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Novel pharmaceutical treatments for minimal traumatic brain injury and evaluation of animal models and methodologies supporting their development

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Abstract

Background—The need for effective pharmaceuticals within animal models of traumatic brain injury (TBI) continues to be paramount, as TBI remains the major cause of brain damage for children and young adults. While preventative measures may act to reduce the incidence of initial blunt trauma, well-tolerated drugs are needed to target the neurologically damaging internal cascade of molecular mechanisms that follow. Such processes, known collectively as the secondary injury phase, include inflammation, excitotoxicity, and apoptosis among other changes still subject to research. In this article positive treatment findings to mitigate this secondary injury in rodent TBI models will be overviewed, and include recent studies on Exendin-4, *N*-Acetyl-l-cycteine, Salubrinal and Thrombin.

Conclusions—These studies provide representative examples of methodologies that can be combined with widely available in vivo rodent models to evaluate therapeutic approaches of translational relevance, as well as drug targets and biochemical cascades that may slow or accelerate the degenerative processes induced by TBI. They employ well-characterized tests such as the novel object recognition task for assessing cognitive deficits. The application of such methodologies provides both decision points and a gateway for implementation of further

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translational studies to establish the feasibility of clinical efficacy of potential therapeutic interventions.

Keywords

Traumatic brain injury; Animal models of TBI; Exendin-4; *N*-Acetyl-L-cycteine; Salubrinal; Thrombin

1. Traumatic brain injury

Concerns about traumatic brain injury (TBI) are rising amidst contemporary military deployments, reports on contact sport athletes, motor vehicle accidents and an aging population. To date, an estimated 1.7 million people sustain a TBI in the USA annually, not including those who seek care outside of the hospital Emergency Room setting or no care at all (CDC 2011). Of these cases, 80–90% fall under the classification of mild TBI (mTBI). Although the least severe classification, the term “mild” refers to the severity of the initial injury and not to the severity of the injury’s consequences, which can include cognitive and behavioral deficits lasting from months to many years (Brain Injury Association of America; Schreiber et al., 2008). Additionally, mTBI has been linked to an increased risk of developing a number of neurodegenerative conditions, such as Alzheimer’s disease and Parkinson’s disease, later in life (Daneshvar et al., 2011), and repetitive mTBI is a conduit to the development of chronic traumatic encephalopathy (Kondo et al., 2015).

TBI can be appreciated as having two separate, but connected injury components. First, an externally derived mechanical injury to the head and second, an internal cascade of molecular mechanisms understood to cause further neurological damage. Mechanisms of the secondary injury are still subject to research, but are known to include inflammatory, excitotoxic, and apoptotic processes (Werner and Engelhard, 2007). In the case of mTBI where the primary injury is substantial but far less severe than other forms of TBI, it is the secondary injury that likely is responsible for most of the ensuing damage. Fortunately, it is also the secondary injury that could prove treatable, should an experimental treatment option be both effective against components that drive the secondary phase and administered early enough to inhibit it. As secondary injury spans minutes to days after the immediate insult, this creates a valuable window of opportunity for mTBI treatment once the time-dependent molecular mechanisms are understood. If effective treatments are identified to minimize the dysregulation resulting from mTBI, then cell death can be minimized to positively affect patient outcomes.

Currently there are no pharmaceutical treatments for mTBI; however, a continually improving understanding of the mechanisms of secondary injury is allowing ever better drug development and repositioning of existing drugs. Activation of antioxidant production and inhibition of pro-apoptotic proteins and pro-inflammatory cytokines are among drug features of recent interest. Using rodent models of mTBI and relevant cognitive and biological tests, numerous treatments with mechanism-based drug features have been reported to ameliorate mTBI induced cognitive deficits. As a recent example among many, we have lately shown that an increased thrombin concentration induced by mTBI may cause amnesia through the

activation of its receptor, the proteases activated receptor 1 (PAR1) (Itzekson et al., 2014, Maggio et al., 2014); thereby exposing a novel therapeutic target potentially underpinning cognitive detriments of trauma. In the following sections, several widely used rodent models of mTBI are highlighted that can be combined with well characterized behavioral assays, epitomized by the novel object recognition (NOR) task for assessing cognitive deficits, to provide a methodological toolbox for evaluating molecular mechanisms involved in mTBI. To this, other techniques can be added, as required, to evaluate cell death or survival (e.g., by the use of immunohistochemistry, Western blotting, etc.), functional assays (e.g., electrophysiology, neuroimaging, etc.) and biomarkers of disease progression or remission (plasma, CSF), to both test hypotheses as well as potential treatment strategies.

