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Current Understanding and Experimental Approaches to the Study of Repetitive Brain Injury

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1. Introduction

Repetitive traumatic brain injury (TBI) occurs in a considerable number of individuals in the general population, such as athletes involved in contact sports (e.g. boxing, football, hockey and soccer), or child abuse victims. Repeated mild injuries, such as concussions, may cause cumulative damage to the brain and result in long-term cognitive dysfunction. The growing field of repetitive TBI research is reflected in the increased media attention given to reporting incidences of athletes suffering multiple blows to the head, and in several recent experimental studies of repeated mild TBI *in vivo*. Experimental reports generally demonstrate cellular and cognitive abnormalities after repetitive injury using rodent TBI models. In some cases, data suggests that the effects of a second mild TBI may be synergistic, rather than additive. In addition, some studies have found increases in cellular markers associated with Alzheimer's disease after repeated mild injuries, which demonstrates a direct experimental link between repetitive TBI and neurodegenerative disease. To complement the findings from humans and *in vivo* experimentation, my laboratory group has investigated the effects of repeated trauma in cultured brain cells using an *in vitro* model of stretch-induced mechanical injury. In these studies, cells exhibit cumulative damage when receiving multiple mild injuries. Interestingly, the extent of damage to the cells is dependent on the time between repeated injuries. Although direct comparisons to the clinical situation are difficult to make, these types of repetitive, low-level, mechanical stresses may be similar to insults received by certain athletes, such as boxers, or hockey and soccer players. As this field of TBI research continues to evolve and expand, it is essential that experimental models of repetitive injury replicate injuries in humans as closely as possible. For example, it is important to appropriately model concussive episodes versus even lower-level injuries (such as those that might occur during boxing matches or by heading a ball repeatedly in soccer). Suitable inter-injury intervals are also important parameters to incorporate into studies. Additionally, it is essential to design and utilize proper controls, which can be more of a challenge than experimental approaches to single mild TBI. These issues, as well as an overview of findings from repeated TBI research, are discussed in this chapter.

2. Overview of TBI

2.1 Occurrence and impact of TBI

Traumatic brain injury (TBI) is an insult to the brain caused by an external physical force, resulting in functional disability. Falls and motor vehicle accidents are the primary causes of TBI, while sports, assaults and gunshot wounds also contribute significantly to these types of injuries (Centre for Disease Control, 2010). TBI is one of the leading causes of death and disability worldwide, including the developing world (Reilly, 2007). In the United Kingdom, an estimated 200-300 per 100,000 people are hospitalized every year due to a TBI (McGregor & Pentland, 1997) and the incidence is reported as even higher in southern Australia and South Africa (Hillier et al., 1997; Nell & Brown, 1991). Although it has been difficult to compile reliable statistics on the prevalence and incidence of TBI in Canada (Tator, 2010), estimates in the United States suggest that between 1.4 and 1.7 million Americans sustain a TBI each year, accounting for 50,000 deaths and 80,000 to 90,000 individuals who suffer from long-term disability (Centre for Disease Control, 2010; Thurman & Guerrero, 1999). In Europe, it is estimated that at least 11.5 million individuals are suffering long-term disabilities related to a TBI (Schouten, 2007). In addition, TBI is considered to be a robust risk factor for the further development of neurodegenerative diseases, such as Alzheimer's disease (Slemmer et al., 2011), leading to additional dysfunction. Financially, the costs of TBI to society are no less distressing. Over two decades ago, an estimated 37.8 billion dollars was spent on direct costs related to hospital care in the U.S., or on indirect costs related to work loss due to disability (Max et al. 1991), and this cost has likely increased substantially. Due to the enormous impact TBI has on human health and health care systems in general throughout the world, understanding the mechanics and pathophysiology involved in TBI is essential for developing successful acute and long-term therapeutic strategies.

2.2 Repetitive mild TBI

TBI is characterized as mild, moderate or severe. Mild TBI, i.e. concussion, accounts for 70-90% of all TBI cases and 15-20% of individuals with a mild TBI have long-term dysfunction (Ryu et al, 2009). Although individuals who have experienced a moderate or severe TBI are certainly at risk of a second insult (Saunders et al., 2009), repetitive injuries occur in a considerable portion of individuals who have experienced a mild TBI. Child abuse victims, as well as victims of spousal abuse, are often subjected to multiple injuries to the head (Roberts et al., 1990; Shannon et al., 1998). Many injuries of these types go unreported, and it is difficult to assess how many insults a patient may have suffered. Arguably, athletes represent the largest group of patients that are at risk for experiencing repeated brain injuries, especially concussions (Guskiewicz et al., 2000; Kelly, 1999; Kelly & Rosenberg, 1997; Powell and Barber-Foss, 1999). Also, in comparison to child or spousal abuse victims, there is generally better documentation of how many brain injuries an individual has sustained due to recreational or sports related activities, making this population easier to study.

