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ORIGINAL PAPER

Stretch-Induced Deformation as a Model to Study Dopaminergic Dysfunction in Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is defined as damage to the brain that consequently disrupts normal function. Neuronal death, a hallmark of TBI, has been related to the development of neurodegenerative disorders like Parkinson's disease (PD), where loss of dopaminergic neurons and dopaminergic dysfunction are observed. To date, no in vitro model exists in which the dopaminergic damage observed in TBI is replicated. In this study, we evaluated the effects of in vitro simulated TBI on human dopaminergic neurons. To simulate TBI, neurons were subjected to 0%, 5%, 10%, 15%, 25% and 50% deformation. 24 h after injury, cell viability and apoptosis were determined by lactate dehydrogenase (LDH) release and DNA fragmentation, as well as ethidium homodimer and caspase 3/7 staining. Dopamine (DA) levels were determined by ELISA. Levels of tyrosine hydroxylase (TH) and DA transporter (DAT) were determined by western blot. Only 50% stretch increased LDH release and ethidium homodimer staining, suggesting the induction of necrosis. On the contrary, 25% and 50% stretch increased DNA fragmentation while 15%, 25% and 50% increased caspase 3/7 staining, suggesting that moderate and severe TBI promote apoptosis. Levels of intracellular DA decreased in a stretch-dependent manner with 15%, 25% and 50% stretch, which were related with a decrease in TH expression. Extracellular DA levels increased only at 50%. Levels of DAT remained unchanged regardless of treatment. These data support the use of stretch as a model to simulate TBI in vitro in human dopaminergic neurons, replicating the acute effects of TBI in the dopaminergic system.

Keywords Traumatic brain injury · Dopamine · Apoptosis · In vitro models

Introduction

Traumatic brain injury (TBI) affects millions of Americans every year as a result of falls, traffic accidents, sports and combat related injuries [1]. TBI occurs due to mechanical forces disrupting normal brain function [2]. These disruptions can be classified as primary and secondary injuries. Primary injury occurs almost immediately as a consequence of mechanical forces applied to the brain and is characterized by irreversible vascular and tissue damage. On the other hand, secondary injury is a consequence of the primary injury and is characterized by an increase in blood–brain barrier (BBB) permeability, neuroinflammation, an increase in the release of excitatory neurotransmitters and neuronal death [3–5]. Studies in animal models have shown that TBI induces neuronal death shortly after injury [6, 7] that still occurs for several days [8] and weeks after TBI [9]. Additionally, recent studies have shown that TBI disrupts the dopaminergic system [10]. Hutson and colleagues demonstrated that TBI decreases the number of tyrosine hydroxylase (TH) positive neurons in the substantia nigra and striatum, 11 days post-injury, and the number decreased even further 26 weeks after the injury [8]. Similar studies also indicate that TBI reduces the number of dopaminergic neurons in the substantia nigra and striatum at different times after injury [11–13]. In this regard, levels of striatal dopamine (DA) were shown to be decreased 24 h after injury in an experimental mouse model of TBI [14]. Moreover, multiple studies have also reported dopaminergic dysfunction

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and a decrease in striatal DA after experimental TBI [9, 10, 15–17]. In addition of its role in TBI, dopaminergic dysfunction and loss of dopaminergic neurons are two of the hallmarks observed in Parkinson's disease (PD), the second most prevalent neurodegenerative disorder [18]. Retrospective studies have analyzed the relationship between TBI and the development of PD. Individuals who suffer TBI episodes early [19] or late in life [20] have an increased risk of developing PD. Recently, a major study that pooled clinical and neuropathological data from three prospective cohort studies found an increased risk of PD in patients with TBI involving loss of consciousness [21]. These studies highlight the importance of studying dopaminergic dysfunction in TBI, due to its possible role in the development of neurodegenerative disorders.

To study TBI in a laboratory setting, animal models that recapitulate diverse mechanisms of injury have successfully been employed [22]. However, in vitro models that simulate TBI are frequently used due to their high reproducibility, ease of use, and well controlled parameters of injury [23, 24]. In vitro models of TBI are typically employed to investigate specific mechanisms of injury or repair in isolated cellular populations or brain regions in order to identify and develop potential therapeutic interventions for TBI [23]. These models also represent different types of injury, including blast-induced injuries [25], focal injuries [26, 27] and penetrating injuries [28, 29]. More recently, models have been developed based on the induction of brain deformation by submitting cells in culture or organotypic brain slices to different degrees of stretch [6, 30–38], simulating the brain biomechanics induced by TBI [39–42]. Even though multiple studies report the use of stretch-induced deformation to model TBI in vitro, only a few studies have attempted to model the dopaminergic damage induced by TBI [31, 43–45]. These studies have focused on the analysis of cell death, but the effect of stretch-induced injury in the production and release of DA has not been studied to date. To be used with confidence in the study of TBI, an in vitro model needs to accurately recapitulate the response to injury observed in vivo. In the present study, we used a stretch-induced deformation model to test if the decrease in DA production and altered release observed in vivo after TBI can also be reproduced in vitro.

