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Duration of ATP reduction affects extent of CA1 cell death in rat models of fluid percussion injury combined with secondary ischemia

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Abstract

Secondary ischemia (SI) following traumatic brain injury (TBI) increases damage to the brain in both animals and humans. The current study determined if SI after TBI alters the extent or duration of reduced energy production within the first 24 h post-injury and hippocampal cell loss at one week post-injury. Adult male rats were subjected to sham injury, lateral (LFPI) or central fluid percussion injury (CFPI) only, or to combined LFPI or CFPI with SI. The SI was 8 min of bilateral forebrain ischemia combined with hemorrhagic hypotension, applied at 1 h following FPI. After LFPI alone adenosine triphosphate (ATP) levels within the ipsilateral CA1 were reduced at 2 h ($p < 0.05$) and subsequently recovered. After LFPI+SI the ATP reductions in CA1 ipsilateral to FPI persisted for 24 h ($p < 0.01$). ATP levels in the contralateral CA1 were not affected by LFPI alone or LFPI+SI. After CFPI alone CA1 ATP levels were depressed bilaterally only at 2 h ($p < 0.01$). Similar to the LFPI paradigm, CFPI+SI reduced ATP levels for 24 h ($p < 0.01$), with bilateral ATP reductions seen after CFPI+SI. Cell counts in the CA1 region at 7 days post-injury revealed no significant neuronal cell loss after LFPI or CFPI alone. Significant neuronal cell loss was present only within the ipsilateral ($p < 0.001$) CA1 after LFPI+SI, but cell loss was bilateral ($p < 0.001$) after CFPI+SI. Thus, SI prolongs ATP reductions induced by LFPI and CFPI within the CA1 region and this SI-induced energy reduction appears to adversely affect regional neuronal viability.

Keywords

Adenosine triphosphate; Cell death; Hippocampus; Rat; Secondary ischemia; Traumatic brain injury

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1. Introduction

A secondary insult, such as hypotension, ischemia or hypoxia, can exacerbate brain damage in both experimental and clinical traumatic brain injury (TBI). Secondary ischemia (SI) after fluid percussion injury (FPI) was initially shown to increase cell death in the vulnerable CA1 region of the hippocampus (Jenkins et al., 1989), and SI after cortical contusion injury (CCI) was found to increase hippocampal cell death and increase cortical contusion volume (Cherian et al., 1996). Multiple reports have now shown that addition of secondary hypotension, ischemia or hypoxia following experimental TBI further decreases cerebral blood flow (CBF) (Giri et al., 2000; Matsushita et al., 2001b), increases contusion volume (Bramlett et al., 1999b; Matsushita et al., 2001a, 2001b), exacerbates cortical and hippocampal cell loss (Bauman et al., 2000; Bramlett et al., 1999b; Jenkins et al., 1999; Yamamoto et al., 1999), and worsens neurological function (Bramlett et al., 1999a; Schütz et al., 2006). In TBI patients, secondary insult is consistently reported to result in poor outcome (Butcher et al., 2007; Chi et al., 2006; Henzler et al., 2007; Manley et al., 2001; McHugh et al., 2007; Sánchez-Olmedo et al., 2005). The precise mechanisms by which secondary insult worsens brain damage are unclear, but might be related to the induction of energy crisis.

Adenosine triphosphate (ATP) is the major high energy phosphate needed to maintain cellular homeostasis, and is continuously produced by mitochondria. Decreases in ATP levels strongly affect cellular functions and can lead to cell death. Several previous studies have indicated that experimental TBI-induced reductions of ATP are generally transient or mild, and rapidly recover to baseline (Buczek et al., 2002; Lee et al., 1999; Lifshitz et al., 2003; Marklund et al., 2006; Mautes et al., 2001; Signoretti et al., 2001; Sullivan et al., 1998; Vagnozzi et al., 1999, 2005). However, the duration or extent of ATP depression following FPI is affected by severity of the initial insult, with ATP decreasing significantly after moderate (2.5 atm), but not mild (1.5 atm) injury (Buczek et al., 2002). In addition, the ATP reductions after FPI and CCI are related to the duration of injury-induced neuronal activation (Lee et al., 1999).