The idea that multiple head injuries in athletes could lead to clinical problems has long been suggested. For example, many clinicians believe that the development of *dementia pugilistica* in professional boxers is caused by the multiple hits to the head that a boxer endures over the course of their career (Jordan, 2000). Also, studies have shown that the number of concussions is inversely related to performance on several neuropsychological tests in soccer players (Matser et al., 1999; 2001), and jockeys that have experienced multiple concussions

generally display more cognitive dysfunctions than those who have had a single injury (Wall et al. 2006). An association between repetitive concussions and cognitive impairment, as well as clinical depression, has been demonstrated in professional football players in the United States (Guskiewicz et al., 2005; 2007). In Canada, the occurrence of concussion in ice hockey has been in the press substantially in recent months. The incidence of concussions in hockey appears to be on the rise not only in the National Hockey League, but also at the junior level (Ackery et al., 2009; Echlin et al., 2010). Many of these players have repeated concussions and suffer from post concussion symptoms such as memory impairment, headaches and depression (Ackery et al., 2009). As with boxers, there is evidence that repeated concussions may increase the risk of developing dementia later in life (De Beaumont et al., 2009). Therefore, it is important to understand the processes underlying the pathology of repetitive TBI.

3. Experimental approaches to the study of repetitive TBI

When studying repetitive brain trauma in athletes, we can gain much information about the pathology and progress of such injuries from the injured athletes themselves, e.g. by measuring changes in cognitive and motor performance. However, these injuries are generally at a mild level, and therefore, except in rare cases when athletes die as a result of the insult, we cannot assess the changes that have actually occurred in the brain at the cellular and sub-cellular levels. In order to compile this type of information, we must turn to experimental models of TBI.

3.1 *In vivo* studies

When discussing experimental studies of repetitive TBI *in vivo*, this does not include studies of secondary insults, such as a mechanical insult to the head followed by a defined duration of ischemia or glutamate exposure. Repeated TBI experimentation consists of an initial mechanical injury to the head followed by another mechanical insult to the head of the same or different degree. Based on these criteria, there were very few of these types of experiments conducted before the year 2000, with only a handful of repetitive injury studies being published (Kanayama et al., 1996; Olsson et al., 1976; Weitbrecht & Noetzel, 1976). Several additional *in vivo* studies of repeated injuries in rodents have now been conducted over the past decade (Allen et al., 2000; Conte et al., 2004; Creeley et al., 2004; DeFord et al., 2002; Friess et al., 2009; Huh et al., 2007; Laurer et al., 2001; Longhi et al., 2005; Raghupathi et al., 2004; Shitaka et al., 2011; Uryu et al., 2002; Yoshiyama et al., 2005). All of these repeated mild injury studies were conducted using rodent models of TBI with the exception of the studies by Friess et al (2009) and Raghupathi et al (2004), which used a pediatric model of repeated injury in pigs.

Repetitive TBI generally occurs at a mild level, therefore experimental models have been used which are minimally invasive and do not require a craniotomy, such as weight drop models or other forms of closed-skull TBI. The models must also be administered at a level that produces minimal, or preferably, no fatality. Individuals who have suffered from a mild TBI often complain of cognitive difficulties post-injury. Therefore, repeated injury studies usually evaluate cognitive function, for example using the Morris water maze (MWM) test, as well as the extent of cellular abnormalities in the cortex and hippocampus. The hippocampus in particular has received significant attention in the study of repeated mild TBI, because it plays a critical role in certain types of learning and aspects of memory

storage. Experimental and clinical data have demonstrated not only the importance of this brain region in learning and memory, but also that the hippocampus is uniquely vulnerable to injury, even after mild brain trauma (Lowenstein et al., 1992; Lyeth et al., 1990). In a study by DeFord et al. (2002), repeated mild injuries were administered to mice (four times every 24 hr), followed by MWM testing and histological analysis. Significant learning deficits were found after repeated injuries, which were not evident after a single injury. These deficits occurred even in the absence of cell death within the cortex and hippocampus. Cognitive deficits after multiple mild TBIs (using MWM analysis) were demonstrated in a similar study using a weight drop model (Creeley et al., 2004). In a recent study, Shitaka et al. (2011) used a controlled cortical impact model in mice and found that animals receiving two injuries 24 hr apart displayed MWM deficits for several weeks. In addition, although no gross histological abnormalities were noted, mice that received two insults had damaged axons in various brain areas, which could underlie the cognitive abnormalities.

In one of the early studies of repeated injury *in vivo*, Laurer et al. (2001) used an injury regimen that they described as "concussive". This model was meant to mimic the type of insult that athletes may receive, and was also used for many subsequent studies (Conte et al., 2004; Longhi et al., 2005; Uryu et al., 2002). In an assessment of cognitive and motor function after repeated injury in mice, Laurer et al. (2001) found that the brain was more vulnerable to a second insult if the second injury occurred 24 hr after the first. Even though no cognitive deficits were demonstrated in mice receiving repeated injuries, there was a decrease in motor function and neuronal loss. The authors also stated that the effects of a second mTBI could be synergistic, rather than additive. To further analyze the effects of lengthening the inter-injury interval, Longhi et al. (2005) investigated repetitive injuries three, five and seven days apart. Animals that received repeated injuries three or five days apart exhibited cognitive dysfunction not evident in sham animals or those injured only once. However, no deficits were observed when the injury interval was extended to seven days. This experimental evidence demonstrating that the brain can recover from a first injury, given sufficient amount of time, is certainly alluring, especially in relation to establishing "return-to-play" guidelines for athletes. Overall, the evidence from these *in vivo* experimental models suggests that repetitive mild TBI causes more cognitive and cellular dysfunction than a single injury, if the brain is not given a sufficient amount of time to recover.