2. Rodent modeling of mTBI

At present, at least a dozen distinct experimental methods exist for modeling TBI in rodents aimed at reproducing the multiplicity of human injury types. These protocols vary widely in their likeness to real-life human injury scenarios. Some are highly comparable and possess strong face validity, as exemplified by open field blast injury mTBI models involving mice that are elevated to the height of a standing human and are placed at a combat relevant distance from an actual TNT explosion (Rubovitch et al., 2011). Others are highly controlled and calculated, like in-laboratory trials of vacuum pulses applied directly to surgically exposed brain tissue (Shreiber et al., 1999). Whereas no animal model can replicate all aspects of the human injury, specific ones have benefits as well as disadvantages in relation to their utility and translational relevance. Nevertheless, across all models of injury, method requirements are to reproduce clinical TBI sequelae reliably, quantifiably, and with adjustable severity (Morales et al., 2005).

Even with all model requirements met, for those using rodents limitations may come from dissimilarities between animal and human brains including brain size, brain complexity, craniospinal angle, and white-to-gray matter ratio (Greig et al., 2014). Still, current methods have successfully replicated many specific human biopathological mTBI features, both focal and diffuse in nature. Three such widely used models are described below that can be valuably integrated with numerous neuroscience techniques.

2.1. Fluid percussion injury (FPI)

The fluid percussion device is one of the most extensively used in animal models of TBI. Initially described by Lindgren and Rinder (1965) in the rabbit, this model has been widely applied to larger and small animal species, including rodents (Kabadi et al., 2010). To induce injury, initial surgery is undertaken that involves a small, defined craniotomy to expose the dura, and the placement of a female Luer lock that is cemented into place. This procedure often is undertaken the day preceding TBI to minimize associated damage. The method of injury is by transient compression/deformation of the underlying brain by a fluid-mediated pressure pulse that is generated by a classical piece of equipment. This comprises of a cylindrical reservoir that is filled with sterile isotonic saline, and on one end has a transducer connected via a tube and male Luer lock to the female Luer lock attached to the skull. Injury is initiated by the development of an acute fluid pulse against the intact dural surface, which

is induced by the release of a pendulum (from a precalibrated height or angle associated with a desired injury severity) that strikes a piston at the other end of the reservoir (Thompson et al., 2005). A number of micro-FPI devices are widely available that possess microprocessor-controlled pneumatically driven devices to accurately generate pressure waves (Kabadi et al., 2010). A sample protocol for a mild FPI injury is the delivery of a 0.9 ± 0.1 atm fluid pressure pulse. Generation of a larger pulse can be induced to create a more moderate to severe form of TBI, as necessary to meet focused research needs. Depending on the type of injury being modeled, the craniotomy may be placed at the midline for focal damage or laterally to generate a coup-contrecoup injury, which will result in both focal and diffuse damage (Morales et al., 2005). An experiment testing axonal damage at five time points within six weeks after a mild FPI found the localization of the damage to move across different brain regions over time, just as is found in human mTBI (Spain et al., 2010).

No TBI model recapitulates the full diverse spectrum of the human condition, with each model mimicking certain clinically translatable aspects. Whereas FPI models have the advantage of inducing graded levels of brain injury and creating axonal injuries and contusions, accompanied by impaired neurologic motor function and cognitive impairments, the fluid pulse that underpins the injury does not directly relate to the type of mechanical impact to the brain associated with TBI in the human scenario. Hence a comparison between tissue injuries generated by a fluid percussion model and that evident in the clinical setting is difficult. Furthermore, fluid flow characteristics within brain likely are dependent on the brain geometry of the animal species under study, making biomechanical analyses across species complex (O'Connor et al., 2011).

2.2. Controlled cortical impact (CCI)

Controlled cortical impact, often referred to as rigid percussion, imparts a solid mechanical injury force directly to the surface of the brain, and thus, like FPI, CCI requires craniectomy before injury induction. Nevertheless, CCI-induced injury force has the most biomechanical control of any model, as it is delivered by a rigid impactor that is precisely adjustable over the parameters of time, velocity and depth (Morales et al., 2005). This method also has no risk of rebound injury associated with other models. An example protocol for a mild injury used by Gao and Chen (2011) in mice is a 0.2 mm deformation delivered at a piston velocity of 2.8 m/s to an intact dura. The injury produced is mostly focal in nature, and is of strong relevance to those that occur in sports collisions or car accidents. Of value, this model – originally developed in the ferret (Lighthall, 1988), is adaptable across animal species – from small (rodents) to large (pigs and nonhuman primates, by varying the diameter size of the impactor tip, its velocity and depth setting (Osier et al., 2015). Important to CCI and other animal TBI models, particularly those involving a surgical procedure such as craniectomy, is to control for the confounding effects of surgery by the use of sham animals that are similarly anesthetized and subjected to surgery/craniectomy. There is increasing cognizance that sham surgery is not benign and may initiate biochemical cascades of relevance to TBI, for example neuroinflammation and/or blood vessel damage, and thereby induce a diversity of pathological responses. Sham animals in CCI studies have been described to have greater deficits than anesthesia only controls (Cole et al., 2011). Additionally, the influences of craniectomy appear to differ contingent on the location and

method selected, whether by trephine or dental drill. Midline procedures for CCI can lead to sagittal bleeding, and hence the lateral procedure is now commonly used.

