

Repeated mild injury causes cumulative damage to hippocampal cells

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Summary

An interesting hypothesis in the study of neurotrauma is that repeated traumatic brain injury may result in cumulative damage to cells of the brain. However, post-injury sequelae are difficult to address at the cellular level *in vivo*. Therefore, it is necessary to complement these studies with experiments conducted *in vitro*. In this report, the effects of single and repeated traumatic injury *in vitro* were investigated in cultured mouse hippocampal cells using a well characterized model of stretch-induced injury. Cell damage was assessed by the level of propidium iodide (PrI) uptake and retention of fluorescein diacetate (FDA). Uninjured control wells displayed minimal PrI uptake and high levels of FDA retention. Mild, moderate and severe levels of stretch caused increasing amounts of PrI uptake, respectively, when measured at 15 min and 24 h post-injury, indicating increased cellular damage with increasing amounts of stretch. For repeated injury studies, cultures received a second injury 1 h after the initial insult. Repeated mild injury caused a slight increase in PrI uptake compared with single injury at 15 min and 24 h post-injury, which was evident primarily in glial cells. However, the neurites of neurones in cultures that received repeated

insults showed signs of damage that were not evident after a single mild injury. The release of neurone-specific enolase (NSE) and S-100 β protein, two common clinical markers of CNS damage, was also measured following the repeated injuries paradigm. When measured at 6 h post-injury, both NSE and S-100 β were found to be elevated after repeated mild injuries when compared with the single injury group. These results suggest that cells of the hippocampus may be susceptible to cumulative damage following repeated mild traumatic insults. Both glial cells and neurones appear to exhibit increased signs of damage after repetitive injury. To our knowledge, this study represents the first report on the effects of repeated mechanical insults on specific cells of the brain using an *in vitro* model system. The biochemical pathways of cellular degradation following repeated mild injuries may differ considerably from those that are activated by a single mild insult. Therefore, we hope to use this model in order to investigate secondary pathways of cellular damage after repeated mild traumatic injury, and as a rapid and economical means of screening possibilities for treatment strategies, including pharmaceutical intervention.

Keywords: brain trauma; hippocampal neurones; *in vitro*; neurone-specific enolase; S-100 β

Abbreviations: FDA = fluorescein diacetate; HBSS = Hanks' balanced salt solution; MTBI = mild traumatic brain injury; NSE = neurone-specific enolase; PrI = propidium iodide; rMTBI = repeated mild traumatic brain injury; TBI = traumatic brain injury

Introduction

Traumatic brain injury (TBI) continues to be a leading cause of death in Western industrialized nations (Jennett, 1996). In the United Kingdom, it is estimated that 200–300 people per 100 000 are hospitalized each year due to TBI (McGregor and Pentland, 1997). In 1999, a National Institutes of Health Consensus Development Panel in the United States compiled an alarming set of statistics on the occurrence of TBI, the cognitive and physical ramifications after TBI, and the

current lack of viable prevention and treatment options (NIH Consensus Development Panel on Rehabilitation of Persons With Traumatic Brain Injury, 1999). For example, as many as 6.5 million individuals may be living with the consequences of TBI in the United States alone. One poorly understood aspect of TBI is mild traumatic brain injury (MTBI). Thurman and Guerrero (1999) found a 51% decrease in hospitalizations in the United States for TBI between 1980

and 1995. They believe that changing admission practices in hospitals, spurred by a fixed number of beds and insurance practices, are excluding many cases of MTBI. Unfortunately, MTBI is difficult to diagnose properly, and the fact that more medical cases are being treated on an outpatient rather than an inpatient basis is troubling (Macciocchi *et al.*, 1993; Robinson, 1996; Thurman and Guerrero, 1999). Similar to human TBI cases, injured animals have demonstrated deficits in memory and learning that correlate with the level of *in vivo* injury (Hamm *et al.*, 1992; Hicks *et al.*, 1993). *In vivo* studies have shown that cell death and tissue damage, commonly found after moderate and severe TBI, are not apparent in MTBI (Dixon *et al.*, 1991; Scheff *et al.*, 1997; DeFord *et al.*, 2002). Although MTBI may not necessarily produce cell death (Kanayama *et al.*, 1996; DeFord *et al.*, 2002), animal models show resultant cellular dysfunction and memory deficits (Lyeth *et al.*, 1990; Kanayama *et al.*, 1996; DeFord *et al.*, 2002; Uryu *et al.*, 2002).

An emerging hypothesis in the study of neurotrauma is that repeated mild traumatic brain injury (rMTBI) may cause cumulative damage to the brain, which could ultimately result in memory and learning dysfunction. Recurrent brain injuries are not uncommon in many sports (Kelly and Rosenberg, 1997). Some sports include blows to the head (contact sports) or use the head as part of the accepted way of playing the game (e.g. 'headers' in soccer). The most frequent brain injury in sports is concussion and accumulation of these MTBIs. In contact sports such as boxing and some martial arts, cumulative concussion is common and inevitable because victory is direct by rendering the opponent unconscious with a grade three concussion. In collision sports, like soccer, ice hockey, rugby and American football, a high frequency of cumulative concussion, due to collisions between players, has been documented (Bailes, 1998). In American football, for example, players with repeated concussions perform worse on neuropsychological tests than those with either a single concussion or no history of concussion (Collins *et al.*, 1999). Moreover, in soccer, a mechanism for potential brain injury is repetitive heading of the ball, a unique aspect of soccer (Matser *et al.*, 2001). Studies have shown that soccer players who regularly head the ball have more brain concussions, and have demonstrated impairment on multiple neuropsychological tests when compared with matched controls (Matser *et al.*, 1998, 1999). These forces on the brain may be mild, but their accumulation may be similar to other medical problems caused by repeated low-level activity, such as lung cancer from smoking cigarettes (Babbs, 2000). Even in non-collision sports, such as horse riding, biking, skiing, ice skating and skateboarding, cumulative concussions are often reported as a result of collision and falls. Most cumulative concussion results in deterioration of planning and memory capacity (Matser *et al.*, 2001), the most common symptom of chronic TBI in athletes.