Other *in vivo* studies have been conducted with a primary interest in discovering more about the pathology of inflicted repetitive brain injury in the pediatric population, such as 'shaken impact syndrome' (Friess et al., 2009; Huh et al., 2007; Raghupathi et al., 2004). In a study by Raghupathi et al. (2004), neonatal pigs were subjected to rapid axial rotations of the head, either once, or twice within 15 minutes. Brains were analyzed at 6 hr post-injury and animals that had received double insults exhibited a wider distribution of injured axons than animals that were injured once. In another study in piglets (Friess et al., 2009), animals were injured (by axial head rotation) either once, twice one day apart, or twice one week apart. Animals injured one day apart had the highest mortality rate. Also, animals receiving two injuries had worse neuropathology and neurobehavioral outcome than those injured only once. Huh et al. (2007) conducted experiments in young rats (11 days old) and administered one, two or three injuries spaced only 5 minutes apart. Animals receiving multiple injuries generally displayed increased axonal damage, which was evident earlier after injury than a single impact. Overall, these studies suggest a graded response to repeated injury in the pediatric brain.

3.2 Studies conducted *in vitro*

Several *in vitro* approaches have now been developed to study traumatic injury, which utilize dissociated brain cells or slices grown in culture (LaPlaca et al., 2005; Morrison et al., 1998; Noraberg et al., 2005; Spaethling et al., 2007; Weber, 2004). For many years, my laboratory group has utilized an *in vitro* model of stretch-induced mechanical injury originally developed by Ellis et al. (1995). We have characterized this stretch injury model in cell cultures composed of neurons and glia from murine hippocampus (Slemmer *et al.*, 2002; Slemmer & Weber, 2005), cortex (Engel et al., 2005), and cerebellum (Slemmer *et al.*, 2004), and currently in cortical cultures from rat pups.

We have previously conducted studies investigating the effects of repeated trauma on cultured hippocampal cells (Slemmer *et al.*, 2002; Slemmer & Weber, 2005), which were intended to complement the findings from humans and *in vivo* experimentation. In these studies, we utilized a mild level of stretch injury that produces some measurable damage to cells when administered a single time. When mild stretch injuries were repeated at either 1-hr or 24-hr intervals, cells exhibited cumulative damage. For example, cultures that received a second insult displayed a significant loss of neurons not evident in cultures that received only one injury (see Figure 1). Additionally, cultures injured twice released a significant level of neuron specific enolase (NSE), which was not observed in cultures injured a single time. Interestingly, the extent of damage to the cells was dependent on the time between repeated injuries. For example, cultures that received a second insult 1 hr after the first injury released more S-100B protein (a biomarker of injury commonly employed in the clinic) than cultures that received a second injury at 24 hr. Cultures injured 24 hr apart also exhibited less staining with the intravital dye, propidium iodide, than those injured 1 hr apart. As demonstrated in some *in vivo* studies, these findings suggest that a level of injury producing measurable damage or dysfunction on its own, may cause cumulative damage if repeated within a certain time frame (Laurer et al., 2001; Longhi et al., 2005).

We also investigated the effects of a very low level of stretch, which produces no overt cell damage (Slemmer and Weber, 2005). This "subthreshold" level of stretch did not cause significant damage or death, even when it was repeated at a 1 hr interval. However, this low level of stretch did induce cell damage when it was repeated several times at a short interval (every 2 min), indicated by increased propidium iodide staining (a marker of cellular injury), neuronal loss, and an increase in NSE release. Although direct comparisons to the clinical situation are difficult to make, these types of repetitive, low-level, mechanical stresses may be similar to the insults received by certain athletes, such as boxers, and hockey and soccer players (Jordan, 2000; Matser et al., 1998; Matser et al., 1999; Webbe & Ochs, 2003; Wennberg & Tator, 2003). This type of *in vitro* model may provide a reliable system in which to study the mechanisms underlying cellular dysfunction following repeated injuries. In addition, this approach could provide a means for rapid screening of potential therapeutic strategies for both single and repeated mild TBI.

Another study of repeated injury *in vitro* used a model of axonal injury (Yuen et al., 2009). Low levels of strain to cortical axons in culture resulted in no obvious pathological changes. By 24 hr however, these axons exhibited increased sodium channel expression. When axons were stretched again at 24 hr, there was a significant increase in intracellular calcium, which led to degeneration of the axons. This finding suggests a possible mechanism underlying the susceptibility of the brain to a second impact within a certain temporal window.

