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Repeated Mild Traumatic Brain Injury: Potential Mechanisms of Damage

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Abstract

Mild traumatic brain injury (mTBI) represents a significant public healthcare concern, accounting for the majority of all head injuries. While symptoms are generally transient, some patients go on to experience long-term cognitive impairments and additional mild impacts can result in exacerbated and persisting negative outcomes. To date, studies using a range of experimental models have reported chronic behavioral deficits in the presence of axonal injury and inflammation following repeated mTBI; assessments of oxidative stress and myelin pathology have thus far been limited. However, some models employed induced acute focal damage more suggestive of moderate–severe brain injury and are therefore not relevant to repeated mTBI. Given that the nature of mechanical loading in TBI is implicated in downstream pathophysiological changes, the mechanisms of damage and chronic consequences of single and repeated closed-head mTBI remain to be fully elucidated. This review covers literature on potential mechanisms of damage following repeated mTBI, integrating known mechanisms of pathology underlying moderate–severe TBIs, with recent studies on adult rodent models relevant to direct impact injuries rather than blast-induced damage. Pathology associated with excitotoxicity and cerebral blood flow-metabolism uncoupling, oxidative stress, cell death, blood-brain barrier dysfunction, astrocyte reactivity, microglial activation, diffuse axonal injury, and dysmyelination is discussed, followed by a summary of functional deficits and preclinical assessments of therapeutic strategies. Comprehensive characterization of the pathology underlying delayed and persisting deficits following repeated mTBI is likely to facilitate further development of therapeutic strategies to limit long-term sequelae.

Keywords

repeated mild traumatic brain injury, pathology, functional deficits, reactive gliosis, oxidative stress, myelin abnormalities

Introduction

Traumatic brain injury (TBI) encompasses structural brain damage or physiological alteration in brain function resulting from an external force.[1] Worldwide, the leading causes of TBI are falls and motor vehicle accidents, resulting in an estimated 10 million deaths and/or hospitalizations annually[2]; TBI is the leading cause of mortality and morbidity for persons under 45 y of age.[3] TBI is a robust environmental risk factor for neurodegenerative disorders,[4] and chronic sequelae may lead to permanent disability and ongoing care and cost.[5] Currently, therapeutic interventions for TBI are lacking.

TBI can be mechanically induced by blunt or penetrating impacts, non-impact blast waves, or inertial loading. While penetrating injuries are typically synonymous with severe TBI, other causes of injury do not necessarily lead to specific injury severity or prognosis. As such, classification systems are employed to delineate TBI severity, based on clinical presentation and structural findings.[6] Clinical severity is determined using the universally accepted Glasgow Coma Scale,[7] which scores ocular, motor, and verbal responses on a scale of 3–15. Mild TBI (mTBI) patients score 13–15, moderate TBI patients score 9–12, while severe injuries score <9. Additionally, traditional neuroimaging techniques such as magnetic resonance imaging and computed tomography are employed to detect the presence of gross lesions, allowing a broad differentiation between focal and diffuse damage.[8] Patients diagnosed with

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moderate or severe TBIs are often grouped together, as they exhibit gross structural damage on neuroimages. Overt abnormalities are typically focal in nature and can include cerebral contusions, extra or subdural hematomas, subarachnoid hemorrhage, intracranial or intraventricular bleeding, or skull fractures.[9] On the other hand, patients diagnosed with a mTBI exhibit normal neuroimaging[10]; however, it is important to note that microscopic damage such as diffuse axonal injury (DAI) is undetectable using traditional neuroimaging techniques.[6] As such, a diagnosis of mTBI is determined on clinical observation or self-reported symptoms; the term concussion is generally used interchangeably to define the clinical syndrome.[11] Hereafter, mTBI will be used to describe these injuries.

Mild and Repeated mTBI

Epidemiological research indicates that 70–90% of all TBIs are mild, with incidence likely to be substantially underestimated.[12] Mild head trauma is common among professional athletes engaged in contact and collision sports[2] and military personnel[13]; this review will focus on models of mTBI more relevant to sports-related injury. The primary cause of mTBI in sports is the application of both linear and rotational acceleration and impact deceleration forces to the brain, inducing nonpenetrating diffuse rather than focal damage.[14-16] Typically, mTBI is characterized by a transient disturbance in brain function, with short-lived neurological symptoms including headache, dizziness, and confusion.[17] Symptoms for most patients generally subside within 10 d of injury[18]; however, they can persist with 10–40% developing postconcussion syndrome,[19-21] associated with long-term cognitive deficits and white matter changes.[22] While a single mTBI may not always result in behavioral impairments, clinical research suggests that further injuries induce cumulative effects, by both increasing the susceptibility for further mTBI and progressing to long-term functional deficits[23,24] and underscoring the importance of "return to play" guidelines in sports. In particular, retired American football athletes with a history of repeated mTBI show elevated rates of cognitive impairment,[25] long-term psychiatric illness, and an increased incidence of chronic traumatic encephalopathy (CTE), a progressive tauopathy.[26-28] This review focuses on potential mechanisms of damage underlying the cumulative and chronic effects of repeated "closed-head" mTBI, referring to single mTBI in the context of studies exploring subsequent injuries. For more detailed discussion of single mTBI, the reader is referred to Dewitt et al.[29] for review. The importance of using clinically relevant experimental models of mTBI is receiving increasing attention and will be touched upon here (see Xiong et al.,[30] Angoa-Pérez et al.,[31] Laplaca et al.,[32] Namjoshi et al.,[33] and Zhang et al.[34] for further insights).

Experimental Models of TBI: Toward Clinical Relevance

In order to develop therapeutic strategies to prevent or ameliorate long-term damage and deficits following repeated mTBI, an understanding of the pathophysiological cascade of events and the mechanistic link between acute and chronic mTBI pathology needs to be elucidated. This can only be achieved using experimental models that suitably approximate the forces behind the primary injury, producing structural and functional deficits akin to human mTBI. Further, there is a threshold for the generation of injury and its potential exacerbation by repeated traumatic insults, with implications for long-term outcome measures. As such, additional considerations in experimental design include severity and number of impacts as well as inter-injury interval. As the majority of studies exploring repeated mTBI have used young adult rats of 2–3 mo of age, this review will focus on studies using adult rodents.

The majority of mechanistic TBI studies have used models incorporating stereotaxic head restraint, anesthesia, a craniotomy, and direct impact onto the brain to induce focal injuries and marked acute behavioral deficits. However, human mTBI features head movement in the absence of dural penetration and structural and functional deficits are subtle. Craniotomies[35] and anesthesia[36] in rodent models of mTBI likely confound damage, particularly if repeated, and do not reflect the human injury. While there are no universally accepted criteria for validity in mTBI models, nonpenetrating mechanical input, without acute focal damage and incorporating linear and rotational forces, is intuitively desirable. Indeed, confirming the absence of skull fracture, hemorrhage and acute cell death, and/or neuronal degeneration following mTBI is becoming commonplace.[29] An absent or mild acute behavioral phenotype and the capacity for repeated injury are further useful attributes of a suitable model of repeated mTBI.

However, given the heterogeneity of TBIs in humans, and inherent lack of face validity of animal models, no single experimental model can mimic the entire complexity of TBI-induced pathology. While closed-head models incorporating both linear and rotational forces are more appropriate to model single and repeated mTBI, it is nevertheless important to consider "open-head" models causing moderate and severe injuries for what they can tell us about mechanisms of pathology.

Open-head Models of TBI

Open-head experimental models, namely, lateral fluid percussion (LFP)[37] and controlled cortical impact (CCI),[38] have been extensively utilized to explore moderate–severe TBI. Within experiments, direct force onto the brain imparts highly reproducible focal damage, though changes in craniotomy position translate to variable outcomes between laboratories.[39] In the LFP model, a pendulum strikes a fluid-filled reservoir, and pressure from a fluid-filled bolus is forced into the epidural space,[37,40] The LFP model typically induces focal damage such as hemorrhage and edema at the site of impact, with progressive subcortical cell death,[41-43] thereby replicating many structural, pathological, and neurobehavioral features of moderate–severe human TBIs. LFP has been used to model single[44] and repeated mTBI,[45-47] by lowering the pendulum height to reduce the pressure pulse and therefore injury severity. However, focal cell loss in control animals receiving a single “mild” TBI may still remain.

In the CCI model, an electromagnetic or pneumatically driven piston directly penetrates the underlying cortex from a known distance and velocity.[38] Deformation of the underlying cortex induces cortical cell loss and subdural hematoma[37] and leads to more diffuse axonal injuries than the LFP model.[48,49] CCI simulates many pathological and behavioral outcomes characteristic of moderate–severe TBI in humans, and injury severity can be graded by adjusting impact depth and velocity.[30] Indeed, CCI is extensively employed to model repeated mTBI,[50-52] including closed-head variations without focal damage[53-57] and incorporating acceleration–deceleration forces.[58,59] However, care must be taken when interpreting findings referred to in publications as mild or repeated mild CCI, as head movement upon impact is still predominantly restricted.