Materials and Methods

Cells

Human primary dopaminergic neuronal precursor cells (HPDNPCs) were purchased from Applied Biological Materials (Richmond, BC). Differentiation to dopaminergic neurons was achieved by culturing the HPDNPCs in culture plates coated with poly-L-lysine at a density of 1×10^4 cells/cm^2 using proprietary PeriGrow IV culture medium. Medium was supplemented with 5% fetal bovine serum and 10 ng/mL of each: basic fibroblast growth factor, epidermal growth factor and dibutyril-cyclic adenosine monophosphate. Prior to the induction of in vitro TBI, HPDNPCs were maintained in differentiation medium for 7 days to achieve full differentiation to neurons.

In Vitro Model of TBI

In vitro simulated TBI was induced by applying biaxial stretch deformation using the cell injury controller II (CIC II) device (Custom Design and Fabrication; Sandston, VA) as previously described [36, 38]. Differentiated neurons were cultured in poly-L-lysine-coated 6-well plates with flexible bottoms (Flexcell International; Burlington, NC) in differentiation medium for 7 days. To induce TBI, pressurized medicinal air was infused for 50 ms to reach pressures ranging from 0.46 to 4.0 psi to achieve 5, 10, 15, 25 and 50% membrane deformation or stretch. Neurons were then returned to the incubator for 24 h before subsequent analysis.

Lactate Dehydrogenase (LDH) Release

Induction of necrosis was determined by measuring LDH released from the cytosol of injured neurons using the Cytotoxicity Detection Kit^{PLUS} (Roche; Indianapolis, IN). Twenty-four h after a single TBI episode, 100 µL of the supernatant were collected and transferred to 96-well plates and then an equal volume of reaction mixture was added and incubated in the dark for 30 min at room temperature. LDH activity was quantified by measuring the absorbance at 490 nm.

Viability Assay

Cellular viability after stretch-simulated TBI was assessed using a commercially available kit (Thermo Fisher; Waltham, MA). Briefly, 24 h after a single stretch, the medium was removed from the culture plates and neurons were washed with Dulbecco's phosphate-buffered saline (DPBS). Then, a solution of 2 µM calcein and 4 µM ethidium homodimer-1 (EthD-1) in DPBS was added and cells were incubated for 30 min at room temperature. After that, micrographs were taken at 4X using a fluorescence microscope. Live cells were detected using a fluorescein isothiocyanate filter (calcein

staining) and dead cells were detected using a tetramethylrhodamine filter (ethidium homodimer-1 staining). Images were analyzed using ImageJ software.

DNA Fragmentation

Detection of DNA fragments in the cytoplasm of injured neurons was measured as an index of apoptosis and was determined using a commercially available kit (Roche; Indianapolis, IN). Briefly, 24 h after a single stretch, neurons were lysed and lysates containing the DNA fragments were transferred to a 96-wells plate coated with anti-histone antibodies. Samples were incubated for 90 min at room temperature and then anti-DNA peroxidase antibodies were added and incubated for another 90 min at room temperature. Peroxidase substrate was added, and absorbance was measured at 405 nm within 20 min.

Caspase 3/7 Assay

The activity of caspases 3 and 7 was determined using a commercially available kit (Thermo Fisher; Waltham, MA). Briefly, 24 h after a single stretch, the medium was removed from the culture plates and neurons were washed with DPBS. After that, a solution of 5 µM of the caspase-3/7 detection reagent was added to the neurons and incubated at room temperature for 30 min. Nuclei were counter-stained using Hoechst. Micrographs were taken at 4× using a fluorescence microscope. Cells with active caspase-3/7 emitted a green fluorescence that was detected using an FITC filter. Images were analyzed using ImageJ software.

Dopamine Content and Release

Concentration of intracellular and extracellular DA were quantified using an ELISA kit (Eagle Biosciences, Nashua, NH), following the manufacturer instructions. Briefly, neurons were lysed, and lysates were filtered through a 0.45 µm filter. DA was acetylated and then extracted from the neuronal lysates and culture medium. DA antiserum and samples containing acetylated DA were then transferred to a 96-well plate with DA bound to its surface and incubated overnight at 4 °C. DA present in the sample and DA fixed in the plate compete for the fixed number of DA antibodies in the antiserum. The DA antibody bound to the DA attached to the surface of the plate is detected using a secondary antibody coupled with peroxidase at a 450-nm wavelength. The absorbance obtained is inversely proportional to the concentration of DA present in the sample.

