Chapter

Early Cell Response to Mechanical Stimuli during TBI

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Abstract

Traumatic brain injury (TBI) refers to brain damage resulting from external mechanical forces such as a blast or crash. The tissue and cell deformations caused by shear forces are the most common pathological features in TBI and lead to long-term symptoms. Our current understanding of TBI derives mainly from *in vivo* studies of poststimulus pathology and the effects on brain function. Little is known about the early responses of brain cells during mechanical stimuli. In this chapter, we evaluate the early cell response to the rapid shear forces *in vitro*. We introduce advanced technologies capable of generating fast shear stimuli mimicking forces occurring in TBI and reporting internal forces in specific proteins at the time of injury. We define the threshold shear forces for calcium influx using an astrocyte model. We describe the spatiotemporal distribution of cytoskeletal forces and correlate them with variations in cell membrane tension. This chapter makes a strong argument that cells' response to external forces is nonlinear. The heterogeneous distribution of cytoskeletal tension and the gradient of protein forces play a key role in the cells' response to mechanical stimuli.

Keywords: traumatic brain injury (TBI), cell mechanics, astrocytes, shear stress, cytoskeletal forces, cell membrane tension

1. Introduction

Traumatic brain injury (TBI) is a form of acquired brain injury that is caused by abrupt external mechanical forces. TBI occurs in both civilians and military veterans from events such as an explosive blast, a blunt impact, and uncontrolled acceleration, deceleration, or rotation of the head. The injury occurs in two phases: a primary injury caused by mechanical forces that occur at the incipient moment of injury and a secondary injury that involves subsequent biochemical and metabolic dysfunction [1–3]. Depending on the type of forces, the injury is normally classified as either a focal injury that results in cerebral contusions in a specific location or a diffuse brain injury that occurs over a widespread area due to shear forces generated by rapid acceleration or deceleration [4–6]. While the focal injuries are common in moderate to severe brain injuries, diffuse injuries are the main cause of mild TBI [7]. The deformations caused by shear forces are more difficult to define because they occur throughout the brain and are often invisible to commonly available imaging techniques at the early stages of injury [8, 9].

Most TBI patients exhibit mild or minimally observable damage upon the initial shock, but serious pathology can develop within hours and days [8, 10, 11]. Cell death may proceed from multiple locations within the brain. Many of these sites contain cells

with no initially observable injury or proximity to the damage site [12]. Thus, brain cells can respond to low mechanical forces that do not cause an immediate structural damage. Understanding early cell response to mechanical stimuli will help provide valuable insight into the origin and evolution of long-term pathological changes.

Our current understanding of TBI is derived from both *in vitro* and *in vivo* studies. *In vivo* studies provide data on the state of cells well after (hours to days) the injury and probably reflect injuries from downstream processing [13–15]. *In vitro* models, using cultured cells, examine cells' response at the expense of normal physiological interactions [2, 16]. The applied forces for *in vitro* models are often not on the same timescale as TBI. Recent research has begun using the so-called next-generation injury assessment tools to study specific cell responses in real time.

This chapter examines the earliest cell responses during TBI as seen in astrocytes via changes in Ca^{2+} levels *in vitro*. In this chapter, we will introduce advanced technologies that can generate fast shear stimuli mimicking forces that cause TBI and can measure internal forces in specific proteins at the very point of time of injury. We will determine the features of force stimuli that are most susceptible to cells and that lead to long-term alterations. The mechanical properties of cells will be evaluated by real-time measurements of forces in specific cytoskeletal proteins and in cell membranes.

2. Brain structure and traumatic brain injury

2.1 Head structure and brain cells

The brain by its very nature is made of soft substances that are submerged in a thin layer of fluid inside the skull. Underneath the skull, there are multiple layers of tissues separated by intracranial spaces containing water-like liquid, called the cerebrospinal fluid (CSF), as shown in **Figure 1**. It is believed that this fluid plays an important role in the shock-absorbing capacity of the brain. Further inwards, it is the brain itself that consists of a network of functional brain cells. These cells are arranged into several specialized areas, each performing distinct physiological functions.

During TBI, the transient external forces cause linear and angular accelerations, resulting in a range of injuries to the brain. Two main types of injuries may occur: the impact injury due to the brain directly hitting the skull at the point of impact that also generates a whipsaw effect at the opposite side of the brain [17]; and the shear injury due to relative movements between the brain and surrounding tissues and between tissues of different densities [18]. For example, with a sudden acceleration, movement of the brain lags behind that of the skull, producing shear stresses at various interfaces between the brain and the cortical tissues. Similarly, in a deceleration injury, the brain continues its inertial path after the skull has been abruptly halted. Shear injuries commonly occur at gray/white matter junctions, but they are also found in the deeper white matter of the corpus callosum, brain stem, and cerebral cortex [19, 20]. The shear strains and stresses are responsible for producing the loss of consciousness during diffuse axonal injury that accounts for ~60% of hospitalized TBI cases [9, 21].

2.2 Brain cells

Two major cell types in the brain are the neurons and glia. Astrocytes are the most abundant glial cells. While the neurons play the role of processing and transmitting information, astrocytes provide the critical link between the circulatory system and the structural support and the maintenance of neurons (tripartite synapses, neurotransmitter processing, etc.) [22]. **Figure 1** (zoom-in panel) illustrates the configuration of the brain cells. During TBI, astrocytes transmit