Secondary insults have been reported to reduce hemispheric ATP levels below those found with experimental TBI alone (Ishige et al., 1987, 1988; Signoretti et al., 2001; Tavazzi et al., 2005). However, these prior studies did not evaluate regional ATP changes, nor have prior studies determined if the extent or duration of ATP reductions affect neuronal viability following TBI and secondary insult. To address these issues the current study examined changes in ATP levels in the hippocampal CA1 region at 2, 6 and 24 h, and CA1 neuronal loss at 7 days, in rat models of FPI with and without the imposition of SI at 1 h post-FPI. Either lateral (LFPI) or central (CFPI) FPI were used to induce primary injury, since the locations of the injury cap for FPI are known to differentially affect cortical and hippocampal responses (Floyd et al., 2002; Vink et al., 2001). We focused on injury-induced changes in the CA1 region of the hippocampus, as this region is known to be vulnerable to SI imposed after mild TBI (Jenkins et al., 1989). The severity of FPI and SI were selected based on preliminary experiments showing no neuronal loss in CA1 after any single injury. Our hypotheses were that FPI-induced ATP reductions would be increased by addition of SI, and that acute ATP reductions would correlate with delayed neuronal cell loss.

2. Results

2.1. Injury Severity

Data for the measures of FPI severity, which included loss of consciousness (LOC; absence of pedal reflex) and apnea after induction of LFPI or CFPI, are shown in Table 1. The LOC ranged from 150–300 sec after LFPI and from 135–300 sec after CFPI. The duration of apnea ranged from 6–28 sec after LFPI and 8–46 sec after CFPI. There were no significant differences between groups for any of these factors.

2.2. Physiological Data

No significant effects of injury condition and survival time were found for arterial pH, PaCO₂ or PaO₂ measures among the 8 experimental groups with SI. The pH, PaCO₂ or PaO₂ measures taken prior to, during and after induction of SI for groups with LFPI or CFPI, collapsing data across the 4 survival times, are shown in Table 2. For these isoflurane-anesthetized rats using O₂ as the carrier, the PaO₂ levels were always greater than 100 mm Hg. No significant sampling time effects were observed for PaO₂ levels. Arterial pH was reduced below normal values prior to induction of SI, but increased during SI and then returned to baseline post-SI. The pH during SI was significantly higher than was the pH pre-SI ($p < 0.001$) or post-SI ($p < 0.001$). The PaCO₂ levels were decreased significantly during SI compared to the pre-SI ($p < 0.001$) or post-SI ($p < 0.001$) levels. These pH and PaCO₂ changes are likely due to the hypoventilation caused by anesthesia throughout the surgery and a hypovolemia-induced reactive hyperventilation observed during the period of SI.

In LFPI and CFPI groups surviving for 7 days the arterial pH, PaCO₂ and PaO₂ measures, taken at times corresponding to pre- and post-SI samples, did not differ from those in LFPI+SI and CFPI+SI counterparts. The pre- and post- values for LFPI were: pH, 7.36 ± 0.05 and 7.34 ± 0.01 ; PaCO₂, 41.50 ± 4.20 mmHg and 40.75 ± 2.22 mmHg; PaO₂, 397.75 ± 20.76 mmHg and 392.75 ± 17.97 mmHg. For CFPI the pre- and post- values were: pH, 7.35 ± 0.03 and 7.36 ± 0.03 ; PaCO₂, 39.50 ± 3.87 mmHg and 38.25 ± 3.40 mmHg; PaO₂, 406.25 ± 20.68 mmHg and 403.25 ± 25.06 mmHg.

2.3. ATP Measurements

The ATP levels in the left and right CA1 region are presented in Fig. 1 and 2, respectively. Relative to control values, LFPI alone significantly decreased ATP levels in the left CA1 region ipsilateral to injury at 2 h ($p < 0.05$), but not at 6 or 24 h. No significant suppression of ATP levels was observed in the CA1 contralateral to LFPI alone. When LFPI was combined with a transient, bilateral SI, the ATP levels were significantly decreased from control levels in the left CA1 region at 2 ($p < 0.01$), 6 ($p < 0.01$) and 24 h ($p < 0.01$). As with LFPI alone, the ATP levels in the CA1 region contralateral to the FPI were not significantly altered at any time point after LFPI+SI.