Western Blot Analysis

Expression of TH and DA transporter (DAT) were evaluated by western blot. Twenty-four hours after injury, cells were collected, and protein was extracted using RIPA buffer. After that, 10 µg of protein extract were loaded into native tris-glycine gels and run for 1 h at 200 V. Proteins were then transferred to polyvinylidene difluoride membranes at 100 V for 1 h. Membranes were then blocked for 1 h using Odyssey blocking buffer-TBS (LI-COR; Lincoln, NE) and then incubated with primary antibodies against TH (Catalog # TYH, Aves Labs Inc.) and DAT (Catalog # 431-DATC, PhosphoSolutions) overnight at 4 °C. Membranes were then washed and incubated with secondary antibodies for 1 h at room temperature. Images were acquired using the Odyssey CLx imaging system. Proteins of interest were quantified using the Image Studio Ver 5.0 software and normalized by total protein using the REVERT total protein stain kit (LI-COR; Lincoln, NE).

Statistical Analysis

Data was collected in triplicate from at least 3 independent experiments and analyzed using one-way ANOVA followed by post-hoc analysis. The data are presented as means ± SEMs and the difference from control was considered significant if $p < 0.05$.

Results

In Vitro Simulated TBI Increases LDH Release Only at High Levels of Stretch

Release of LDH from the cytoplasm of injured cells was measured as an index of necrosis. Twenty-four h after stretch injury LDH release was only increased by 34.9% at 50% stretch, compared to 0% stretch (control). Stretch at lower magnitudes did not promote LDH release (Fig. 1).

In Vitro Simulated TBI Decreases Viability Only at High Levels of Stretch

In agreement with the LDH release data, only 50% stretch increased the number of dead cells (EthD-1 positive cells) to 5.3 per field, compared to 1 at 0% stretch (Fig. 2). Lower levels of stretch did not increase the number of dead cells, compared to control.

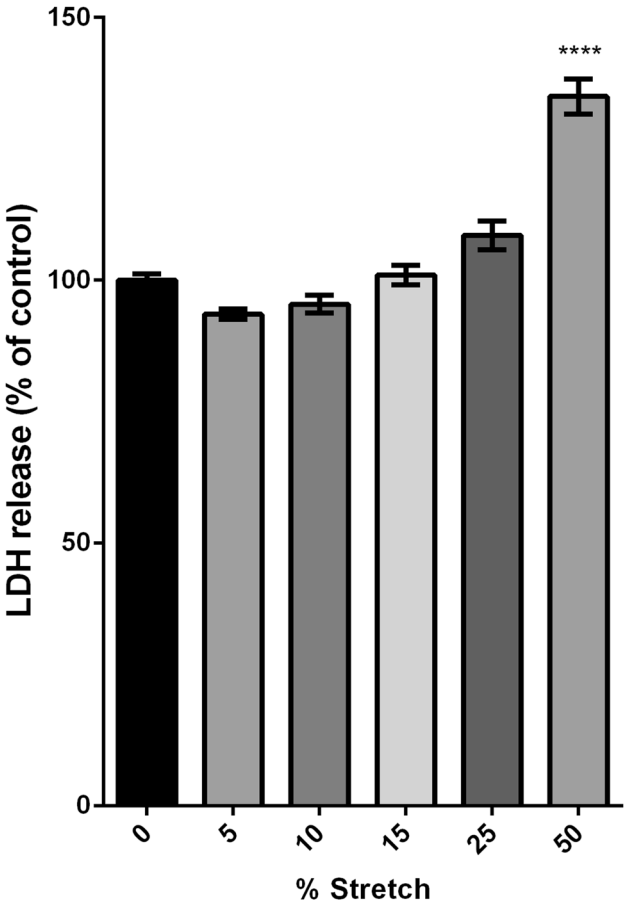

Fig. 1 In vitro simulated TBI increases LDH release. Human dopaminergic neurons were submitted to biaxial stretch-induced deformation at 0%, 5%, 10%, 15%, 25% and 50% for 50 ms. After 24 h, LDH release was measured as an index of necrosis. Each value represents the mean ± SEM of 5 independent experiments run in triplicate. ****$p < 0.0001$ versus control

In Vitro Simulated TBI Increases the Activity of Caspases 3 and 7 at Mid and High Levels of Stretch

To analyze if stretch-simulated TBI induces apoptosis, activity of caspases 3 and 7 was measured. Compared to damage of membrane integrity which was only observed at 50% stretch, an increase in the activity of caspases 3 and 7 was observed at 15%, 25% and 50% stretch. The number of caspase positive cells per field increased from 1.6 at 0%, to 6.5, 9.8 and 18.3 at 15%, 25% and 50% stretch, respectively (Fig. 3), with no changes observed at lower levels of stretch.

In Vitro Simulated TBI Increases DNA Fragmentation at High Levels of Stretch

To evaluate if an increase in the activity of caspases 3 and 7 was related to an increase in apoptosis, the presence of DNA fragments in the neurons' cytoplasm was measured. An increase in DNA fragments was detected at 25% and 50% stretch, increasing by 20.1% and 46.4%, respectively, compared to 0% stretch (Fig. 4). Lower levels of stretch did not increase DNA fragmentation.