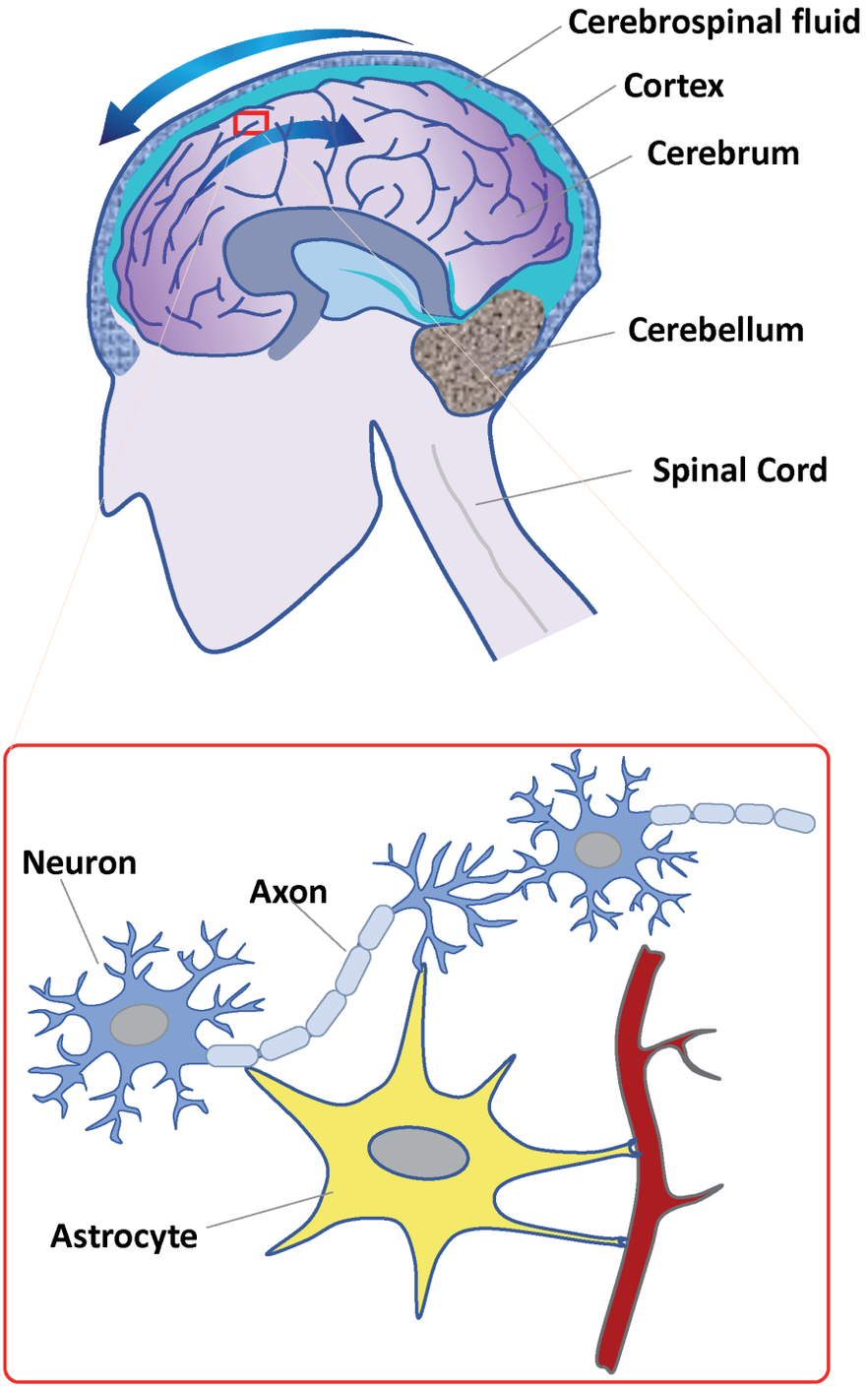

Figure 1.
Schematic illustration of brain structure and brain cells.

mechanical forces to neurons and (by Newton's third law) accept forces from them. They can also propagate damage signals via Ca^{2+} waves, signal to neurons through neurotransmitters, and alter ion concentrations in the extracellular space [22, 23]. By applying a blast-like overpressure to brain slices, it has been shown that astrocyte injury *precedes* the neuronal injury. This raises the possibility that astrocyte Ca^{2+} may be one of the earliest responses modulating the brain function [24].

2.3 Earliest cellular events in injury models

Two early events that are caused by external forces are known: a Ca^{2+} influx and an associated neurotransmitter release [25–27]. An increase in intracellular Ca^{2+} is universally seen in brain cells that have been subjected to mechanical perturbations

regardless of the nature of the forces [28–30]. There are many Ca^{2+}-dependent processes that can be activated during TBI, including cell swelling [31–33], cytoskeletal disruption [34–36], membrane poration [37, 38], failure of ATP-dependent membrane pumps [39, 40], and mitochondrial dysfunction and apoptosis [41–44]. The Ca^{2+}-loaded or injured neurons release neurotransmitters including glutamate [45, 46]. The release of glutamate and other excitatory amino acids, in turn, can lead to cytotoxic injury and cell death [47–49]. These occurrences clearly show that the early responses of brain cells to mechanical stimuli are responsible for the subsequent pathology.

3. Shear forces and their assays

3.1 TBI-induced internal forces

The external force results in the development of internal forces within the skull, causing brain injury. Several types of internal forces can occur depending on the type of incidents. For instance, a blast-generated shockwave initially changes the pressure inside the skull. The abrupt motion of the head causes the acceleration or deceleration to generate compression, stretching, and shear forces at multiple places inside the skull [2].

In vitro studies using cultured cells show that excessive pressure (above 10 atm) is required to stimulate a cellular response. This level of pressure is much higher than the overall pressure measured *in vivo* within the brain [50]. Using a blast chamber to apply rapid pressures and shear forces to cell cultures, Raven et al. showed that cells are more responsive to shear forces than to hydrostatic pressures [51].

Tensile forces are primarily localized at impact points and have been commonly studied using stretching methods. In stretching models, cell cultures or tissues are placed on a flexible substrate that can be stretched with a vacuum pulse [52] or by piezoelectric actuators [53]. In these studies, cells produced a measurable Ca^{2+} response only when they experienced very large strains (up to 40%) [54–56]. In contrast, an overall cell deformation of ~4% due to shear pulses was sufficient to cause a similar Ca^{2+} response [57]. These studies show that brain cells are more susceptible to shear forces than other types of internal force.

Most of the studies that have investigated diffusive brain injury due to shear forces have focused on the injury of neurons. A sliding between two tissue layers can break the long thin nerve fibers (called axons) of neurons by their extension across the layers [58, 59]. However, shear stresses have been found to induce drastic responses in cells (neurons and astrocytes) even without any noticeable damage to the axons, leading to cell damage and cell death [60]. In other words, subcellular signaling is likely to be a more causal pathway than obvious physical damage in these cells.

3.2 Microfluidic assays for shear forces

Earlier studies variously used a rotating disk, air blow, or pulsed media to apply shear stresses to the cells [61–63]. Following the application of force stimuli, it was found that transient shear deformations tended to increase the membrane permeability to Ca^{2+} and small dye molecules and decrease the cell viability [64]. A blast chamber was also used to apply rapid pressure and shear forces to cell cultures. It was found that Ca^{2+} response was more sensitive to shear forces than to hydrostatic pressures [51]. These methods provide controlled shear stimuli, followed by live cell measurements. In real scenarios of TBI, the forces increase in milliseconds, and small elastic deformations in cells occur in real time [16]. Thus, the challenge for the shear stress assays is the ability to rapidly ramp the shear forces and an ability to reliably measure cells' response in real time.

