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Current *ex Vivo* and *in Vitro* Approaches to Uncovering Mechanisms of Neurologic Dysfunction after Traumatic Brain Injury

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Abstract

Traumatic brain injury often leads to progressive alterations at the molecular to circuit levels resulting in epilepsy and memory impairments. *Ex vivo* and *in vitro* models have provided a powerful platform for investigating the multimodal alteration after trauma. Recent *ex vivo* analyses using voltage sensitive dye imaging, optogenetics, and glutamate uncaging have revealed circuit abnormalities following *in vivo* brain injury. *In vitro* injury models have enabled examination of early and progressive changes in activity while development of three-dimensional organoids derived from human induced pluripotent stem cells have opened novel avenues for injury research. Here, we highlight recent advances in *ex vivo* and *in vitro* systems, focusing on their potential for advancing mechanistic understandings, possible limitations, and implications for therapeutics.
1| Introduction

Traumatic brain injury (TBI) results in a spectrum of neurocognitive impairments including memory deficits, post-traumatic stress disorder (PTSD), chronic encephalopathy, seizures, and neurodegeneration (1). The effects of trauma on brain structure and function takes place in two phases. Direct mechanical disruption to the brain from an external force results in the primary injury characterized by necrotic cell death and damage to blood vessels, neurons, and glia. This acute phase is followed by the secondary injury, which is catalyzed by excessive excitatory neurotransmitter release and immense calcium influx, leading to genetic changes, free radical production, and apoptotic cell death in processes that vary over time (2). Together these changes disrupt neuronal networks, alter excitability, and reshape the connectivity of surviving neurons, altering circuit function (3). The secondary injury phase is an area of intense research as understanding the mechanisms underlying post-traumatic circuit remodeling during this period presents a potential therapeutic window.

Here, we will discuss advances in approaches to investigate trauma outside of the organism including ex vivo preparations following in vivo injury, which are used to explore cellular and circuit pathology, as well as emerging tools to model trauma in vitro. Acute brain slices from animals subjected to in vivo injury have been widely used to gain insights into injury-induced changes in neuronal excitability, plasticity, and epilepsy (4). While the various established methods of in vivo TBI have been reviewed elsewhere (2) and are beyond the scope of this review, we will highlight recent advances and the opportunity for further circuit dissection using modern ex vivo approaches. Next, we will discuss organotypic brain slices and dissociated primary neuron systems, which are prepared from naïve animals, and can be used to model trauma either during the process of establishing the in vitro system or by subsequent mechanical
injury to the established culture system (5). Finally, we touch upon the unique opportunity posed by the use of human induced pluripotent stem cells (hiPSCs) derived from human somatic cells (e.g., fibroblasts) to generate human brain cells \textit{in vitro}. HiPSC-derived neurons can be analyzed in a two-dimensional system or grown into three-dimensional organoids for \textit{in vitro} injury and have the potential to offer unique insights into the consequences of human TBI (6). We will discuss advances in post-traumatic circuit dissection from the last five years (2015-2020) across \textit{ex vivo} and \textit{in vitro} experimental platforms and highlight recent studies (2018-2020) of particular interest, which will be denoted with an asterisk*.