Following CFPI alone ATP levels decreased significantly below control values at 2 h in both the left ($p < 0.05$) and right ($p < 0.05$) CA1 regions, but did not differ significantly from controls at either 6 or 24 h. The combination of CFPI+SI resulted in significantly decreased ATP levels in the left CA1 regions at 2 ($p < 0.01$), 6 ($p < 0.05$) and 24 h ($p < 0.01$) as well as in the right CA1 regions at 2 ($p < 0.01$), 6 ($p < 0.05$) and 24 h ($p < 0.01$).

2.4. Neuronal Cell Damage in CA1

Group data for counts of surviving CA1 neurons at 7 days after LFPI or CFPI alone and for the LFPI+SI and CFPI+SI conditions are shown in Fig. 3. No significant differences were found for CA1 neuronal counts in the left or right hemispheres of LFPI alone versus CFPI alone groups. Neurons in the LFPI+SI group were significantly reduced in the left ($p < 0.001$) CA1 compared to the LFPI alone group, but not in the right CA1. In the CFPI+SI group, there was a significant loss of cells in the left ($p < 0.001$) and right ($p < 0.001$) CA1 regions, compared to cell counts in the CFPI alone group. Photomicrographs of the hippocampal CA1 damage 7 days after SI alone (from preliminary studies), and 7 days after CFPI+SI or LFPI+SI, are presented in Fig. 4.

Examination of data for left CA1 neuronal counts (ranging from 80 to 145 neurons) and the LOC (range of 150 to 253 sec) in the 4 rats assigned to the initial LFPI+SI combined injury group suggested there might be a relationship between severity of the LFPI and extent of CA1

damage (Pearson $r = -0.700$, $p > 0.05$). To further explore this possibility, we added 4 animals to this combined injury condition. These latter rats were intentionally subjected to a milder LFPI (LOC ranging from 65 to 115 sec) followed by SI (see section 4.4), and the CA1 neurons were counted after 7 days survival. With data from all 8 of these LFPI+SI animals there was a significant correlation between the severity of the initial LFPI (assessed by LOC) and neuronal cell loss in the ipsilateral (Pearson $r = -0.912$, $p < 0.01$), but not contralateral (Pearson $r = -0.246$, $p > 0.10$), CA1 at 7 days after LFPI+SI.

3. Discussion

In a previous study we assayed both cerebral glucose utilization and ATP levels acutely after mild-moderate LFPI or moderate CCI in rats (Lee et al., 1999). After LFPI ATP was significantly (31.1%) reduced within the primary injury site at 30 min, but not at 2 h, which corresponded with significantly increased glucose utilization at 30 min, but not at 2 h. In contrast, after CCI the ATP in injured cortex was significantly (>50%) reduced at both 30 min and 2 h, while cortical glucose utilization was significantly increased at both time points after injury. Histological evaluation revealed cell death in the CCI model, whereas no cell death was seen in the LFPI model. Based on the differential results in the two injury models, we suggested that the concomitant depletion of ATP with increased duration of energy demand might be crucial indices between a reversible (LFPI) and an irreversible (CCI) cellular injury.

This concept of an injury-induced energy crisis being responsible for cellular damage following TBI has received some support. Recently we reported that a forced increase in neuronal activity, induced by low dose kainic acid (without any effect by itself) at 1 h after mild LFPI, resulted in increased glucose metabolism and a marked loss of ipsilateral hippocampal neurons at 7 days post-injury (Zanier et al., 2003). Based on the preceding observations, we hypothesized that one mechanism by which SI induces cell death is related to the extent and/or duration of post-traumatic energy crisis. The results of the current study are amenable with this hypothesis.

3.1. ATP Changes and CA1 Damage Following TBI

The relative duration of hippocampal ATP decrease seen in the current study after FPI alone are in general agreement with transient depression of ATP reported in other experimental models of TBI. For example, Sullivan et al. (1998) reported moderate CCI in rats significantly decreased ATP in the injured cortex at 10 min and in hippocampus at 30 min, but ATP recovered to baseline levels in both regions at 6 h. In a weight drop model of TBI, cortical ATP levels significantly decreased at the injury site at 4 h, but not at 12 h, and remote cortex showed significant reductions at 12 h, but ATP recovered to baseline levels by 24 h after injury (Mautes et al., 2001). Verweij et al. (2000) analyzed a therapeutically removed brain sample from TBI patients and also found a decrease in ATP production.