The CCI model has had much success with biopathological mimicry. Common clinical findings of TBI: brain edema/blood-brain barrier breakdown, elevated intracerebral pressure, and decreased cerebral blood flow have all been demonstrated in testing (Cernak, 2005). A disadvantage to CCI is that the subject brains are so sensitive to the injury that even the lowest levels frequently result in intracranial contusions. This presentation is inconsistent with human mTBIs, which generally do not manifest with clear morphological defects (DeWitt et al., 2013), unlike more severe forms.

2.3. Weight drop

The weight drop model, said to be the original animal model of TBI, involves dropping a free-falling weight guided by metal tubing to impact the temporal brain region of an anesthetized animal. Sufficient anesthesia for the weight drop model is best achieved by a short-acting gaseous anesthetic (for example, isoflurane) delivered just before the injury, contributing to this method's ease of preparation. In accordance with TBI model requirements, the severity of the focal injury can be readily adjusted by changing the mass of the weight or the height of the drop. To impart a mild injury to a mouse, protocols use weights of between 30 to 50 g from a height of approximately 80 cm (31.5 in.). This range of weight generates an injury that is free from gross neuroanatomical changes (Tweedie et al., 2007a,b) and skull fraction complications that can occur with heavier weights; although, there is still some associated risk of rebound injury (Morales et al., 2005). In general, the anesthetized mouse is placed below the guide tube through which the weight is dropped, aligned so that it will strike the skull on the temporal side, between the corner of the eye and the ear (predefined on either the left or right side). The head rests on a supportive foam sponge positioned to allow antero-posterior motion without any rotational head movement at the moment of impact (Rachmany et al., 2013a,b; Baratz et al., 2015; Milman et al., 2005; Tashlykov et al., 2009).

As is the case for other TBI models, a number of variations of the weight drop mTBI model exist to allow its adaption to meet specific needs. An example of this among many is the model used by Lu and Zhuo (Kondo et al., 2015), where anesthetized mice are placed on a piece of tissue paper that is positioned directly below the weight guide tube, with the animal's tail held firmly. A weight (54 g) is then released that impacts the dorsal aspect of the skull, which results in rotational acceleration of the animal through the tissue paper in a manner that risk of rebound injury is negated. Further examples are the adaption of the technique to rats (Marmarou et al., 1994; Foda and Marmarou, 1994), involving a larger weight and use of a metal disk placed over the skull to prevent bone fracture, a technique likewise used in mice by Hall (1985).

Like all other methods described here, weight drop replicates a dynamic loading injury, which is the most prevalent injury type. Significantly, it is the only method of the impact kind that can be performed noninvasively. At the time of the injury the anesthetized mouse may be un-operated, or have skull exposed, or have the dura exposed, depending on the requirements of the study (Bolouri and Zetterberg, 2015). The option to replicate a closed

head injury, scalp intact, is a meaningful feature of this model considering the vast majority of clinically presenting mTBI injuries occur under these conditions.

The biopathology of the weight drop method has been shown to induce neurodegeneration in all observable cortical areas using weights as light as 10–15 g. TUNEL-positive cells appear in cortical and hippocampal regions with a threshold weight of 15–20 g, indicating late stage apoptosis as is seen in post-traumatic human brain tissue (Tashlykov et al., 2007). Even weights as light as 5 g have been shown to upregulate pro-(p53) and anti-apoptotic markers (Tashlykov et al., 2009; Greig et al., 2014). At the level of transcription, mild weight drop injury prompts upregulation of mRNA for genes like Ccl3, Gfap, and Lyz2 associated with microglia, astrocyte, and phagocyte activation, respectively. These transcription increases are associated with stages of apoptosis as well as immune defense and healing functions – in particular inflammatory processes and chemokine signaling – that can now be targeted in further research (Israelsson et al., 2009). In the Israelsson et al. (2009) study it was found that all but one of the 37 genes upregulated by 3-fold or more in the weight drop model were found upregulated to the same degree in the CCI model. This consistency amongst TBI models indicates promisingly that pharmaceuticals developed for identified gene targets in any of the focal TBI models will be applicable across many types of head injury. In addition to promising biopathological similarities, the weight drop model produces cognitive deficits shown to last up to at least 90 days as well as neuronal loss within key brain regions, such as the dentate gyrus, associated with cognition (Greig et al., 2014).