Closed-Head Models of TBI

In addition to closed-head mild CCIs, weight-drop (WD) models are capable of delivering a diffuse injury through the intact skull. While the first WD models mainly induced focal damage, with[60] or without a craniotomy,[61,62] subsequent closed-head models were developed to produce more diffuse damage.[63,64] In Marmarou’s WD impact-acceleration model, a free-falling weight is guided down a tube, striking a steel disk placed on the rodent’s exposed skull, preventing skull fracture.[63] As the rat rests on a piece of foam, slight movement of the head is allowed, thereby transmitting some acceleration forces. The result is widespread damage of neurons and axons alongside severe compression of the cranial vault, suitably modeling non-penetrating moderate–severe TBI.

In recent years, the heaviness of the weight and the drop height have been modified and titrated to eliminate focal cortical injury as an acute feature.[65-69] Increasing amounts of head movement have also been incorporated,[70-72] to more closely approximate human head kinematics following mTBI.[73] As such, WD models are increasingly utilized to model repeated mTBI. To incorporate rapid translational and angular acceleration forces, the animal is rested on a Kimwipe,[72] aluminum foil[70,71] or traversable “trap door”[74] suspended on a hole in the center of the apparatus stage. The impact results in unrestricted movement of the head and body as the animal readily penetrates the material upon impact and free falls onto a padded cushion below.

Various other models have been developed to increase rotational acceleration[75] and employ momentum-exchange principles in a frontal impact model[76,77] through the use of a pendulum striker[78,79] as well as projectiles.[80,81] Mechanical input parameters and subsequent outcomes can be more variable in closed-head models incorporating rotational head movement.[33] However, resulting tissue strains are greater than those produced by the pure translational forces that define open-head models[16,82] and are more reflective of human head movement following impact-related mTBI.

Mechanisms of Pathology Following TBI

TBI is traditionally characterized by primary and secondary injury phases, both contributing to the extent of damage.[83] The primary injury represents acute disturbances and/or damage induced at the moment of impact, while secondary injury mechanisms, collectively known as secondary degeneration, involve a cascade of downstream interacting pathophysiological mechanisms.[15] In mild and repeated mTBI, however, there is no clear spatial separation between primary and secondary injury, and mechanisms of pathology remain insufficiently characterized. In a single mTBI, a dynamic restorative process likely underpins the transient alteration in brain function.[84] In contrast, the long-term sequelae of repeated mTBI are more reminiscent of moderate–severe injuries, suggesting that similar underlying cellular and metabolic events are occurring in repeated mTBI, albeit to a reduced degree and in a starkly different temporal progression.[85,86] It remains to be elucidated whether this worsening of long-term outcome is due to a cumulative effect of subsequent mTBIs or the independent or synergistic action of secondary processes exacerbating outcome. Herein, mechanisms of damage known to occur in moderate–severe TBI are described, with specific reference to evidence from repeated mTBI literature where available. Table 1 provides further study-specific information of known pathology in the various repeated mTBI models.

Excitotoxicity and Cerebral Blood Flow-Metabolism Uncoupling

In moderate–severe TBI, the initial impact mechanically disrupts axolemma and neuronal plasmalemma protein channels, causing immediate depolarization and dysregulated ionic homeostasis.[87] Indiscriminate release of excitatory amino acids, particularly glutamate, exacerbates potassium (K^+) efflux in a severity-dependent fashion.[88] Overactivation of glutamate receptors and voltage-gated calcium (Ca^{2+}) channels facilitates Ca^{2+} influx, triggering mitochondrial Ca^{2+} sequestration, Ca^{2+}-dependent Ca^{2+} release from intracellular stores, and dramatically elevated cytosolic Ca^{2+}.[89] Further, excessive Ca^{2+} influx can initiate cell death pathways[90] and lead to compaction of neurofilaments, microtubule disassembly, and impaired axonal transport, coupled with eventual swelling and axotomy.[91] In moderate–severe TBI, Ca^{2+} accumulation as measured by isotope-labeled Ca^{2+} can persist for up to 1 wk, concomitant with memory deficits in the Morris water maze (MWM).[92] Additionally, Ca^{2+}-induced depolarization of the mitochondrial membrane allows electron leakage to oxygen in the electron

Table 1. Summary of outcome measures in repeated mTBI studies in adult rodents.

Study	Animal, age	Injuries (number)	Interval	Time analyzed	Outcome measures	Key findings
Controlled cortical impact with craniotomy						
50	Male rats Sprague-Dawley 2–4 mo	Single Repeat (2) Bilateral	3 or 7 d	Acute, subacute + chronic (radiology) Subacute (cellular)	Tissue integrity (MRI) Extravascular blood deposition Lesion/oedema/blood volumes Astrocyte reactivity Microglial activation	Overt tissue damage Damage exacerbated with 7-d injury interval, with bilateral mTBIs Lesion composition depends on injury interval (oedema: 3 d; blood: 7 d) *Repeat*: reactive astrocytes at second injury site only *Repeat, 3 d*: ↑ activated microglia *Repeat, 7 d*: ↑↑ activated microglia
52	Male rats Sprague-Dawley 65–75 d	Single Repeat (2)	1, 3, or 7 d	Acute Subacute Long term (behavior)	Behavior Tissue integrity (MRI) Extravascular iron deposition Astrocyte reactivity Microglial activation	Overt tissue damage: cortical tissue loss Damage exacerbated with 3-d interval, followed by 1 d Lesion composition depends on injury interval (oedema: 1 d; blood: 3 d) *Single + repeat:* ↑ subacute microglial reactivity *Repeat, 3 d:* ↑↑ Extravascular iron deposition ↑ Astrocyte reactivity ↑↑ Activated microglia ↑ Long-term learning deficits + Anxiety-like behavior
51	Male rats Sprague-Dawley 2–4 mo	Single Repeat (2)	7 d	Long term	Axonal injury White matter integrity (DTI) White matter ultrastructure	No difference in neurofilament 200 *Single + repeat:* ↓ Myelin thickness *Repeat:* ↑ Radial diffusivity, g-ratio, axon calibre + CC size Progressive myelin sheath abnormalities and axonal damage
Controlled cortical impact without craniotomy						
Skin intact						
53	Male mice C57BL/6 10 wk	Single Repeat (5)	48 h	Acute (all) Subacute (behavior, subset of single)	Physiologic measurements Behavior Tissue integrity Astrocyte reactivity Microglial activation Axonal injury Myelin integrity	No overt tissue damage ↓ Apnoea duration with subsequent injuries *Single:* Acute cognitive deficits, transient Mild progressive astrocyte reactivity Microglial activation, persisting ↑↑ APP immunoreactive axonal profiles in CC, transient Repeat: ↑↑ Cognitive deficits ↑ Astrocyte reactivity Acute microglial activation ↑ APP immunoreactive axonal profiles in CC + brainstem
214	Male + female mice C57BL/6 +Tg hτ 18 mo	Single Repeat (5)	48 h	Subacute	Tissue integrity Apoptosis Astrocyte reactivity Microglial activation Axonal degeneration Neurodegeneration	No overt tissue damage No axonal degeneration *Single:* ↑ Astrocyte reactivity *Repeat:* Cell death in cortex ↑↑ Astrocyte reactivity ↑ Microglial activation ↑ Phosphorylated τ immunoreactivity
151	Male mice C57BL/6 +FVB/N Tg (GFAP$_{luc}$) 2–3 mo	Single Repeat (2, 3, 5) Three impact speeds for mild characterization in reporter mice	24 h	Acute (in *vivo* imaging) Subacute + long term + chronic (behavior) Chronic (behavior, cellular)	Behavior Tissue integrity Apoptosis Astrocyte reactivity Signaling pathway Neurodegeneration	No overt tissue damage No motor deficits, anxiety-like behavior or cell death Dose dependent ↑ bioluminescence signal for 1–3 mTBIs *Repeat:* ↑ Locomotor activity, transient Long-term cognitive deficits, persisting ↑ Astrocyte reactivity ↓ p-CREB immunoreactivity ↑ p-τ immunoreactivity

(continued)

Table 1. (continued)