The current literature for experimental TBI indicates that the decreases in ATP levels due to primary injury alone are relatively brief and transient, with most studies reporting recovery to baseline values in almost all regions examined (see citations in Introduction). Comparison of the results from the current study and these prior reports indicate that the extent and/or duration of TBI-induced ATP changes may depend upon the type and severity of TBI, the rodent strain used, as well as the brain region included in the assays. Importantly, the current results indicate that even though ATP levels in the CA1 are significantly decreased for at least 2 h in the CA1 ipsilateral to LFPI, and at least 2 h in both the left and right CA1 after CFPI, these primary FPI models alone did not result in CA1 neuronal loss by 7 days.

3.2. ATP Changes and CA1 Damage Following TBI and SI

In the current study addition of SI after the primary injury led to a more prolonged significant reduction of ATP levels in the CA1 (to 24 h) than was observed after FPI alone (2 h), but the magnitude of ATP reductions at the earliest time point (2 h) was not significantly increased by the addition of SI. These findings, in conjunction with results showing that only the combination of FPI+SI produced significant CA1 neuronal loss by 7 days post-injury, suggest that the duration of ATP reductions may impact on neuronal survival. A similar relationship between duration of ATP decreases and markers of cell damage are reported for focal cerebral ischemia (Hata et al., 2000; Mies et al., 1991, 1999). It should be noted, however, that peak ATP depression may have occurred prior to or after the 3 time points assessed after FPI alone or FPI+SI. For example, Ishige et al. (1988) have reported that the combination of hypovolemic hypotension [30 mmHg of mean arterial blood pressure (MABP)] and FPI produced an immediate decrease in ATP only after the onset of SI (with FPI or SI alone producing no ATP effects). Thus, while the regional and temporal ATP changes in our study appear correlated to cell death, indicating that ATP depression for more prolonged periods is detrimental, we cannot rule out the possibility that combined FPI+SI induced a more substantial decrease in ATP during the period of SI.

The ipsilateral effects seen after LFPI and the bilateral effects seen after CFPI suggest that only those hippocampal regions underlying the initial FPI site were affected by our primary injury models. The fact that ATP levels and numbers of CA1 neurons were not decreased contralateral to the LFPI, and that these same results were obtained in the LFPI+SI group which experienced bilateral carotid occlusion, indicates the SI alone was induced at a magnitude which did not induce cell damage in the right CA1 (confirming preliminary studies showing no damage with SI alone).

The ATP reductions in CA1 regions within 2 h following FPI alone cannot be explained by CA1 neuronal cell loss, given the lack of cell loss at 7 days after the LFPI alone or CFPI alone. Since FPI+SI groups were found to have significant cell loss at 7 days post-injury, we cannot rule out the possibility that the later (e.g. 6 and 24 h) ATP reductions in the combined injury groups could be due to some CA1 cell loss within the first 24 h. However, the ischemia literature suggests that reductions in ATP levels due to cell loss are most likely to occur at 48 h or later post-injury. In a gerbil model of transient ischemia Katayama et al. (1997) reported that ATP levels in the hippocampus were decreased acutely, but recovered to pre-ischemic levels by 1 day following reperfusion. These authors also observed a delayed, secondary reduction of ATP levels in the hippocampus at 3 days after reperfusion. Other studies in ischemia models have also reported delayed reductions of ATP levels, occurring between 2 to 4 days following reperfusion (Arai et al., 1986; Mies et al., 1990). These delayed reductions in ATP after ischemia, rather than the early reductions, are likely due to frank cell loss at the later time points after injury. In this study, we have only analyzed CA1 to focus on the relationships between regional ATP level and cell viability, because the FPI+SI model is known to induce damage in this area. However, it is possible that other brain regions (e.g. cortex or other hippocampal regions) would show either similar or different patterns for changes in ATP levels which might affect cell viability after FPI and SI. Further study is required to answer this question.