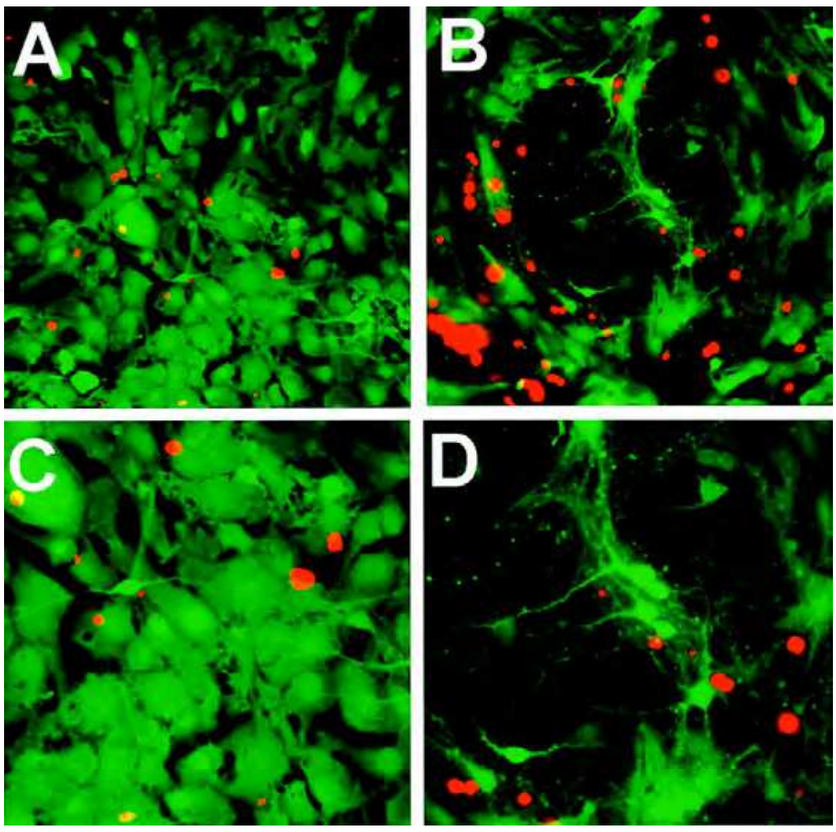

Fig. 1. Effect of repeated stretch injury on hippocampal cells in culture. Cell injury was assessed using the two dyes fluorescein diacetate (FDA) and propidium iodide (PrI). FDA stains healthy, viable cells and fluoresces green, while PrI does not pass through intact cellular membranes. If membranes are damaged, however, cells lose their ability to retain FDA and PrI will enter the cell and bind to the nucleus, fluorescing red. (A) PrI uptake following mild stretch injury at 1 h post-injury (B) A double mild insult increased PrI uptake when evaluated immediately after the second injury. Note that many cells also have beaded neurites. (A and B) Magnification: 100X. (C and D) Enlargements of A and B, respectively. Magnification: 200X. Modified from Slemmer et al. (2002). Reprinted with permission from Oxford Press, 2002.

3.3 The preconditioning phenomenon

Several studies have indicated that an initial, very mild insult to either cultured cells or to the brain itself, may provide some protection from a second, more severe insult, a finding that has been termed "preconditioning". Ischemic preconditioning, in which a brief exposure to ischemia renders the brain more resistant to subsequent longer periods of ischemia, has been well described (for review, see Schaller & Graf, 2002). There is also evidence of preconditioning cross-tolerance. For example, brief ischemia lessens damage following TBI *in vivo* (Perez-Pinzon et al., 1999). More recently, several other types of pretreatments have been demonstrated to improve outcome and pathology after experimental TBI, such as a low dose of *N*-methyl-D-aspartate (Costa et al., 2010), exposure to lipopolysaccharide (Longhi et al., 2011) or glucagon (Fanne et al., 2011), hypothermia (Lotocki et al., 2006), as well as exposure to hyperbaric oxygen (Hu et al., 2008; 2010).

Another interesting phenomenon is that heat acclimation (chronic exposure to moderate heat) can also provide resistance to subsequent TBI (Shein et al. 2007; 2008; Umschwief et al., 2010).

In our *in vitro* studies using mechanical stretch, we observed a novel form of mechanical preconditioning. When hippocampal cultures were administered a subthreshold level of stretch 24 hr prior to a mild stretch, there was a significant decrease in released S-100B protein compared to cultures that were injured at a mild level alone (Slemmer & Weber, 2005). This observation suggests some form of protection initiated by this low level of stretch. A similar finding *in vivo* was reported by Allen et al. (2000). In their study, rats received a series of mild injuries spaced three days apart using a weight drop model. Some of these animals received a severe injury after the repetitive mild injuries. Motor function deficits were evident in severely injured animals, but not in animals that received repeated mild injuries or repeated mild injuries followed by a severe injury. This last observation suggests a preconditioning effect.

An important question is how do we utilize this information for beneficial means? One can imagine the ethical implications of suggesting to people that a mild insult to their brains may in fact protect them from worse insults in the future. We still have much to learn about preconditioning. For example, what is the threshold for mechanical insults between initiating protective versus damaging mechanisms in the brain? A clear understanding of the mechanisms by which this protection is elicited holds potential for the management of mild TBI. The fact that a wide variety of stressors can protect the brain from TBI (i.e. cross-tolerance) suggests that the same, or similar mechanisms are responsible for the endogenous protection. Increasing the expression of these protective systems could not only be a reliable way for managing mild TBI, but could also provide resistance in individuals who may be at risk of sustaining an additional head injury, such as athletes. Both *in vivo* and *in vitro* models could provide reliable systems in which to study the mechanisms underlying the preconditioning phenomenon.