Study	Animal, age	Injuries (number)	Interval	Time analyzed	Outcome measures	Key findings
[180]	Male mice C57BL/6 9–15 mo	Single Repeat (5)	48 h	Chronic	Behavior Tissue integrity Astrocyte reactivity Microglial activation Axonal injury Neurodegeneration Myelin integrity	No changes in τ, no chronic motor deficits *Single* + *repeat:* Learning deficits ↑ Astrocyte reactivity ↑ Microglial activation ↓ CC thickness *Repeat:* Exacerbated astrocyte reactivity, microglial activation + CC thinning ↑↑ Learning + memory deficits, worsening ↑ APP immunoreactive profiles in CC Chronic WM degradation
[265]	Male mice C57BL/6 8–10 wk	Repeat (5)	48 h	Long term Chronic	Visual function Tissue integrity Morphology (ON + retina) Morphometry (ON) Astrocyte reactivity (ON) Microglial activation (ON) Myelin integrity	No overt tissue damage *ON:* ↓ Diameter ↑ Cellularity + local areas of demyelination ↑ Astrocyte reactivity + microglial activation *RGC:* ↓ RGC numbers + amplitude of visual response
[58]	Male mice C57BL/6 12 wk	Single Repeat (42) Helmet No anesthesia	2 h @ 6/d For 1 wk	Acute, subacute + long term (neurologic, behavior) Long term (sleep) Chronic (behavior)	Neurologic function Behavior Sleep	*Single* + *repeat:* Neurologic + motor deficits, transient Subacute anxiety-like behavior Long-term learning deficits + sleep disturbances *Repeat*: Acute memory deficits ↑ Long-term depression-like behavior ↑ Long-term risk-taking, persisting
[59]	Male mice C57BL/6 12 wk	Single Repeat (42) Helmet No anesthesia	2 h @ 6/d For 1 wk	Acute (tissue) Long term + chronic (tissue, cellular)	Tissue integrity BBB breach Astrocyte reactivity Microglial activation Neurodegeneration	No overt tissue damage *Single*: ↑ Acute astrocyte reactivity ↑ Long-term phosphorylated τ, subsiding *Repeat*: ↑↑ Widespread astrocyte reactivity, resolved long-term, chronic reactivation ↑↑ Widespread microglial activation, persisting ↑↑ Phosphorylated τ, persisting
Skull exposed						
[54]	Male mice C57BL/6 12 wk	Single Repeat (2)	24 h	Acute (all) Subacute (axonal) Long term (behavior, cellular)	Physiologic measurements Neurologic function Behavior Tissue integrity BBB breach Axonal injury Neurodegeneration	No overt tissue damage, no cognitive deficits, Aβ or τ *Single*: ↓ acute neurologic function, transient Acute BBB breach *Repeat*: ↓↓ Neurologic function, persisting ↑ Acute motor deficits, persisting ↑↑ Widespread BBB breach ↑ Subacute axonal injury ↓ Regional MAP2 immunoreactivity
[132]	Male + female mice B6D2/F1 +Tg APP 9–12 mo	Single Repeat (2)	24 h	Acute + subacute + long-term (cellular) Chronic (neurologic function, behavior, cellular)	Neurologic function Behavior Tissue integrity Astrocyte reactivity Oxidative stress Axonal injury Neurodegeneration	Iron deposits (single) + mild oedema (repeat) *Single* + *repeat*: Persisting astrocyte reactivity No Aβ deposits in wild-type *Repeat in Tg mice:* ↑ Cognitive deficits ↑ Long-term Aβ and Aβ deposition, persisting, higher burden in females ↑ Long-term urinary isoprostanes, persisting

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Table 1. (continued)

Study	Animal, age	Injuries (number)	Interval	Time analyzed	Outcome measures	Key findings
212	Male mice C57BL/6 6–8 mo	Single Repeat (2)	3, 5, or 7 d	Acute	Physiologic measurements Behavior Tissue integrity Axonal injury Neurodegeneration	No overt tissue damage Axonal injury + behavioral deficits exacerbated at 3 d, then 5-d interval *Single + repeat:* ↓ MAP2, axonal injury, transient motor deficits *Repeat, 3 d:* ↑↑ Motor + cognitive deficits ↑↑ Diffuse axonal injury
215	Male + female mice B6D2/F1 +Tg τ T44 12 mo	Repeat (16)	4/d @ 1 d/wk for 4 wk	Chronic	Neurologic function Behavior Tissue integrity Extravascular iron depositions Astrocyte reactivity Axonal degeneration/injury Neurodegeneration	No overt tissue damage No chronic behavioral deficits *Repeat in one Tg T44 mouse* Extensive τ pathology: ↑ insoluble τ, widespread NFT, cerebral atrophy, ↓ cortical thickness, dilated lateral ventricles, ↓ neurological function/ cognitive deficits, ↑ reactive astrocytic processes, ↑ axonal degeneration
56	Male mice C57BL/6 2–3 mo	Single Repeat (2)	24 h	Acute + long term	Behavior Tissue integrity Microglial activation Axonal degeneration/injury Ultrastructural analyses	No overt tissue damage *Repeat*: ↑ Cognitive deficits, persisting ↑ Microglial activation in close proximity to injured axons, transient in gray matter, persisting in WM tracts ↑ Acute cytoskeletal abnormalities ↑ Acute axonal degeneration, persisting in WM tracts
55	Male mice C57BL/6 J 6–8 wk	Repeat (2)	24 h	Acute (all) Subacute (all)	Microglial activation Axonal injury (DTI) White matter integrity Ultrastructural analyses	↑ Subacute microglial activation ↑ Acute axial + radial + mean diffusivity in cortex ↓ Subacute axial + mean diffusivity in WM ↑ Subacute axonal injury Silver staining density in WM tracts correlates with relative anisotropy
57	Male mice C57BL/6 Age not reported	Single Repeat (4)	24 h	Acute (physiologic, perfusion, behavior) Subacute (cellular) Chronic (behavior)	Physiologic measurements Cerebral blood perfusion Behavior Tissue integrity Astrocyte reactivity Microglial activation Axonal degeneration	No overt tissue damage *Single*: ↓ Acute cerebral perfusion, transient ↑ Acute vestibulomotor, motor, cognitive deficits, transient *Repeat*: ↓ Acute cerebral perfusion, (transient 1-3x; persisting 4x) ↑↑ Acute vestibulomotor (transient), cognitive impairments (chronic) ↑ Prolonged microglial activation, astrocyte reactivity ↑ Axonal degeneration
266	Male mice C57BL/6 6 wk	Repeat (2)	24 h	Acute + subacute (all) Long term (cellular)	Behavior Microglial activation	Synergistic effects of repeat + postinjury foot shock stress *Repeat*: ↑ Social deficits, anhedonia-like behavior, depressive-like behavior ↑ Microglial activation, delayed *Repeat + postinjury foot shock stress:* ↑↑ Social deficits + ↑↑ depressive-like behavior
150	Male mice C57BL/6 + Tg τ 2–3 mo	Single Repeat (5) Six impact depths to characterize mTBI	24 or 48 h	Acute (physiologic, cellular) Subacute (cellular)	Physiologic measurements Tissue integrity Astrocyte reactivity Microglial activation Axonal injury Neurodegeneration	No overt tissue damage No τ hyperphosphorylation *Single + repeat 48 h*: ↑ astrocyte reactivity *Repeat 24 h:* Neuronal degeneration/haemorrhagic lesions ↑↑ Astrocyte reactivity ↑ Microglial activation

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Table 1. (continued)