In Vitro Simulated TBI Alters Neuronal DA Content

DA content in the cytoplasm of the neurons and DA released into the culture medium were quantified after stretch-induced injury. Intracellular levels of DA were decreased from 67.12 pg/mg at 0% stretch, to 37.93 pg/mg, 30.46 pg/mg and 24.42 pg/mg at 15%, 25% and 50%, respectively (Fig. 5a). On the other hand, extracellular levels of DA increased from 152.24 pg/mg at 0% stretch, to 313.61 pg/mg at 50% stretch (Fig. 5b) without significant changes observed at lower levels of stretch.

In Vitro Simulated TBI Decreases Levels of TH

Levels of TH were determined to evaluate if the decrease in intracellular DA was related with a decrease in its synthesis or an increase in its release. Control cells (0% stretch) presented values of 3174 arbitrary units (AU), while 15%, 25% and 50% stretch had significantly lower levels of TH with values of 1910, 1444 and 1493 AU respectively. No changes were observed at 5% and 10% (Fig. 6a, c). Levels of DAT were not changed by any of the stretch levels (Fig. 6a, b).

Discussion

In vitro models that simulate different characteristics of TBI pathology are increasingly being used to study specific mechanisms of injury or to evaluate potential therapeutic interventions [23, 24]. To confidently use these types of models in research, it is imperative to corroborate that the damage observed in vivo after TBI can successfully be replicated in vitro. In the present study we aimed to replicate the dopaminergic damage