Damage to the hippocampus following TBI has received particular attention. Lowenstein and colleagues

found that an increased degree of impact correlated with increased hippocampal damage (Lowenstein *et al.*, 1992). They theorized that this damage could provide a link between brain injury and resultant disorders such as memory loss and epilepsy. Jenkins and colleagues subjected rats to either a single or double insult paradigm; after 7 days, only those animals that received the double insult had significant neural loss, specifically in hippocampal area CA1 (Jenkins *et al.*, 1989). Although there was no axonal injury in any animals when brain slices were examined at the light microscopic level, a definite threshold of damage was crossed by a double injury that was not crossed by a single injury. It has been suggested that post-injury sequelae may differ between single and repeated brain traumas (Olsson *et al.*, 1971). Therefore, our objective was to characterize better the cellular degradation and dysfunction that occurs after single and repeated MTBI using cultured mouse hippocampal cells.

Although few studies have dealt with the issue of rMTBI, recent reports have examined repeated injury in the whole animal (Kanayama *et al.*, 1996; Laurer *et al.*, 2001; DeFord *et al.*, 2002; Uryu *et al.*, 2002). *In vivo* studies of repetitive insults are advantageous, in that changes in animal behaviour post-injury can be measured and correlated to cell damage. These studies, however, can only postulate the underlying mechanisms of cognitive impairment at the cellular level following rMTBI. Thus, it is important to supplement *in vivo* studies with well designed *in vitro* studies. In the current study, we used an established *in vitro* model of mechanical injury (Ellis *et al.*, 1995; Weber *et al.*, 1999) to address rMTBI-related cell damage. This model replicates the acceleration-deceleration stresses incurred during motor vehicle accidents (Schreiber *et al.*, 1995) and sport-related concussions (Powell and Barber-Foss, 1999). We used two fluorometric dyes that indicate cell viability and damage, as has been reported previously (McKinney *et al.*, 1996; Weber *et al.*, 1999; Pike *et al.*, 2000; Zhao *et al.*, 2000). We also measured the release of two commonly used clinical markers of brain damage following cardiac surgery (Ali *et al.*, 2000) and TBI: neurone-specific enolase (NSE) (Persson *et al.*, 1987; Herrmann *et al.*, 2001; Pleines *et al.*, 2001; Woertgen *et al.*, 2001; Berger *et al.*, 2002) and S-100 β protein (Persson *et al.*, 1987; Waterloo *et al.*, 1997; Raabe and Seifert, 1999; Romner *et al.*, 2000; Herrmann *et al.*, 2001; Pleines *et al.*, 2001; Berger *et al.*, 2002). Using a combination of these approaches, we are able to make comparisons with clinical data and to investigate directly the underlying cellular damage that may contribute to findings described at the behavioural level. This model therefore provides an *in vitro* correlate to what is described following repeated injury, both experimentally *in vivo* and in the human clinical situation.

Methods

Animals

All experiments were conducted in accordance with the European Communities Council Directive and were approved by the animal welfare committee of Erasmus Medical Center Rotterdam. Throughout these studies efforts were made to minimize animal suffering and the number of animals used.

Cell culture

Primary hippocampal cultures were prepared from E18 FVB/N mouse embryos. Embryonic cultures were used in order to optimize neuronal and glial growth. Pregnant female mice were killed by rapid cervical dislocation and the embryonic sacs were surgically removed. Embryonic mouse brains were dissected out of the skulls and placed on ice in 15 ml ice-cold Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) supplemented with 10 µg/ml gentamicin (Sigma, St Louis, MO, USA). The hippocampi were separated from the cortices and placed in 10 ml of fresh HBSS. The tissue was washed and then centrifuged at 200 *g* at 4°C for 3 min; the supernatant was removed, 10 ml fresh HBSS was added, and the tissue was washed and centrifuged again. The supernatant was discarded and 2 ml 0.25% trypsin–EDTA (Invitrogen) in HBSS was added, supplemented with 8 ml fresh HBSS. The tissue was incubated at 37°C for 15 min, followed by centrifugation at 200 *g* at 4°C for 3 min. The supernatant was removed and the tissue was washed with 10 ml BME (Basal Medium Eagles; Invitrogen) growth media containing 10% horse serum (Invitrogen), 10 µg/ml gentamicin, 0.5% glucose (Sigma), 1 mM sodium pyruvate (Sigma) and 1% N₂ supplements (Invitrogen) and then centrifuged at 225 *g* at 4°C for 5 min. The cells were triturated with 4 ml fresh growth media using a 10 ml plastic pipette until all large aggregates were dissociated, followed by further trituration with the same pipette equipped with a 100 µl plastic pipette tip. The suspension was filtered through a 70 µm nylon cell strainer, the cells were counted, and the suspension was diluted with growth media to a concentration of 500 000 cells/ml. Cells were plated in 1 ml aliquots onto collagen-coated six-well FlexPlates (FlexCell, Hillsborough, NC, USA) coated overnight with poly-L-ornithine (500 µg/ml; Sigma). Cultures were maintained in a humidified incubator (5% CO₂, 37°C). Neuronally enhanced cultures were obtained by replacing half of the media at 2, 6 and 9 days *in vitro* with serum-free culture media [BME containing 10 µg/ml gentamicin, 0.5% glucose, 1 mM sodium pyruvate, 1% N₂ supplement and 2% B27 supplement (Invitrogen)]. Glia formed a confluent monolayer that adhered to the membrane substrate. Anti-MAP-2 (microtubule-associated protein 2) immunostaining was initially performed on cultures to verify the existence of morphologically distinct neurones. MAP-2 staining revealed neuronal phenotypes growing in the upper layer of cultures that adhered to the underlying glial layer, which is consistent with previous reports in hippocampal cultures (Pike *et al.*,