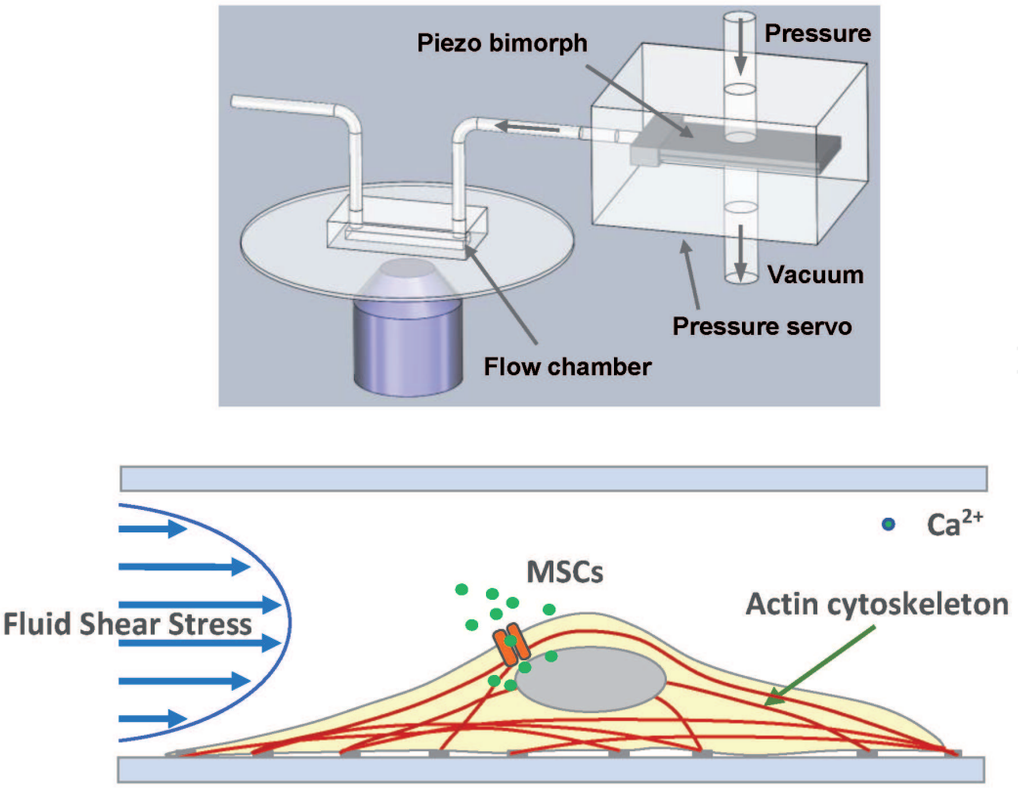

Figure 2.
Schematic of the pressure servo-driven microfluidic chamber. High-speed pressure servo is connected to a microfluidic chamber to generate controlled fluid shear stimuli on cultured cells.

In this chapter, we introduce a high-speed programmable microfluidic assay tool to apply precise shear forces to cultured cells [30]. The fluid shear is controlled by a fast pressure servo [65] that is capable of generating pressure pulses with a time resolution of ~1 ms in the microfluidic chamber [30, 65]. The millisecond resolution allows the ability to program arbitrary waveforms emulating the waveforms recorded in TBI [66]. The microfluidic chamber allows high-resolution optical microscopy, which enables *in situ* measurements of changes in intracellular Ca^{2+} and protein forces in single cells during the very application of forces. **Figure 2** displays the schematic of the experimental setup.

4. The response of cells to transient shear forces

The severity of TBI is conventionally classified as mild, moderate, or severe, depending on the extent of the damage to the brain tissue. In the 1950s, the head injury tolerance to external forces began to be evaluated by dropping embalmed corpse heads on a rigid surface and examining the resulting profile of the skull fracture. Linear skull fracture came to be used as the criterion of injury. These studies led to the first quantitative tolerance curve to be established, now known as the Wayne State Tolerance Curve (WSTC) [67, 68]. This curve describes the influence of magnitude and duration of external loads on the severity of injury to the head.

However, the internal forces are not directly proportional to the external load, which comprise a more complex load profile depending on the type of forces. It is now also known that most cells show a physiological response at much lower internal force levels that are below the injury criteria. Therefore, the thresholds for specific internal forces need to be defined.

4.1 Shear stress thresholds

Using the previously described high-speed servo to generate well-defined shear stresses in a microfluidic chamber, the threshold shear stress to cell response in cultured astrocytes was analyzed. Ca^{2+} rise being the earliest measurable signal in cells, the Ca^{2+} rise in the cells was measured in real time. The thresholds were defined with multiple input parameters including magnitude, duration, and load rise time.

The cells responded reliably to a shear stimulus of 23 dyn/cm^2 with a transient Ca^{2+} increase, but they did not respond up to a pulse of 11.5 dyn/cm^2, thereby setting an effective threshold [30]. However, the amplitude of the force stimuli is not the only parameter, and a variety of relaxation times has been observed. Changes in pulse duration and rise time also affect the subthreshold responses. For example, a low amplitude of short duration (11.5 dyn/cm^2, 10 ms) did not trigger a measurable Ca^{2+} elevation, whereas a pulse width of 1000 ms of the same amplitude did. Together, the two parameters establish a multidimensional threshold matrix for pulses with a square profile, as shown in **Figure 3**. The relative significance of each stimulus

