Chapter 12

Weight Drop Models in Traumatic Brain Injury

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Abstract

Weight drop models in rodents have been used for several decades to advance our understanding of the pathophysiology of traumatic brain injury. Weight drop models have been used to replicate focal cerebral contusion as well as diffuse brain injury characterized by axonal damage. More recently, closed head injury models with free head rotation have been developed to model sports concussions, which feature functional disturbances in the absence of overt brain damage assessed by conventional imaging techniques. Here, we describe the history of development of closed head injury models in the first part of the chapter. In the second part, we describe the development of our own weight drop closed head injury model that features impact plus rapid downward head rotation, no structural brain injury, and long-term cognitive deficits in the case of multiple injuries. This rodent model was developed to reproduce key aspects of sports concussion so that a mechanistic understanding of how long-term cognitive deficits might develop will eventually follow. Such knowledge is hoped to impact athletes and war fighters and others who suffer concussive head injuries by leading to targeted therapies aimed at preventing cognitive and other neurological sequelae in these high-risk groups.

Key words Mice, Closed head injury, Weight drop, Concussion, Cognitive deficits, Morris water maze, Diffuse injury, Mild traumatic brain injury

1 Introduction

The creation of clinically relevant animal models of traumatic brain injury (TBI) has proven difficult given the biomechanical and pathophysiologic complexity of the injury process. Most models cannot simulate the entire spectrum of human TBI or replicate common mechanisms of injury. All models are confounded by the inherent variability in injury severity and neurologic outcome. These limitations make molecular and translational studies challenging.

Weight drop models are a relatively nascent area of investigation, but the models are gaining momentum given their similarities to human TBI. Weight drop models can simulate the full spectrum of TBI, ranging from mild concussion to severe TBI. Common models of TBI, such as fluid percussion and controlled cortical impact produce a focal brain contusion with little axonal injury. Conversely, weight drop models aim to reproduce diffuse brain

Firas Kobeissy et al. (eds.), *Injury Models of the Central Nervous System: Methods and Protocols*, Methods in Molecular Biology, vol. 1462, DOI 10.1007/978-1-4939-3816-2_12,

injury. In the first portion of this chapter, we summarize the characteristics of some of the well-known weight drop models. In the second part, we describe the development of our own weight drop models of repetitive mild traumatic brain injury.

1.1 Review of Weight Drop TBI Models

Dr. Anthony Marmarou proposed the first weight drop model of closed skull TBI in rats [1]. This model produces shear stress and diffuse axonal injury, as opposed to prior TBI models that produced focal brain contusion. The scalp of anesthetized mice is shaved, and an incision is made to expose the periosteum. A stainless steel helmet is fixed to the skull with dental acrylic. The helmet distributes kinetic energy over the brain, thereby preventing focal injury. The head injury device consists of a column of brass weights that fall freely through a plexiglass column. The falling weight ranges from 50 to 500 g. The rat is placed on a foam bed of known spring constant. The weights are dropped from a set height to induce a reproducible injury. Data from the initial description of the model demonstrates a mortality rate of 44% and a skull fracture rate of 12.5% when a 450-g weight is dropped from 2 m. The injury is accompanied by seizures, apnea, and hypertension. The use of mechanical ventilation significantly improved survival [2]. Postmortem analysis demonstrated brain edema; ventriculomegaly; and widespread damage to neurons, axons, and microvasculature. Diffuse axonal injury was found in the brain stem, optic tracts, corpus callosum, internal capsule, and the cerebral and cerebellar peduncles [2]. A biomechanical analysis of impact dynamics estimated that the 450-g weight dropped from 2 m produced a peak acceleration of $900 \times g$.

Adelson et al. described a modification of the Marmarou model that produces diffuse cortical swelling [3]. The authors found that the 450-g weight used by Marmarou produced too great a mortality, and therefore studied a 75- and 100-g weight dropped from 2 m. The 75- and 100-g injury severity levels produced mortality rates of 18.2% and 38%, respectively. Pathological examination of the brains from the severely injured animals demonstrated neuronal death, vascular disruption, and diffuse cerebral edema. No gross contusions were noted. Of Note, this model produced a brain stem injury that may be responsible for at least some of the observed mortality.