3. Novel object recognition as a means of assessing mTBI

Clinically, one of the chief symptoms for mTBI diagnosis is memory dysfunction, which can be self-reported or observed (Morales et al., 2005). In the animal model where typical written and verbal assessment methods are void, tests must be designed around an observable, quantifiable behavior to which memory is intrinsic. Current tests vary in the types of memory and learning they assess; for example, the passive avoidance test represents classical Pavlovian conditioning whereas the water T maze tests short-term executive function, and the Morris water maze tests spatial learning and memory. This variation in testing is valuable as we determine more precisely what memory is impacted by head injury and how. As yet, all of these methods have shown impaired performance in mTBI mice (Zohar et al., 2003; Milman et al., 2005).

Novel object recognition (NOR) is one task that utilizes a rodent’s short-lived, unconditioned novelty preference during exploration that lasts the duration of the encoding process (Ennaceur, 2010). Given the presentation of a novel and a familiar object, we may infer from heightened interaction with the novel object that there is an encoded memory of the familiar object and, conversely, that more equitable exploration denotes a deficit in recognition.

Briefly, the protocol for NOR includes three parts: a habituation phase, a familiarization phase and a testing phase. The intervals between these phases can be modified to test short- or long-term memory. All trials take place in a rectangular, open-field arena where first, the rodent is allowed to explore the arena without any objects; second, with two identical objects

placed in opposite and symmetrical corners of the arena; and third, with one of the two identical objects replaced by a novel object. Between all trials the arena is cleaned with ethanol to remove any odor cues. Additional precaution is taken by selecting an object pair with multiple discriminating factors to make allowances for our incomplete understanding of rodent sensory modalities, but with no evident differences that would make one object preferable over the other (for example one object being easier to climb). In the third phase, the time the animal spends exploring each object is recorded and calculated into a preference index. Although the definitions of exploration may change from study to study, most consider it the directing of the nose at the object at between 1 and 4 cm of distance (Antunes and Biala, 2012). It is noteworthy that the task uses neither rewarding nor painful stimuli but, rather, activities common to rodent behavior.

Preferential exploration of the novel object in NOR testing is seen to decrease systematically in mTBI mice (Saykally et al., 2012; Eakin et al., 2014; Rachmany et al., 2013a,b). This is true not only for weight drop-challenged mice, but across multiple TBI models. In the FPI model of mTBI in rats, failing NOR performances were seen linked to reduced hippocampal bursts and reduced firing of novel-object-specific pyramidal cells (Munyon et al., 2014). Additionally, in the CCI mouse model, Zhao et al. (2012) found that NOR effectively distinguished non-spatial learning and memory impairment from moderate and severe TBI with only a one hour gap between familiarization and testing phases. Inadequate NOR task performance reveals a memory deficit that could be likened to episodic memory without the temporal component, which depends highly on the hippocampus (Ennaceur, 2010; Antunes and Biala, 2012). Deficits in episodic memory are observed consistently in moderate to severe TBI cases and also in mild TBI in the case of divided attention (Mangels et al., 2002). Regaining these functions are a critical component of returning to school and work and, as such, recovery of NOR performance by use of experimental treatments is an encouraging measure of trial drugs in the mouse model.

4. Pharmaceutical treatments for weight drop-induced damage to NOR

Given the myriad of symptoms that compose mTBI neuropath-ology (brain edema, neuroinflammation, free radical generation, DNA damage, glutamate-induced excitoxicity, and others), a successful treatment could target any number or combination of factors. An efficacious treatment would also need to produce its therapeutic effects when administered at a delayed time from the injury that is feasible for medical intervention. Hence when targeting a biological cascade triggered by TBI as a therapeutic approach, it is both essential to engage the target while it is mechanistically relevant to the ensuing damage, as well as to select a target and intervention time that is translatable to the human condition. Using the nonsurgical weight-drop model of mTBI and decreased NOR performance as an indication of cognitive deficit, our collaborative group has found a number of promising therapeutic agents targeting different secondary injury features.