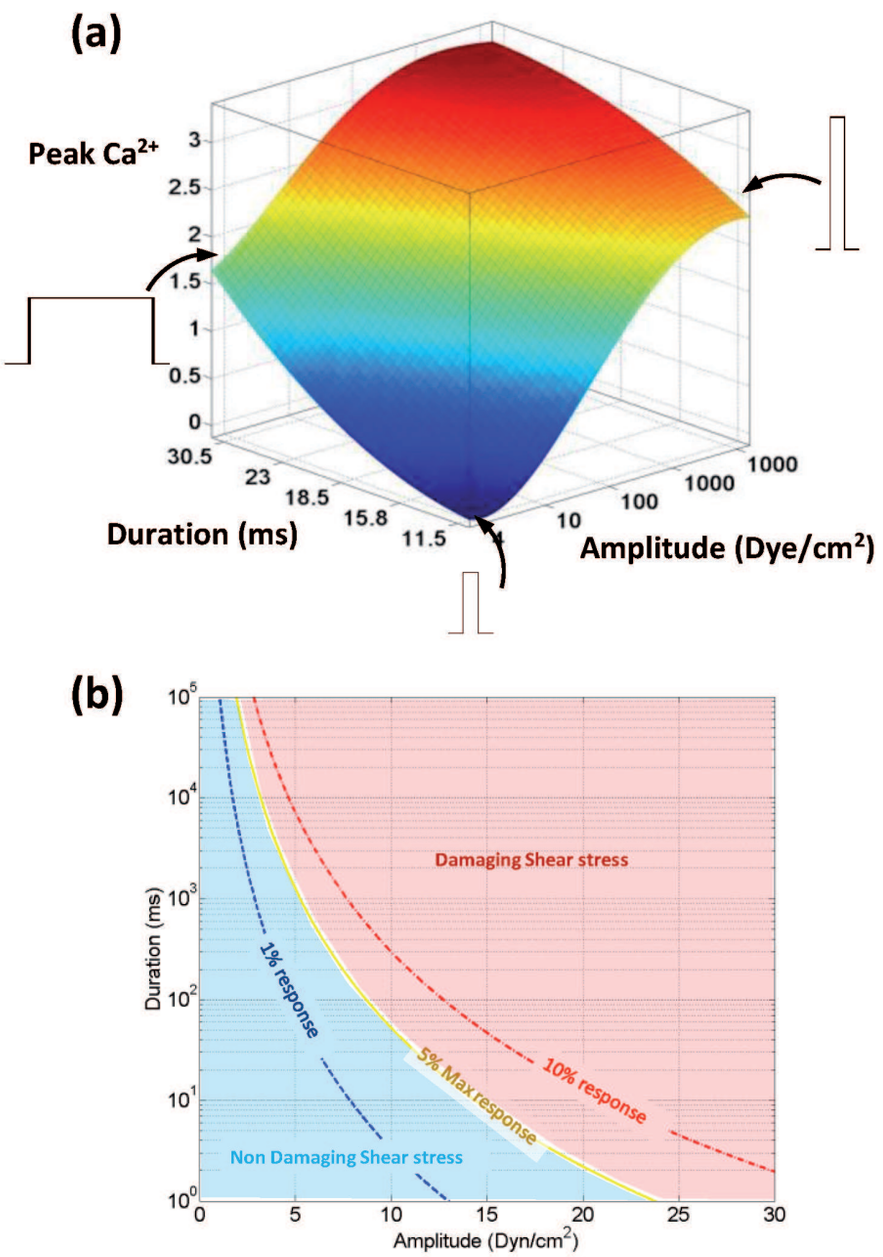

Figure 3.
(a) Profile of shear stimulus thresholds. (b) The damage threshold is defined by a 5% maximum response.

parameter was evaluated by fitting the profile of Ca^{2+} response with a two-state Boltzmann equation, which shows the amplitude is the primary determinant [30].

4.2 Cell response is nonlinear

The cell response to shear stimuli is nonlinear. The nonlinear characteristic of Ca^{2+} response is demonstrated in **Figure 4**. For example, an abrupt increase in shear stress caused the Ca^{2+} rise, but a slow increase to the same amplitude failed to activate the cells, as shown in **Figure 4(a)**. The cell sensitivity decays rapidly with an increase in force rise time. Moreover, a brief mechanical shock is more important than a sustained shear force. As shown in **Figure 4(b)**, at short intervals of 10 ms, the pulse train caused a response that was three times higher than a single pulse of the same amplitude and a total pulse duration of 100 ms [30]. Thus, the total energy input of the stimulus is not the only determinant in Ca^{2+} response; the kinetics of the stimulus (force loading rate) also plays an essential role. This emphasizes the viscoelastic/plastic nature of the force transduction processes in cells [64, 69]. By using sine wave stimuli of different frequencies, it has been shown that cells' response is also frequency dependent; the peaks in response occur at a higher frequency (>80 Hz) or a lower frequency (<20 Hz) [30].

The strain rate dependence of cell injury has been observed in 3D matrix cultured neurons and astrocytes, showing an increase in membrane permeability to small molecules and an increase in post-insult cell death [70, 71]. However, this effect was not observed in hippocampal tissues under biaxial stretch at strain rates ranging from 0.1 to 50 s^{-1} [72]; this rate of ramping is likely outside the sensitivity

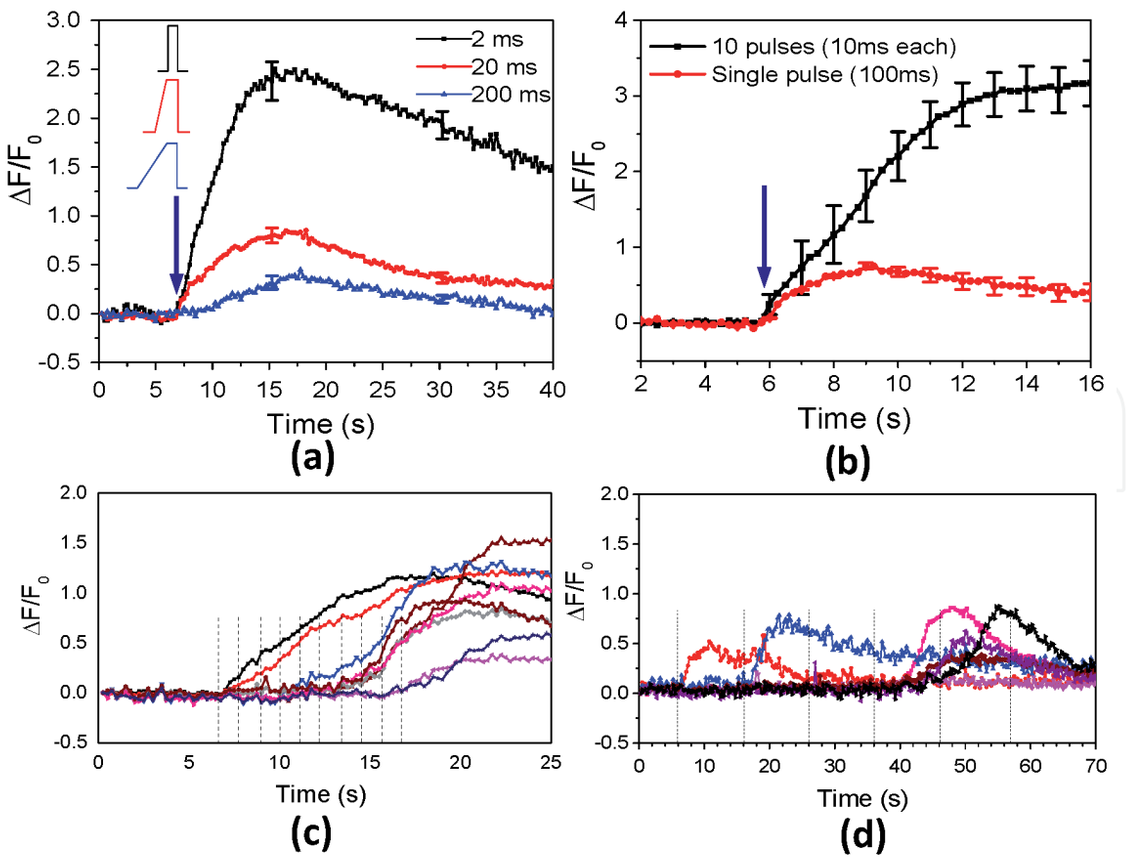

Figure 4.
Nonlinear Ca^{2+} response of cells. (a) Ca^{2+} response to shear pulses of different rise times. (b) Ca^{2+} response to a train of 10 narrow consecutive pulses (10 ms wide) is three times higher than a single broad pulse (100 ms wide) of the same amplitude. (c) and (d) Ca^{2+} response to repeated pulses (11.5 dyn/cm², 10 ms) with intervals of 1 s and 10s, respectively, showing that the response is nonlinear.

range of the cells [73]. In addition, different cell types have been found to exhibit different properties, which is not surprising.

