane and the magnitude and timing of the applied pressure.

V. CONCLUSIONS

A simple, reproducible *in vitro* model of discrete axonal stretch injury of cultured primary neurons is presented, allowing, for the first time, the controlled application of mild to very mild strain injuries. When used for applying very mild (4.3 μm upward deflection, 0.5% strain) or mild (14.1 μm upward deflection, 5% strain) axonal stretch injury to neurons at 7–9 DIV, cellular responses similar to those found in the *in vivo* models of traumatic brain injury were observed. This device is unique in the small size and magnitude of the applied strain, but surprisingly immunostaining and fluorescent imaging still revealed several pathological alterations characteristic of DAI *in vivo* following application of these low level strains. The device allows specific alterations in axons to be investigated. With the fluidic isolation of the axonal and somal compartments enabling targeted exposure to potential therapeutic agents, this system provides a valuable tool for studying the degenerative and regenerative neuronal responses induced by acute axonal stretch injury for testing potential therapeutic agents for traumatic brain injury.

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