3.3. Mechanisms Contributing to ATP Depression and CA1 Damage Following TBI and SI

The current findings of SI-induced CA1 neuronal death after mild-moderate LFPI or CFPI replicate and expand upon the earlier report of Jenkins et al. (1989) where the combination of mild CFPI and ischemia, but not CFPI or ischemia alone, resulted in cell death in the CA1 region of the hippocampus. The mechanisms by which these combined injuries increase neuronal injury are speculative, but our findings suggest that more prolonged decreases in ATP

may result in delayed neuronal death after FPI+SI, which is compatible with several other mechanisms proposed.

Some investigations of secondary insult effects have focused on CBF alterations (Dietrich et al., 1998; Giri et al., 2000; Ishige et al., 1987; Matsushita et al., 2001) and, while TBI can impair the ability to regulate CBF (Lewelt et al., 1980), this is not always the case in rodent models of FPI (Bedell et al., 2004). When autoregulation is lost after an initial injury, a further decrease in CBF by secondary insult might lead to prolonged ATP depression that could lead to cell damage. In the present study we attempted to minimize the influence of CBF alternations by utilizing both mild-moderate models of FPI and a profound but fairly brief period of SI (Bedell et al., 2004; Davis et al., 1998; DeWitt et al., 1995). However, although mild-moderate FPI induces only mild and transient reductions of CBF (Yamakami and McIntosh, 1989; Yuan et al., 1988), vascular autoregulation may have been impaired at the 1 h post-FPI time point when we administered SI. All rats in our study showed respiratory alkalosis which may also have altered vasoreactivity and possibly CBF. The fact that the severity of the primary injury in our LFPI+SI group was correlated with CA1 cell loss at 7 days suggests there may be corresponding FPI severity effects on impairment of autoregulation that are “unmasked” when an equal intensity of SI is given.

Other possible mechanisms for prolonged ATP depression after combined FPI+SI are the ionic dyshomeostasis and metabolic alterations which increase energy demand. In the acute phase (<1 h) after TBI there is a transient increase in extracellular level of excitatory amino acids (EAA), an indiscriminate efflux of potassium, and accumulation of calcium (Faden et al., 1989; Fineman et al., 1993; Katayama et al., 1990; Osteen et al., 2001). The release and subsequent binding of EAA to receptors (primarily the N-methyl-D-aspartate receptor) are responsible for the pronounced increase in cerebral glucose metabolism in the early period after FPI (Kawamata et al., 1992; Yoshino et al., 1991). More prolonged increases in extracellular EAA concentrations are reported when experimental TBI is combined with hypoxia (Geeraerts et al., 2008; Matsushita et al., 2000). Recent clinical investigations also reported that a secondary ischemic event was strongly associated with high extracellular glutamate levels (Bullock et al., 1998; Vespa et al., 1998). Accordingly, it is likely that the ionic and metabolic disturbances contributed by the SI are additive with those induced by initial injury. This prolonging of ionic dyshomeostasis and neuronal activation by SI could thus decrease ATP production due to the ongoing increase in energy demands.

Another possible explanation for prolonged ATP depletion after SI is mitochondrial dysfunction. A TBI-induced increase in EAA leading to accumulation of calcium in mitochondria can induce mitochondrial permeability transition (Lifshitz et al., 2004; Sullivan et al., 2005). Loss of mitochondrial membrane potential can reduce ATP synthesis, increase reactive oxygen species production, and lead to the release of cytochrome c and pro-apoptotic proteins (Brustovetsky et al., 2002; Jiang et al., 2001; Lifshitz et al., 2004; Opii et al., 2007; Sullivan et al., 2005). Lifshitz et al. (2003) have clearly shown that mitochondria are morphologically damaged after experimental TBI, with functional damage also evidenced as decreased ATP production in both cortex and hippocampus for at least 3 h post-injury. The addition of SI after TBI likely increases mitochondrial injury, resulting in further or more prolonged depression of ATP levels. For example, Signoretti et al. (2001) reported that ATP, and N-Acetylaspartate which is also synthesized by mitochondria, were both depressed to a greater extent after TBI+SI (hypoxic hypotension, PaO₂ = 44.5 mmHg, MABP = 35.5 mmHg) than they were after TBI. Thus, mitochondrial dysfunction, leading to more severe or prolonged impairments of oxidative metabolism, may underlie the more enduring ATP reductions and delayed cell death following FPI+SI compared to FPI alone.

