Repetitive stretching enhances the formation of neurite swellings in cultured neuronal cells

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Abstract

Repetitive mild traumatic brain injury (r-mTBI) has been gaining increasing attention from the researchers since several studies have reported that the cognitive dysfunctions after single mTBI become measurably long-term deficits, such as delayed speed of processing and memory dysfunction, after r-mTBI, contributing to the emerging hypothesis that r-mTBI may cause cumulative damage to the brain, and in the absence of cell death, could result in cognitive deficits which may ultimately progress to memory and learning dysfunction. Studies also associated r-mTBI with "second-impact syndrome" and chronic traumatic encephalopathy (CTE) as possible consequences of r-mTBI. However, the potential injury mechanisms involved in r-mTBI still remain unclear and research on r-mTBI is still in early stages. Thereby, in this study an in vitro uniaxial stretching model was used to investigate the r-mTBI related cell damage for clarifying the pathology and post-injury sequelae of r-mTBI in comparison with single mTBI. 3 types of stretching experiments were conducted including the single loading groups that were subjected once to stretching with a strain of 0.1 at a strain rate of 5 s⁻¹, the repetitive loading groups that were subjected again to the same stretching 1 h after the initial stretching, and the sham control groups that were set in and removed from the uniaxial stretching device without receiving mechanical loading. Results shows that even though initial insult induced some level of swelling formation, swelling formation increased by second stretching confirming that r-mTBI causes increased amounts of cellular damage when compared with single insults of the same magnitude. Moreover, the absence of progression to cell death at 24 h post injury is detected after swelling formation by repetitive stretching. These data indicate that if injured neurons are subsequently subjected to low strain at the same level, the rate of injury significantly increases confirming the emerging hypothesis on repetitive mTBI exacerbating traumatic axonal injury.

Background

To this day, traumatic brain injury (TBI) continues to be a leading cause of mortality and morbidity worldwide, making TBI a significant public health problem [1]. In the United States alone, the estimated number for annual occurrence of TBI is 1,700,000 [2-3] whereas in Asia 300-400, and in European countries (average of the 23 countries) 200-300 people per 100,000 are hospitalized annually due to TBI [4-6]. Moreover, majority of these cases categorized as mild TBI (mTBI) or concussion [2,7] while, in spite of the name "mild", approximately 15% of mTBI patients suffering persistent cognitive dysfunction in the United States alone [8]. Furthermore, several studies on professional athletes and military personnel have reported that these cognitive dysfunctions become measurably long-term deficits, such as delayed speed of processing and memory dysfunction, after "repetitive" mTBI (r-mTBI), contributing to the emerging hypothesis that r-mTBI may cause cumulative damage to the brain, and in the absence of cell death, could result in cognitive deficits which may ultimately progress to memory and learning dysfunction [9-17]. Aiding to the human cases, studies on animal models have also demonstrated a worsened outcome with repetitive TBI, where cell death and tissue damage are common consequences of moderate and severe TBI, not mTBI, yet cellular dysfunction and memory deficits are overt after mTBI [18-22].

Additionally, increasing evidence suggests that mTBI patients suffer from diffuse axonal injury (DAI), which is one of the most common pathology of TBI, associated with rapid brain deformation, stretching, inertial forces occurring as a result of traumatic incidents such as accidents, falls and assaults resulting in the stretching of neuronal axons [23-27] where primary damage to axons progressively develops into secondary cascades such as neuronal degeneration and axonal cytoskeletal disconnection [28-31]. Considering that DAI is involved in the immediate loss of consciousness after TBI and several studies have been shown white matter abnormalities consistent with DAI in mTBI patients [32-34], there is a strong possibility of a potential mechanism which leads to vulnerability with a repeat injury since phenotypic or physiologic changes of the injured axons would likely influence outcome following r-mTBI and that DAI has an important role in r-mTBI. Studies also associated r-mTBI with "second-impact syndrome" where individuals displaying post-concussion symptoms after initial TBI, which can include visual, sensory or motor dysfunctions or mental difficulty such as cognitive and memory problems, and devastating brain swelling resulting in semi-comatose situation with dilating pupils, loss of eye movement and respiratory failure after a second TBI occurring days or weeks after the first injury [35]. More recently, similar pathology, referred to as chronic traumatic encephalopathy (CTE), a neurodegenerative brain disorder, has been gaining growing awareness as another consequence of r-mTBI [36-40].

However, the potential injury mechanisms involved in r-mTBI still remain unclear and research on r-mTBI is still in early stages. Although in vivo models contribute substantially in understanding pathological and physiological sequelae on macroscopic and microscopic levels, complementing these with in vitro studies that simulate specific

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aspects of r-mTBI is crucial to address questions concerning post-injury sequelae and r-mTBI-related sub-lethal cellular dysfunction at the cellular and subcellular levels [41].