4.3 Response to repeated challenges

It has been known for a long time that repeated low-amplitude shocks that never had any clear damage to the cells could be lethal in animal models of TBI [74–76]. This suggests that low subthreshold mechanical forces can be registered and accumulated by the cells. In other words, cells have a long-term pernicious "memory" to repeated stimuli. To study this memory effect, cells were exposed to repetitive low-amplitude stimuli that alone are not able to produce a Ca^{2+} rise. The response to a pulse train with 1 s intervals of 10 ms duration revealed that individual cells were activated at different times. About 20% of the cells responded to the second pulse, while 60% of the cells responded to the sixth and subsequent pulses (**Figure 4(c)**). Reducing the frequency sufficiently can eliminate the response (**Figure 4(d)**). There are two possibilities for this behavior: the plastic mechanical deformation due to cytoskeletal bonds break and reform, or the accumulation of second messenger (Ca^{2+}) pools. Recent studies on cytoskeletal protein forces show that rapid shear stress can generate prolonged cytoskeletal tension, implying that the cytoskeletal deformation plays a key role.

As discussed in detail in the following sections, cells withstand mechanical loading through support from the cytoskeleton consisting of dynamically cross-linked structural proteins [77–80]. The deformation of a cell depends on the intrinsic elastic deformation of fibrous cytoskeletal proteins and plastic deformation involving reversible cross-linking and cytoskeleton reorganization [81, 82]. Slow shear loading engages the plastic processes that significantly modify the local forces around the Ca^{2+} transducers.

The nonlinear response of cells to external forces provides evidence that cells use complex force transduction mechanisms to register the forces. Understanding the force transduction pathways is an important step toward understanding the effectors that lead to TBI pathology.

5. Force transduction mechanisms

While fluid shear stresses act on the apical surface of the cells, this force can be transmitted to force-sensitive molecules that trigger ionic trafficking such as Ca^{2+} influx and other biochemical reactions via a variety of mechanosensitive mechanisms, a process called mechanotransduction. Recent studies suggest that shear stress transduction is mediated by the cytoskeleton, a filamentous network that connects different regions of the cells and holds the mechanical structure of cells [83–85].

5.1 Role of actin cytoskeleton in force transduction

The cytoskeleton is the universal mechanical structure of animal cells and responsible for cell shape, topography, and facilitating cell locomotion [86]. Cells resist the external mechanical forces via the cytoskeleton. The cytoskeleton consists of filamentous proteins that are interlinked to form filaments. Actin filaments are the smallest type of filaments. Single filaments of actin bind together via cross-linking proteins, such as α-actinin, forming a network that connects different regions of the cells. Actin bundles are found across the cell body; actin mesh is also found beneath the cell membrane, supporting the plasma membrane.

Like many other subcellular organelles, the actin cytoskeleton can be affected during TBI. Cytoskeleton damage has been observed in various moderate to severe

TBI conditions [87, 88]. A number of studies now suggest that trauma-induced neuronal cell death may be preceded by the disruption of the neuronal cytoskeleton [89, 90]. Through its connections, the cytoskeleton can also transmit forces to different functional elements of the cell to alter their functions [86, 91–93]. Among them are the mechanosensitive channels (MSCs) that permeate cation ions such as Ca^{2+}.

5.2 Mechanosensitive channels and Ca^{2+} signaling

Mechanosensitive ionic channels are transmembrane proteins that form a pore structure across the cell membrane. These channels are linked to the cytoskeletal proteins; thus, changes in cytoskeletal stresses may open the mechanosensitive channels [94, 95]. Similarly, changes in membrane tension could also alter the channel configuration.

Several MSCs have been identified in astrocytes that are members of the transient receptor potential (TRP) family including TRPV4, TRPC1, TRPC5, and TRPA1 [96–98, 99]. Studies of sensory neurons have suggested that Piezo channels play an important role in brain cells [100, 101]. Piezo-type MSCs are also present in astrocytes that can be inhibited with specific Piezo channel inhibitor [102]. Using an astrocyte model, a recent study has shown that N-Methyl-D-aspartic acid receptors (NMDARs) are the primary Ca^{2+} source in astrocytes and fluid shear stimuli can activate NMDARs in the absence of agonists [57].

The fluid shear forces can alter the channel activities via several mechanisms. They can modify the cytoskeletal stresses, and the cytoskeleton under high tension can pull the channel proteins via their links. Many MSCs are known to link with the actin cytoskeleton with cross-linking proteins [103–105]. In astrocytes and neurons, α-actinin binds to NMDA receptors, providing a mechanical link between NMDA receptors with the underlying cytoskeleton [106]. Shear stress can also cause transient deformation and bending of the lipid bilayer, altering the MSCs directly [107–109]. Most likely, both mechanisms are correlated.

6. Heterogeneous force distribution in single cells subjected to shear pulses

The shear-induced strains and stresses in the cytoskeleton could be the core of the mechanical response of a cell. Since the cytoskeleton is an anisotropic collection of dynamically cross-linked structural proteins, the cytoskeletal stresses are intrinsically nonuniform. This heterogeneity could be responsible for the nonlinear behavior of cells in response to shear stresses.

6.1 Cytoskeletal force measurements

The recent development of force probes based on fluorescence resonance energy transfer (FRET) technique [110–113] provides a powerful tool to observe cytoskeletal protein forces in live cells. The probe consists of two mutant fluorophores, a donor and an acceptor, linked with an elastic structure. Mechanical forces change the distance and/or the angle between the donor and the acceptor, resulting in a change in the energy transfer efficiency from donor to the acceptor [112, 113]. These probes were genetically encoded in cross-linking proteins, such as α-actinin and actin, and they are being used to report the strains in cytoskeletal linking proteins when cells are subjected to shear pulses. Using FRET probes, the distribution of protein forces induced by fluid shear stress has been mapped in real time [114].

6.2 Heterogeneous force distribution in single cells

By inserting the FRET-based force probes in actin-binding protein (α-actinin) in astrocytes, the cytoskeletal protein response to shear stress was measured for the first time. Results show that rapid shear stimuli generate nonuniform protein forces in single cells. **Figure 5** demonstrates that a narrow square shear pulse produced an immediate increase in force in α-actinin at the upstream end of the cell and a small negative force at the downstream end, resulting in a force gradient along the flow direction. In contrast, a slow ramp to the same force amplitude caused a minimal and more uniform change in actinin force. Moreover, the cytoskeleton structure and its prestress states also influence the cell response. The shear pulse produced significant internal force gradients in softer cells that have fewer bundled actin filaments [114]. These experiments demonstrate that uniform shear stress can generate heterogeneous cytoskeletal forces in single cells. Importantly, the time dependence of the stimulus plays a critical role.

While fluid velocity in the flow chamber was precisely controlled, the body stresses produced in the cells depend on cell geometry and the distribution of stresses within the cytoskeleton (we now know that stress distribution is heterogeneous). This heterogeneity likely accounts for the observed variability between cells. Thus, an averaged impact force cannot be used as a parameter to specify the activation of cell activities; the stress at local points can be an order of magnitude higher than the average.