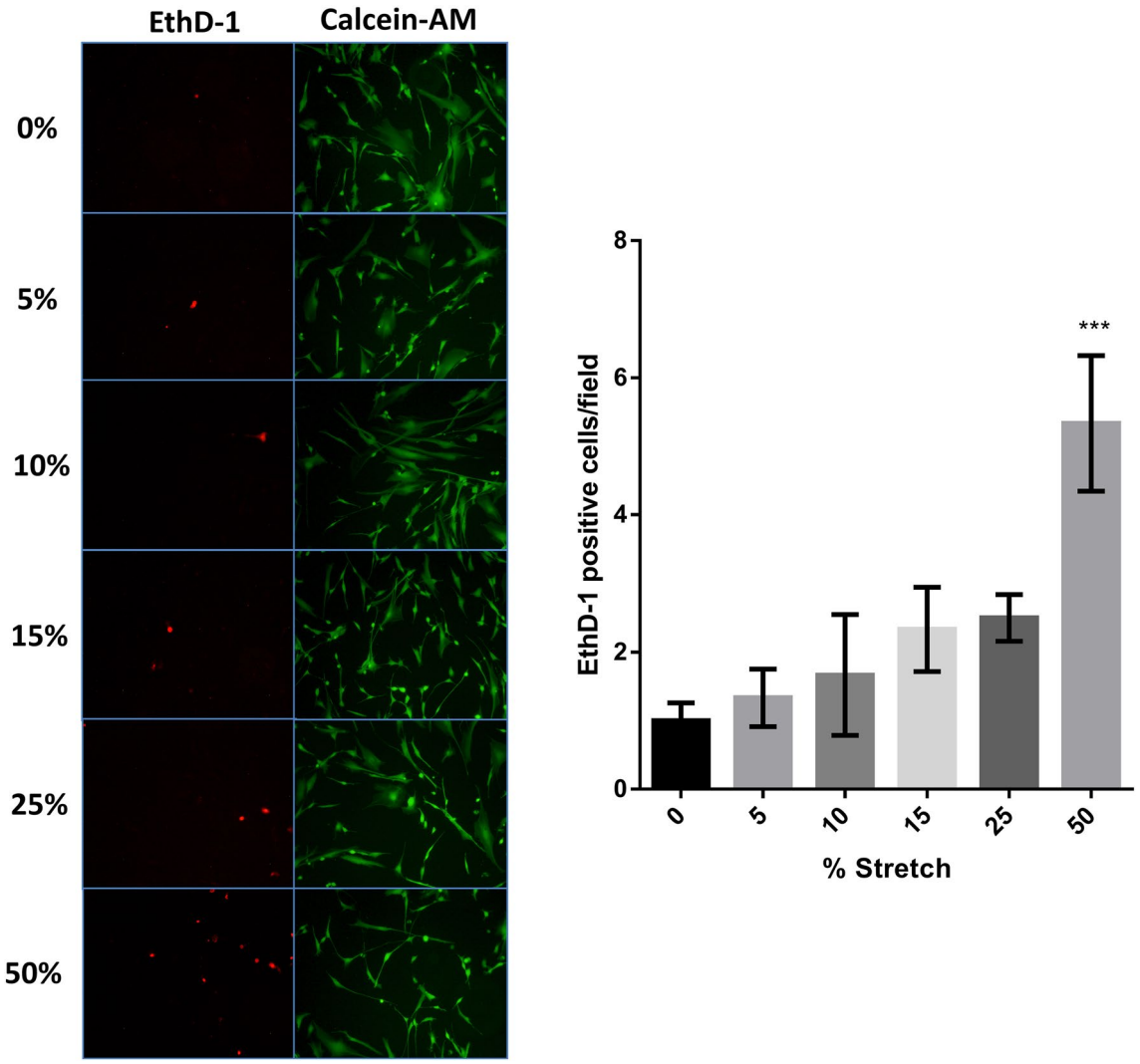

Fig. 2 In vitro simulated TBI decreases neuronal viability. Human dopaminergic neurons were submitted to biaxial stretch-induced deformation at 0%, 5%, 10%, 15%, 25% and 50% for 50 ms. The number of live (green) and dead (red) cells was determined using a commercially available kit, 24 h after injury. Images were captured at 4× magnification and the number of dead cells was determined using the ImageJ software. Each value represents the mean ± SEM of 6 different fields analyzed in 3 independent experiments. ***$p < 0.001$ (Color figure online)

observed after TBI using an in vitro model based on stretch-induced deformation of human dopaminergic neurons in culture. To model different severities of TBI we employed a wide-range of stretch-induced deformation of our neuronal cultures, reaching a maximum of 50%. These magnitudes of deformation are relevant to human TBI since published reports based on laboratory simulated events that result in TBI (traffic accidents, falls from ground level and sport-related injuries) have shown that the human brain experiences region-specific deformations of up to 60% in cases of brain trauma [46]. Therefore, the levels of deformation used in this study fall within the range observed in human TBI.

Neuronal death is one of the hallmark events in human TBI [2] and has also been successfully replicated in animal models [6, 8, 47]. In the present study we found that only very high levels of stretch-induced deformation (50%) induce necrosis, as evidenced by an increase in