2000; Zhao *et al.*, 2000). Cells were used for experiments within 9–13 days *in vitro*.

Cell injury

Primary mouse hippocampal cultures were injured using a model 94A Cell Injury Controller (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA, USA) as described previously (Ellis *et al.*, 1995). In brief, the Silastic membrane of the FlexPlate well is rapidly and transiently deformed by a 50 ms pulse of compressed nitrogen, which deforms the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure. The extent of cell injury—produced by deforming the Silastic membrane on which the cells are grown—is dependent on the degree of deformation, or stretch. Based on previous work (Ellis *et al.*, 1995), three levels of cell injury were chosen (5.5, 6.5 and 7.5 mm deformations) and defined as mild, moderate and severe, respectively. These degrees of membrane deformation result in a biaxial strain or stretch of 31, 38 and 54%, respectively. This range of cell stretch has been shown to be relevant to what would occur in humans after rotational acceleration/deceleration injury (Schreiber *et al.*, 1995). Uninjured control wells were contained in the same FlexPlates as injured wells, and thus underwent the same manipulations, except that they did not receive rapid deformation of the Silastic membrane.

Cell viability

Cell injury was assessed using the two dyes fluorescein diacetate (FDA; Sigma) and propidium iodide (PrI; Sigma) as reported previously (McKinney *et al.*, 1996; Weber *et al.*, 1999; Pike *et al.*, 2000; Zhao *et al.*, 2000). FDA is known to stain healthy, viable cells and fluoresces green, while PrI cannot pass through intact cellular membranes. If membranes are damaged, however, cells lose their ability to retain FDA. In addition, PrI will enter cells and stain the nucleus, resulting in a bright red fluorescence. Stock solutions of FDA (20 mg FDA/ml acetone) and PrI [5 mg PrI/ml phosphate-buffered saline (PBS)] were created, from which a working solution was prepared (10 µl FDA stock and 3 µl PrI stock, diluted in 10 ml PBS). The culture medium was removed from the well and replaced with 1 ml PBS and 200 µl FDA/PrI working solution. The working solution was added to the culture well immediately after injury, or the injured cells were returned to the incubator and the solution was added immediately before measurement (for experiments conducted at 1 and 24 h). The cells were stained for 3 min at room temperature, and the PBS and stain were removed. A 15-mm glass coverslip was centred over the stained cells, and images were captured using red (Texas Red) and green (fluorescein isothiocyanate) filters on a Leica DMRBE fluorescence microscope, equipped with a Hamamatsu C4880 CCD camera. FDA and PrI images were taken separately, pseudocoloured and overlaid. Images were adjusted for contrast and brightness using Adobe