\textbf{2| Ex Vivo Analysis of \textit{in Vivo} Injury Models}

\textit{In vivo} injury models of TBI have been extensively reviewed with fluid percussion injury (FPI) (7), controlled cortical impact (CCI) (8), weight drop (9), blast (10), and penetrating ballistic-like models being the most widely used (2). \textit{In vivo} injuries have been modeled in rodents and provide a close approximation to the human condition. Using \textit{ex vivo} brain slice analysis of \textit{in vivo} injuries (as shown in Figure 1A), recent studies have shown changes in circuit excitability (11–15), neuro-immune interactions (16) and cell-specific changes (17,18). Our recent slice physiology studies after FPI identified a role for the innate immune receptor, toll-like receptor 4 (TLR4), in enhancing calcium-permeable AMPA receptor currents early after FPI. Based on mechanistic insights from \textit{ex vivo} studies, we were able to show that treatment with TLR4 antagonists early after FPI could limit the development of epilepsy and memory deficits (16*, 19). These findings highlight the potential of \textit{ex vivo} systems to elucidate trauma-induced pathology, uncover basic functional properties of neurons and neural circuits when injured, and inform treatment strategies.
While most studies rely on rodents for their TBI animal models, larger animals with gyrencephalic brains, particularly swine, ferrets, and non-human primates, may be more clinically relevant models for investigating rotational brain injury (20). Swine have been utilized to simulate human rotational injury biomechanics and have addressed circuit-level questions using slices (21,22). Across species, ex vivo analyses enable the use of cutting-edge techniques such as voltage sensitive dyes (11,23), glutamate uncaging (24), and optogenetic stimulation (25). In particular, use of voltage sensitive dye imaging in acute slices enabled Folweiler and colleagues to visualize the spatial and temporal spread of focally evoked activity in the hippocampus and investigate how this activity was altered following trauma (11)*. We propose that the research ground is fertile for further investigations using optogenetic (using transgenic mice expressing light activated opsins) and chemogenetic (utilizing designer receptors exclusively activated by designer drugs, or DREADDs) approaches to probe neural circuits (26,27).

It should be noted that while ex vivo analysis of post-traumatic circuits has great promise for scientific discovery and developing novel therapies, it is not without limitations. While larger animal models may more closely approximate a human injury, they suffer from high cost, limited ability for genetic manipulations, and low throughput. Ex vivo approaches, in general, are not conducive to detailed tracking of circuit pathology over time or real-time examination of the processes occurring during injury. Moreover, animal injuries are complex and heterogenous, which complicates analysis. For example, like in humans, only a subset of injured animals goes on to develop spontaneous recurrent seizures (SRS) (28). Thus, ex vivo investigations shortly after injury, before SRS, cannot control for this variability within the cohort. Similarly, while blood brain barrier (BBB) compromise associated with animal models of TBI (1,2) simulates
human TBI, the potential infiltration of peripheral immune cells across a compromised BBB and inflammatory response cannot be isolated from the changes in neuronal circuitry due to the primary injury.

3| In Vitro Injury Platforms Using Animal Models

While *in vitro* systems are not intended to replace *in vivo* animal studies, culture systems are powerful in that they allow for controlled and reproducible neuronal injuries that bypass much of the variability seen in *in vivo* systems. They also offer the unparalleled ability to visualize and probe cellular and network function during trauma and to track progressive changes over time. Because tissue for culture systems is typically obtained from naïve animals, confounds from peripheral immune responses across a leaky BBB may be avoided. Further, culture systems are advantageous in that they are faster paced and allow for greater throughput than *in vivo* experimentation. However, the caveat of these systems for the study of injury is that the process of generating the cultures involves mechanical dissociation or sectioning which are traumatic processes that could confound interpretation.

3.1| Organotypic Slice Culture Injury

Organotypic slice cultures offer a system that retains the layer-specific structure of neuronal connectivity in slices while providing the ability to assess real-time and progressive changes in circuits (5) (as shown in Figure 1B). Previous studies have delivered blast injuries to organotypic slice cultures (29), enabling analysis of the effects of primary blast injury on neuronal circuits outside of the animal and without the confounds of systemic responses. Recently, Campos-Pires and colleagues (30)*, described a novel blast model for hippocampal organotypic slices in which a simplified tissue culture insert system was used to connect cultured
slices to the end of a shock tube. This system enabled fast and simple processing of samples allowing for greater throughput and less risk of tissue hypothermia associated with lengthy procedures where the tissue is outside of the incubation chamber (30)*. This system could accelerate efforts to isolate and understand the cellular effects of blast injury. Apart from blast injury, organotypic slices have been used to examine the effects of tissue deformations by stretching the entire slice (31). Such a system coupled with a stretchable microelectrode array (MEA) can facilitate real-time and layer-specific examinations of electrical activity in circuits as they undergo deformations (32) and allow for multiple stretch injuries to be administered to model mild repetitive injury (33). Organotypic slices further enable circuit analysis using chemogenetic (34), optogenetic (35), and real-time calcium imaging approaches during injury which offer great promise for understanding the early phases of primary mechanical injury. Additionally, it is possible to obtain human sections for organotypic slices. However, the availability of human tissue is limited to that resected for medical reasons, such as from the epileptic foci of patients with medically refractory epilepsies (36,37), which may not be ideal for studying TBI.