If the local cytoskeletal stresses are the direct cause of Ca^{2+} rise, then they should be correlated in time and space. This correlation cannot be resolved by simple statistical comparisons. The distribution of forces in the structural proteins and the changes in Ca^{2+} was measured simultaneously using a Quad-View optical imaging system. It was found that the activation of Ca^{2+} began in regions of higher strain,

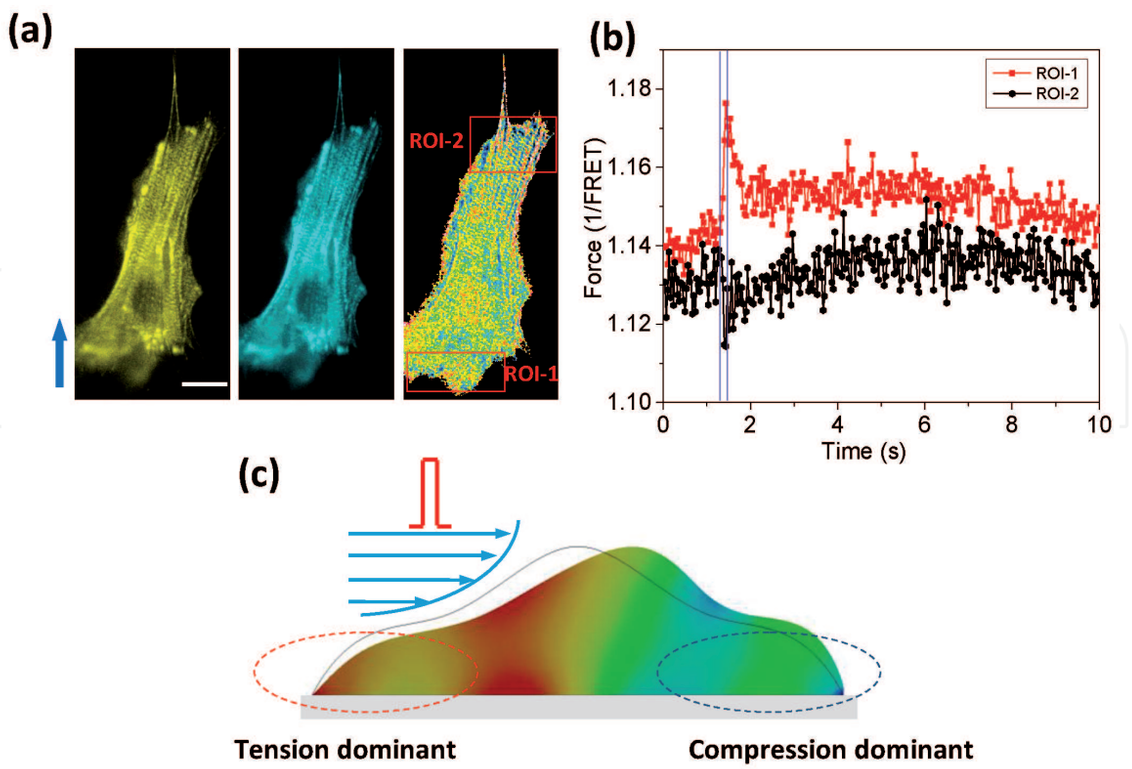

Figure 5.
Rapid fluid shear pulse generates nonuniform force distribution in astrocytes. Panel (a) shows fluorescent images (YFP, CFP) and inverse FRET ratio representing actinin forces in an astrocyte cell. (b) Changes in actinin force in two ROIs in response to a square shear pulse (23 dyn/cm², 15 ms), showing that the narrow shear pulse produce tension in actinin at the upstream edge and compression at downstream edge. (c) Finite element analysis model of a viscoelastic cells under fluid shear stress.

which normally occurred at the upstream edge of the cells and propagated from the edge to the somata of the cell as a wave [114]. In animal models, the NMDAR subunits are found primarily in the astrocytic processes [115]. This finding supports the earlier speculations that applied force may primarily affect the processes where the cytoskeletal structure is most pronounced.

We have noticed that shear stresses above the Ca^{2+} threshold did not disrupt cell adhesions nor produced any visible changes in cytoskeletal structures, indicating that the activation of Ca^{2+}-permeable ion channels require much lower forces than breaking of the bonds or irreversible deformations of the cytoskeleton.

7. Shear-induced cell membrane tension

Since the mechanosensitive ion channels are membrane bound, changes in cell membrane tension inevitably affect the ion channel configurations that mediate Ca^{2+} influx. Indeed, it is known that mechanosensitive channels can be opened by membrane tension [116, 117].

7.1 The cell membrane

The cell membrane consists of a lipid bilayer incorporating the membrane proteins including integral proteins such as transmembrane ion channels and receptors and peripheral proteins that loosely attach to the outer side of the cell membrane. Through the functional proteins, the cell membrane selectively controls the transport of ions, water, and macromolecules between the intracellular and extracellular compartments. Inside the cell, the lipid bilayer intimately adheres to the cortical cytoskeleton that provides the support for membrane topography and integrity [118]. On the outside is a hair-like structure called the glycocalyx. Depending on the cell type and local environments, the cell membrane may have tension at its resting state, called pretension. Several factors contribute to the pretension, including internal forces exerted by the cytoskeleton, osmotic pressure from the cytosol, and the forces resulting from cell-substrate interactions at adhesions that can be passed by cytoskeleton [119].

7.2 Effect of shear stress on cell membrane tension

The bilayer tension can be measured using lipid-soluble molecular rotor probe FCVJ [120] whose mobility is commonly used to extract the lipid bilayer fluidity or viscosity. An increase in bilayer tension increases the fluidity in cell membrane, causing a decrease fluorescent intensity of probes [121]. We measured the bilayer tension in astrocyte membranes using the molecular rotor probe FCVJ with the above-described microfluidic chip. As demonstrated in **Figure 6(a)**, a square pulse of fluid shear (23 dyn/cm^2, 400 ms) generates a gradient in the membrane tension, with higher tension at the upstream edge of the cell and a lower tension (compression) at the distal edge. Both tension and compression recover back to the initial state within ~30 ms. In comparison, the same shear pulse generated a much longer-lasting tension in actinin at the upstream edge of the cell. **Figure 6(b)** and **(c)** illustrates these different characteristics. Interestingly, the membrane tension at the front edge increased much slower than compression at the downstream edge, suggesting that there exists a pretension in the membrane probably via the cortical actin cytoskeleton. The pretension resists the effect of shear force at the upstream edge. In addition, buckling (rapid compression) can occur at the downstream edge. It has been shown that buckling of the lipid membrane can occur at a similar timescale that takes ~150 ms to saturate [122].