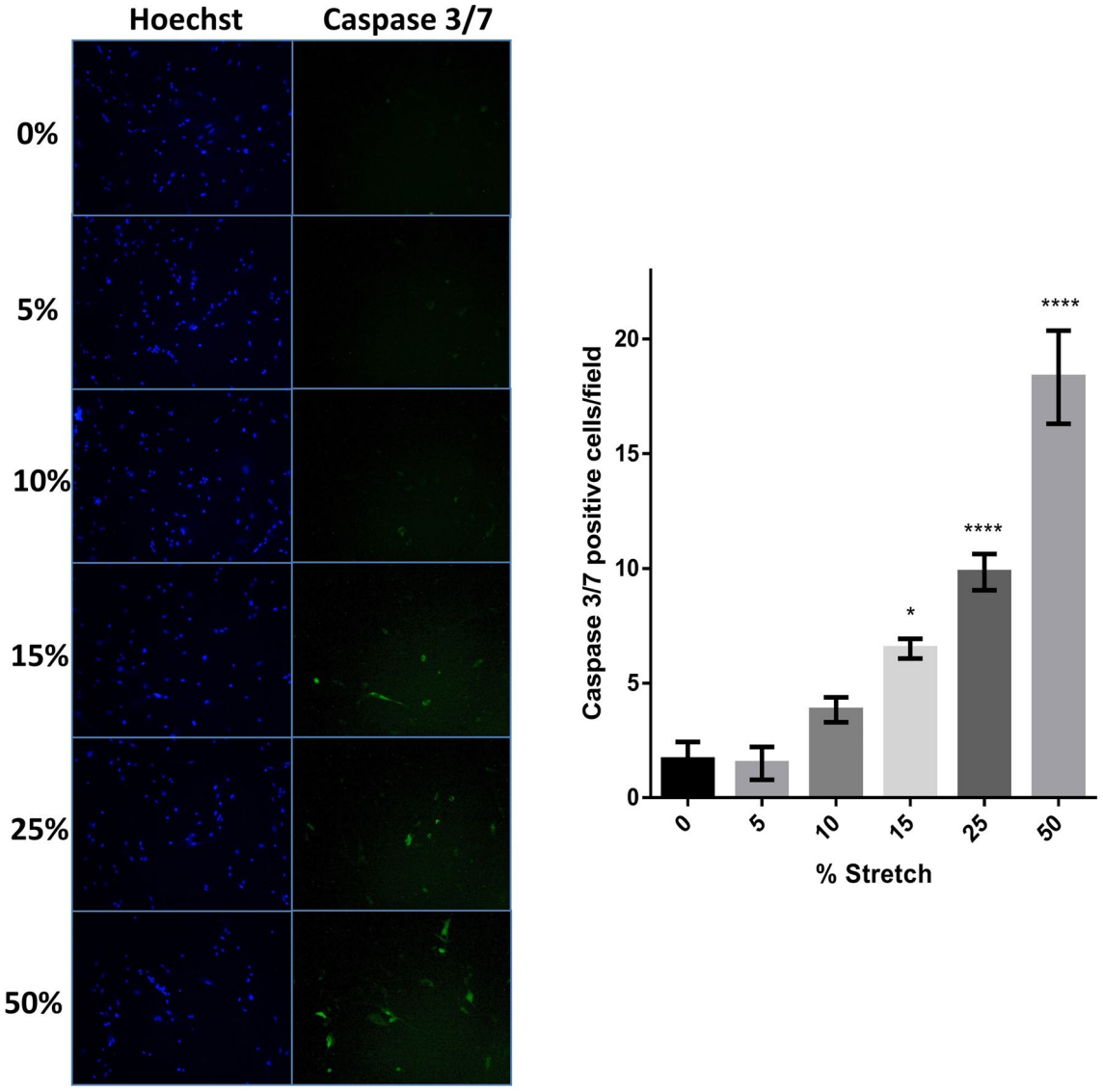

Fig. 3 In vitro simulated TBI increases the activity of caspases 3 and 7. Human dopaminergic neurons were submitted to biaxial stretch-induced deformation at 0%, 5%, 10%, 15%, 25% and 50% for 50 ms. Neurons with active caspases 3 and 7 (green) were detected using a commercially available kit, 24 h after injury. Nuclei were counter-stained using Hoechst (blue). Images were captured at 4× magnification and the number of caspases 3 and 7 positive cells were obtained using the ImageJ software. Each value represents the mean ± SEM of 6 different fields analyzed in 3 independent experiments. *$p < 0.05$ and ****$p < 0.001$ (Color figure online)

LDH release and EthD-1 staining, most likely a consequence of the mechanical primary injury. On the other hand, an increase in the activity of caspases 3 and 7 was observed starting at 15% deformation and increased in a magnitude-dependent manner. The increase in the activity of caspases 3 and 7 was related to an increase in neuronal apoptosis starting at 25% deformation. The discrepancy between the levels of stretch that induce necrosis versus apoptosis may be a consequence of different mechanisms of stretch-induced damage. The increase in LDH release (a hallmark of necrosis) is almost certain to be a consequence of the mechanical stretch experienced by the neurons, causing a disruption of the plasma membrane and subsequent LDH leakage.

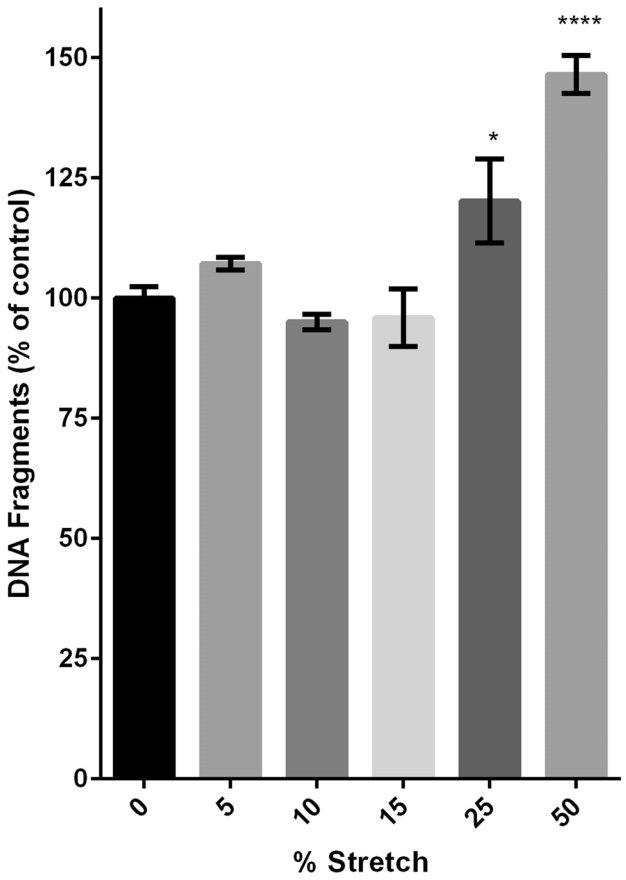

Fig. 4 In vitro simulated TBI increases DNA fragmentation. Human dopaminergic neurons were submitted to biaxial stretch-induced deformation at 0%, 5%, 10%, 15%, 25% and 50% for 50 ms. After 24 h, DNA fragments in the cytoplasm of the cells were measured as an index of apoptosis. Each value represents the mean ± SEM of triplicates from 3 independent experiments run in triplicate. *$p < 0.05$ and ****$p < 0.001$ versus control

On the contrary, in injured neurons with undamaged plasma membranes a secondary response to injury was triggered resulting in the activation of caspases that led to neuronal death by apoptosis.