4.1. Neuroinflammation and apoptosis

A key target following any form of TBI is the rapidly ensuing neuroinflammation, which is a key component of secondary injury. In this regard there are numerous anti-inflammatory

approaches and, as recently reviewed by Bergold (2015), whereas many have been evaluated in animal models, few have translated to effective clinical development. An interesting agent under recent evaluation by our collaborative group is the small synthetic candidate drug 3,6′-dithiothalamide that inhibits the synthesis of tumor necrosis factor-alpha (TNF-α) (Zhu et al., 2003; Greig et al., 2004). TNF-α is an early proinflammatory cytokine identified as initiating and regulating molecular cascades contributing to inflammation, and has been suggested as a primary mediator of neurotoxicity following closed head injury. Whereas initiation of an inflammatory response can be essential to promote neuroreparative mechanisms in response to a physiological insult (whether TBI, stroke or a bacterial infection), if this is excessive or unregulated, it can augment neuronal dysfunction and degeneration by inducing a self-propagating pathological cascade of neuroinflammation that can ultimately drive pathological processes (Tweedie et al., 2007a,b; McCoy and Tansey, 2008; Frankola et al., 2011). Shortly following TBI, substantial synthesis and release of proinflammatory cytokines occur from microglia and astrocytes, in particular TNF-α, with its mRNA and protein levels becoming acutely elevated within as little as 17 min after injury, as evaluated in post-mortem brains from patients expiring shortly after a TBI (Frugier et al., 2010). An analogous rapid sequence has been reported in rodent TBI animal models in which an elevation in brain TNF-α precedes the appearance of subsequent cytokines (Shohami et al., 1997; Lu et al., 2009; Yang et al., 2010). Contingent on signaling pathways activated, TNF-α can exacerbate trauma and oxidative stress within the brain and contribute to glutamate release and blood-brain barrier disruption that then instigates further influx of inflammatory factors from blood to brain to drive pathological processes (Tuttolomondo et al., 2014). In this regard, increased mRNA expression of TNF-α by up to 30-fold as well as up regulation of the protein itself has been shown in the rodent model as the result of closed head injury (Shohami et al., 1997). Such a rise in brain TNF-α is often transient and, in the case of weight drop mTBI, becomes rapidly elevated immediately following injury, peaks at 12 h and declines by 18 h (Baratz et al., 2015) When the TNF-α synthesis inhibitor 3,6′-dithiothalamide was administered, even at a low dose of 28 mg/kg up to 12 h post weight drop injury, the drug fully mitigated the rise in brain TNF-α, ameliorated the diffuse neuronal cell loss that occurred consequent to mTBI, and protected against mTBI-induced NOR impairment evaluated at 7 and 30 days later. In contrast, when treatment was delayed beyond 12 h (initiated at 18 h post mTBI – i.e., 6 h beyond the mTBI-induced spike in brain TNF-α levels) no mitigation of neuronal loss or cognitive impairment was evident (Baratz et al., 2015). These results are clearly promising for translation to clinical studies in light of elevated levels of TNF-α post-TBI in human studies, the wide therapeutic window of 12 h to initiate treatment (Baratz et al., 2011; Baratz et al., 2015), and the effectiveness of this agent and treatment strategy across neurodegenerative disorders such as Alzheimer's disease and stroke in which there, likewise, is a neuroinflammatory component centered on TNF-α (Tweedie et al., 2012; Belarbi et al., 2012; Russo et al., 2012; Yoon et al., 2013).

The therapeutic window for target engagement after TBI is clearly critical to response and efficacy. To mechanistically evaluate this window and to separate cells undergoing immediate necrotic cell death from potentially reversible apoptotic cell death, one can utilize small molecular weight p53 inactivators (tetrahydrobenzo thiazoles and oxazoles) (Zhu et al., 2002) that have become widely used as pharmacological tools in the neurosciences to

both inhibit and characterize apoptosis (Luo et al., 2009; Culmsee et al., 2001; Duan et al., 2002; Plesnila et al., 2007). In animal models of stroke (transient middle cerebral artery occlusion), for example, studies have demonstrated that infarct volume can be reduced by >50% by p53 inhibition, resulting in improved neurological outcome with a window of opportunity of 3 h (Leker et al., 2004), and a newly developed neuronal specific conditional p53 KO (CamcreTRP53$^{loxP/loxP}$) mouse in which neuron p53 ablation reduces stroke volume by 50% (Filichia et al., 2015), recently confirms these studies. Our studies across mild (weight drop, Rachmany et al., 2013a,b) and moderate (CCI, Yang et al., 2015) TBI rodent models with p53 inhibitors, likewise, demonstrate that a substantial portion of dying neurons undergo apoptotic cell death and, importantly, are amenable to rescue. Critically, such rescue mitigates TBI-induced cognitive impairment, as evaluated by NOR and other quantitative cognitive measures, with a window of 5–7 h in moderate and up to 12 h in mild TBI – thereby defining the time-dependent opportunity for clinical intervention and human translational studies.