Shohami and colleagues developed a weight drop model in the rat that utilizes a free-falling rod rather than weights [4]. This injury produces blood–brain barrier disruption, cerebral edema, and neurological deficits as well as focal contusion and cell death. In this model, the scalp is incised, and a free falling, silicone-tipped rod delivers a cranial impact over the left hemisphere (1–2 mm lateral to midline). Blood–brain barrier permeability peaks in 4 h and is present up to 4 days [4, 5]. Brain edema peaks at 18 h postinjury [5]. The investigators developed a neurological severity score (NSS) to assess motor function and cognitive deficits after injury. The NSS correlates closely with the pathologic severity of brain damage [5]. The Shohami laboratory has

extensively characterized the functional and biochemical response to injury in their model. Closed head injury is accompanied by the rapid production of eicosanoids (5-HETE and prostaglandin E2) [6] and cytokines (IL-6 and TNF-alpha) [7]. Several therapeutics, including endothelin antagonists [8], acetylcholinesterase inhibitors [9, 10], cannabinoids [11, 12], and TNF-alpha modulators [13], have proven protective in this model.

Given the emergence of genetically modified mice, the Shohami group adapted their rat model to produce a similar mechanism of injury in mice [14]. Similar to the rat model, the severity of brain injury could be modulated by the falling height and the mass of the weight. Using a weight of the falling rod between 333 and 1600 g, a 2-cm drop height is associated with mild injury, while a 3-cm drop height is associated with severe injury. There is an increased probability of skull fractures with increased injury severity. This model consistently produces disruption in the blood–brain barrier, cerebral edema, and neuronal cell death below the contusion site and remotely in the hippocampus. There is 13% mortality in the immediate postinjury period, with an additional 13% mortality in the subsequent 24 h [14].

Feeney et al. used a weight drop device to generate graded, focal cortical contusions [15]. The contusing apparatus consisted of a 40 cm stainless steel tube attached to a circular footplate that was positioned over the exposed dura of rats. Contusions were created in the hindpaw region of the brain that has both motor and somatosensory functions. Behavioral deficits were observed in the contralateral hindlimb. Mild forces (50 g/cm) did not produce surface hemorrhaging, but more severe forces (200–1000 g/cm) produced surface hemorrhaging and cortical disorganization in some cases. Necrotic cavitation with subcortical cell loss was observed 24 h postinjury with 200 and 600 g/cm forces. After 2 weeks, these cavitary lesions were lined with macrophages and stained positive for acid phosphatase. Animal performance on a balance beam demonstrated trauma–dose relationship, and a persistent deficit was observed at 90 days postinjury.

1.2 Review of Other Closed Head TBI Models

Nedergaard and colleagues developed a "Hit and Run" model of closed head injury that did not require stereotactic fixation of the head or preparation of a cranial window, as in other models [16]. This model induces a closed head injury, which creates cerebral edema and intracranial hypertension commonly observed in human TBI. The device used in this model is a modification of the pneumatic cortical impact device. The instrument is rotated and mounted 90° such that the metal impact rod is oriented horizontally. After anesthesia, mice are hung vertically from their incisor teeth from a metal ring. The impactor rod strikes the mouse skull laterally between the eye and ear. The mouse head is free to move in response to the impact. The velocity of the rod can be varied to create different severities of injury. "Mild injuries" (impact speed 4.8 m/s) were

characterized histologically by diffuse cortical reactive gliosis without gross tissue disruption. "Moderate injuries" (impact speed 5.2 m/s) were associated with cortical disruption and the formation of a glial scar. The impact depth and contact times were held constant between groups. Both levels of severity were associated with a loss of myelination; this effect was more prolonged in the "moderate injury" group. Axon degeneration and loss of white matter were also observed in both mild and moderate injury groups. Neither injury severity level was associated with early death, and delayed death was very rare. No mice suffered skull fractures. Of Note, the authors only described a single-hit model; the effect of repetitive injuries has not been reported.