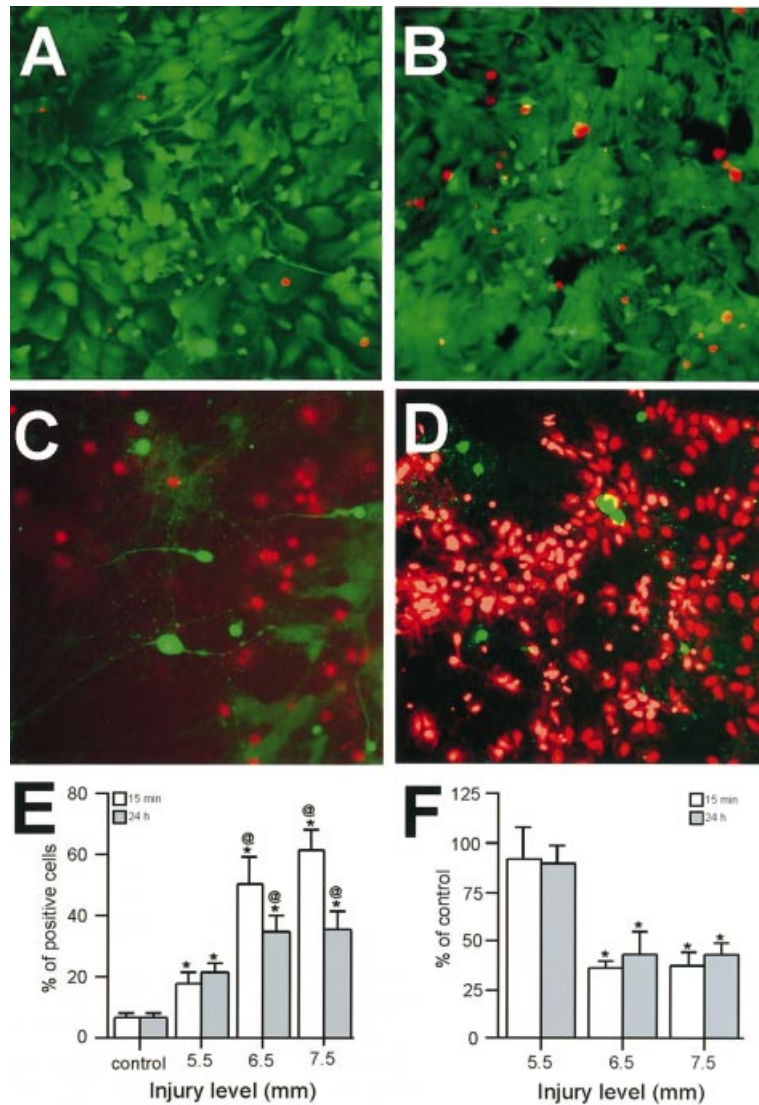


Fig. 1 Effect of stretch injury on hippocampal cultures. FDA and PrI images were taken separately, pseudocoloured and overlaid. Images were adjusted for contrast and brightness using Adobe Photoshop 4.0. (A) Control, uninjured cultures demonstrate retention of FDA and minimal PrI uptake, indicating healthy, viable cells. (B–D) Images taken 15 min post-injury: (B) mild injury, 5.5 mm deformation; (C) moderate injury, 6.5 mm deformation; (D) severe injury, 7.5 mm deformation. Cultures demonstrated an increased amount of PrI staining, the majority of which occurred in the underlying monolayer, suggesting that it was glial in origin. Note that the neuronal somata appear intact following injury; however, neuronal processes appear beaded at the higher injury levels, as shown in C. (A–D) Magnification: $\times 100$. (E) PrI uptake is positively correlated to the degree of stretch. Data shown are at 15 min and 24 h post-injury. Uptake of PrI increased with mild (5.5 mm; $n = 5$), moderate (6.5 mm; $n = 3$) and severe (7.5 mm; $n = 4$) levels of stretch at 15 min post-injury compared with control ($n = 5$). At 24 h, PrI levels remained elevated at all levels of injury (5.5 mm, $n = 5$; 6.5 mm, $n = 5$; 7.5 mm, $n = 3$) compared with control ($n = 5$). Moderate and severe levels of stretch caused a decrease in total cell number at 15 min and 24 h post-injury due to detachment of cells from the membrane that did not occur after mild injury. $*P < 0.01$ compared with control; $^{\circ}P < 0.05$ compared with 5.5 mm injury. (F) Neuronal cell phenotypes in injured hippocampal cultures. Data are shown at 15 min and 24 h post-injury. Mild injury caused only a slight reduction, while moderate and severe levels of stretch caused a significant reduction in neuronal phenotypes at 15 min and 24 h post-injury. $*P < 0.05$ compared with control (n is same as E).

Photoshop 4.0 (Adobe Systems, San Jose, CA, USA). Five sequential $100\times$ images were counted and averaged per well. All images were taken from the centre portion of the well, as this region was previously shown to receive equal impact

from the cell injury controller (Ellis *et al.*, 1995). All PrI and FDA cell counting was performed blind. Data are expressed as the percentage of total cell number that stained positively for PrI. In control cultures, PrI staining was low, accounting

for ~5% of total cell number, consistent with previous reports (Zhao *et al.*, 2000).

NSE and S-100 β assays

NSE and S-100 β protein levels were measured in culture media at 6 and 24 h post-injury using lumino-immunometric (LIA-mat) assay kits (Sangtec Medical, Bromma, Sweden). These kits have been used previously to detect NSE and S-100 β levels in experimental animals and human patients (Romner *et al.*, 2000; Herrmann *et al.*, 2001; Woertgen *et al.*, 2001). NSE is found in the cytoplasm of neurones, concentrations of which can be measured in CSF and serum once it has been released from the cytoplasm by cell damage. S-100 β is a calcium-binding protein found in astrocytes and Schwann cells (Herrmann *et al.*, 2001). All experiments were completed using cultures at 9–10 days *in vitro*. Following the assays, the remaining culture medium was removed, and the cells adhering to the Silastic membrane were solubilized with 1 ml 0.1% NaOH to determine protein concentration. Total protein count was determined by the bicinchoninic (BCA) assay (Pierce, Rockford, IL, USA). Final results are expressed as nanograms of NSE or S-100 β released into the culture media per milligram of protein. NSE levels were determined in three separate culture preparations, while the S-100 β assay was performed on four separate culture preparations.

Data analysis

The data were analysed using the statistical program GB Stat (Dynamic Microsystems, Silver Spring, MD, USA). Data were computed as means \pm standard error (SE) values. Statistical significance was established by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Data are considered significant at $P < 0.05$.

Results

Increased level of stretch injury correlates to increased PrI uptake

Prototypical pseudocolour images of control and injured mouse hippocampal cultures at 15 min post-injury are presented in Fig. 1A–D. The microscope required that FDA and PrI images be taken separately, pseudocoloured and overlaid. The neuronal layer was chosen to be the focal plane, and thus the underlying glial monolayer appears slightly blurred. As shown in Fig. 1A, uninjured cultured cells show high retention of FDA and present normal cellular morphology; the same was true for control cultures at 24 h (images not shown). There is little PrI staining, indicating that most cells are healthy and viable, with intact cell membranes. Fifteen minutes after injury, however, cultures demonstrated an increasing amount of PrI staining, the majority of which occurred in

the underlying layer, suggesting that it was primarily glial in origin (images at 24 h not shown). The increased staining was minimal after mild injury (5.5 mm; Fig. 1B), where FDA retention appears unchanged, especially in the neuronal layer. Moderate injury (6.5 mm; Fig. 1C) created increased PrI staining in the glial layer and 'beaded' neurites in the neuronal layer. However, the neuronal somata appear intact after injury. Moderate injury also caused detachment of some cells from the Silastic membrane, which resulted in a decrease in total cell number measured at 15 min post-injury. After severe injury (7.5 mm; Fig. 1D), most cells are stained with PrI, possibly in both the neuronal and glial layers. As with moderate injury, severe stretch led to an even greater detachment of cells from the membrane at 15 min post-injury, evident by a decrease in total cell number and the empty gaps not found in control cultures.