4.2. Neurotrophic and neuroprotective agents

Balancing pro-apoptotic pathways leading to cell dysfunction and death following a brain insult are multiple biochemical cascades that promote cell survival. In this regard, increasingly well-characterized neurotrophic/protective actions have been achieved by glucagon-like peptide-1 receptor (GLP-1R) activation in cellular and animal models of acute and long-term neurological injuries (for review: Salcedo et al., 2012). This drug target, the GLP-1R, has clinical relevance to type 2 diabetes mellitus and is gaining increasing interest in neurological disorders (Salcedo et al., 2012; Campbell and Drucker, 2013; Duarte et al., 2013; Greig et al., 2014; Hölscher, 2014). GLP-1 is an endogenous 30 amino acid insulinotropic peptide that, with glucose-dependent insulinotropic polypeptide (GIP) of 42 amino acids, regulates blood glucose levels by activation of their cognate receptors on pancreatic β-cells (Campbell and Drucker, 2013). These insulinotropic actions are glucose-dependent and thus incretin-based therapies are not associated with hypoglycemia, in contrast to most other anti-diabetic drug classes (Gallwitz, 2013). Interestingly, GLP-1 not only provides insulinotropic actions at pancreatic β cells but also acts as a trophic agent, inducing pancreatic β-cell proliferation and neogenesis, and inhibiting β -cell apoptosis (Campbell and Drucker, 2013). This spurred the development of long-acting GLP-1R agonists for treatment of type 2 diabetes mellitus, where such agents (Exendin-4 (Ex-4), also known as exenatide, and liraglutide) are well tolerated and widely used (Campbell and Drucker, 2013; Gallwitz, 2013). Interestingly, the GLP-1R is expressed on numerous non-islet cells throughout the body and, notably, on neurons throughout the central and peripheral nervous system. GLP-1 also is generated in brain, chiefly in the nucleus of the solitary tract in brainstem (Alvarez et al., 2005), as well as within M2 phenotype (anti-inflammatory) microglia too (Kappe et al., 2012), and systemic GLP-1 and associated longer-acting peptide analogues readily enter the brain (Salcedo et al., 2012; Greig et al., 2014; Hölscher, 2014).

The GLP-1R is a G protein-coupled receptor that is coupled to the cAMP second messenger pathway, increases in which are associated with neuroprotection (Perry and Greig, 2003; Stiles et al., 2014) – a function very different from its prior known role in brain in the

regulation of food intake and satiety (Campbell and Drucker, 2013). Numerous recent cellular and animal studies of GLP-1 and analogues have demonstrated promising actions across a number of neurodegenerative disease models (Perry et al., 2002a,b, 2007; Perry and Greig, 2003, 2005; Li et al., 2009, 2010a,b, 2012a, b, 2015; Salcedo et al., 2012), suggestive of the efficacy of this potential treatment approach in TBI.

We hypothesized that GLP-1R agonists would be useful to mitigate TBI on two complementary levels: (i) as neuroprotective/neurotrophic agents to mitigate neuronal cell death and cognitive impairment, and (ii) as anti-hyperglycemic agents (Greig et al., 2014) – since TBI-induced hyperglycemia is associated with increased mortality and morbidity in humans (Moppett, 2007). To vigorously this hypothesis and provide a translational basis for clinical TBI studies, 3 complimentary models of TBI (weight drop mTBI in mice (Rachmany et al., 2013a,b; Tweedie et al., 2013), CCI moderate TBI in rats and blast-induced mTBI in mice (Tweedie et al., 2015) were evaluated utilizing clinically translatable doses and routes of administration of the long-acting GLP-1R agonist, Ex-4. The dose appraised in rodents was selected as equivalent to 0.3 to 0.5 the FDA approved human dose in a 60–85 kg subject for diabetes treatment, after normalization of body surface area between species, in accord with FDA guidelines. Across all of these models, whether administration was initiated prior to or following TBI, Ex-4 significantly mitigated TBI-induced deficits in cognition (Rachmany et al., 2013a,b; Tweedie et al., 2013, 2015). It also reduced neuronal cell death in hippocampus and, notably, largely reversed many gene pathways up- and down-regulated by TBI (including those associated with oxidative stress, neuroinflammation, ribosomal and electron transport chain, neurogenesis and, remarkably, AD), returning them back to control levels (Tweedie et al., 2013, 2015). Notably, this occurred at both the pathway analysis and individual gene level (Tweedie et al., 2013, 2015). Taken together these data suggest a strong beneficial action of Ex-4 in managing TBI secondary damage across animal models. To cross-validate this, a clinically translatable dose of liraglutide was evaluated in mice subjected to weight drop mTBI, and cognitive impairments were, likewise mitigated (Li et al., 2015).