The Wayne State model reported by Kane et al. uses a 95 g weight dropped down a guide tube from a height of 1 m onto the head of a lightly anesthetized mouse suspended on aluminum foil [17]. The foil allows for unrestricted movement of the animal at the time of impact, and a string tied to the weight prevents it from bouncing and hitting the mouse a second time. After a single hit, mice do not experience seizures or paralysis. The authors have studied mice after repeated hits (1 hit per day for 5 days) and demonstrated a reproducible cognitive deficit compared to control mice. In particular, the mice had deficits in motor coordination that recovered over time. In injured mice 30 days after injury, there is evidence of gliosis, astrocytosis, and elevated phosphorylated Tau. There was no microglial activation, disruption of the blood–brain barrier, or extensive loss of cortical white matter. Preliminary studies did not find evidence of β-amyloid deposition.

The Wayne State model shares several similarities with the Harvard weight drop model described later: both models allow for unconstrained movement of the head and body after impact, and both models can be adapted for multiple impacts. Neither model induces skull fractures, cerebral edema, or intracranial hemorrhage. In the section that follows, we describe our own model of closed head concussive brain injury.

2 Development of a Mild Closed Head Injury Model of Repetitive Sports Concussions

2.1 Considerations for Development of a Rodent Model of Sports Concussions

2.1.1 Choice of Mouse as the Species for Model Development

In considering which species to use for development of a new sports concussion model, we considered rats versus mice. Advantages of the rat include its excellent performance in the Morris water maze, a gold standard test of learning and memory applied to almost all TBI models; its relatively cheap cost and ease of maintenance; ease of line placement for intravenous drug injections; and availability of antibodies and PCR reagents to detect a wide range of rat proteins and RNA species. The large size of the rat head is a distinct advantage when considering positron emission

tomography (PET) imaging, as well as functional magnetic resonance imaging (fMRI) and magnetic resonance spectroscopy, as most magnets can successfully incorporate coils to fit the head of a rat. Mice are more challenging, and spatial resolution of most imaging studies is not as good as rats because of the smaller brain size. On the other hand, mice are much cheaper than rats, many strains perform well in behavioral tests including the Morris water maze, and the existence of genetically modified mice allows for investigation of mechanisms of secondary brain injury associated with concussion. Based mostly on the latter consideration, we chose to develop our weight drop concussion model in mice.

2.1.2 Anesthetic Considerations

A number of anesthetic agents have been used in experimental TBI models including inhaled agents (isoflurane, sevoflurane, halothane), intravenous agents (ketamine/xylazine, barbiturates, propofol), and others such as chloral hydrate and avertin. Almost all anesthetics have neuroprotective effects in models of brain injury including antiapoptosis, anti-inflammatory, and energy sparing effects [18]. Several studies have shown dose- and exposure time-dependent neurodegenerative effects in developing rodent brains, which may influence anesthetic choice in pediatric concussion models; however, no anesthetic agent has a clear advantage over others in terms of potential for neuroprotection that might confound model development [18]. Another important issue is how a particular anesthetic agent might interact with the secondary response to concussive brain trauma; for example, sevoflurane anesthesia was associated with a decrease in reduction of loss of consciousness after multiple concussions compared to a single injury in mice, an effect that was thought to be an interaction between sevoflurane and repetitive injury [19]. For our purposes, we chose to use isoflurane/nitrous oxide to facilitate quick recovery from anesthesia and thereby accurately measure loss of consciousness time. The decision to use a given anesthetic is not trivial, as any agent that interferes with the natural history of concussive injury in the mouse will necessarily limit translation to humans. For this reason, at least one group has chosen to avoid the use of anesthesia all together in a multiple hit concussion model in mice [20].

2.1.3 Choice of Injury Mechanics

To best model sports concussions, we wanted a weight drop model in which the head was free to move downward after impact, to mimic both impact and rapid head acceleration forces experienced by athletes. Studies of impacts experienced by football players suggest that angular and rotational forces may significantly influence whether or not a collision produces a concussion [21–27] (and reviewed by [19]). In primates, loss of consciousness is more efficiently produced by impact plus acceleration forces rather than by whiplash injury alone [28, 29] or striking a head fixed in place [30]. These considerations ruled out controlled cortical impact and fluid percussion injury models in which

the head is held fixed, as well as rotational acceleration models that lack impact which often result in severe brain injury [31].