Study	Animal, age	Injuries (number)	Interval	Time analyzed	Outcome measures	Key findings
Lateral fluid percussion with craniotomy						
267	Male rats Wistar 2–3 mo	Single Repeat (7)	24 h	Subacute Long term	Behavior Neurodegeneration	No overt tissue damage *Repeat:* ↑ Locomotor activity with ↑ number + ↓ inter-injury intervals ↑ Subacute MAP2 + p-NFH, persisting and spreading ↑ τ-1 Immunoreactivity in neurons ↓ Efficiency habituating to new environment
268	Male rats Long-Evans 59–64 d	Single Repeat (2, 3)	10–14 d	Acute Subacute	Behavior	No motor deficits ↑ Acute cognitive deficits with ↑ Number mTBI
46	Male rats Long-Evans Age not reported	Single Repeat (3, 5)	5 d	Acute Long term	Physiologic measurements Behavior Tissue integrity Microglial activation	*Single:* acute cognitive deficits *Repeat, 3x:* Persisting cognitive dysfunction ↑ Cortical damage *Repeat, 5x:*↑↑ Cortical damage Persisting cognitive dysfunction + microglial activation Acute microglial activation Anxiety + depression-like behaviors
45	Male rats Sprague-Dawley 10–12 wk	Single Repeat (3)	48 h	Acute (neurologic, behavior) Subacute (all)	Neurologic function Behavior Tissue integrity Microglial activation Synaptic plasticity	Overt tissue damage in cortex *Single:* ↑ Cognitive deficits *Repeat:* ↑ Microglial activation ↑↑ Cognitive deficits Failed long-term potentiation Attenuated NMDA-receptor-mediated synaptic transmission
Weight-drop with craniotomy						
269	Male rats Sprague-Dawley Age not reported	Single Repeat (3, 3 + severe) Two impact heights + weights (mild + severe)	3 d Severe 3 or 5 d After final mTBI	Acute Subacute (behavior, cellular) Subacute (behavior)	Behavior Tissue integrity BBB breach Heat shock proteins Astrocyte reactivity	Overt tissue damage in cortex Conditioning effect: motor deficits in severe but not repeat + severe *Repeat + single severe:* ↑ Heat shock protein 27 ↑ Astrocyte reactivity
69	Male rats Wistar Age not reported	Single Repeat (2) Two impact heights (mild + severe)	3 or 5 d	Acute	Mitochondrial function Oxidative stress	*Repeat:* ↓ NAA, ADP, ATP/ADP (5 d vs. sham, not mild single) ↓↓ NAA, ADP + ATP (3 d vs. mild single, but not severe single)
107	Male rats Wistar Age not reported	Repeat (2)	1, 2, 3, 4, or 5 d	Acute	Gene expression Mitochondrial function	Cerebral metabolism modulated by injury interval: progressive changes maximal at 3-d interval, near control at 5-d interval, persisting to 7 d *Repeat, 3 d:* ↑ Sum oxypurines (hypoxanthine + xanthine + uric acid) ↑ Sum nucleotides (ATP + ADP + AMP) ↑ *ASPA* expression, AMP, ADP ↓ ATP, ATP/ADP ratio, NAA, NAAG, NAD^+, NADH, CoA-SH, acetyl-CoA, NAD^+/NADH, $NADP^+$
119	Male rats Wistar Age not reported	Repeat (2)	1, 2, 3, 4, or 5 d	Acute	Oxidative stress Nitrosative stress	Oxidative and nitrosative stresses modulated by injury interval: progressive changes maximal at 3-d interval, near control at 5-d interval ↑ Malondialdehyde, nitrite + nitrate ↓ Ascorbic acid, glutathione + reduced/oxidized glutathione (GSH/GSSG)

(continued)

Table 1. (continued)

Study	Animal, age	Injuries (number)	Interval	Time analyzed	Outcome measures	Key findings
Weight-drop without craniotomy						
With no or limited head movement						
65	Male mice B6C3F1 8 wk	Single Repeat (4) Three impact weights Two impact heights for heaviest weight	24 h	Acute (physiologic, BBB, cellular) Subacute (behavior, cellular)	Physiologic measurements Neurologic function Behavior Tissue integrity BBB breach Axonal injury	No overt tissue damage Characterized mild injury: not largest weight + height combination No BBB breach, axonal injury ↑ Cognitive impairment at 2 heavier weights (vs. heaviest weight with single mTBI)
66	Male mice C57BL/6 7–8 mo	Repeat (3)	24 h	Acute (physiologic, cellular, behavior) Subacute (behavior)	Physiologic measurements Behavior Tissue integrity Axonal degeneration	Contra-coup injury near skull and in ventral brain structures Learning deficits
211	Male rats Sprague-Dawley 3–6 mo	Single Repeat (2,3) Three impact heights	2 (0.5 m) at 3 h 3 (0.5 m) at 2 h 2 (0.75 m) at 3 h 2 (1.0 m) at 3, 5 or 10 h	Acute	Physiologic measurements Vascular reactivity Axonal injury	No axonal or microvascular change with repeated subthreshold impacts *Repeat:* ↑ Axonal damage + ↑ vascular dysfunction with ↑ height + ↓ interval
68	Male mice Swiss Webster 2–3 mo	Single Repeat (2)	3 or 20 d	Subacute	Behavior Tissue integrity Glucose metabolism Inflammatory gene expression Astrocyte reactivity Microglial activation Axonal degeneration	No overt tissue damage *Repeat:* ↑ Inflammation: *itgam*, astrocyte reactivity, microglial activation *Repeat 3 d:* Failed transient acute ↑ local cerebral glucose utilization = energy crisis ↑ Cognitive deficits ↑ *IL-1* + *TNF* ↑ Axonal degeneration
270	Male mice C57BL/6 3 mo	Single Repeat (3) Helmet Three impact heights	24 h	Acute (sleep) Subacute	Physiologic measurements Behavior Sleep	↑ Mortality with ↑ heights No anxiety-like or depression-like behavior *Single + repeat:* ↑ sleep disturbances *Repeat:* ↑ Cognitive deficits ↓ Motivation, apathy
271	Male mice C57BL6/J 5–6 wk	Single Repeat (4, 12) Helmet Three impact heights	4 @ d 0, 1, 3, 7 12 @ 3/d (2 h then 3 h) at 0, 1, 3, 7	Acute (neurologic, BBB) Subacute Long-term Chronic	*Brain + RGC/ ON:* Neurologic function Tissue integrity BBB breach Microglial activation Axonal degeneration/injury Neurodegeneration	No overt tissue damage Axonal/ON degeneration + RGC loss ↑ with weight, number + frequency *Single:* ↑↑ BBB breach ↑↑ Axonal injury in ON *Repeat:* ↑ BBB breach ↑ Axonal injury in ON No long-term phosphorylated τ immunoreactivity
With rotational acceleration						
72	Mice Sex, species + age not reported	Repeat (4, 5, 10)	4 in 2 d @ 6h 5 + 10 at 24 h	Acute + long-term (motor) Subacute (balance, cellular)	Physiologic measurements Behavior Tissue integrity BBB breach Astrocyte reactivity Microglial activation Axonal injury Neurodegeneration	No overt tissue damage No microglial activation, BBB breach ↑ Deficits with ↑ injury + ↓ inter-injury interval ↑ Acute motor activity deficits in repeat 4x + 5x *Repeat, 10x:* ↑ Subacute astrocyte reactivity ↑ Long-term tau phosphorylation

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Table 1. (continued)

Study	Animal, age	Injuries (number)	Interval	Time analyzed	Outcome measures	Key findings
71	Male mice C57BL/6 2–3 mo	Single Repeat (3, 5, 10)	3, 5 + 10 in 24 h 5 in 1w + 1 mo	Acute + long-term (behavior, neuronal degeneration) Chronic (behavior)	Physiologic measurements Behavior Tissue integrity Vascular damage BBB breach Axonal injury	No overt tissue damage No axonal degeneration, BBB breach ↑ Cognitive deficits with ↑ injuries, ↓ inter-injury interval + ↑ drop height *Repeat, 5x*: No cognitive deficits with 1-mo interval Cognitive deficits persist long-term (1-wk interval) + chronic (24-h interval)
70	Male mice C57BL/6 + Tg apoE 2–3 mo	Single Repeat (5,7)	5 at 24 h, 1 wk, 2 wk + 1 mo 7 in 9 d	Acute + subacute (behavior) Chronic (all)	Behavior Tissue integrity (MRI) Brain volume Astrocyte reactivity Microglial activation Axonal injury Neurodegeneration White matter integrity	No overt tissue damage ↑ Intervals protects against long-term cognitive deficits *Repeat*: Cognitive deficits independent of ↑ AB/τ phosphorylation Cognitive outcome not influenced by ApoE4 status Long-term cognitive deficits associated with ↑ astrocyte reactivity
74	Male mice CD-1 8 wk	Repeat (5)	5 in 3 d or 5 in 15 d	Acute Subacute Long-term	Lateral ventricle volumes Astrocyte reactivity Microglial activation Axonal injury Neurodegeneration Myelin integrity	No τ phosphorylation, axonal injury or myelin changes *Repeat, 3 d:* ↑ Acute astrocyte reactivity, persisting long-term ↑ Acute microglial activation, transient *Repeat, 15 d*: ↑ Acute astrocyte reactivity, transient
Miscellaneous models with rotation						
81	Male rats Wistar, Age not reported	Single Repeat (3) Helmet Two impact weights 3 projectile speeds	6 h	Acute Subacute	Injury parameters Tissue integrity	↑ Bleeding areas/ focal contusions with ↑ weight + ↑ speed Fine petechial hemorrhage on brain surface in parenchyma and meninges nearest impact in least severe impact
80	Male rats Wistar, Age not reported	Repeat (3) Helmet 2 impact weights 3 projectile speeds	3 min	Acute Subacute	Physiologic measurements Intracranial pressure Behavior	↑ Cognitive deficits with ↑ weight + ↑ speed *Most severe weight + speed*: ↑ Acute transient intracranial pressure ↑ Acute memory deficits Anxiety-like behavior, persisting
76	Male + female mice C57BL/6 + Tg τ (±, ±, ±) 4–6 mo	Single (CCI, frontal) Repeat (2) (frontal)	48 h	*Frontal*: Acute (radiology) Subacute + long-term (behavior) Chronic (cellular) *CCI*: subacute (behavior)	Behavior (only CCI outcome) Neuronal degeneration Tissue integrity (MRI) Astrocyte reactivity Microglial activation Axonal degeneration/injury Neurodegeneration	No overt tissue damage *Repeat (frontal)*: ↑ Long-term cognitive deficits (WT + $\tau^{+/+}$) No persisting astrocyte reactivity or microglial activation No motor, anxiety- or depressive-like behavior Partial τ reduction ↓ axonal injury + cognitive deficits *CCI:* Acute memory but not learning deficits in $\tau^{+/+}$
75	Male mice C57BL/6 4 mo	Repeat (2)	24 h	Acute (all) Subacute (behavior, axonal, inflammation)	Physiologic measurements Neurologic function Behavior Cytokines Microglial activation Axonal degeneration Neurodegeneration	Cognitive deficits, persisting Acute ↑ TNF-α + ↑ IL-1β Persisting microglial activation in WM Persisting axonal degeneration in CC + contrecoup regions ↑ endogenous τ phosphorylation