4.3. Oxidative stress and antioxidant approaches

A further key target to potentially mitigate TBI-induced neuronal damage and impairment is to ameliorate the oxidative stress that ensues. In this regard, *N*-acetyl-L-cycteine (NAC), the active agent in the FDA approved medication Mucomyst, has been found to have neuroprotective effects when tested in multiple TBI models (Ellis et al., 1991; Xiong et al., 1999; Hicdonmez et al., 2006; Eakin et al., 2014). The therapeutic mechanism that underpins this appears to be two-fold, combating both oxidative stress and inflammation. Not only is NAC an antioxidant itself, but also it is a precursor of the important endogenous antioxidant glutathione, which prevents damage from reactive oxygen and nitrogen species, particularly at the level of mitochondria (Xiong et al., 1999). A 100 mg/kg dose of NAC combined with a 30 mg/kg dose of top-iramate (an anti-epileptic medication), administered one hour following a mild weight drop injury was shown to resolve cognitive damage (Eakin et al., 2014). In this representative study, whereas the mTBI mouse group had a markedly reduced performance from the sham vehicle (control) group at 7 days and 30 days in both a NOR and Y-maze paradigm, the mTBI treatment group was not found to significantly differ

from the sham vehicle group at either time. In the same study, rats challenged with FPI and treated with NAC, likewise, demonstrated mitigation of cognitive impairment when evaluated in the Morris water maze; demonstrating activity across TBI models, species and behavioral paradims (Eakin et al., 2014). Notably, a parallel dose given to active duty military personnel in the first study of its kind was found to improve auditory, vestibular, and cognitive function sequelae following an mTBI blast injury (Hoffer et al., 2013), providing an excellent example of successful translation, as well as a preclinical methodological strategy to approach human studies.

An alternative approach to administering an exogenous antioxidant is to up regulate endogenous antioxidant levels. *Tert*-butylhydroxiquinone (tBHQ) is a chemical activator of nrf2, a basic leucine zipper (bZIP) protein that functions as a transcription factor which, in turn, is known to activate antioxidant and oxidative stress genes that protect against oxidative damage triggered by injury and inflammation. Under normal physiological, unstressed conditions, Nrf2 primarily exists in the cytoplasm where it interacts with Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3 that rapidly degrade Nrf2 by ubiquitination (Itoh et al., 1999) (half-life 20 min (Kobayashi et al., 2004)). Under oxidative stress, Nrf2 is not degraded, but instead translocates to the nucleus where it binds to a DNA promoter and initiates transcription of antioxidative genes and their proteins. tBHQ is able to cross the blood-brain barrier and disassociate nrf2 from its cytoplasm protein interactions to augment its levels and nuclear translocation. Mice deficient of Nrf2 have previously shown increased apoptosis and inflammatory signaling post moderate TBI. A 2012 study determined a dose of 33.4 mg/kg, to be effective in rescuing a NOR deficit 7 days post injury and showed a positive trend but not significant difference at day 30 (Saykally et al., 2012). tBHQ and other drugs that stimulate the nrf2 pathway are being evaluated across a number of neurological and systemic disorders involving oxidative stress (Saso and Firuzi, 2014).

The phosphatase inhibitor Salubrinal targets endoplasmic reticulum (ER) stress, a leading mechanism leading to apoptosis, via the eIF2α arm of the unfolded protein response. By inhibiting de-phosphorylation of the translation initiation factor, one can envisage that less proteins are produced within mTBI-induced over stressed cells; and this may result in their sparing from apoptotic cell death. To test this hypothesis, a dose of 1 mg/kg Salubrinal was administered 24 h post injury and was found to be effective at preventing neuronal degeneration 72 h post injury and reversing NOR deficits at both 7 and 30 days later (Rubovitch et al., 2015).

4.4. Protease-activated receptors and antagonists

Our collaborative group recently demonstrated that SCH79797, a protease-activated receptor 1 (PAR1) antagonist, is able to alleviate trauma-induced amnesia in mice that have undergone mTBI. Thrombin is a serine protease that plays an essential role in the blood coagulation cascade (Siller-Matula et al., 2011). Subsequent to its formation, following the enzymatic cleavage of prothrombin by activated Factor X, thrombin regulates a cascade of proteolytic events that ultimately lead to the formation of blood clots (Lippi et al., 2012). Lately, however, novel signaling cascades mediated by thrombin have been discovered