2.1.4 Histopathology Requirements

Human concussion TBI is defined as a complex functional alteration in brain activity rather than structural damage detectable by routinely used computerized tomography or structural magnetic resonance imaging sequences [32]. No doubt this definition will change as more sensitive noninvasive methods are developed to detect potential subtle histopathological features of concussion, and when functional imaging becomes more commonplace. Our goals for histopathology in a concussion model were lack of structural brain damage such as contusion, hemorrhage, blood–brain barrier disruption, edema, and acute cell death. A schematic of the gross pathology of the experimental closed head injury is shown in Fig. 1. Diffusion tensor imaging studies have suggested an association between concussion TBI and white matter injury [33], and we expected to see some evidence for axonal injury in a concussion model. Other histopathological features that we sought were diffuse gliosis [34] and increase in phosphorylated tau species, as well as increased beta amyloid protein and plaque formation, brain atrophy [35] and ventricular enlargement, consistent with development of chronic traumatic encephalopathy (CTE) seen in younger athletes with a prior history of sports concussions [36–44]. On the other hand, it is possible that debilitating neurological symptoms of concussion can be produced independently of tau and beta amyloid pathology. The histopathology of a concussion model is a critical issue because clinical case series and autopsy studies alone cannot be used to prove that concussions eventually lead to development of CTE in athletes [45]. Animal models are needed to directly test this hypothesis and provide a direct link [19].

Fig. 1 Gross pathology of closed head injury. Compared to contusion injury generated by controlled cortical impact (**a**), the brain is structurally normal after closed head injury (**b**)

2.1.5 Single Versus Repetitive Injuries

Human concussion presents with cognitive dysfunction early after injury, and repetitive concussions in humans are thought to increase neurological deficits over time. Therefore, we hoped that we could develop a mouse concussion model that would exhibit early functional deficits after a single injury, and more pronounced deficits and perhaps histopathology after multiple injuries [46–52]. If so, the model would allow us to test several clinically relevant hypotheses regarding human concussion, such as whether the number of concussions directly correlate with cognitive deficits, whether an injury-free time interval exists between concussions that mitigates permanent cognitive dysfunction, and whether increasing the level of injury might lengthen the vulnerable period between concussions, defined as that period of time in which additional injury may lead to increased neurological and psychological deficits associated with human concussion such as depression and anxiety [53–57]. As will be discussed later, in a multiple hit concussion model one might not want cognitive deficits after one or even the first few injuries in order to model milder forms of injury that synergize to produce cognitive and other deficits over time. Finally, by modulating the injury level (changing either the bolt weight or the drop height, see below) we hoped that we could extend an adult concussion model to immature mice [19], since a high percentage of sports-related concussions occur in children and adolescents [58, 59].

3 Materials

3.1 Development of a Single Hit Concussion Model in Mice

The materials needed for execution of our concussion model are the following:

1. A metal guide tube (this can be made of other materials as well, such as plastic, fiberglass, etc.) 66 in. long.
2. Tape to attach the guide tube to a wall or other solid structure.
3. Metal bolts of various weights (54, 83 g) that fit through the guide tube. Our initial goal was to develop a single hit weight drop concussion model in mice that resulted in rapid onset cognitive deficits and no structural brain injury. Male C57Bl/6 mice (2–3 months of age, 25–30 g) were anesthetized in 4.5% isoflurane/70% nitrous oxide/balance oxygen for 45 s and placed face down on a Kimwipe napkin with the experimenter grasping the mouse by the tail on the Kimwipe (Fig. 2).
4. Conceptually, the head and upper body of the mouse would be free to accelerate downward through a tear in the Kimwipe after the bolt struck the head, thus providing a whiplash component of injury relevant to sports concussion.

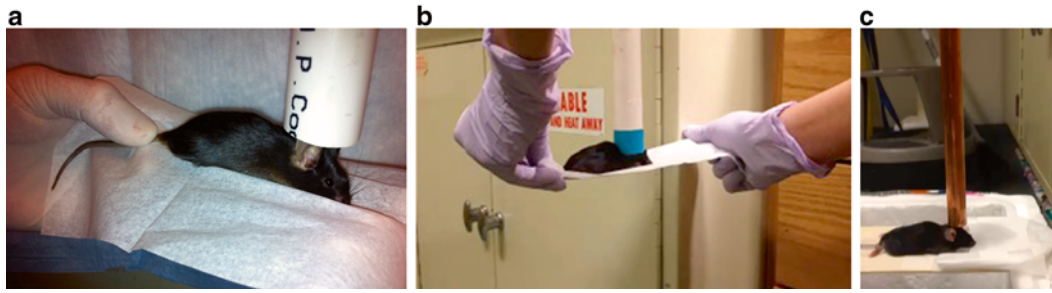

Fig. 2 Description of the weight drop closed head injury model. (**a**, **b**) Mice are gripped by the tail on a Kimwipe napkin and the head is placed under a guide tube. (**c**) Alternatively, a platform can be used to hold the mouse in place, allowing for a single operator to perform the model