4. Repetitive injury and neurodegenerative disease

A correlation between the occurrence of TBI and the further development of neurodegenerative disease later in life has been recognized for several years, and TBI is considered to be one of the most robust risk factors for developing Alzheimer's disease (AD; Szczygielski, et al., 2005; Slemmer et al., 2011). There is also evidence that genetic predisposition may increase one's risk of developing AD, such as possession of the apolipoprotein E ε4 allele (Isoniemi et al., 2006). A phenomenon known as chronic TBI occurs in a significant amount of professional boxers (Jordan, 2000), with the most serious form, the neurodegenerative disorder *dementia pugilistica*, resulting in severe cognitive and motor dysfunctions. A potential link between TBI and Parkinson's disease has also been suggested (Masel and DeWitt, 2010). It is generally accepted that the pathology of AD and *dementia pugilistica* are quite similar (Geddes et al., 1999; Schmidt et al., 2001). Although epidemiological data linking TBI and neurogenerative diseases are quite strong, only a modest amount of experimental work has been conducted in order to achieve a mechanistic link between repeated mild TBI and the development of either AD or *dementia pugilistica.*

In addition to cognitive symptoms, dementias such as *dementia pugilistica* and AD are associated with specific types of neuropathological markers. In fact, AD in humans can only

be fully confirmed post-mortem via the presence of extracellular senile plaques, which are abnormal amyloid β (Aβ) protein deposits, and abnormal tau protein aggregation in specific brain regions (Price et al., 1991). The tau protein is an important functional component of the cytoskeleton in healthy neurons, but it is also a predominant component of neurofibrillary plaques found in AD and *dementia pugilistica* (Schmidt et al., 2001). Therefore, the development of abnormal tau protein pathology is a potential molecular link between TBI and dementia. In a study by Kanayama et al (1996), rats were injured with a mild impact once a day for seven days. Analysis showed an increase in abnormal tau protein deposits by one month after injury. Yoshiyama et al. (2005) used a robust injury paradigm in an attempt to model human *dementia pugilistica* in transgenic mice expressing the shortest human tau isoform (T44). Mice were subjected to four injuries a day, once a week, for four weeks, resulting in each mouse receiving a total of 16 injuries, and surprisingly, they could find only one mouse that displayed pathology of *dementia pugilistica* at nine months of age. Partly for this reason, the vast majority of animal studies have focused on the deposition of Aβ, or the intracellular processing of amyloid precursor protein (APP), from which Aβ is derived. Although high levels of Aβ have clearly been demonstrated in AD patients, the exact function of amyloid protein has not been established. Interestingly, deposition of Aβ has not been observed in the majority of nontransgenic animal studies after trauma (Laurer et al., 2001; Szczygielski, et al., 2005), and as a result, many of the current models used to investigate traumatic dementia are derived from transgenic rodents that were originally created to investigate AD. For example, the transgenic mouse Tg2576, which is characterized by AD-like amyloidosis by nine months of age, has been used in several investigations of repetitive mild TBI, and has become a popular animal model for traumatically-induced dementia.

In a study by Uryu et al. (2002), Tg2576 transgenic mice subjected to repeated, but not to single mild TBI, displayed cognitive deficits and Aβ deposition. As shown in Figure 2, Aβ deposition did not occur in these mice at either 9 or 16 weeks post-sham injury. In contrast, brain slices from Tg2576 mice that underwent repeated mild TBI displayed evident Aβ deposition (in the form of senile plaques) at 16 weeks post-injury. The appearance of senile plaques followed a delayed time-scale, which is not surprising, as dementia is often manifested in humans long after TBI. This study also demonstrated that the transgenic background alone was not sufficient to induce marked amounts of Aβ deposition in these aged mice, which is in line with a "two-hit" hypothesis proposed by Nakagawa et al. (1999). In this case, the first-hit is the genetic predisposition, which enables an individual to produce high amounts of abnormal proteins such as Aβ, and the second-hit is the TBI. However, a single mild injury alone was not enough to produce AD-like pathology. It is therefore possible that more than one mild TBI is necessary to lead to dementia later in life, whereas a single moderate or severe TBI on its own may lead to dementia. Increased incidence of dementia in humans is obviously associated with increased age, and recent evidence links aging with the overproduction of free radicals via oxidative stress (Slemmer et al., 2008). TBI is also known to dramatically increase free radicals and reactive oxygen species (Slemmer et al., 2008, Weber, 2004). Repetitive, but not single mild TBI, has been previously shown to increase oxidative stress in Tg2576 mice (Uryu et al., 2002), which could be reduced by supplementing the rodent chow with vitamin E, a known antioxidant (Conte et al., 2004). Therefore, oxidative stress may be a major contributing factor leading to the development of neurodegenerative disease following TBI.