In summary, the mechanisms by which SI increases brain injury are no doubt multifactorial, but the current study indicates that the duration of ATP depression is a potentially important factor in the development of neuronal loss. ATP decreases are caused by the mismatch between ATP production and energy demands, and thus SI appears to place more prolonged energy demands on the injured brain and/or impair energy production after TBI. The present report shows that the injured brain carries an increased vulnerability to damage from SI. This vulnerability may include enhanced susceptibility, such as the loss of vascular reactivity, as well as increased sensitivity, such as a decrease in the ability of brain cells to withstand SI (DeWitt et al., 1995). The present report can not identify the precise form of vulnerability in our FPI+SI models, since we did not include additional measures of CBF, metabolism or the potential influence of excitotoxicity. However, the addition of a brief SI at 1 h after either LFPI or CFPI did prolong ATP depression in the CA1 region and this effect was correlated with subsequent cell death in this region. These findings suggest that, for TBI patients, even a brief period of SI during the acute post-injury period may have adverse effects on outcomes.

4. Experimental Procedure

4.1. Preliminary Experiments

In order to work out the injury parameters for this study, preliminary experiments were conducted to identify appropriate levels of FPI and SI where combined FPI+SI, but not FPI or SI alone, resulted in CA1 neuronal damage. FPI (1.6–2.0 atm; see section 4.3) was induced using parameters our laboratory has previously shown to induce no overt morphological damage in young adult rats (Fineman et al., 1993; Gorman et al., 1996; Katayama et al., 1990). The SI (see section 4.4) was initially studied using hemorrhagic hypotension with MABP reduced to 50 mm Hg for 6 or 8 min. With our experimental conditions this level of SI produced no evidence of CA1 damage, assessed using either Fluoro Jade B stain at 2 days or cresyl violet stain at 7 days, after FPI+SI or SI alone. We then used the same FPI parameters, but induced SI using hemorrhagic hypotension where the MABP was reduced to 35 mm Hg for 8 min. This level of SI produced no evidence of CA1 cell loss in sham injury controls but the ipsilateral CA1 in rats with LFPI+SI, and both the left and right CA1 in rats with CFPI+SI, showed evidence of neuronal cell loss by 7 days post-injury.

4.2. Subjects

All experimental procedures reported herein, and the care and use of experimental animals, were reviewed and approved by the University of California at Los Angeles Chancellor's Committee for Animal Research.

A total of 77 adult (250–350 g) male Sprague-Dawley rats were used for the initial experimental studies. Sixty-one animals were used to assess ATP levels in left and right CA1 regions of the hippocampus at 2, 6 or 24 h after surgery. These animals were assigned to groups with LFPI alone (n=4, 5 and 5, respectively, at 2, 6 and 24 h), LFPI+SI (n=5 per group), CFPI alone (n=4 per group) or CFPI+SI (n=4 per group). Eight non-operated rats (anesthesia only) served as controls (n=2, 3 and 3 per time point) for the ATP study. Sixteen rats were used to assess CA1 neuronal viability at 7 days after LFPI alone (n=4), LFPI+SI (n=4), CFPI alone (n=4) or CFPI+SI (n=4).

As is described in section 2.4, 4 rats with intentional induction of a milder LFPI were added to the initial LFPI+SI group surviving for 7 days (final n=8) to examine the relationship between severity of LFPI followed by a constant SI on the extent of CA1 damage.