Organotypic slice cultures preserve a high degree of native circuitry and enable controlled manipulations, however, the trauma of sectioning tissue to create an organotypic slice system is sufficient to progressively reorganize circuits and produce epileptiform activity as seen following trauma in vitro (38–40). Specifically, Staley and colleagues have suggested that organotypic slice cultures spontaneously develop epileptiform activity after one week in culture and may be treated as a model of post-traumatic epilepsy (38). This epileptiform activity was found to be only modestly affected by slice culture media and is believed to be the result of the injury processes initiated during preparation of the slices (39). As with cortical undercut models in
vivo, the trauma of slicing could simulate the pathological processes of penetrating injuries and has been used to investigate epileptiform activity (40). However, whether circuit-level reorganization in organotypic slices, which are completely isolated from their input circuits and adjacent lamellar networks, will replicate the cellular and network reorganization observed after penetrating injuries in vivo remains an open question. Another limitation of the system is that organotypic cultures usually require younger animals for slice preparation. Younger animals have key differences in synaptic physiology, including potential depolarizing actions of gamma-aminobutyric acid (GABA) receptor-mediated currents rather than the hyperpolarizing effects observed in adults, and greater synaptic plasticity and resilience to injury (5), which limit the generalizability of these systems to trauma in adult circuits.

3.2 Neuronal Injury in Dissociated Cultures

Stepping down a level of complexity, dissociated neuronal cultures may be used to investigate trauma at the single cell level, as illustrated in the schematic in Figure 1C. Specifically, uniaxial neuronal stretch (41,42), biaxial neuronal stretch (43), and axotomy using microfluidic chambers (44–46) have been implemented in dissociated neuronal cultures to examine the distinct processes involved in dendritic and axonal responses to trauma. Recent advancements in microfluidic chambers have optimized the procedures for in vitro axonal injury and can be reviewed here (47,48). In vitro culture systems have been used to investigate changes in neuronal excitability (44), connectivity (46), and health following injury (42,43). Using a commercially available uniaxial stretch injury system (STREX ST-150), a recent study visualized axonal calcium signals in cultured primary mouse cortical neurons subjected to stretch injury and demonstrated that the rate of strain greatly influences calcium load, cytoskeletal
damage, and neuronal death providing fundamental insights into processes underlying diffuse axonal injury (42)*. In a complementary approach, Van de Wijdeven and colleagues have developed a microfabricated system in which cultured neurons are grown in microfluidic chambers to impose a network structure (49). Coupling this system with an MEA can facilitate analysis of network activity and reorganization of connectivity in response to axotomy (46)*.

The power of single-cell systems, similar to the organotypic slice culture systems discussed above in section 3.1, is the potential ability to manipulate and interrogate individual neurons and networks outside of the body without complexities associated with systemic injuries. Studying neurons in isolation allows for better analysis of effects of primary injury and to determine cell- or process-specific responses to injury, such as axonal beading or altered calcium dynamics (42). However, these systems have clear limitations in reflecting the complexity of the in vivo circuit in terms of connectivity, circuit development, and maturity.