Note: Key outcome measures and findings in repeated mTBI studies using adult rodents. Study descriptors are included where available; interval refers to inter-injury interval. Time analyzed is defined as: <7 d = acute, ≥7d to <1 mo = subacute, ≥28 d to <3 mo = long-term, and ≥3 mo = chronic. Aβ, amyloid beta; ADP, adenosine diphosphate; ApoE, apolipoprotein E; APP, amyloid precursor protein; ASPA, acetylaspartate acylase; ATP, adenosine triphosphate; BBB, blood-brain barrier breach; CC, corpus callosum; CCI, controlled cortical impact; d, days; CREB, cAMP response element binding protein; DAI, diffuse axonal injury; DTI, diffusion tensor imaging; h, hours; IL-1β, interleukin 1β; mo, months; m/s, meters per second; MAP2, microtubule-associated protein-2; MRI, magnetic resonance imaging; MWM, Morris water maze; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartyl glutamate; NAD, nicotinamide adenine dinucleotide; NS, neuroscore; ON, optic nerve; P-NFH, heavy neurofilament; RGC, retinal ganglion cell; TNF-α, tumour necrosis factor-α; WT, wild type; WM, white matter; mTBI, mild traumatic brain injury.

transport chain, uncoupling oxidative phosphorylation, and suppressing adenosine triphosphate (ATP) synthesis in a CCI model.[93] ATP-dependent pumps are engaged to restore TBI-induced ionic imbalances, resulting in a transient increase in cerebral glucose uptake.[94] In a closed-head model of diffuse TBI, mitochondrial dysfunction, as measured by ATP and *n*-acetyl aspartate reductions, positively correlates with injury severity.[95,96] Concomitant with hyperglycolysis, acute decreases in cerebral blood flow (CBF) have been well-documented in experimental models,[97,98] and this failure to meet increased energy demands induces a severity-dependent metabolic crisis.[99] A subsequent period of hypoglycolosis can ensue.[90,97,100] While complete recovery typically occurs within 10 d,[101] the precise longevity correlates with severity,[102] behavioral deficits,[103] and progressive white matter damage above a certain threshold.[90] Interestingly, effects of applying a secondary insult such as ischemia or bilateral carotid occlusion following CCI[99,104,105] and WD[106] suggest that the critical temporal CBF-metabolic uncoupling period reflects a window of vulnerability of increased susceptibility. In a repeated moderate TBI WD study, an inter-injury interval of 1–3 d induces maximal damage in a range of metabolic outcome measures.[107] The state of metabolic depression reflects an altered cerebral state that is associated with functional deficits and has been proposed to reflect a "window of vulnerability" or vulnerable cerebral state.[107]

CBF-Metabolism Uncoupling Following Repeated mTBI

Aligning with the acute temporal profile of ionic fluxes and metabolic events characterizing moderate TBIs,[91] it has been suggested that acute ionic imbalances and energy dysregulation following a single mTBI also represent a temporal window of vulnerability that is associated with acute behavioral deficits[107,108] and increased susceptibility to damage with further insults.[54,69,71] Indeed, while acute cerebral hypometabolism, mitochondrial dysregulation, and cognitive deficits resolve within 1 wk following a single WD mTBI, a second injury delivered after a 3 but not 20 d interval, resulted in exacerbated metabolic dysregulation concomitant with cumulative cognitive deficits.[68] While the implications of these findings on long-term outcome remain unexplored, it is possible that these earlier chemical vulnerabilities may be the impetus for longer-lasting metabolic cascades.

Oxidative Stress

There is a highly interactive relationship between glutamate excitotoxicity, intracellular Ca^{2+} accumulation, metabolic depression, and reactive oxygen species (ROS) production,[109-111] and the latter is thought to mediate neurotrauma-induced secondary degeneration.[112] TBI is characterized by increases in both ROS and reactive nitrogen species production as a consequence of excessive[113] intracellular Ca^{2+} as well as decreases in enzymatic or nonenzymatic antioxidants such as manganese superoxide dismutase glutathione peroxidase, ascorbic acid, and glutathione.[114-116] When excess ROS and reactive nitrogen species overcome endogenous antioxidant capacities, the state of metabolism is referred to as oxidative stress.[117] Subsequent oxidation of lipids, proteins, and DNA to toxic metabolites causes cellular dysfunction[118]; ROS-mediated tissue damage has been positively correlated with TBI severity.[119,120]

Oxidative stress after TBI predominantly manifests as lipid peroxidation, likely attributable to the brain's high polyunsaturated fatty acid (PUFA) content.[121] In a rat focal contusion model, a progressive increase in lipid hydroperoxides is observed following an immediate post-traumatic burst in hydroxyl radical formation.[122] ROS-mediated lipid peroxidation, measured by 4-hydroxynonenal[123] and malondialdehyde concentrations,[124] respectively, is similarly increased following moderate CCI and WD TBI. Additionally, lipid peroxidation has been associated with blood-brain barrier (BBB) damage following CCI TBI.[122] Acute increases in protein nitration[125] and DNA damage[123] can also occur following TBI, while excessive ROS may also trigger caspase-dependent[126] and -independent cell death pathways.[127]

In a repeated moderate WD study, increases in reduced glutathione/oxidized glutathione, and nitrate and nitrite stressors, together with decreases in the antioxidant ascorbic acid were observed 48 h after final injury, when 2 moderate TBIs were given 1–3, but not 5 d apart.[119] While studies are limited, these findings provide further support for an acute temporal period of compromised cellular defenses in the brain, with modulation of oxidative and nitrosative stressors by injury interval and a cumulative effect of increased ROS production following repeated moderate TBI implicating oxidative stress in the proposed window of vulnerability. Beyond this, oxidative stress following moderate–severe TBI feeds back to and propagates Ca^{2+}-induced glutamate excitotoxicity and mitochondrial dysfunction. Further, long-term oxidative stress plays a pivotal role in neurodegeneration[128,129] and potentially in the pathogenesis of neurodegenerative diseases.[130,131]

Oxidative Stress Following Repeated mTBI

Given the progressive nature of pathology and long-term negative outcomes following repeated mTBI, oxidative stress is implicated as a driver of damage. However, studies exploring oxidative stress in the context of repeated mTBI are scarce. A transgenic mouse model of Alzheimer's disease-like amyloidosis has been used to explore the relationship between repeated mTBI and neurodegenerative disease.[132,133] A transient increase in isoprostanes, a product of free radical peroxidation of PUFAs, is reported following a single mild CCI, and a subsequent injury given after 24 h results in exacerbated lipid peroxidation that persists to 4 mo. Increased isoprostanes is associated with greater cognitive impairment and accelerated brain amyloid beta (Aβ) protein accumulation and deposition[132]; similar findings have been reported elsewhere.[133]

Cell Death

Injury-induced neuronal and glial cell death likely occurs along a continuum of necrotic (passive) and/or apoptotic (programmed) mechanisms,[134,135] resulting in removal of injured and dysfunctional cells, but also progressive neuronal degeneration and exacerbated functional deficits.[136,137] Necrotic cell death occurs under conditions of excitotoxicity and metabolic failure particularly prevalent at the site of impact immediately following focal TBI, while surviving cells spatially separated from the primary necrotic injury can undergo delayed and programmed cell death.[3] Caspase-3 triggers cell death in CCI[138] and LFP[139] TBI models, while in a moderate–severe rat CCI study, protein unfolding following endoplasmic reticulum stress activates capase-12[140] and elevated capsase-12 messenger RNA in a severity-dependent manner.[141] In a severe CCI model, conditions of impaired mitochondrial respiration and oxidative/nitrosative stress are associated with apoptosis-inducing factor mediating cell death via poly(adenosine diphosphate ribose) polymerase-1-induced apoptosis, 6 h post-injury in the hippocampus.[142] The maintenance of intact mitochondrial membrane potential is a critical factor in determining propensity toward apoptotic instead of necrotic mechanisms.[143,144] It follows that the type, extent, and temporospatial distribution of cell death is closely related to injury type and severity.[145] Further, significant reductions in mature oligodendrocytes in white matter tracts are observed acutely,[44] subacutely,[146,147] and persisting to 1 mo[148,149] following moderate LFP and CCI TBI. Concomitant temporal and spatial association with increased caspase-3 expression[146,148] indicates oligodendrocyte vulnerability to TBI-induced apoptosis in subcortical white matter that may underlie dysmyelination and contribute to secondary axonal injury.