A summary of the effects of increasing levels of stretch magnitude on PrI uptake in hippocampal cultures at both 15 min and 24 h post-injury is represented in Fig. 1E. At 15 min post-injury, the number of cells stained with PrI increased with increasing levels of stretch ($n = 3-5$). At 24 h post-injury, the amount of PrI-positive cells was elevated compared with matched controls at all levels of injury ($n = 3-5$). Moderate and severe levels of stretch exhibited a decrease in PrI-stained cells compared with 15 min post-injury. There was no change in the total cell number at 24 h compared with 15 min after injury, suggesting that the decrease in the percentage of injured cells was not due to glial proliferation. Therefore, it appears that many glial cells regain their capacity to exclude PrI by 24 h post-injury.

In addition to the total number of injured cells after injury, we also investigated the effects of injury on the number of neuronal cell phenotypes, as summarized in Fig. 1F ($n = 3-5$). FDA-stained neuronal cells growing in the upper layer of the cultures were identified as having a morphology consistent with that of MAP-2 immunostaining (as described in Methods). Cell density in the cultures was consistent, as there was no significant difference between the number of neuronal cell phenotypes in uninjured control images between 15 min and 24 h, or from different culture preparations. There was no change in the number of neuronal cells after mild (5.5 mm) injury, indicating minimal, if any, neuronal death, or of cells lifting from the membrane. Following moderate and severe injury, however, the number of neuronal cells was decreased dramatically, indicating either a loss of FDA retention or lifting of the neurones from the cell membrane. The latter explanation is more likely, considering the fact that cell lifting from the membrane was evident at these levels of injury as described above. There was no further change in neuronal cell phenotypes at 24 h compared with 15 min, suggesting no further increase in neuronal cell death or of cells lifting from the membrane.

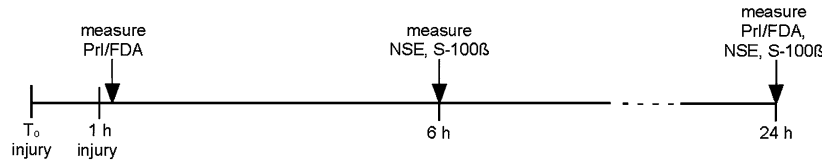


Fig. 2 Timeline for repeated injury experiments. Cultures that received a double insult were injured 1 h after the initial injury. Experimental measurements were conducted at the indicated time-points.

Repetitive mild stretch injury causes cumulative cell damage

In this set of experiments, cultures that received a double insult were injured 1 h after the initial injury. Cultures that received a single insult were evaluated at 1 h post-injury, while cultures receiving repeated insults were evaluated immediately after the second insult. Cultures were also evaluated at 24 h post-injury (or 23 h after the second insult, see experimental timeline represented in Fig. 2).

Prototypical pseudocolour images of mouse hippocampal cells that received either one or two stretch injuries are presented in Fig. 3A–D. As shown in Fig. 3A, PrI uptake following mild injury (5.5 mm) at 1 h post-injury was similar to that seen at 15 min post-injury (see Fig. 1B), while a double insult increased PrI uptake (Fig. 3B). In cultures that received two mild injuries, many of the neurones showed beaded neurites as seen previously only after higher levels of injury (see Fig. 1C). The marked difference between single and double mild (5.5 mm) injury can be better seen in Fig. 3C and D, respectively.

The effects of repeated insults on cultured hippocampal cells are summarized in Fig. 3E. At both 1 and 24 h post-injury, PrI uptake was significant after a single mild (5.5 mm) insult compared with matched controls ($n = 7$ for all conditions). In addition, there was an increase in PrI-positive cells following a double mild (5.5 mm) insult as compared with a single injury. Although a significant reduction in neuronal cell phenotypes was not evident initially after double injury, as shown in Fig. 3F, we found a significant decrease of neuronal cell phenotypes of $32 \pm 6\%$ compared with controls at 24 h following a double insult ($n = 7$ for all conditions). This suggests a reduction in neuronal FDA retention or detachment of some neurones from the membrane, which could be indicative of a delayed neuronal injury or death.

Release of NSE and S-100 β after single and repeated mild injuries

We initially measured NSE and S-100 β release at 1 h after injury. In the case of NSE, there was no elevation of release following any injury level versus control (data not shown). For S-100 β we did not find reportable levels of this protein after any degree of stretch. We then measured NSE and S-100 β release at 6 and 24 h post-injury, consistent with time points previously reported *in vivo* (Woertgen *et al.*, 2001).

Figure 4 summarizes the release of NSE into the culture media in nanograms per milligram of protein. NSE release was significant compared with controls after both single and double 5.5 mm injury; the release of NSE after double insults was also significant compared with a single injury at 6 h post-injury. NSE levels remained elevated 24 h after both single and repeated injuries ($n = 5$ for all conditions).