(Siller-Matula et al., 2011). Specifically, through the activation of the PARs, thrombin appears to directly affect the activity of multiple cell types and regulate a variety of biological functions that include inflammation, leukocyte migration, cellular proliferation, vascular permeability and tone, edema formation, and other processes related to tissue repair (Coughlin, 2000, 2001; Sambrano et al., 2001; Chen and Dorling, 2009; Schuepbach et al., 2009; Spiel et al., 2011). PARs belong to a distinctive family of G protein-coupled receptors (Luo et al., 2007). Their activation is instigated by an irreversible site-specific proteolytic cleavage within the *N*-terminal extracellular region of the protein. The uncovered *N*-terminal region then acts as a tethered ligand that activates the receptor (Gingrich and Traynelis, 2000). As recently reviewed by Maggio et al. (2013a, b) and Ben Shimon et al. (2015), PARs are present within the nervous system and whereas PAR-2 acts as a class of trypsin/tryptase-activated receptors, PAR-1, PAR-3, and PAR-4 are most efficiently triggered by thrombin (Gingrich and Traynelis, 2000). Within brain, PAR-1 is expressed on neurons and astrocytes, with stronger immunore-activity evident on astrocytces in human brain – particularly in the hippocampus, cortex, and striatum (Junge et al., 2004). Albeit the molecular cascades triggered by PAR-1 within astrocytes and neurons have yet to be fully characterized as are its various roles and modes of regulation, PAR-1 activation is reported to modulate synaptic transmission and plasticity by augmenting *N*-methyl-D-aspartate (NMDA) receptor currents (Gingrich et al., 2000; Lee et al., 2007; Maggio et al., 2008), and impacting long term potentiation (LTP). Studies recently reviewed by Reiner and Sapir (2014) indicate a key of PAR-1 role in axon formation in primary hippocampal neurons as well as neuron migration. Notably, PAR-1 knockout animals present with substantial impairments in hippocampus-dependent learning and memory processes (Almonte et al., 2007, 2013). In synopsis, PAR-1 has a key role in memory formation and synaptic plasticity (Maggio et al., 2013a,b, 2014), which can be regulated in a concentration-dependent manner by thrombin under physiological and pathological conditions. Interestingly, thrombin concentrations rise in the brain just a few minutes following a mTBI (Itzekson et al., 2014; Maggio et al., 2014), with such an increase in concentrations related to a poor NOR performance in animals challenged by brain trauma. Notably, injection of the PAR1 antagonist SCH79797 prevents memory deficit and rescues LTP in injured mice. These results may therefore indicate a potential new therapeutic strategy aimed at PAR-1 inhibition for alleviating amnesia and possibly other cognitive and behavioral deficits following mTBI in humans. Modulating this pathway may also have a beneficial effect on the long-term consequences of mTBI, and provide an interesting avenue for further research. It is important to note, however, that this potential therapeutic strategy possess potential risks that warrant preclinical investigation, as thrombin and/or PAR-1 inhibition may complicate trauma in the event that excessive bleeding occurs within the brain. Like other strategies involving potential therapeutic targets that have concentration-dependent roles in both physiological and pathological processes, a fine balance needs to be maintained in relation to both time-and concentration-dependence in an attempt to optimize beneficial actions and minimize potentially adverse ones, as well as optimizing the approach towards a patient population/disease state that may better respond.

5. Synopsis

Although no single animal TBI model perfectly mimics the human condition, a broad number have been developed and characterized that favorably recapitulate defined aspects evident clinically. For example, the non-invasive weight drop mTBI model (Baratz et al., 2011; Milman et al., 2005; Rachmany et al., 2013a,b; Tashlykov et al., 2009) has features relevant to human concussive head injury that can arise from traffic accidents, sports injuries, and falls. This involves diffuse axonal injury that can occur throughout the brain leading to diffuse neuronal loss and neuroinflammation, which are features predominant in a large proportion of human mTBIs in which no contusion and focal site of tissue loss or lesion is evident (Tweedie et al., 2007a,b). By contrast, for more severe human conditions where induction of a cortical contusion, generally involving focal destruction of brain tissue with micro haemorrhages, occurs – most frequently in the frontal and temporal regions and often also in brain regions opposite to the initial impact (contre-coup contusion) during the initial period post-TBI – this too can be broadly mimicked by a well characterized TBI model, such as CCI. Based on the heterogeneity of TBIs occurring clinically and the complexity of the associated injury mechanisms involved, the availability of well-characterized models is of clear value to aid elucidate both resulting deficits and strategies for their resolution. Importantly, as animals are temporarily anesthetized during the TBI procedure, potentially confounding effects associated with post-traumatic stress are thereby limited.

TBI research remains a major area of research in order to find novel therapeutic tools to potentially alleviate brain damage of the exposed population. In this regard efficacy across TBI models, in which common biochemical/pathological cascades are triggered, is likely more valuable than evaluation of efficacy within a single model alone. Clearly, the precise timing of treatment within the appropriate therapeutic window for target engagement is critical, as are such factors as age and gender. While several promising therapeutic targets have been revealed in recent years, additional studies are needed to validate these options as having restorative brain function under pathological conditions in translational studies where drugs are administered as both treatments and tests to evaluate mechanism-based hypotheses.

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