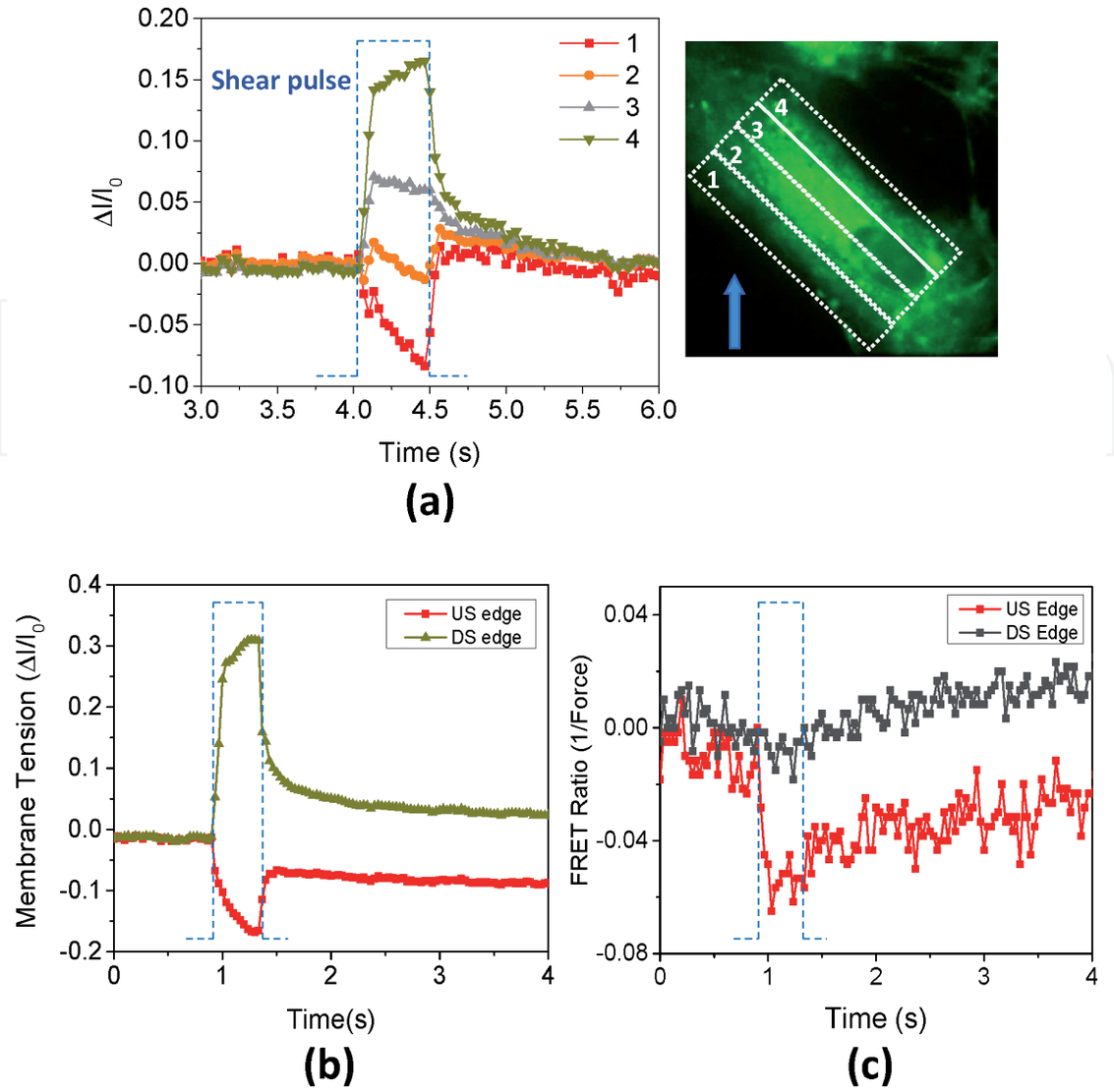

Figure 6.
Change in membrane tension measured using FCVJ molecular rotor incorporated into the astrocyte membrane. (a) Left panel: Membrane tension in response to a shear pulse (23 dyn/cm^2, 400 ms), where downward inflection indicates tension and upward inflection compression. Labels 1–4 correspond to the regions shown in the image in the right panel. (b) and (c) comparison of membrane tension and actinin force, respectively, in response to a same shear pulse. They show membrane tension at upstream edge increases much slower than actinin force.

A rapid shear pulse generates a gradient in the membrane tension. The spatio-temporal distribution of tension is dependent on the rise time of the shear force. When subjected to a slow ramp-up shear stress, the tension gradient was reduced significantly.

7.3 Membrane tension gradients are coupled to cytoskeletal forces

It has been shown that mechanosensitive channels can be activated by bilayer tension in the lipid vesicles without the cytoskeleton [123]. However, membrane tension measurements show that the membrane tension at the upstream edge increases rather slowly compared with the Ca^{2+} rise. This suggests that the Ca^{2+} channel could not have been activated by the bilayer tension alone and additional mechanisms are likely involved. Slowly ramping the shear stress was not able to change the membrane tension at the upstream edge until it reaches a shear stress threshold. This threshold exactly matches the threshold observed for the rise in the cytoskeletal tension. Therefore, both cytoskeleton and membrane tensions are involved.

Time-dependent analysis shows that shear pulse generates faster and longer-lasting tension in actinin at the front edge of the cell compared to membrane tension, suggesting the cytoskeleton imposes the limiting force at the upstream edge of the cells. In the presence of cytochalasin D that disrupts F-actin, this limit is lifted, and a more predominant membrane tension is seen at the upstream edge compared with controls. While disruption of F-actin diminished tension gradients in the cytoskeleton, it also eliminated the fast influx of Ca^{2+} at the upstream edge of the cell [114]. Thus, cell membrane tension is modulated by the cytoskeleton stresses since the bilayer alone has a minimal ability to bear a large tension gradient.

Moreover, fluid shear by itself is not likely to affect the cell membrane directly since most of the velocity gradient is absorbed by the glycocalyx, leaving little friction at the bilayer [124]. Fluid drag applied to the cell body will pull on the cytoskeleton, thereby increasing its stress [116, 125].

7.4 Effect of shear stress on cell membrane

The effect of shear stress on the lipid membrane response has been variously modeled. The generally proposed model for lipid bilayers is the fluid mosaic model [126]. This model describes the structure of the plasma membrane as a mosaic of components including phospholipids, cholesterol, proteins, and carbohydrates that give the membrane a 2D viscous fluid characteristic. Since the lipid membrane is expected to be intrinsically incompressible, the membrane tension changes could not be explained by solely considering the pure fluid characteristics. Hence, lipid bilayer and MSC interaction via a fluid, or instability of curved stress states of a non-lamellar lipid bilayer, is not easily justifiable within the scope of the fluid mosaic model [127]. Subsequent studies suggest that the lipid membrane is more mosaic rather than fluid [128]. Other models such as the surface model [129] could predict the interaction of lipids and proteins better by allowing both compression and tension changes in the membrane. Based on such models, interactions of lipid and MSCs could be explained by an increase in distance of lipid hydrophobic head groups in the vicinity of proteins and a decrease in outer layer viscosity of membrane [130]. However, without an input from underneath cytoskeleton, the tension gradient in bilayer is minimal.