### 4| Human Induced Pluripotent Stem Cell Systems

The use of animal models in research has potential limitations in translation to humans. The advent of strategies to induce pluripotency in cells from human tissue has enabled the ability to study hiPSC-derived neurons. This grants researchers the advantage of investigating human neurons in a culture setting without the need for invasive retrieval of neuronal tissue from patients, which is limited in supply and utility as human brain tissue can only be obtained post-mortem or from “pathological” tissue resected for medical reasons. HiPSCs provide a promising platform to generate specific neuronal types and to create two-dimensional neuronal cultures (similar to primary neuronal cultures described in section 3.2) or three-dimensional organoids which form layered structures (see Figure 1D). Indeed, hiPSCs can be programmed to
differentiate into a number of non-neuronal cell types (namely, neural stem cells, astrocytes, oligodendrocytes, microglia, and brain vascular endothelial cells pericytes) and neuronal cell types (namely, motor neurons, dopaminergic neurons, GABAergic neurons, cortical neurons, serotonergic neurons, hippocampal neurons, and nociceptors) for analysis (6).

Two-dimensional hiPSC systems have been used to study stretch injury in hiPSC-derived neuronal cultures. The benefit of this technique is that it can be performed in a 96-well plate for high throughput screening and can examine neuronal tissue of human origin (50). Human neuronal organoids (also known as cortical spheroids or mini brains) have been created from hiPSCs and developed into brain-like tissue with non-reactive astrocytes and physiologically mature neurons with spontaneous activity (51). Notably, hiPSC-derived neuronal organoids can be molecularly modified and subsequently used for live imaging and electrophysiological experiments with optical stimulation (52). A study by Pașca and colleagues used hiPSC lines to generate neuronal organoids to model infant hypoxic brain injury and examine changes in gene expression profile and cell density in the hiPSC-derived brain organoids (53)*. HiPSC-derived brain organoids have even been used recently to identify how membrane damage and oxidative stress are increased with increasing peak pressure of a primary blast wave (54). In an intriguing approach, a recent study has shown that plating brain organoids at opposite ends of a hydrogel column leads to the development of three-dimensional axons tracts (55), which could enable investigation of diffuse axonal injury in a more biologically relevant arrangement. The studies to date using organoids and hiPSCs are still in the development phase when it comes to modeling TBI. However, there are exciting opportunities to use human three-dimensional organoids in conjunction with modern optogenetic, chemogenetic, and imaging tools coupled with realistic blast, compression, or stretch injuries to gain novel insights into injury mechanisms. While there
is power in using human-derived tissue, and developing cultures and organoids from human subjects with brain injuries has the potential to lead to personalized medicine for trauma patients, it should be noted that organoids are quite artificial and do not faithfully model any particular circuit in the brain and so do not represent the best model for circuit analysis.

5| Conclusions

TBI induces an array of molecular and physiological changes to brain tissue, which can be modeled in a number of ways. Figure 1 shows an overview schematic of the approaches discussed here. In vivo injury models (Figure 1A) have the benefit of working with an intact organism which allows for a more-clinically-relevant model of injury, especially in large animals, however, does not grant the same level of environmental control that in vitro injury systems do. Stretch and blast injuries have been implemented in organotypic brain slice cultures (Figure 1B) which maintain intact neural circuits and have enabled MEA recordings and molecular manipulations in a more controlled setting. Organotypic slices, however, have been shown to exhibit spontaneous epileptiform activity, suggesting that the act of preparing slices itself is a form of trauma that presents as a form of post-traumatic epileptiform activity within a week after preparation. Subsequent in vitro injuries in organotypic slices produce further damage, however, which can be used to elucidate underlying mechanisms in neuronal injury. Further, dissociated cultures (Figure 1C) allow for detailed analysis of neuronal injury, such as axonal shearing and repair in real-time. This system, however, is limited by the inability to replicate complex, layer-specific circuits. Both organotypic and dissociated neuronal cultures in animal models tend to rely on embryonic to neonate animal preparations which may skew findings and may not adequately represent the responses of mature brains to injury. Lastly,
hiPSC-derived neurons and neuronal organoids allow researchers to investigate properties of human neurons in a controlled setting and without invasive or bias tissue collection, e.g., as the result of resection of a seizure focus from a patient. HiPSC systems are powerful in their genetic relatability to humans, however, clearly lack the human circuitry that would be advantageous to study for trauma-induced circuit remodeling. Improved future neuronal organoid models may be able to approximate functional circuits, such as the tri-synaptic loop through the hippocampus, which would create a powerful platform for in vitro trauma investigations. Importantly, each of the four levels of TBI modeling presented here can be used for electrophysiological and molecular investigations to elucidate post-traumatic neuronal alterations and can complement each other for elucidating basic neuronal and circuit alterations and lead to the development of new and improved therapeutics for the treatment and prevention of TBI sequelae.
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**Figure Legend**