Cell Death Following Repeated mTBI

Histological stains such as cresyl violet, hematoxylin and eosin, and fluorojade are used to assess cell death as an acute outcome following mTBI, with cell death is typically absent following single mTBI.[54,56,65,70] One repeated mTBI study reported significant acute neuronal death in the entorhinal cortex and around hemorrhagic lesions, following 5 CCI mTBI given at 24 h intervals.[150] Findings were deemed an outcome of both injury number and inter-injury interval, as a 48-h interval prevented neurodegeneration[150]; however, a study using similar injury parameters and experimental design did not report neurodegenerative changes.[151] While a threshold for injury severity exists, the typical absence of overt neuronal cell death at both acute and chronic time points suggests that neuronal dysfunction and diffuse axonal injury are greater contributors to the progressive nature and chronic sequelae of mTBI and repeated mTBI than death of neurons; little is known regarding the death of glial cells following repeated mTBI.

Blood-Brain Barrier (BBB) Dysfunction

The BBB is a highly dynamic system comprising a network of non-fenestrated endothelial cells connected by tight junctions surrounded by astrocytic end feet and pericytes that physically separate the intra- and extravascular central nervous system (CNS) content.[152] In response to perturbations in the neurochemical microenvironment, BBB tight junctions, transporters, and enzymes are regulated to protect the brain from noxious circulating stimuli while ensuring nutrient supply.[153,154] Following focal moderate–severe TBI, the BBB is breached, resulting in immediate infiltration of peripherally circulating leukocytes into the brain parenchyma. Together with the initiation of transcriptional changes in the neurovascular network, infiltrating cells aggravate the resident neuroinflammatory response,[155,156] culminating in neuronal dysfunction and neurodegeneration and a feed forward loop of further neuroinflammation.[157] Excessive excitatory amino acids, ROS, nitric oxide (NO) production,[158] and upregulated proinflammatory cytokines[159] contribute to and exacerbate BBB dysfunction and subsequent developing pathology.[160,161]

Primary mechanical injury may also damage endothelial cells, leading to capillary albumin extravasation and an increase in small vessel permeability.[157,162] Temporal progression varies between animal models; BBB permeability increases immediately at the site of LFP injury with a hasty resolve,[163] while an acute biphasic response is observed in a CCI model.[164] Further, increased cerebral vascular permeability is reported 4–6 h following focal closed-head WD injury, with concomitant widespread protein leakage[61,165] persisting for up for 4[166] and 7 d.[61] Such an extended opening of the BBB can exacerbate posttraumatic invasion of leukocytes[167] and neutrophils.[159] Detachment of vascular pericytes and migration into the parenchyma also occurs within 24 h following a moderate WD injury.[168] While there are a multitude of factors contributing to the probability, severity, and longevity of negative long-term TBI-induced sequelae,[169] there is increasing evidence of chronic inflammatory states in animal models of diffuse TBI, characterized by less pronounced leukocyte recruitment,[3] and persisting microgliosis in white matter tracts,[170,171] implicating dysfunction of the BBB in continuing pathology.

BBB Dysfunction Following Repeated mTBI

There are few reports of BBB dysfunction following repeated impact-related mTBI. Assessment of BBB integrity via permeability to immunohistochemically detected intracerebral mouse immunoglobulin G (IgG) and has indicated limited BBB breach. Specifically, following a single mild CCI, a small focal BBB breach was observed up to 48 h after injury, while a second injury given 24 h later resulted in increased cortical and white matter IgG immunoreactivity, with associated intraparenchymal serum extravasation that spread to white matter tracts.[54] Although not measuring BBB disruption directly, 5 mild CCI TBIs delivered at either 24- or 48-h intervals revealed major histocompatibility complex class II-associated antigen-labeled macrophages in hemorrhagic lesions.[150] Other repeated mTBI studies revealed no BBB compromise in CCI,[59] WD,[65] or Kimwipe/aluminum foil models,[71,72] although all analyses were conducted at acute time points.

Astrocyte Reactivity

Astrocytes are critical early responders to TBI-induced extracellular changes, becoming reactive in a process known as astrogliosis and exerting complex heterogeneous responses including altered gene expression, hypertrophy, and proliferation.[172] Astrocytes regulate the inflammatory response and can subdue the spread of damage. Through membrane protein channels and engagement of ATP-dependent pumps, astrocytes recycle excitatory amino acids to reduce glutamate excitotoxicity and restore K^+, Ca^{2+}, and Na^+ ionic homeostasis.[173] Buffering excess extracellular K^+, glutamate and ATP levels allow for provision of substrates for ATP synthesis and/or neuronal consumption to counter ROS-induced mitochondrial dysfunction and scavenge free radicals.[174] However, astrocytes can also release free radicals and proinflammatory cytokines and exacerbate ATP-induced ATP release, triggering microglial activation and propagation of Ca^{2+} waves via the astrocytic syncytium.[175,176] These dual neuroprotective and neurotoxic responses have been observed following LFP and CCI TBI and the balance between responses depends on the nature and severity of the injury.[177-179] However, how astrocytes interact with surrounding cells to influence the progression of response to repeated mTBI is yet to be fully elucidated.

Astrocyte Reactivity Following Repeated mTBI

While astrocyte responses typically increase with mTBI severity, number, and decreased inter-injury interval,[150,151] there is variability in reported time courses of response. A single mTBI can lead to a rapidly resolving[151] or mildly progressive[53,150] astrogliosis in a closed-head CCI model. When 4 mTBIs at 24-h intervals are delivered in both WD rotational and CCI models, the astrocytic response is exacerbated at 1,[53,150] 7,[72] and 14 d,[57] persisting to 6 mo, with concomitant cognitive deficits.[70] Increasing the inter-injury interval to 1 wk results in no observable response,[70] indicating that longer inter-injury intervals may be protective. Intermediate inter-injury intervals of 48 h yield variable outcomes.[150,180] Intriguingly, however, there is relative consistency in the progressive spread of the astroglial response from cortical to hippocampal to white matter domains in repeated mTBI.

Microglial Activation

Microglia are spread throughout the brain parenchyma in their quiescent state and are the primary immune effector cells of the CNS.[181] TBI-induced release of astrocyte-derived ATP triggers microglial recruitment.[175] Microglia proliferate and infiltrate toward the injury site, phagocytosing necrotic tissue, cellular debris, and toxic substances,[182] with the time course dependent upon the nature of injury.[183] Also depending on the nature of the TBI, microglia upregulate cell surface marker expression, enhance pro-inflammatary cytokines (interleukin [IL]-1β, IL-6, and TNF-α) and oxidative metabolites (NO, ROS) release and increase protease secretion, thereby exacerbating oxidative stress, neuroinflammation, and axonal pathology.[184] Sustained microglial activation and chronic inflammatory states contribute significantly to the spread of secondary degeneration,[185] playing a pivotal role in long-term and progressive axonal injury, neurodegeneration and neurological impairments[155,180,182,186,187] via mechanisms that include lipid peroxidation and apoptosis.[185,188] Indeed, there is increasing evidence of chronic microglial activation in the cortex, corpus callosum (CC), and thalamus up to 1 y after injury following moderate–severe TBI.[182] Alternatively, microglia can assume a reparative role by releasing anti-inflammatory cytokines such as IL-10 that inhibit proinflammatory functions.[189] Numbers of microglia along the cell death (M1) and repair promoting (M2) phenotypic spectrum depend on TBI severity and kinetics of regulation.[188,190]

The "immunoexcitotoxicity" theory suggests an alternative to the traditional and functionally distinct M1-M2 phenotypic polarization. Microglia are said to move from their resting and ramified state to one, where they swell with proinflammatory cytokines, remaining "primed" for action in the absence of inflammatory resolution.[191] With further triggers, microglia become increasingly aggressive in their pro-inflammatory cytokine and free radical release, propagating downstream cascades that exacerbate damage and deficits, resulting in increased vulnerability to subsequent stimuli.[191] The immunoexcitotoxicity theory may therefore provide a potential mechanistic link between the progression of acute to chronic pathology following repeated mTBI.