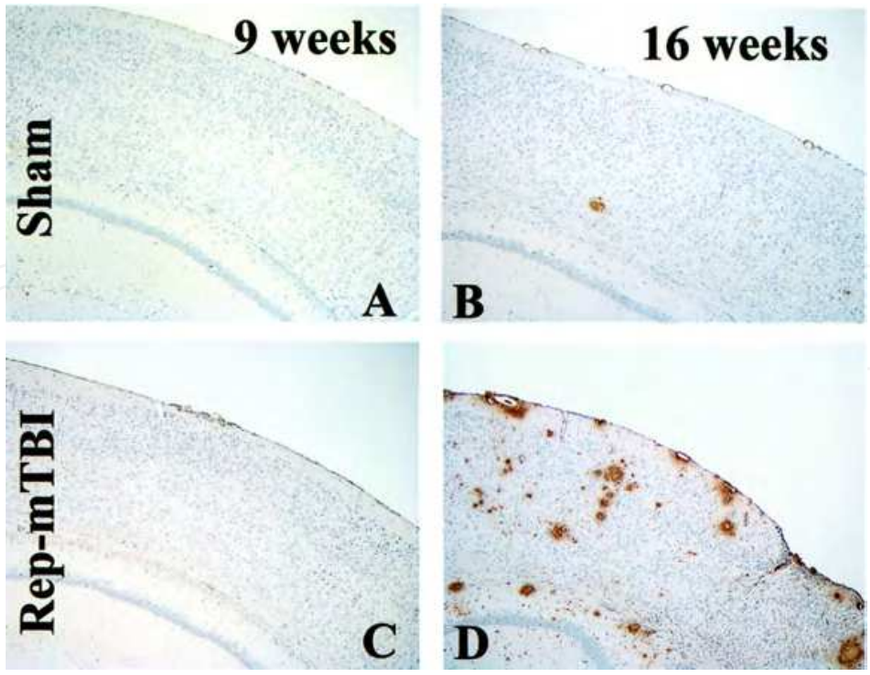

Fig. 2. Amyloid deposition in Tg2576 mice with sham (A, B) or repetitive mild TBI (C, D) with 4G8 immunohistochemistry at 9 (A, C) and 16 (B, D) weeks after mild TBI. Senile plaques increased in an age-dependent manner in both sham and injured mice, but the largest number of Aβ-positive plaques are evident in the 16-week repetitive mild TBI mice (D). Modified from Uryu et al. (2002). Reprinted with permission from the Society for Neuroscience, 2002.

The overall findings of these *in vivo* studies are quite significant, because they can demonstrate a direct experimental link between repeated mild TBI and the development of AD-like pathology, as well as other forms of dementia. Generally, it takes many years before the onset of symptoms of neurodegenerative disorders is evident, after an individual has experienced a TBI. Therefore, it requires an exceedingly long amount of time to gather this type of epidemiological data from the human population. This area of research, in particular, is where experimental models could truly help decipher the mechanisms by which neurodegenerative disease may be triggered by repetitive brain injury, and to identify potential therapeutic strategies.

5. Future directions

5.1 Potential new experimental directions

The current lines of research in repetitive TBI should certainly be continued, such as attempting to firmly establish the link to neurodegenerative disease, as well as demonstrating appropriate recovery times after a mild injury. However, new avenues also need to be explored. For example, much experimental evidence suggests that animals demonstrate cognitive deficits and cellular dysfunction after repetitive mild TBI, even though the injury may not necessarily lead to cell death (DeFord et al., 2002; Kanayama et al., 1996). Therefore, rather than trying to prevent cells from dying after repeated injuries, it may be more useful to learn how to restore normal cellular physiology after a traumatic episode. Combining studies

at the cellular and behavioral levels is crucial for attaining this goal, and one area of potential interest is the evaluation of the effects of repeated TBI on synaptic plasticity in the cortex and hippocampus. The ability of neurons to undergo changes in synaptic strength, such as long-term potentiation (LTP), is postulated to be a cellular correlate of learning and memory (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999). Several studies have reported impaired hippocampal LTP after TBI *in vivo* (see Albensi, 2001; Weber, 2004). One area of future research could focus on restoring mechanisms of synaptic plasticity after injury (such as LTP), as well as correlated hippocampal-mediated behavioral tasks.

The hippocampus shares neuronal projections with areas of the cerebral cortex, which undoubtedly also contributes to memory formation and storage. Indeed, alterations in synaptic plasticity may also occur directly in the cortex after repeated mild TBI. Therefore, although the hippocampus may play a central role in the cognitive dysfunction observed after mild TBI, it is important not to overlook contributions from other brain areas as well. Since some repeated injury studies demonstrate motor impairment, it may also be appropriate to investigate cellular physiology and synaptic plasticity in the cerebellum (see Hansel et al., 2001; Weber et al., 2003; Slemmer et al., 2005) after repetitive TBI. These types of investigations could involve electrophysiology measurements as well as analysis of intracellular calcium dynamics. Intracellular calcium is extremely important to the normal function of neurons and can be considerably altered even in cells that do not go on to die (Weber, 2004; Yuen et al., 2009).

5.2 Experimental design considerations

Although deciding on appropriate research directions is of paramount importance to developing potential therapeutic strategies for repetitive TBI, the utilization of proper parameters for repeated injury studies may be just as crucial. For example, what are the best inter-injury interval, or intervals, to use? Although 24 hr between injuries is the most common (and perhaps practical) interval in the laboratory (Conte et al., 2004; Creeley et al., 2004; DeFord et al., 2002; Friess et al., 2009; Kanayama et al., 1996; Laurer et al., 2001; Shitaka et al., 2011; Uryu et al., 2002; Weitbrecht & Noetzel, 1976; Yoshiyama et al., 2005), is it the most appropriate in mimicking what occurs in humans? Also, how many injuries should a researcher administer? If one is attempting to model concussive episodes, then two or three may be enough, as this may closely mimic a true situation, especially with athletes. However, when attempting to recreate *dementia pugilistica* (Yoshiyama et al., 2005), the number of injuries should certainly be increased, and perhaps be 'subthreshold' levels of injury, i.e. a level of injury which produces no overt damage on its own.