This study introduces an in vitro model in which subsequent injuries were induced by using a uniaxial stretching device for realization of the post-injury sequelae of r-mTBI. Proposed in vitro uniaxial stretching model provides a reliable environment to study the mechanisms underlying cellular dysfunction and for a better characterization of the cellular degradation and dysfunction following both single and repeated injuries. Results showed that swellings along neurites stretched twice at an hour interval increased immediately after stretching and were sustained for 24 h whereas swellings along neurites stretched once transiently increased within several hours following stretching. However, the neurites of neurons in cultures that received repeated insults showed signs of damage that were not evident after a single mild injury. Furthermore, no significant cell death was detected after r-mTBI.

These data indicate that if injured neurons are subsequently subjected to low strain at the same level, the rate of injury significantly increases confirming the emerging hypothesis on repetitive mTBI exacerbating traumatic axonal injury.

Methods

Uniaxial stretching device

The uniaxial stretching device mainly consists of a servo actuator, a linear sensor for measuring tensile displacement, a load cell for measuring tensile loading and a polydimethylsiloxane (PDMS) chamber, and is shown in Figure 1. A full description of the device configuration and loading mechanism has been published [42]. The Green-Lagrange strains of the culture substrate in the PDMS chamber were calculated by microscope images before and after the stretching. The strains on the central point of the culturing substrate in the stretching direction were obtained for every 0.5 mm displacement to 4 mm in total and plotted in Figure 2A. In this study, the history of stretching direction were obtained for every 0.5 mm displacement to 4 mm in total and plotted in Figure 2A. In this study, the history of the displacement shown in Figure 2B was applied to the chamber with a strain of 0.10 at a strain rate of 5 s$^{-1}$. The strain rate was obtained by dividing the maximum strain by the time to maximum strain. The curves stood up in the first 10 msec by stretching, and converged in the last 10 msec by releasing with the same ratio as the standing up. It is expected that the neuron on the substrate is stretched/compressed with a similar deformation history through the substrate.

Cell culture

PC12 cells—tumor cells derived from adrenal chromaffin cells of a rat [43] —were provided by the RIKEN Cell Bank (Tsukuba, Japan). After exposure to nerve growth factor (NGF), the cells extend neurites, which are structurally axon-like cylindrical protrusions, and differentiate into cholinergic sympathetic neurons [44]. The cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10% horse serum (horse serum), 100 U/mL penicillin (Sigma-Aldrich), and 0.1 mg/mL streptomycin (Sigma-Aldrich) under conditions of 5% CO$_2$ and 100% humidity at 37°C. The cells were seeded into the PDMS chamber at 1500–3000 cells/cm$^2$ in the presence of 100 ng/mL NGF (Invitrogen) for 5 days. As a sham control, neurons matched in age to those subjected to strains were cultured in the PDMS chamber, after which they were placed in and removed from the uniaxial stretching device without mechanical loading. The medium in the PDMS chamber was not removed during the experiment, which was completed within 5 min. The surrounding temperature of the device was held at 37°C. The PDMS chamber was returned to the CO$_2$ incubator after the experiment.

Repetitive stretching experiment

3 types of stretching experiments were conducted. The single loading groups were subjected to initial stretching with a strain of 0.1 at a strain rate of 5 s$^{-1}$, the repetitive loading groups were subjected again to the same stretching 1 h after first stretching with a strain of 0.1 at a strain rate of 5 s$^{-1}$. The sham control groups were set in and removed from the uniaxial stretching device without receiving mechanical loading. The medium in the PDMS chamber was not removed during the experiment which was completed within 5 min. The temperature of the device and surroundings were kept at 37°C. The PDMS chamber was returned to the CO$_2$ incubator after the experiment.

Morphological observation

Cells were observed using an inverted fluorescence microscope (FSX100, Olympus, Tokyo, Japan). Phase-contrast images were obtained immediately before stretching and at 5 min, 1 h, 3 h, 6 h and 24 h after first stretching. The number of cells and length of neurites were manually measured with ImageJ (National Institutes of Health,
A neurite swelling was defined as a thick part in a neurite. Injured neuron was defined as the rate of neurons that have neurite swellings.

**Cell viability**

Cell viability was assessed using fluorescent probes (LIVE/DEAD Viability/Cytotoxicity Assay Kit; Lonza, Walkersville, MD, USA) for distinguishing live and dead cells. Cells were rinsed with Dulbecco’s phosphate buffered saline (D-PBS) and were incubated with 4 μM ethidium homodimer-1 (EthD-1; excitation and emission wavelengths of 528 and 617 nm, respectively) and 2 μM calcein-AM (excitation and emission wavelengths of 494 and 517 nm, respectively) at 37°C for 30 min. After rinsing with D-PBS, fluorescence images were obtained at 24 h after injury using the FSX-100. Live and dead cells were manually quantified using 5 randomly selected regions per experiment.