3.2 Methods

1. We used a hollow guide tube and a metal bolt of various weights (54, 83 g) dropped from various heights (ranging from 28 to 66 in.) to modulate the injury level (*see* **Note 1**).
2. Mice were grasped on the Kimwipe by the tail and the head was centered underneath the guide tube (10 mm diameter). The bolt was dropped through the guide tube onto the head by one investigator while another held the Kimwipe and mouse under the opening (*see* **Note 2**).
3. In the beginning, it took a fair amount of practice to overcome the natural tendency to pull the mouse away from the tube opening just prior to impact with the bolt.
4. Using a bolt weight of 83 g and drop height 66 in., we experienced high mortality rates from apnea. Interestingly, if we rotated the head slightly left or right so that the bolt struck over the right or left ear, death from apnea was virtually eliminated. Moreover, we found that mortality rates seemed to depend in part on how snugly the mouse head was placed within the guide tube: if the head was held up to the guide tube with upward pressure, mortality was higher than it was when the mouse was held a few millimeters under the guide tube opening without upward pressure.
5. It was noticed that in order to achieve consistency between operators, it would be ideal to strike the mouse on the center of the head, so we reduced the bolt weight to 54 g. Using a drop height of 66 in. and bolt weight 54 g, loss of consciousness (defined as return to spontaneous ambulation in our initial studies, but later changed to return of righting reflex in later studies) was 450 ± 20 s in injured mice compared to 36 ± 1.8 s in sham-injured mice (mice subjected to anesthesia without weight drop, $p < 0.0001$), suggesting a robust injury [60].
6. Mortality, mainly from apnea, in this model was approximately 20%. In a subset of five mice we placed femoral arterial lines using p10 tubing and measured blood pressure and blood gases

before and after injury. We found a transient, modest increase in blood pressure at 2 min that returned to baseline values by 4 min [60]. As expected with apnea, closed head injury (CHI) caused a transient drop in PaO_2 and a modest increase in $PaCO_2$ that resolved by 4 min. However, no mice were hypoxic and all mice recovered blood pressure and blood gases similar to preinjury values within a few minutes after CHI [60].

4 Results

We next examined whether CHI caused gross structural brain injury using 53 g and 66 in. injury parameters. We were pleased that none of the mice had skull fractures or intraparenchymal hemorrhages and no gross structural brain damage. We assessed brain edema using the wet-dry/wet weight method (brains were weighed and dried in an oven at 90 °C for 48 h then weighed dry) and found no increase in brain water content in either hemisphere at 24 or 48 h compared to sham-injured mice. We assessed blood–brain barrier permeability to Evans blue albumin over the first 24 h after injury and found no increase in injured mice compared to shams. For this experiment, 2% Evans blue in PBS was injected intravenously (5 ml/kg) before CHI and 24 h later mice were transcardially perfused with PBS and brains were placed in *N*,*N* dimethylformamide for 3 days. Evans blue extracted by *N*,*N* dimethylformamide was measured spectrophotometrically. Alternatively, we performed immunohistochemistry to detect mouse IgG in brain tissue sections [61]. The finding of lack of edema, BBB damage, and structural brain damage satisfied many of our histological criteria for a mouse concussion model (*see* **Note 3**).