Microglial Activation Following Repeated mTBI

Microgliosis has been observed predominantly in the CC in closed-head models of single and repeated mTBI. Mild microgliosis in the CC is seen in the first 2 wk following a single mTBI[53,57,75]; longer-term outcomes were not assessed. However, 2 injuries delivered at 24-h intervals result in prominent acute microglial responses that persist in white matter until 7 wk.[56] Interestingly, when 4 mTBIs are given, exacerbated microglial hypertrophy and increased immunoreactivity are observed at acute[150] and subacute,[57] but not chronic[70] time points. No acute or subacute microglial inflammation is observed when inter-injury interval is increased to 48 h in both CCI[150] and WD aluminum foil models.[72] In contrast, persisting microglial responses are described in more severe, albeit still mTBI.[53,180]

Diffuse Axonal Injury (DAI)

DAI is a hallmark of TBI of all severities, in part due to anisotropically arranged axonal projections in white matter tracts being particularly susceptible to compression, tension, and torsion forces during rapid acceleration/deceleration.[109,192-194] The degree of axonal injury is dependent on injury severity[195] and correlated with the plane of mechanical loading and decelerating force.[192] Diffuse axonal injury is typically characterized by axonal stretching, mitochondrial swelling, and transport dysfunction.[193] In moderate–severe TBI, the mechanical loading induces focal perturbations in the axolemma[196] that can disrupt voltage-gated sodium (Na^+) channels, reverse the Na^+/Ca^{2+} exchanger, open voltage-gated Ca^{2+} channels, and facilitate excessive Ca^{2+} influx.[197] Secondary messenger cascades activate protein kinases, phospholipases, and proteases, which within 6 h leads to either neurofilament instability via phosphorylation or neurofilament collapse via calpain-mediated proteolysis of side-arms.[198] Ca^{2+}-mediated microtubule disassembly ensues,[199] and cytoskeletal disorganization often persists,[200] with silver staining used to visualize the punctate structures and argyrophilic fibers that are observed. Proteins accumulate, leading to multifocal axonal swellings that hinder axonal transport,[201] often detected as accumulations of amyloid precursor protein (APP).[202] Protein accumulation can initiate downstream cascades associated with secondary disconnection of the axon cylinder.[197] While the detached distal segment undergoes Wallerian degeneration, the proximal axonal segment and associated neuronal soma of origin swells, but does not necessarily die.[203] In contrast, ionic restoration can lead to axonal recovery,[204] while a host of secondary injury mechanisms likely contribute to progressive axonal degeneration.[200,205] Hyperphosphorylation of tau also occurs in TBI,[206] resulting in reduced microtubule binding,[207] which causes disassembly of microtubules and thus impaired axonal transport, leading to compromised neuronal and synaptic function.[208] Increased tau aggregation into insoluble fibrils and larger aggregates in the form of insoluble fibrils, tangles, and neuropil threads are also observed[209] and have been associated with subsequent neurodegenerative disease.[27]

Diffuse Axonal Injury Following Repeated mTBI

DAI is considered to be a key feature of pathology following mTBI[210] and is typically exacerbated with repeated injury.[54,211] Repeated mTBI in adult mice worsens diffuse axonal injury and cognitive function with inter-injury intervals of 1–5, but not 7 d.[150,151,212] Reducing the inter-injury interval to hours rather than days results in axonal injury, providing the injury severity is not sub-threshold.[211] Similarly, motor function and spatial learning deficits, as well as increases in cytoskeletal damage and axonal injury indicated by increased APP, are more prominent in animals with repeated mTBI separated by 3 d than following single mTBI or a 7 d inter-injury interval.[212] Following 2 CCI mTBIs given 24 h apart, increases in APP develop subacutely, subsiding by 56 d.[54] Further, 5 repeated CCI mTBIs, with a 48-h inter-injury interval, also result in increased APP-immunoreactive axonal profiles in the CC[53] that persist chronically.[180] Increases in microtubule-associated protein-2 are observed following 2 mTBIs with a 24-h inter-injury interval, persisting chronically.[151] However, no chronic axonal pathology is observed following 5 closed-head mTBIs delivered using the Kimwipe model at 24-h or 1-wk intervals, despite persisting cognitive deficits.[70,71]

Axonal degeneration detected by silver staining has been observed following 4 CCI mTBIs given at 24-h intervals, acutely[213] and subacutely.[57] Further, acute cytoskeletal abnormalities and intra-axonal organelle compaction detected by ultrastructural analysis, that persist long-term in white matter tracts, is temporospatially coincident with a prominent microglial response following 2 CCI mTBIs,[56] with similar outcomes in a model featuring rotational acceleration.[75] Activated microglia form extended cytoplasmic processes in direct contact with injured axons to form a potential barrier between the healthy and injured tissue, suggesting that microglial activation is a response to the axonal damage.[56,175]

Phosphorylated tau and Aβ have been explored in repeated mTBI studies,[54,72,151] particularly using transgenic animal models,[132,214,215] given their associations with CTE.[26,216] Although repeated mTBI is thought to exacerbate secondary injury mechanisms that accelerate the development of chronic neurodegenerative diseases, the mechanistic link between repeated mTBI and CTE pathobiology is yet to be elucidated, and further prospective and longitudinal studies are required.[28] Indeed, cognitive deficits after repeated mTBI can occur in the absence of increased tau phosphorylation or Aβ,[70] and transgenic studies of specific tau isoforms indicate further complexities.[217] Table 1 provides further information on studies assessing CTE-like pathology in repeated mTBI.

Dysmyelination

While axons and myelin forming fiber tracts are consanguineous, it is suggested that their pathologies following TBI are distinct,[147,218] although studies exploring TBI-induced myelin pathology are relatively limited. Demyelination can occur as a result of several mechanisms, including primary axonal damage and subsequent Wallerian degeneration, or death of myelinating cells. Subacute loss of myelinated axons[171,219] and myelin decompaction and redundancy[218] have been reported following moderate TBI in rats. In contrast, transient subacute axonal dysfunction has been observed in the absence of myelin abnormalities, following moderate TBI (referred to as mild in the literature).[220-222]

Oligodendrocytes produce large amounts of ROS[223,224] and have low antioxidant capacity.[225] Indeed, oligodendrocytes, oligodendrocyte progenitor cells (OPCs), and myelin are particularly sensitive to glutamate excitotoxicity, Ca^{2+} overload, oxidative stress, and altered metabolism that occur following neurotrauma,[226-228] with sensitivity thought to be maturation dependent.[229] Extensive and sustained calpain-mediated degradation of myelin basic protein was reported in a moderate CCI TBI model, with intact proteins returning to baseline levels 3–5 d after injury.[171] Additionally, myelin debris can stimulate inflammatory cells, and activated microglia and astrocytes can likewise promote myelin phagocytosis via ROS release[230] and activate OPC recruitment.[231] OPCs can rapidly respond to white matter injury and produce matrix metallopeptidase 9 that appears to open the BBB and trigger secondary cascades of cerebrovascular injury and demyelination.[159,169] Indeed, perturbations in the BBB are known to be a critical part of white matter pathology in a wide range of CNS disorders.[232] However, transient upregulation of mature oligodendrocyte genes by OPCs[147] as well as localization of OPCs to brain regions exhibiting neuronal damage[170] suggests an acute regenerative response. Nevertheless, dysmyelination and demyelination persist and progress up to 1 y following injury,[233] occurring simultaneously with prolonged reactive microgliosis and astrogliosis,[147,234] indicating ongoing stimulation by myelin debris.

Dysmyelination Following Repeated mTBI

Studies exploring myelin abnormalities in animal models of repeated mTBI are scant. Subcortical white matter tract damage is seen in single mTBI[54,212,221,235,236] and a long-term experimental study of mTBI using both single and repeated injuries reported CC thinning accompanied by neurological deficits 1 y following injury.[180] Progressive myelin pathology has been indicated by decreased radial diffusivity[55] and double concentric myelin sheaths[56] following 2 CCI mTBIs delivered at a 24-h interval. More persistent and severe myelin pathology with evidence of remyelination was observed with a repeated “mild” TBI; however, acute focal lesions and blood deposition suggest the injury was of moderate severity.[51] These findings likely reflect cognitive and behavioral dysfunction following mTBI[237,238]; however, behavioral assessments in these studies were lacking. Further studies assessing the effects of injury interval, number and severity on chronic myelin pathology together with behavior are warranted.

Behavioral Deficits Following Repeated mTBI

The most commonly used assessments to determine long-term behavioral outcome in TBIs include neurological severity tests to assess gross motor deficits; beam walking and rotarod performance which assess sensory, motor, and sensorimotor domains; and various MWM paradigms to assess learning and memory as a correlate to cognitive function.[239] Tests assessing psychological sequelae have also been employed to detect anxiety- and depressive-like behavior. Behavioral impairments have been consistently revealed in closed-head animal models of repeated mTBI, incorporating various degrees of head movement and inter-injury intervals. Mice sustaining 2 mTBIs at 24-h intervals had acute learning and memory deficits in MWM[56] and Barnes maze performance[75] that persisted to subacute time points. However, variability exists between studies of similar design, with some animals exhibiting longer-term balance deficits in the absence of persisting cognitive impairments.[54] When total injury numbers are increased to 5, transient balance and motor coordination deficits in the rotarod test are described, while locomotor hyperactivity persists to 1 mo.[72] In similar studies, measurable cognitive deficits persist beyond 3 mo.[57,71,151] As such, when repeated mTBIs are delivered within the “window of vulnerability” (described in Mechanisms of Pathology section), cumulative cognitive deficits persist and may be permanent.[54,65,71] Interestingly, longer inter-injury intervals up to 1 mo produce no deficits,[68,71] suggesting that longer time intervals may confer protection against subacute[212] and long-term functional sequelae.[70] Additionally, studies titrating mechanical input have revealed a threshold of injury severity required to elicit deficits.[56,65] Thus, it appears that behavioral deficits significantly associate with the number of mTBIs, inter-injury interval, and severity of impact.