**Statistical analysis**

Results were expressed as the mean ± standard deviation (SD) of 4 independent experiments. 100–200 neurons per a PDMS substrate were analysed totally. Means were compared by Steel’s multiple comparison test. A p value of less than 0.05 was considered significant.

**Results**

Neurite swellings were observed in PC12 cells exposed to stretching (Figure 3); however, they were present in low amounts in the sham control groups and at pre-stretching time points (Figure 4). The formation of neurite swellings in the single loading groups increased significantly immediately after stretching and reached a peak at 3 h, but there was no significant difference at 24 h compared to the sham control group. However, swelling formation in the repetitive loading groups increased two times after second stretching more than after first stretching and was significantly higher at all time points than that in the sham control group.

The length of neurites, the number of cells and the percentage of cell viability at 24 h after loading are shown in Table 1. There was no significant difference in the neurite length and the cell number among all groups. Although cell viabilities of all groups were significantly unchanged, dead cells were observed only in the repetitive loading groups shown in Figure 5. Additionally, neurites of the repetitive loading groups were not stained by calcein-AM even without swelling formation.

**Discussion**

Repetitive mild traumatic brain injury (r-mTBI) recently gaining more interest from the researchers yet the pathology or the underlying mechanisms of detrimental effects of multiple mTBI remains unclear. To address this gap in the literature, several studies have proposed animal models in which studies indicated more persistent behavioral impairments and axonal injury compared to single injury without gross pathological injury or neuronal cell loss, behavioral deficits without apparent neuronal loss, neuronal cytoskeletal abnormalities, accelerated β-amyloid deposition and a potential “vulnerable state” lasting approximately between 3 and 5 days following the first TBI [18-19,21,45-48]. Although these in vivo studies provided valuable information for understanding that the changes in behavior post-injury is possible to be measured and correlated to cell damage, the extent of these studies can only reach to theorizing the underlying mechanisms of cognitive impairment at the cellular level under r-mTBI [41]. Therefore, it is crucial to complement in vivo studies with...
thoroughly devised in vitro studies to address the post-injury sequelae at the cellular level.

Moreover, despite several in vitro models on axonal injury induced by severe, moderate and mild TBI have been proposed ranging from direct axonal transection, transient axonal stretch injuries using pressurized fluid deflection, uniaxial in vitro models controlled by air pulse, focusing on the electrophysiological responses of neural cells, axonal swelling formation, neurofilament structure, changes in ionic homeostasis, and axolemma permeability [29,49-62], even fewer in vitro studies exist for investigating r-mTBI mainly focusing on the inter-injury interval, pathophysiological response, dependence on the severity of insult and characterization of the cellular degradation and dysfunction [41,48,63-64]. Thereby, in this study an in vitro uniaxial stretching model was used to investigate the r-mTBI related cell damage for clarifying the pathology and post-injury sequelae of repetitive mild TBI in comparison with single mTBI. The uniaxial stretching model is chosen since uniaxial stretching of the axon is clinically more relevant to axonal injury than partial stretching [65-67]. Furthermore, the widely employed models involving fluid or air pressure induced deformation is nonuniform where the strain at the center of the membrane is being assumed as the representative value which can lead to inconsistencies when relating cell injury to the amount of applied strain [56]. 3 types of stretching experiments were conducted including the single loading groups that were subjected once to stretching with a strain of 0.1 at a strain rate of 5 s⁻¹, the repetitive loading groups that were subjected again to the same stretching 1 h after the initial stretching, and the sham control groups that were set in and removed from the uniaxial stretching device without receiving mechanical loading. Results show that even though initial insult induced some level of swelling formation, swelling formation increased by second stretching confirming that r-mTBI causes increased amounts of cellular damage when compared with single insults of the same magnitude. Moreover, the absence of progression to cell death at 24 h post injury is detected after swelling formation by repetitive stretching.

In conclusion, the proposed model in this study is a promising method for investigating 8 secondary pathways of damage, combination with the directional control of axonal elongation therefore accurate control and observation of axonal injury, the effects of injury on cultures from various cell types, the long term consequences of r-mTBI, and the influence of different numbers of injuries and different inter-injury intervals which also can be listed as the future work of this study.

Repetitive mild TBI covers a substantial portion of all TBI cases although the attention given to experimental repetitive TBI studies has remained scarce until recent years. However, recent research involving both in vivo and in vitro studies seems promising for interpreting the pathology of r-mTBI. The identification of molecular targets specific to mTBI and ultimately the development of novel, effective therapeutics will be enabled after a thorough recognition of mTBI.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

HN designed the study. EK and SS carried out the stretching test and observed the cell morphology and viability. SA conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors have read and approved the final manuscript.

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