**Figure 1. Overview of models for *ex vivo* and *in vitro* investigations of traumatic brain injury.**

A) shows a naïve animal being exposed to a TBI (Injury), such as from fluid percussion injury (7), controlled cortical impact (8), weight drop (9), or blast (10). Acute slices are then prepared from injured animals at an experimentally defined time after injury. A hippocampal slice is shown as representative, but there is no restriction on circuitry that can be studied in this fashion. Alternatively, naïve animals can be used to create organotypic slice cultures (B) or primary neuronal cultures (C), which can then be used as a platform for studying brain injury. Specifically, blast (29,30) and stretch (31–33) have been applied to organotypic slices (B) and neuronal stretch (41–43) and axotomy (44–46) injuries have been applied to primary neuronal cultures (C). The only non-invasive human neuronal investigations involve the use of human induced pluripotent stem cells (hiPSCs) (D). Human somatic cells (e.g., from skin fibroblasts) can be reprogrammed to induce pluripotency and these hiPSCs can then be differentiated into neurons and glia and grown into three-dimensional functional organoids, which can be used to investigate neuronal trauma, e.g., hypoxia (53) or blast (54). Cell lines may also be used for cell culture preparations in C and D (not shown). Finally, there is potential to reprogram somatic cells from TBI patients to advance personalized medicine, as represented by the injured human (D, right). CA3, Cornu Ammonis (CA) area 3; CA1, Cornu Ammonis (CA) area 1; DG, Dentate Gyrus. Not drawn to scale.
References


Using a voltage sensitive dye, the authors demonstrate that mild fluid percussion injury impairs the ability of the dentate gyrus to gate excitation flowing to CA3, resulting in CA3 hyperexcitability. This work is a clear demonstration of the utility of modern tools in elucidating changes in network function in ex vivo slices after trauma.


This article is the first to report that fluid percussion injury increases calcium-permeable AMPA receptor currents via neuronal TLR4 signaling. This work demonstrates how investigations of ex vivo slices from established in vivo models of brain injury continue to uncover mechanisms of brain responses to trauma.


This work demonstrates a substantial improvement in the organotypic slice culture blast injury model. This technique notably increases throughput and decreases the risk of organotypic slice culture hypothermia.


**This study highlights how single-cell neuronal stretch injury can inform researchers on mechanistic processes involved in axonal response to trauma and subsequent changes in axonal calcium dynamics in real time.**


**This article illustrates how cultured neurons can be grown in microfluidic chambers in combination with microelectrode arrays in order to measure network activity and neuronal connectivity following axotomy injury.**


This research demonstrates how human induced pluripotent stem cell-derived neurons can be used to generate three-dimensional organoids and subsequently used for investigating hypoxic injury in a complex network comprising of human neurons.

Conflict Statement

On behalf of the authors, I declare that this manuscript is not under review elsewhere. The manuscript has been reviewed and accepted by all coauthors and none of the coauthors have any financial or other interests that could pose a potential conflict of interest.