Toward Clinical Management of Repeated mTBI

Pharmacological therapies for TBI in humans are lacking.[83] Therapeutic strategies in development are targeted toward secondary injury pathways that are potentially modifiable and therefore amenable to treatment[43] and are generally focused on facilitating neuroprotection or inhibiting neurotoxicity. Given the subtle acute pathology following mTBI, treatment options for repeated mTBIs hinge on decreasing the progression of secondary damage and improving long-term functional outcomes. As described above, the first injury appears to place the brain in a vulnerable metabolic state. Therefore, appropriate immediate treatment following a single mTBI may facilitate acute physiological recovery and reduce the potential for cumulative damage with further injury. Further, treatments administered beyond the acute time point may be useful for targeting specific elements of secondary degeneration. However, a greater understanding of the mechanisms of damage following repeated mTBI is likely to be required to facilitate effective development of therapeutics as well as inform return to play guidelines.

Therapeutic Strategies for Repeated mTBI

Preclinical studies assessing efficacy of therapeutics for repeated mTBI have been reported, and current targets focus specifically on reducing inflammation, oxidative stress, axonal injury, and associated neurodegenerative-like pathology. Targeting microglial activation via anti-CD11d,[47] progesterone,[240] and Valganciclovir[213] to ameliorate the inflammatory response following repeated mTBI has shown some promise. Rats given 3 mild LFP injuries at a 5 d inter-injury interval, and treated with anti-CD11d antibody, exhibit reduced neutrophil and macrophage infiltration, lipid peroxidation, astrocyte activation, APP accumulation and neuronal loss, concomitant with improved performance on cognitive, sensorimotor, and anxiety tasks, relative to controls.[47] Using a similar study design, the steroid hormone progesterone decreases lipid peroxidation and microglial and astrocytic markers of neuroinflammation, while long-term cognitive and sensorimotor outcomes are improved.[240] For both of these studies however, acute damage and deficits were potentially dampened given the 5 d inter-injury interval. Valganciclovir-induced macrophage depletion decreases the microglial population in the CC and external capsule, as expected, but doesn't alter the extent of acute axonal injury after 2 CCI mTBIs at 24-h intervals.[213]

Rosemary extract (20% carnosic acid) administered following 3 mTBIs at a 24-h inter-injury interval reduces astrocytosis, oxidative stress, inflammatory cytokines, and degenerating neurons in the hippocampus and restores cognitive deficits.[78] However, significant numbers of degenerating neurons in the hippocampus following the repeated mTBI suggest that a more severe injury was induced. The immunosuppressant FK506 (Tacrolimus) or moderate hypothermia (32–33°C for 1 h) following 2 impact-acceleration mTBIs at a 3-h inter-injury interval significantly attenuates axonal and cerebral microvascular changes by inhibiting calcineurin, free-radical and metabolically mediated cascades.[241] Following 2 closed-head mTBIs incorporating rotational acceleration, given on consecutive days, the liver X receptor agonist GW3965 improves cognition, axonal integrity, and Aβ clearance in an Apolipoprotein E (ApoE)-dependent manner.[67]

Preventing tauopathy or decreasing the risk of developing neurodegenerative disease has been attempted using various models and treatment targets. Vitamin E, a potent exogenous antioxidant, was administered for 12 wk to aged transgenic mice, exhibiting Alzheimer's disease-like amyloidosis. Prevention of Aβ peptide accumulation and reduction in brain lipid peroxidation and behavioral deficits following 2 CCI mTBIs[133] suggest antioxidants may reduce the putative risk of repeated mTBI-associated Alzheimer's disease. Following 3 closed-head CCI mTBIs given at 24-h intervals, inhibition of monoacylglycerol lipase, an endocannabinoid 2-arachidonoylglycerol metabolizing enzyme, significantly reduces CTE-like neuropathological changes, proinflammatory cytokines, astroglial reactivity, and cognitive deficits.[242] Additionally, using transgenic and knockout tau mice in a Kimwipe mTBI model, *cis* p-tau antibody treatment prevents the temporospatial progression of tauopathy and cognitive decline by blocking axonal microtubule and mitochondrial disruptions.[243] Finally, sodium selenate treatment of 3 LFP mTBIs at a 5 d inter-injury interval results in tau dephosphorylation and ameliorates cognitive decline via protein phosphatase 2A 55 kDa regulatory B subunit upregulation.[244]

Effects of pre-treatment of repeated mTBIs using fish oil[245] and androgenic steroids[246] have also been explored. Rat chow enhanced with 6% omega-3 fatty acids was provided for 4 wk prior and 2 wk following 2 LFP mTBIs at a 24-h inter-injury interval; recovery of body weight was improved, with a trend toward increases in hippocampal neurons and cognitive performance.[245] Interestingly, a combination of testosterone, nandrolone, and 17α-methyl testosterone increases axonal injury and microgliosis,[246] suggesting athletes who use these agents may suffer from detrimental effects following mTBI.

Barriers to Clinical Translation

While swine[247,248] and cell culture approaches[249,250] have recently been used to model repeated mTBI, the majority of studies use rodents. There are, however, inherent structural and behavioral differences in rodents that challenge the emulation of human mTBI, in terms of mechanical input, and structural and behavioral output. Mass, white to gray matter proportions, cerebral convolutions, presence of cerebrospinal fluid and craniospinal angle influence the nature of TBI-induced tissue strain.[201] Choosing the intervals between injuries in experimental models is confounded by difficulties relating rat age to humans,[251] which is important when determining return to play time frames following sports-related mTBI. Additionally, strain-dependent behavioral and histological responses have been revealed in rodents.[252] Further, studies using non-transgenic female rodents are lacking. While acute neuroprotective effects of oestrogen have been suggested in a rodent WD impact-acceleration model,[253] exacerbated outcomes following repeated mTBIs in female soccer players indicate complexities.[254] Finally, pediatric populations exhibit different responses to adults experiencing a similar head trauma,[255] and limited studies have been conducted assessing younger populations.[235,236,256,257]

Conclusions

Long-term cognitive impairments following CNS injury and in neurodegenerative diseases have been associated with prolonged oxidative stress conditions[131,258] and impaired signal conduction along dysmyelinated axons.[259] Further, persisting behavioral deficits after TBI have been associated with progressive activation of astrocytes[260] and microglia.[261] While mild and repeated mTBI can impair long-term function,[262] studies are yet to simultaneously measure behavioral, oxidative, neuroinflammatory, and myelin integrity outcomes at acute and chronic phases. As such, key mechanistic insights needed to design therapies tailored to limit chronic deficits following repeated mTBI are lacking. Therapies designed to stabilize and improve metabolic status in the acute time period after injury may protect neurons and glia, translating to significant improvements in long-term functional outcome. As more is learned about BBB regulation following repeated mTBI, further opportunities may emerge to target the brain endothelium to maintain health and facilitate recovery. Diffuse axonal injury and white-matter damage is increasingly understood to underlie progression of cognitive impairments and better understanding of myelin pathology using both advanced imaging and ultrastructural analyses following repeated mTBI will also likely contribute to improved therapeutic opportunities. While elucidating the mechanisms of damage underlying cell dysfunction following repeated mTBI is crucial to develop therapeutic strategies, it is also important to appreciate cell regenerative processes known to occur in moderate–severe TBI.[263] The hippocampus is implicated in persisting memory deficits following repeated mTBI and complex forms of hippocampal-mediated learning require adult-born neurons.[264] Following repeated LFP mTBI, long-term potentiation deficits and failed *N*-methyl-D-aspartate-receptor-mediated hippocampal synaptic excitation suggest a lack of adaptive plasticity.[45] Therefore, therapeutic strategies designed to enhance neurogenesis and functional plasticity following repeated mTBI may also be required.

Authors' Contribution

Brooke Fehily researched and wrote the manuscript. Melinda Fitzgerald edited and critically evaluated the manuscript. Both authors read and approved the final manuscript.

Statement of Informed Consent

There are no human subjects in this article, and informed consent is not applicable.

Declaration of Conflicting Interests

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