The release of S-100 β after injury is depicted in Fig. 5. Unlike NSE, in which there were detectable levels of protein in culture media in the majority of uninjured control wells, we found detectable levels of S-100 β (at least 0.2 ng/ml) in only 31% of control wells. After injury, however, there were detectable levels of S-100 β in the culture media of 65% of the wells. Similar to previous studies (Romner *et al.*, 2000), for the purpose of statistical analysis, we report S-100 β levels after injury from culture wells that are S-100 β positive (at least 0.2 ng/ml). At 6 h post-injury there was a significant increase in release of S-100 β after repeated mild injury compared with single injury. At 24 h post-injury, S-100 β levels were still elevated; however, the level of S-100 β following repeated injury was not significant compared with single injury ($P = 0.08$) due to an increased standard error ($n = 8$ for all conditions).

Discussion

Cognitive impairment is one of the most devastating deficits after moderate and severe TBI, and greatly influences the quality of life of survivors. Deficits in cognitive ability are also seen after MTBI (McAllister, 1992). Not only has experimental and clinical data demonstrated that the hippocampus plays a critical role in learning and memory, studies also indicate that the hippocampus is uniquely vulnerable to injury following even mild brain trauma (Lyeth *et al.*, 1990; Lowenstein *et al.*, 1992). An emerging hypothesis in the study of neurotrauma is that rMTBI may cause cumulative damage to the brain, and in the absence of cell death, is believed to contribute to ensuing cognitive deficits. The investigation of rMTBI is in its infancy, and while *in vivo* models provide essential information regarding behaviour, and pathological and physiological sequelae on macroscopic and microscopic levels, they cannot easily address questions concerning dysfunction at the cellular and subcellular levels. Important *in vitro* studies are required to complement *in vivo* research in order to understand better the cellular mechanisms that contribute to rMTBI-related sub-

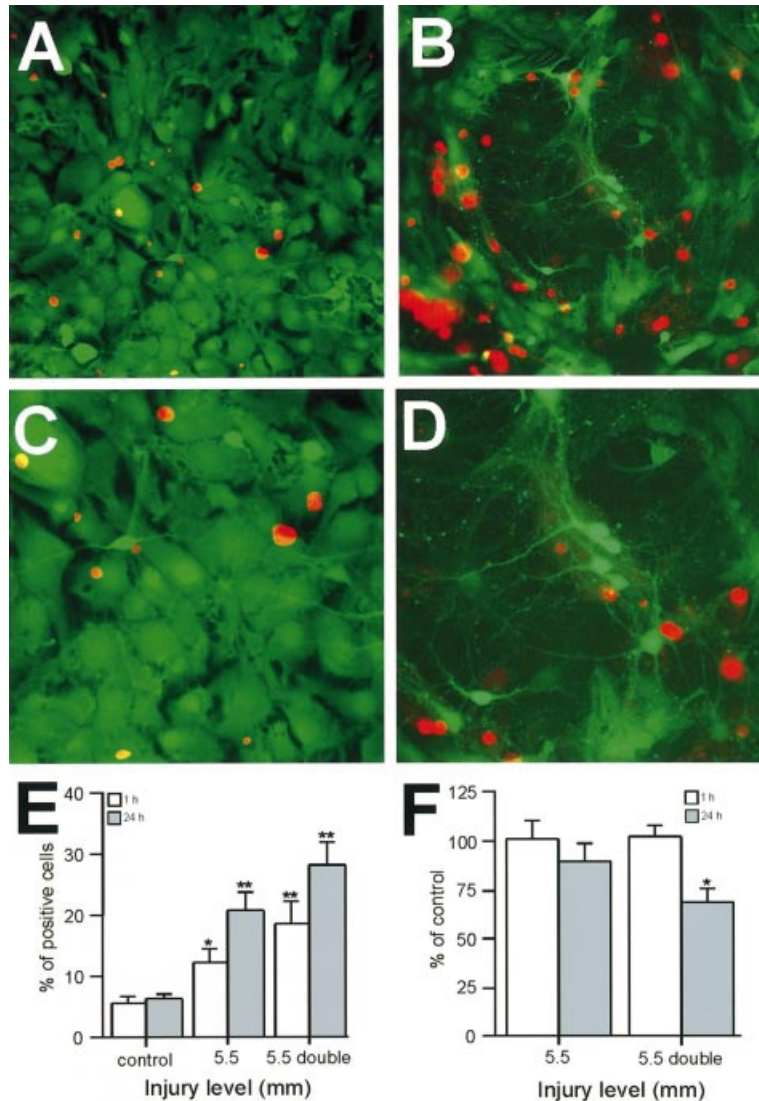


Fig. 3 Effect of repeated stretch injury on hippocampal cultures. Cultures that received a single insult were evaluated at 1 h post-injury, while cultures receiving repeated injuries were evaluated immediately after the second insult. FDA and PrI images were taken separately, pseudocoloured and overlaid. Images were adjusted for contrast and brightness using Adobe Photoshop 4.0. (A) PrI uptake following mild injury at 1 h post-injury was similar to that at 15 min post-injury (see Fig. 1B). (B) A double mild insult increased PrI uptake. Note that many cells have beaded neurites that resemble cultures that received a higher level of injury (see Fig. 1C). (A and B) Magnification: $\times 100$. (C and D) Enlargements of A and B, respectively. Magnification: $\times 200$. (E) PrI uptake following repeated stretch injury. Data shown are at 1 and 24 h post-injury. $*P < 0.05$ compared with control; $**P < 0.01$ compared with control ($n = 7$ for all conditions). (F) Neuronal cell phenotypes after repeated injury of hippocampal cultures. Data are shown at 1 and 24 h post-injury. There was a significant reduction in neuronal phenotypes after 5.5 mm double injury at 24 h. $*P < 0.05$ compared with control ($n = 7$ for all conditions).

lethal cellular dysfunction. In the current report we used an *in vitro* model of stretch-induced injury (Ellis *et al.*, 1995) to examine cellular events following rMTBI.