Previous studies have evaluated the effects of stretch on the viability of neurons in culture. In primary cortical neurons 40% stretch induces apoptosis related to the activation of caspase-1 [6]. Similarly, cortical neurons subjected to stretch injury presented an increase in LDH release only at 54% stretch, but not at lower percentages [48]. These results are in accordance with our findings where only 50% stretch (but not lower levels) induced necrosis in human dopaminergic neurons. In a more comprehensive study analyzing the effects of a wide-range of injury conditions, Sherman and colleagues reported that neurons derived from human induced pluripotent stem cells show an injury phenotype characterized by cell death, shortened neurites and changes in neuronal shape starting at 35% stretch. Higher percentages of stretch increased the number of dead neurons, reaching a saturation at about 70% stretch [49]. In the present study and due to the limitations of the Cell Injury Controller II to induce injury, a maximum of 50% stretch was induced; however, we believe that higher percentages of stretch will induce higher levels of neuronal death and injury. Supporting this hypothesis Skotak and colleagues utilized the human dopaminergic SH-SY5Y cell line to determine the effect of stretch-induced injury on the viability of these DA-producing cells, reaching percentages of stretch of up to 140% [43]. They found that starting at 35% stretch, SH-SY5Y cells presented a decrease in viability, approaching a saturation point at 75%. This study was able to identify 3 different damage profiles, where mild injury is observed at < 30% stretch, moderate injury between 30% and 55% stretch, and severe injury at > 55% stretch [43]. These results are in agreement with the results presented here, as 50% stretch, the maximum utilized in this study, induced moderate levels of cell death.

Induction of cell death in dopaminergic neurons contributes to the dopaminergic dysfunction observed after TBI, which is also characterized by reduced striatal DA levels [50]. In addition, moderate to severe TBI can induce dopaminergic abnormalities in humans, as observed by single proton emission computer tomography, indicating that DA transport is impaired and suggesting reduced striatal DA as a consequence of TBI [13]. In the present study we observed an increase in extracellular DA only at 50% stretch. This effect may be attributed to the exit of DA through the compromised plasma membrane caused by this high magnitude stretch. On the other hand, a decrease in intracellular DA was observed at 15%, 25% and 50% stretch, depleting the intracellular storage of DA, leaving less DA available for its release. The decrease in intracellular DA was related with a decrease in TH, the rate-limiting enzyme in the synthesis of DA at the same percentages of stretch. The decrease in TH expression suggest that the decrease in intracellular DA is due to a decrease in its synthesis, rather than an increase in its release. In this regard, animal studies have shown that TBI suppresses the release of DA from the striatum [16] and nucleus accumbens [15, 51] and that the magnitude of this effect depends on the severity of injury.