Most of the existing concussive TBI models induce some degree of acute cell death. One of our primary goals was to produce a model lacking acute cell death in order to study mechanisms of cognitive dysfunction without this confounding factor. Moreover, it is generally thought that human concussion is a "mild" form of TBI that probably does not induce significant neuronal death, although this is impossible to prove in clinical studies that do not include autopsy results. To determine whether our model involved acute cell death, mice were injured and brain sections obtained at 24, 48, or 72 h after injury and subjected to fluoro-jade B staining (a marker of acute neuronal degeneration), in vivo propidium iodide staining (a marker for loss of membrane integrity), TUNEL (a marker of double strand DNA damage and cell death), hematoxylin and eosin (H&E) staining, GFAP staining (astrocytosis) and IBA-1 staining (microgliosis) [60]. Amyloid precursor protein immunohistochemistry and electron microscopy were used to assess axonal injury. At later times (60 days) after injury, hippocampal cell loss and brain atrophy were assessed using

image analysis of H&E stained brain sections. No acute cell death or cell loss was observed at any of the time points assessed. Axonal injury was only occasionally detected by electron microscopy, and there was no brain atrophy at 60 days [60]. Strikingly, there was robust astrocytosis and microgliosis at 48–72 h in cortex and hippocampus of injured (but not sham-injured) mice, demonstrating an acute inflammatory response to concussion [60]. Similar findings have been reported in autopsy studies of humans with TBI at acute and chronic stages, suggesting that our model recapitulates at least some of the features of concussive TBI reported in clinical case studies [34, 43, 62].

To examine possible biochemical mechanisms of concussion-induced inflammation, we performed reverse transcriptase polymerase chain reaction (RTPCR) and nuclear factor kappa B activity assays on brain tissue obtained within 24 h of CHI. We found biochemical evidence of acute inflammation with early increases in TNF alpha and Fas mRNA, and TNF alpha protein followed by increased activation of NFkB [60]. Importantly, CHI produced deficits in motor performance (wire grip test) and cognitive deficits (Morris water maze performance) within 1–3 days after injury. Mice deficient in TNF and Fas had increased cognitive deficits suggesting that TNF/Fas induction in the concussion model is a protective response to injury, although a limitation of the experimental design (use of knockout mice) did not allow us to dissect early versus later effects of TNF/Fas inactivation. Nonetheless, this was an important result because TNF/Fas antagonism was protective in a contusion TBI model [63], and the results in our concussion model provided evidence for the concept that individual pathways activated in different pathoanatomic subtypes of TBI (e.g., focal contusion vs. diffuse concussion) may influence outcome in opposite ways. We believe these findings have significant implications for treatment trials of patients with focal vs. concussive TBI [63].

Taken together, the aforementioned data suggested that we had developed a mouse model of human concussion that featured an early cognitive deficit in the absence of gross and microscopic brain damage that was associated with a neuroinflammatory response and manipulable by TNF/Fas antagonism. However, a significant weakness of the model was that cognitive outcome was highly operator dependent and cognitive deficits were not always apparent from one operator to the next (also *see* **Note 4**). This was a difficult problem that was not solved by increasing the bolt weight to 83 g, which resulted in higher mortality but not always increased cognitive deficits (*see* **Note 5**). We tried recovering the mice at 37 °C to maintain normothermia after recovery from anesthesia, but that did not make a difference with respect to consistent cognitive outcome. Another caveat with our findings is that Morris water maze testing was performed at a time when motor deficits were present (between 1 and 3 days after CHI). Although motor

function has not been shown to correlate with MWM performance in TBI models, ideally we would have waited until motor deficits had resolved to perform MWM testing. However, we wanted to model the clinical scenario in which cognitive deficits appear early (within 24 h) after concussion so we began MWM testing the day after injury. Lastly, we initially started model development using CD1 mice; however, uninjured mice from this outbred strain did not perform consistently in the MWM so we developed the model using C57Bl/6J mice from Jackson laboratories (Bar Harbor, ME).

5 Development of a Multiple Hit Mouse Concussion Model

After establishing a single hit concussion model, we next set about developing a multiple hit concussion model of sports concussions. Factors that we considered in the planning stages included all of those identified for the single hit model (such as lack of overt brain damage and measurable cognitive deficits). Additional features that we wanted in a multiple hit model included (1) no detectable cognitive deficit after a single hit (and no structural or microscopic brain injury); (2) a reproducible increase in cognitive deficits with increasing numbers of injuries; (3) presence of psychiatric manifestations of concussion such as anxiety and depression [53, 57, 64]; (4) a measurable vulnerable period within which additional concussions lead to worse cognitive deficits, and conversely injuries outside the vulnerable period do not lead to long-term cognitive dysfunction; (5) an increase in phosphorylated tau species and emergence of beta amyloid plaques and tau tangles as mice age, recapitulating features of chronic traumatic encephalopathy as reported in the brains of athletes diagnosed via autopsy findings [44, 64].