The proper controls and endpoints to use for repeated injury studies also need to be carefully considered. For *in vivo* studies analyzing the effects of a single TBI, the issue of controls is fairly straightforward. Sham animals are treated at an equivalent time as injured animals, and the analysis, cellular or behavioral, is also performed at the same time-point. However, when comparing uninjured animals to animals that have received more than one injury, what is the proper comparison? For example, if an animal receives an injury on day one, and an additional injury on day two, and analysis takes place on day three, does one compare the data with sham animals from day one, or from day two (or both, see figure 3A)? The issue is further complicated when comparing repeatedly injured animals to animals that have received a single TBI. If the comparison concerns animals that undergo four injuries or a single injury, are the single insult animals injured at the same time as injury one in the repeated group, or at the same time as the fourth injury (see figure 3B)? This decision will affect the endpoint as well.

For example, if animals or tissue are analyzed one day after the fourth injury, then four days will have passed for the single injury group if those animals were injured on day one. This difference in time could affect the observations. One could argue that if a long enough period of time passes after the injuries, such as weeks or months, then the effect of when the single insult animals were injured will be negligible. Admittedly, this would be more proper for comparison to the human situation in which the effects of mild TBI can be manifested for weeks, months, or even years. However, this is often not practical for many laboratories, as the costs of housing animals for months can at times be prohibitive. Also, conducting long-term experiments *in vitro* is limited, since the cells generally remain viable for only a few weeks. This raises a critical point as to the relevance of repeated injury studies *in vitro*. I strongly believe that *in vitro* experiments can deliver information about the cellular mechanisms of repeated injury that are difficult to obtain *in vivo*, and that it is essential to combine data derived from *in vitro* experiments with those conducted with animals *in vivo*. However, I am unsure how to directly compare the data. For example, is a 24 hr injury interval *in vitro* equivalent to 24 hr *in vivo*? The greater consensus that exists on these issues with individuals who conduct repeated injury studies, the easier it will be to compare the data, and the stronger a case can be made for showing unequivocally, that repeated mild TBI could lead to long-term dysfunction in humans.

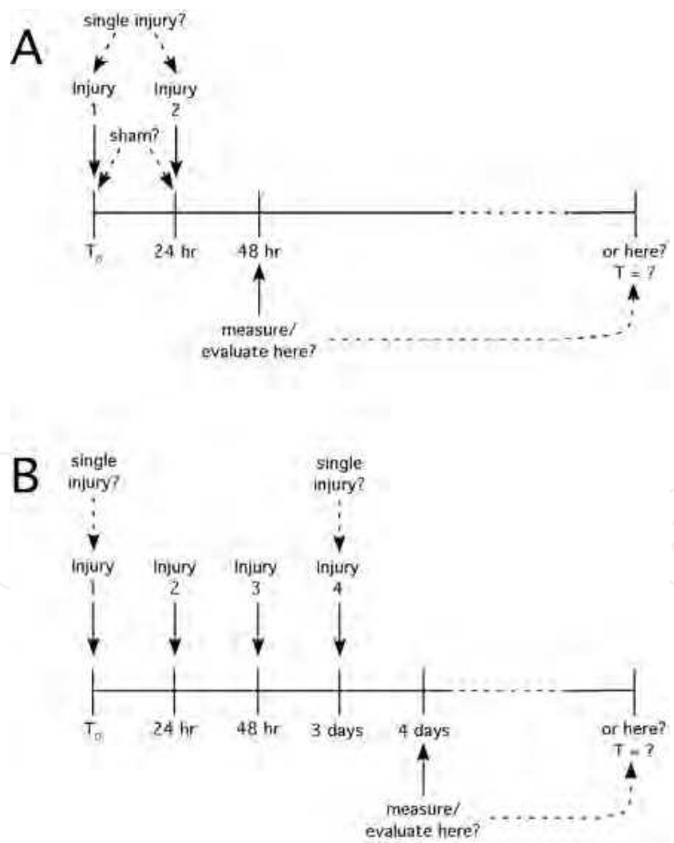

Fig. 3. Issues for consideration when designing repeated TBI experiments (i.e. choosing proper timepoints for controls and behavioral/tissue analysis). T = time.
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5.3 Possible therapeutic interventions

Perhaps one of the best, and most logical, therapeutic interventions that physicians can make especially when athletes are concerned is to not allow these individuals to return to play until they seem to have fully recovered from a mild injury/concussion. This would obviously stop the individual from being in a position of acquiring a second injury in a vulnerable period. Return to play and treatment guidelines have been established by a consensus statement on concussion in sport at the 3rd International Conference on concussion in sport in Zurich in November of 2008 (McCrory et al., 2009). Diagnosis of concussion and recovery involves a wide assessment of an individual including physical signs, behavioral abnormalities, balance, sleep and cognition (Echlin et al., 2010; McCrory et al., 2009). Neuropsychological assessments and tests such as the Sideline Concussion Assessment Tool 2 (SCAT2) and the Immediate Post-Concussion Assessment and Cognitive Test (ImPACT) should also be routinely used (Echlin et al., 2010; McCrory et al., 2009), and players should have no signs of neurological deficits or syndromes before returning to play (Ackery et al. 2009). There will likely still be players that will not comply with return to play advice, but these individuals need to be made aware that lack of compliance may put them at higher risk for experiencing another concussion as well as suffering potential permanent brain damage and disability (Ackery et al., 2009).