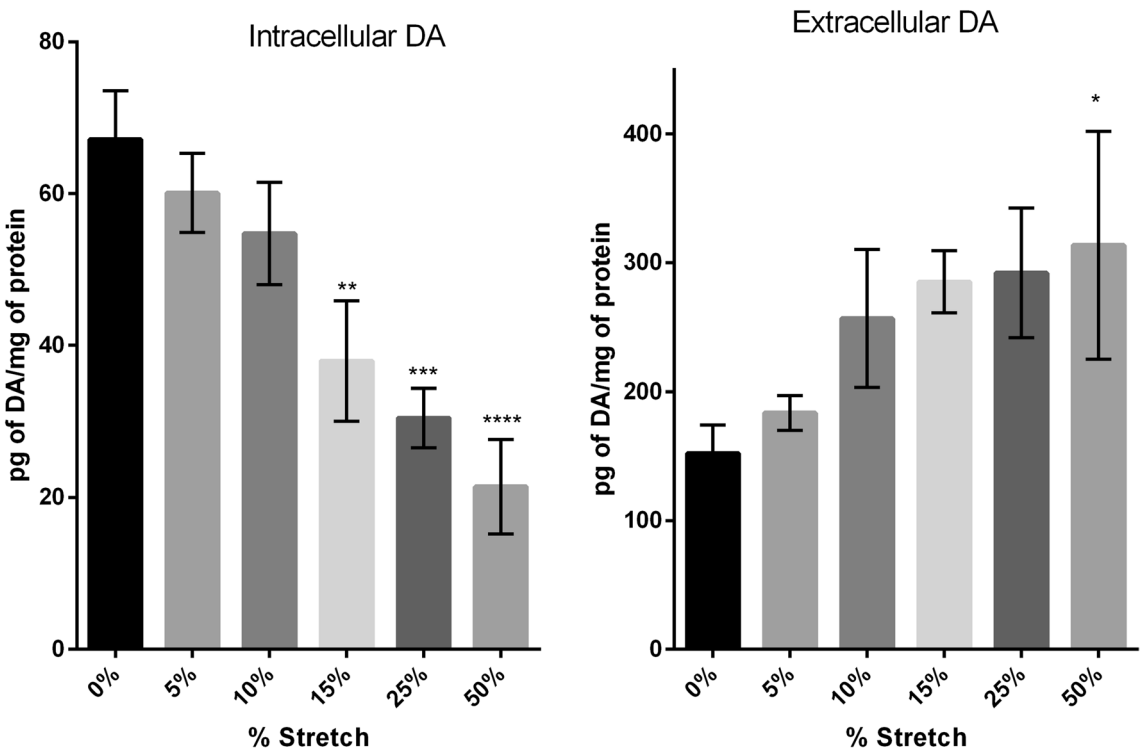

Fig. 5 In vitro simulated TBI alters neuronal dopamine content. Human dopaminergic neurons were submitted to biaxial stretch-induced deformation at 0%, 5%, 10%, 15%, 25% and 50% for 50 ms. After 24 h, intracellular dopamine (**a**) and dopamine in the culture medium (**b**) were quantified by ELISA. Each value represents the mean ± SEM of 6 independent experiments run in duplicate. *p < 0.05, **p < 0.01 and ***p < 0.001 versus control

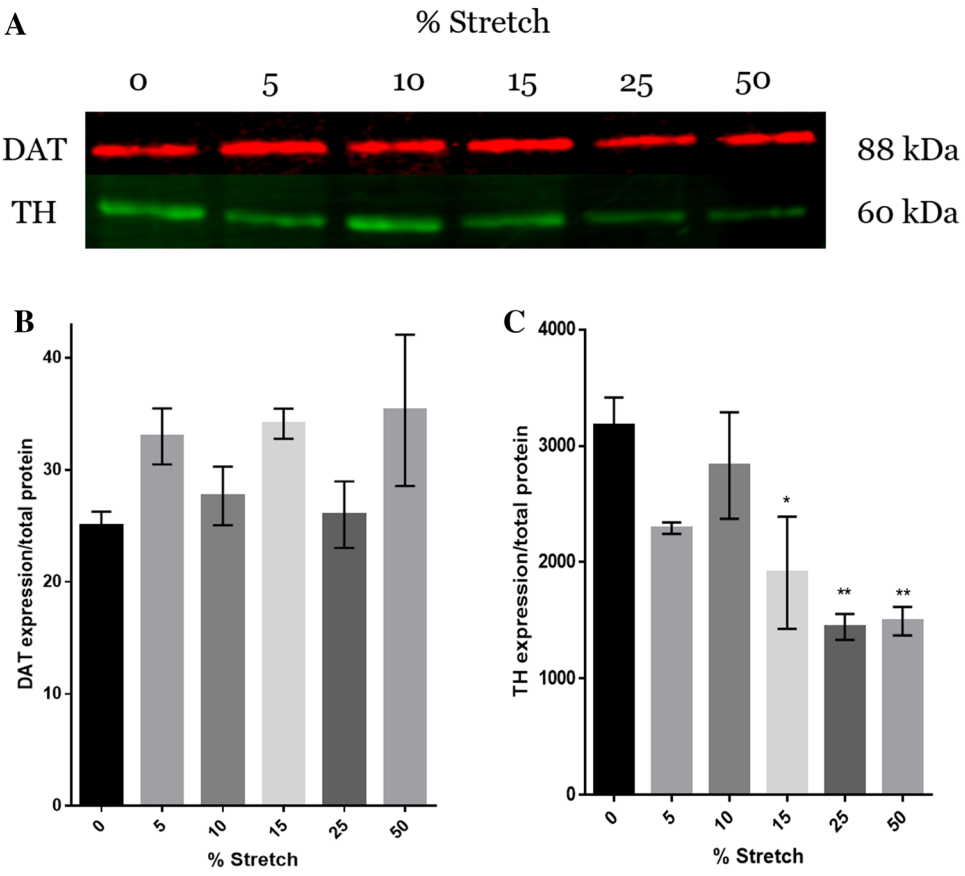

Fig. 6 In vitro simulated TBI decreases TH levels. Human dopaminergic neurons were submitted to biaxial stretch-induced deformation at 0%, 5%, 10%, 15%, 25% and 50% for 50 ms. After 24 h, levels of DAT (**a**, **b**) and TH (**a**, **c**) were evaluated by western blot. Intensity values were normalized by total protein. Each value represents the mean ± SEM of 3 independent experiments. *p < 0.05, **p < 0.01 versuss control

In summary, we have demonstrated that stretch-induced deformation can be used to model the effects of TBI in vitro using human dopaminergic neurons. We were able to replicate the induction of cell death and apoptosis observed in mild and moderate TBI. In addition, and to the best of our knowledge, this is the first report in which the decrease in intracellular DA observed after TBI has been modeled using cells in culture. Therefore, we propose the use of stretch-induced deformation using dopaminergic neurons as a tool to model the effects of TBI on the dopaminergic system. This model can be of particular use for initial screening of potential neuroprotective agents for TBI and also to study the effects of TBI on dopaminergic neurodegeneration that can lead to the development of Parkinson's disease and other neurodegenerative conditions.

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