Because the injury parameters of the aforementioned single hit model (66 in. drop height) produced a cognitive deficit after 1 hit, we lowered the drop height to 38 or 42 in. and injured mice either once, three times daily (1 hit per day for 3 days), five times daily, or 10 daily hits. MWM testing was begun 24 h, 1 month, and/or 1 year after the final injury; repeat MWM testing was done by placing the goal platform in a different quadrant for each repeated test. Data obtained in repeat MWM tests are somewhat limited because procedural learning persists and is already present for subsequent tests. Nonetheless, repeat MWM testing can be used to assess new spatial learning in injured mice. The 38-in. drop height model produced no cognitive deficit after 1 hit, modest deficits after 5 hits, and severe cognitive deficits after 10 hits at either 38 or 42 in. drop heights. Moreover, hidden platform deficits persisted at 1 month and 1 year after injury. When we tested additional groups of mice injured five times with injuries separated by a week or a month, mice injured weekly (but not biweekly or monthly) still performed worse than sham-injured mice, suggesting a safe rest interval in this model between 1 week and 1 month.

Repetitive injury did not lead to structural brain damage, acute cell death, brain edema, or blood–brain barrier damage assessed by IgG immunostaining [61]. Thus, the 38-in. drop height repetitive CHI model satisfied nearly all of our criteria for modeling human concussion. However, we noticed that approximately 25 % of mice had convulsions after injury. We initially reported these events as seizures [61], but in retrospect these limb movements, which occur several seconds after impact and last for less than a minute, may also be due to electrocerebral silence induced by concussion with transient loss of cortical inhibition of spinal cord tracts resulting in limb movement. We are planning EEG studies to distinguish between these two possibilities, but the occurrence of seizure-like activity prompted us to test even lower drop heights to produce a model of repetitive injury devoid of convulsions.

To determine a drop height that eliminated convulsions but still led to cognitive deficits we tested several heights between 38- and 18-in.. Pilot studies suggested that a 28-in. drop height would satisfy both conditions, but lower drop heights did not produce cognitive deficits using a 54 g weight. Mice (3 months old males, Jackson labs) were anesthetized for 45 s in isoflurane as earlier and subjected to sham injury or CHI by dropping the bolt on the vertex of the head (*see* **Note 6**). This model did not produce loss of consciousness in injured compared to sham-injured mice (*see* **Note 7**), nor did it produce convulsions, similar to the majority of athletes who suffer concussions [65].

We next determined that a 5 or 7 hit model (1 hit daily for 5 or 7 days) did not produce acute cell death or overt brain damage, and we were now poised to examine cognitive deficits in a truly mild multiple concussion model. Using a 5 hit daily injury model, we performed experiments with 1 hit CHI and shams, 5 hit daily CHI and shams, 5 hit weekly CHI and shams, 5 hit biweekly CHI and shams, and 5 hit monthly CHI and their respective shams. All injured groups were compared only to their respective sham-injured mice because the groups were injured at different times, making direct comparisons among injured groups problematic.

We tested the hypothesis that a safe rest interval exists such that repeated injuries would not lead to long-term cognitive deficits, and found that indeed mice injured biweekly or monthly had no long-term cognitive deficits whereas mice injured daily or weekly had deficits compared to sham at 6 months (*see* **Note 8**). Because these were long-term experiments that could not be repeated in a reasonable time frame, we powered most of our studies with $n = 12–16$ mice per group and obtained tightly clustered data that allowed confident determination of statistically significant results. With these studies, we confirmed the important concept that a safe rest interval existed in our repetitive concussion model, thus in part validating the concept that rest between concussions may mitigate development of permanent cognitive deficits [32].