One aim of the present study was to characterize successfully a model of stretch injury (Ellis *et al.*, 1995) in mouse hippocampal cells that can be reliably used to investigate the pathology of repeated injury at the cellular level. We first demonstrated that cultured mouse hippocampal cells respond to stretch injury in a manner similar to rat cortical (McKinney

et al., 1996; Weber *et al.*, 1999) and rat septo-hippocampal cultures (Pike *et al.*, 2000). As with these other reports, we found that an increase in PrI uptake was a reliable indicator of injury severity. Pike and colleagues found similar differences between mild and moderate/severe degrees of stretch in rat septo-hippocampal cultures (Pike *et al.*, 2000). At these higher injury levels, cultured cells had a tendency to detach from the cell membrane, to develop beaded neurites, and to have a reduced number of neuronal phenotypes. Also similar

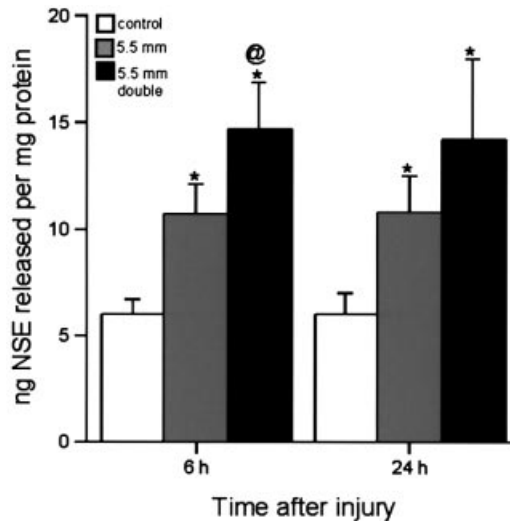


Fig. 4 Release of NSE following single and repeated mild injury. NSE release is shown for control wells, after 5.5 mm single injury and after 5.5 mm double injury, at both 6 and 24 h post-injury. NSE is represented as nanograms released into the culture media per milligram of protein in the culture well. * $P < 0.05$ compared with control; [@] $P < 0.05$ compared with 5.5 mm single insult ($n = 5$ for all conditions).

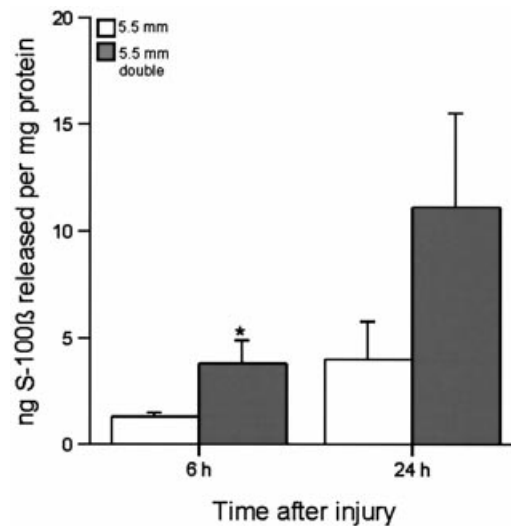


Fig. 5 Release of S-100 β following single and repeated mild injury. S-100 β release is shown for 5.5 mm single injury and 5.5 mm double injury at both 6 and 24 h post-injury. S-100 β is represented as nanograms released into the culture media per milligram of protein in the culture well. * $P < 0.05$ compared with 5.5 mm single insult ($n = 8$ for all conditions).

to previous studies (McKinney *et al.*, 1996; Weber *et al.*, 1999), we found a reduction in PrI-positive cells 24 h following moderate and severe levels of injury compared with 15 min post-injury, suggesting that there may have been glial proliferation after injury, which would decrease the percentage of cells that would stain positively for PrI. However, FDA staining indicated that there was no change in total cell number between 15 min and 24 h post-injury. Therefore, the most likely explanation for this finding is that injured glial cells may have gained the ability to repair their cellular membranes by 24 h after injury, which has been suggested previously by other investigators (Ellis *et al.*, 1995; Rzigalinski *et al.*, 1997).

To our knowledge, this is the first study to investigate repetitive mechanical injury on cells of the brain using an *in vitro* system of trauma. There were few examples of *in vivo* reports in the literature with which to determine the injury time points used in the current study; therefore we injured cells 1 h after an initial insult, similar to a previously reported double insult paradigm (Jenkins *et al.*, 1989). Single and repeated mild injury caused a significant amount of PrI uptake at 1 h and at 24 h post-injury, which appeared to be present primarily in the glial layer, suggesting no immediate, overt damage to neuronal membranes. In addition, repeated insults caused a slight increase in PrI uptake compared with a single insult. Although the majority of neurones after repeated insults had normal-appearing somata, neurites appeared beaded and damaged, a finding that was not observed following a single mild insult. The damaged neurites following repeated injury were similar to dendritic abnormalities observed in hippocampal neurones following mod-

erate TBI *in vivo* (Folkerts *et al.*, 1998), and they strongly resembled those observed after greater magnitudes of stretch. These results suggest that there is cumulative damage to cultured hippocampal neurones following repetitive injury.