This leads to the conclusion that shear stress generates tension gradients in the cell cortex and that membrane tension gradients are coupled to cytoskeletal forces to mediate Ca^{2+} influx. The time-dependent membrane tension gradient is modulated by the intact cytoskeleton forces.

8. Evaluation of astrocytic Ca^{2+} response to shear in brain slices

In the brain, neurons and astrocytes are intimately connected and function through a three-dimensional circuit that passes information waves. The interplay between them is evident in bidirectional glutamatergic astrocyte-neuron signaling in a Ca^{2+}-dependent fashion. A common consequence of TBI is the alternations of this information flow.

While *in vitro* experiments described above permit high-resolution measurements, the environment differs from *in vivo*. To better approximate the *in situ* conditions, and to see how much of the *in vitro* results are applicable *in vivo*, similar sets of experiments can be performed to brain slides, since slides would contain the native cell types and their local environments as *in vivo*. To a closer approximation, a modified shear chamber was used to apply fluid shear stress to mechanically stimulate the slices.

The Ca^{2+} response in acute slices from rats is demonstrated in **Figure 7**, which shows how shear stimuli modulate Ca^{2+} response in cells under physiological

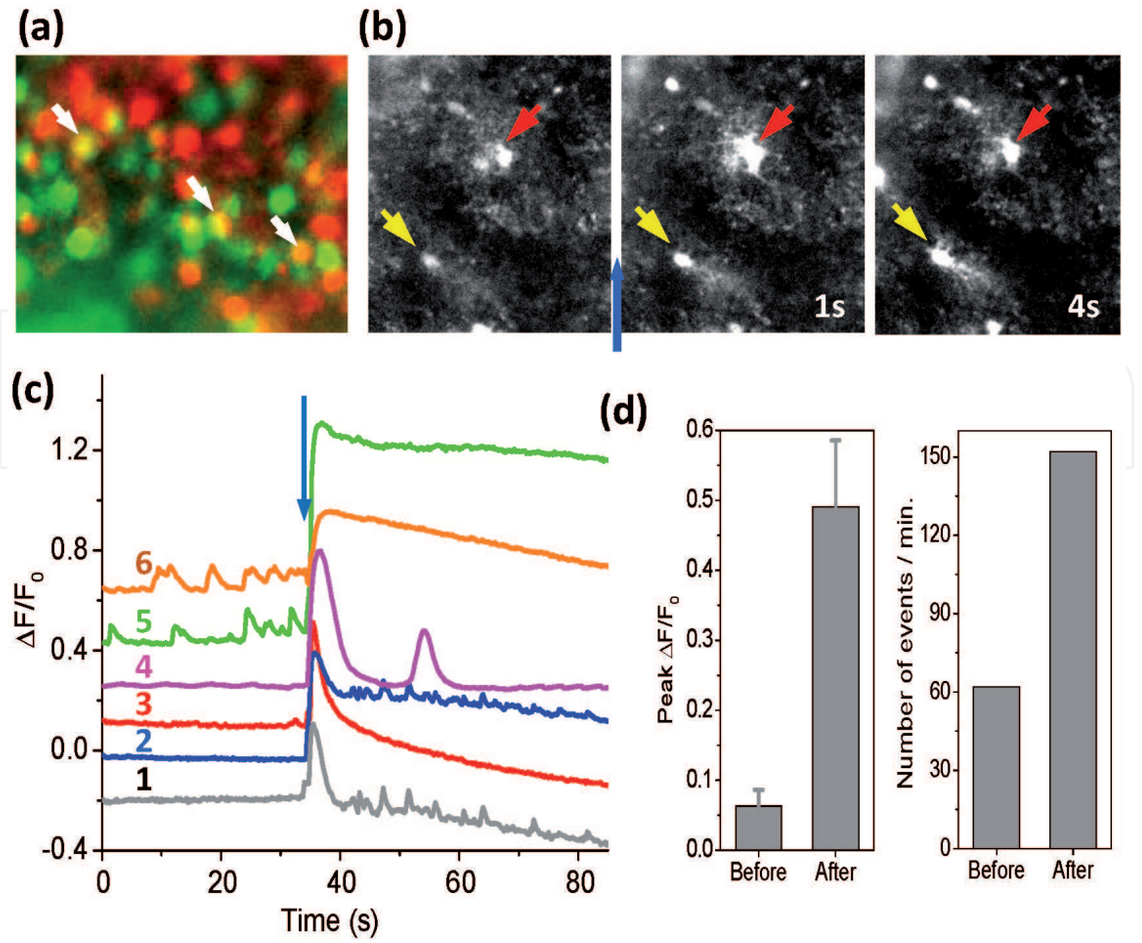

Figure 7.
Astrocyte Ca^{2+} response to a shear pulse in a hippocampal slice. (a) The slice is co-loaded with Flou-4 (green) and SR101 (red). (b) Time sequence of Ca^{2+} images showing Ca^{2+} peaks at different times in selected cells. (c) Typical traces of astrocyte Ca^{2+} response of individual cells. (d) Statistics of peak amplitudes and frequency.

conditions (with the caveat that these may be treated as samples of extreme TBI). To discriminate the astrocytes from neurons, the slices were loaded with SR101 that serves as a marker for astrocytes, as shown in **Figure 7(a)**. Shear stimulated slices showed an acute Ca^{2+} increase in selected cells that peaked in 1 to 4 s and returned to baseline levels within 20 s, consistent with observations in cell cultures. Most of the cells showed one dominant peak, but some (~20% of cells) responded with multiple peaks (trace 4, **Figure 7(c)**). The average peak Ca^{2+} was much higher than the spontaneous Ca^{2+} transients that were 10–20% of the shear-induced peak (traces 5 and 6, **Figure 7(c)**). These Ca^{2+} peaks were eliminated with 10 μM Gd^{3+}, which is a nonspecific MSC blocker. This confirms the observations that shear stress-induced transient Ca^{2+} peaks are via MSCs.

It is worthwhile pointing out that the fluid shear stress generates well-controlled forces on the apical surface of cell cultures. In the slide experiments, once the deformation reaches a deeper layer of the cells, the poroelastic nature of the tissue will also modify the forces, so the stimulus profile itself may change as it propagates.

9. Conclusion

Using advanced technologies that can generate fast shear stimuli mimicking forces that cause TBI, we have demonstrated that cell response to mechanical stimuli is nonlinear and the features of the stimuli play a critical role. Using FRET-based

force probes, the nonlinearity is shown to be a direct result of nonuniform force distribution within the cytoskeleton. Rapid shear pulses generate a heterogeneous distribution of cytoskeletal forces in cells, in both time and space. The cytoskeletal forces and their modulation on cell membrane tension open MSCs that mediate Ca^{2+} response. These early response signals can be small and transient. However, the integration of these signals leads to pathology and the progression of TBI.

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