Interestingly, we found a chronic astrocytosis at 6 months in mice injured daily for 5 days, but no overt neuronal loss. Contrary to published studies in adults with TBI, mice expressing human APOE4 did not have worse cognitive deficits compared to WT suggesting no contribution of astrocytic APOE4 in this model. Somewhat disappointingly, we did not see evidence of CTE in terms of phosphorylated tau species or beta amyloid assessed by ELISA and immunohistochemistry. However, it may require more than 6 months for these processes to become manifest in mice, and more sensitive immunohistochemical reagents may yield positive findings in future studies. Alternatively, it is possible, and even likely, that mechanisms of short-term neurological deficits differ from those associated with development of CTE, which may represent the most severe end of the spectrum for patients with repetitive concussions and severe neurodegeneration [36].

Another finding in this model that may be incongruent with human studies is that cognitive deficits are detectable within a few days after a threshold number of injuries, and these deficits remain even at 6 months after the last injury; thus, rather than develop over the course of time, permanent cognitive deficits produced by our repetitive CHI model are present very early on. It might be more interesting for a concussion model to produce increasing cognitive deficits over the course of several months (or longer with aging)—and this might be the case if we were to test these mice over longer periods of time. Very long-term studies may not be feasible however, as 4/4 mice subjected to 7 daily concussions (28 in., 54 g) died at 14 months after injury compared to 0/4 shams, suggesting that multiple concussions might shorten the lifespan of injured mice. Although this hypothesis requires formal testing with larger numbers, it is an interesting observation that might be applicable to humans with repetitive concussions as well. Alternatively, it may be a shortcoming of the mouse model.

6 Future Considerations

Arguably the development of our CHI model, and similar weight drop models [16, 17] that have been published subsequent to Khuman et al. [60], represent forward steps in modeling human concussion that will facilitate discovery of relevant disease mechanisms. A major question for us and others in the field [19] is whether inhaled anesthetics interact with injury models to reduce neurological deficits, as LOC times decrease with increasing number of injuries in our model (unpublished observations) and in others [19]. One group has managed to avoid the issue all together by subjecting unanesthetized mice to repetitive TBI [20]. Studies examining effects of noninhaled anesthetics are currently underway in our laboratory. Another caveat of our repetitive hit models is that similar to

the single hit model, cognitive outcome and mortality in the 5 hit daily model is highly operator dependent for reasons that remain incompletely defined. In the future, we would like to develop a holding device that is operator independent to ensure consistency in the injury level produced by CHI. Another future direction for our laboratory is development of an adolescent mouse concussion model that recapitulates symptoms of sports concussions in high school and college athletes. Finally, measurements of inflammation, cerebral blood flow, and brain metabolism will be important components of concussion model development as these mechanisms are hypothesized to underlie long-term neurological deficits in patients with neurological degeneration and history of TBI [41–43, 50].

7 Notes

1. There are two ways to increase the level of injury—raising the drop height or increasing the dropped weight. Each lab should experiment with both approaches, calibrating to no structural brain damage and a robust cognitive deficit in the Morris water maze or other behavior test of choice.
2. Although we started with a two person operator system (one to hold the mouse and the other to drop the bolt), the apparatus can be standardized better by using a platform to hold the mouse, thus making injury independent of the second operator. This approach will ensure greater consistency of injury over time and among different operators.
3. The model lends itself to cerebral blood flow testing because of the lack of structural brain injury. We have used diffuse correlation spectroscopy in the past for this, and it is also possible to use laser speckle and laser Doppler flowmetry as well.
4. There may be marked gender effects in the closed head injury model but before examining this issue it is important to use age- and weight-matched male and female mice, as the lighter female mice may sustain less inertial injury after impact.
5. Use of a guide tube that is approximately the diameter of the mouse head will result in more accurate hits with the dropped weight, which ideally should be cylindrical in shape and should easily pass through the guide tube but fit snugly within it. Some groups use metal, others use plastic, or other transparent materials in order to follow the course of the dropped weight.
6. Younger (adolescent) mice may have increased mortality from apnea that can be prevented by injuring over one side of the head or the other.
7. Because repeated injuries may alter the loss of consciousness time for subsequent injuries, it is recommended to measure loss

of consciousness time (defined as righting reflex) at very least after the first in a series of repetitive injuries rather than wait until several injuries to begin measurement of awakening.

8. It is critical to use a mouse strain that performs well in cognitive tests such as the Morris water maze. We have found that C57Bl/6 mice work well (Jackson Laboratories, Bar Harbor, ME). CD1 and other outbred strains may not perform consistently.

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