Another potential type of intervention is genetic screening. As previously mentioned, individuals with the apolipoprotein E ε4 allele generally show poorer outcome after injury than others without this genetic polymorphism (Isoniemi et al., 2006). Also, individuals with a genetic alteration in neprilysin, which is the enzyme that degrades Aβ protein, may be at greater risk of Aβ plaque formation after TBI as well as the development of AD (Johnson et al, 2009). At present, there are no specific therapeutic interventions that are routinely used for these individuals. However, these persons could at least be advised that they may be at a much higher risk of developing AD if they sustain a TBI or repetitive mild TBIs. Therefore, they could make an informed decision about whether they would participate in activities where they may be at high risk of experience a TBI, such as specific types of sports.

TBI is known to increase free radicals and reactive oxygen species, leading to oxidative stress (Slemmer et al., 2008, Weber, 2004), and this may be a prevalent means of damage even after mild TBI. Therefore, specific agents that could be useful for treating mild TBI are antioxidants. In a study mentioned earlier (Conte et al., 2004), vitamin E, a known antioxidant, increased cognitive function and decreased Aβ deposition after repetitive concussive injury. In addition to supplementation, individuals could potentially increase the amount of antioxidant species in their body through diet, as several foods have high amounts of antioxidants (Ferrari & Torres, 2003). Of course, these compounds would have to cross the blood-brain barrier in order to provide protection from TBI. In fact, many of these species do cross into the brain. For example, Andres-Lacueva et al. (2005) demonstrated that compounds present in blueberries were found in rat brain cells after feeding them a diet with blueberry extract. In addition, Sweeney et al. (2002) showed that rats fed blueberries for six weeks were protected from stroke. This raises the possibility that an individual on a diet high in antioxidant species may be somewhat protected from a mild trauma and may have better outcome following a second mild TBI should it occur.

Another interesting prospect in the field of treating repetitive mild TBI is the potential use of cognitive enhancers, such as ampakines, which were and still are touted as therapeutic agents for neurodegenerative conditions such as Alzheimer's disease (Lynch and Gall, 2006). They are now gaining popularity as safe drugs to improve memory and concentration in

healthy individuals. Ampakines positively modulate the AMPA-type of glutamate receptors in the brain (Lynch & Gall, 2006). Glutamate receptors are known to be involved in a wide variety of processes in the nervous system, one of which is memory. Their activation appears to be imperative for memory consolidation. For example, the activation of AMPA receptors is known to facilitate LTP in the hippocampus. Ampakines are peripherally administered drugs known to cross the blood-brain barrier and can potently facilitate LTP, as demonstrated in rodents (Staubli et al., 1994). These drugs also improve memory performance in rodents and humans (Lynch, 1998; Lynch & Gall, 2006).

Ampakines have now been evaluated in clinical trials in humans. One of these drugs in particular (CX516) has demonstrated enhanced memory and cognitive performance in healthy young adults (Ingvar et al., 1997; Lynch et al., 1996). Similar positive cognitive effects were found with CX516 in healthy elderly subjects (Lynch et al., 1997). In these studies, no changes in heart rate, mood or motor performance were found. Another study in healthy elderly volunteer subjects with another ampakine (farampator) showed improvements with short-term memory (Wezenberg et al., 2007). At higher doses, farampator caused side effects such as nausea, headache and drowsiness. Overall, these drugs produce cognitive enhancement with either no, or very mild side effects. This raises the possibility of treating athletes with these drugs after they have sustained a concussion, as well as treating child and spousal abuse victims who have repetitive injuries.

6. Conclusions

Repetitive mild TBI constitutes a significant portion of all TBI cases and the incidence of repeated TBI appears to be on the rise. Overall, there has been surprisingly little attention given to experimental repetitive TBI studies. However, more researchers have conducted studies in this field in recent years. Research involving both *in vivo* and *in vitro* experimentation holds promise for unraveling the pathology of repetitive mild TBI, which may differ from that of single TBI at various levels. A greater understanding of how long the brain takes to recover after a mild injury will aid in determining return to play guidelines for athletes. In addition, further experimentation and monitoring of mild TBI sufferers will assist in developing treatment strategies for decreasing damage should a second injury occur.

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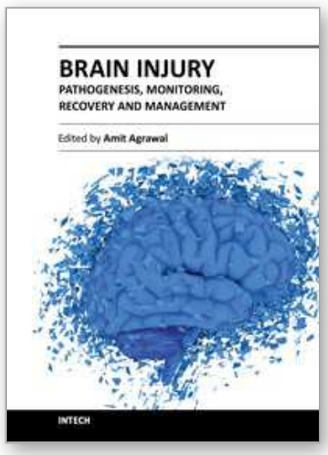

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The present two volume book "Brain Injury" is distinctive in its presentation and includes a wealth of updated information on many aspects in the field of brain injury. The Book is devoted to the pathogenesis of brain injury, concepts in cerebral blood flow and metabolism, investigative approaches and monitoring of brain injured, different protective mechanisms and recovery and management approach to these individuals, functional and endocrine aspects of brain injuries, approaches to rehabilitation of brain injured and preventive aspects of traumatic brain injuries. The collective contribution from experts in brain injury research area would be successfully conveyed to the readers and readers will find this book to be a valuable guide to further develop their understanding about brain injury.

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