In agreement with this evidence of sublethal cellular damage, we found that NSE levels were elevated after repeated insults compared with a single insult 6 h after injury, a time-point at which peak levels of NSE have been reported following TBI *in vivo* (Woertgen *et al.*, 2001). Levels of NSE remained elevated compared with uninjured controls 24 h after injury. This finding is clinically relevant, as elevated levels of NSE can persist for several hours or days after MTBI and increased NSE levels are often correlated with neuropsychological dysfunction (Herrmann *et al.*, 2001). Also similar to clinical reports (Waterloo *et al.*, 1997; Raabe and Seifert, 1999; Ali *et al.*, 2000; Herrmann *et al.*, 2001; Pleines *et al.*, 2001; Berger *et al.*, 2002), we found elevated levels of S-100 β following injury *in vitro*. Levels of S-100 β increased after repeated injury compared with single mild insults. S-100 β can also remain elevated for several days post-injury *in vivo* (Pleines *et al.*, 2001). Although the mechanisms for elevated S-100 β are unknown following TBI, increased S-100 β levels in peripheral blood after trauma may be indicative of damage to the blood-brain barrier or could indicate the activation of secondary damage pathways (Raabe and Seifert, 1999; Herrmann *et al.*, 2001). In addition, S-100 β stimulates glial proliferation (Reeves *et al.*, 1994), which could possibly lead to swelling of the brain after trauma. However, it is also possible that S-100 β is playing a somewhat protective role; for example, S-100 β may be an important mediator of glia-neuronal interactions, and it has been shown to stimulate neurite extension (Reeves *et al.*,

1994). Therefore, glial cells may release S-100 β in an attempt to save, or to repair, dying or damaged neurones.

Collectively, the data suggest sublethal cellular damage to the majority of hippocampal cells. This is an important finding in light of the fact that following TBI *in vivo*, memory function can be impaired in the absence of hippocampal cell death (Lyeth *et al.*, 1990). Although neuronal death was not readily apparent following single or repeated mild injury, we cannot rule out the possibility that at least some of the neurones following repeated injury go on to die, based on the finding that there was a reduction in neuronal phenotypes 24 h post-injury. Other types of traumatic insults, such as elevated glutamate exposure, trophic factor withdrawal or ischaemia, can cause fragmented and beaded neurites, reportedly due to apoptotic enzyme activation (Mattson and Duan, 1999; Mattson *et al.*, 2000; Zhao *et al.*, 2000). This could eventually lead to withdrawal or degeneration of neurites, which could then cause the neurones to lift from the membrane. Also, neurites can become fragmented and beaded under necrotic conditions, and plasma membranes can become compromised. Therefore, lifting of neurones from the membrane or a delayed uptake of PrI could be responsible for the reduction in neuronal phenotypes 24 h after repeated injury. However, further detailed studies of cell death potential, whether apoptotic or necrotic, need to be carried out in this model. It should also be noted that, in these experiments no hypoxia was superimposed on the cells. As ischaemia is often a major contributor to cellular damage following TBI, another important set of experiments in the future will be to investigate single or repeated mechanical injury in addition to a secondarily produced ischaemic insult. This may lead to further findings elucidating interactions between mechanical and ischaemic damage at the cellular level.

Repeated brain injuries occur in a number of populations. Perhaps the most relevant population is that of athletes. Collision sports such as American football, ice hockey, soccer, rugby and boxing present a population of athletes of all ages who encounter rMTBI. In soccer players, for example, the number of concussions was inversely related to the performance on several neuropsychological tests (Matser *et al.*, 1999); these athletes, when compared with matched controls from non-contact sports, had lower scores on memory, planning and visuoperceptual tests (Matser *et al.*, 1998). In addition, repeated MTBI can increase the susceptibility to chronic TBI as well as neurodegenerative diseases (Jordan, 2000; Uryu *et al.*, 2002). For example, there is established evidence of the development of dementia pugilistica in professional boxers (Jordan, 2000). The reasons for these findings could start to be unravelled by using a combination of studies at the cellular and whole-animal level.

As with human MTBI, the occurrence of haemorrhage or blood–brain barrier compromise in animal models following MTBI or rMTBI is controversial. Clinical studies describe MTBI patients who present no signs of haemorrhage but who still perform poorly on neuropsychological examinations. In fact, it is the MTBI patient population that presents with few

or any signs of medical complications (e.g. haemorrhage on CT scan) yet demonstrates cognitive deficits that have the medical and scientific community puzzled (Macciocchi *et al.*, 1993). In the presence or absence of blood–brain barrier compromise, cytoskeletal alteration has been reported following MTBI and rMTBI (Jenkins *et al.*, 1989; Dixon *et al.*, 1991; Kanayama *et al.*, 1996; Folkerts *et al.*, 1998; Saatman *et al.*, 1998; Laurer *et al.*, 2001). In addition, it appears that the effects of a second MTBI may not be additive but synergistic (Kanayama *et al.*, 1996; Laurer *et al.*, 2001; DeFord *et al.*, 2002; Uryu *et al.*, 2002).

In conclusion, our overall results suggest that repeated mild injury causes increased amounts of cellular damage when compared with single insults of the same magnitude. It is possible that pathways of cellular degradation differ between single and multiple TBI. The stretch-injury model used in our study, as well as similar models that cause secondary damage (Adamchik *et al.*, 2000), could provide a relatively fast and economical method for investigating secondary pathways of damage or for screening potential pharmacological treatments for rMTBI. Also, because we have now characterized this model in mouse hippocampal cells, it is possible to investigate the effects of injury on cultures from various transgenic mice in the hopes of gaining further information on possible treatment strategies. Although acute TBI has received much more attention, both clinically and experimentally, an increasing number of studies on repetitive TBI are currently being conducted. We are optimistic that better prevention and intervention treatments for rMTBI will soon be devised based on both *in vivo* and *in vitro* experimentation.

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