4.3. Fluid Percussion Injury

After general anesthesia induction in a chamber with 4% isoflurane (in 100% oxygen, 1.5–2.0 L/min) anesthesia was maintained via nose-cone in spontaneously respiring animals using 1.5–2.0% isoflurane. Core body temperature was monitored continuously, and maintained between 37.0 and 38.0°C with a thermostatically-controlled heating pad. All surgical areas were shaved and disinfected. After securing the head in a stereotaxic frame, a midline skin incision was made to expose the pericranium. Under an operating microscope a single 3 mm diameter craniotomy was made, centered 3 mm posterior to bregma, using a high-speed drill. For LFPI the craniotomy was centered 6 mm lateral (left) of midline, and for CFPI the craniotomy was centered directly over the midline. The skull adjacent to the craniotomy was thoroughly dried, and a plastic injury cap was affixed over the craniotomy using silicone adhesive, cyanoacrylate and dental cement. The injury cap was filled with 0.9% saline after the dental cement was hardened. Anesthesia was discontinued and the animal was removed from the surgical frame and attached to the fluid percussion device. The FPI (1.6–2.0 atm fluid pulse into the epidural space over approximately 20 msec) was administered as soon as the animal exhibited a hind paw pedal reflex (elicited by toe pinch). The duration of apnea (resumption of spontaneous respiration) and LOC (return of the pedal reflex, assessed every 15 sec) were recorded. After return of the pedal reflex the animal was returned to a surgical level of anesthesia, the injury cap was removed and the scalp was sutured closed. The animals assigned to receive a SI underwent additional surgery for vascular catheter placements and SI induction, described below. The animals assigned to LFPI or CFPI only groups were maintained under anesthesia for 70–75 min after FPI. Those groups surviving for 2, 6 or 24 h had no further surgical procedures, but LFPI and CFPI groups surviving for 7 days had a catheter placed to enable arterial blood sampling (for blood gas assays at 50 and 75 min post-FPI). The control rats used for ATP assessments were anesthetized for the same duration as the injury groups (2 to 2.25 h), but no surgical procedures were administered. After the surgical and/or anesthesia procedures, animals were placed in a heated recovery cage until ambulatory, and then returned to their home cage.

4.4. Secondary Ischemia

Parameters of SI were chosen based on earlier work by Jenkins et al. (1989) investigating the effect of similar SI following TBI. Minor modifications were examined during preliminary experiments (section 4.1), using our anesthesia and experimental protocols, to establish a level of SI resulting in no CA1 cell loss in rats surviving for 7 days. Thus, in our study we used values in the contralateral (right) CA1 of LFPI+SI animals to evaluate effects of SI in relatively non-injured brain.

One femoral artery was cannulated with PE-50 tubing to enable continuous monitoring of MABP (Model BPA; Micro-Med, Inc., Louisville, KY). The other femoral artery and one femoral vein were catheterized with PE-50 tubing to enable blood sampling and infusion. After all catheters were in place, surgery was performed to expose both carotid arteries. Ten minutes prior to SI (50 min post-FPI) an arterial blood sample was taken and assayed for pH and blood gases (238 pH/Blood Gas Analyzer; Ciba Corning Diagnostics, Ltd., Halstead, UK). Additional arterial samples for pH and blood gas analyses were taken 4 min after starting and 10 min after completion of the SI. A brief (8 min duration) period of forebrain ischemia was induced 1 h after LFPI or CFPI by applying arterial clips to both carotid arteries. Simultaneous hemorrhagic hypotension was induced by withdrawing arterial blood into heparinized syringes to maintain MABP between 35–40 mm Hg over the 8 min period. To terminate SI the arterial clips were removed and all withdrawn blood was returned via the venous catheter to restore MABP to baseline values. The neck incision was then sutured closed. Following the final blood sample collection all catheters were removed, the femoral vessels were cauterized, and the femoral

incision sites were sutured closed. Animals were placed in a heated recovery cage until ambulatory, and then returned to their home cage.

4.5. ATP Measurements

At their assigned survival time-point each animal was rapidly anesthetized in an anesthesia chamber, placed in a restraining tube, and sacrificed by a focused microwave beam (1.8 kW for 5–7 sec depending on body weight) to the head (Model No. 4104; Thermex Thermatron, Inc., Louisville, KY). The brain was removed, frozen in 2-methylbutane and dry ice, and frozen (–20 °C) coronal sections (20 µm thick) were cut in a cryostat. Sections were thaw mounted onto frozen, subbed slides and quickly refrozen and stored in a –70 °C freezer.

ATP images were obtained using a modification of the luciferin-luciferase method of Kogure and Alonso (1978). To enable bioluminescence imaging, a 20 µm section of frozen normalizing standard (described below) was placed on each slide adjacent to a frozen tissue section. A 100 µm section of frozen enzyme buffer (described below) was layered over the frozen tissue and standard sections and the slide was quickly placed inside a Fluor-S Max MultiImager (Bio-Rad Laboratories, Inc., Hercules, CA). Slide images were digitally captured and integrated for 30 min. An ATP standard curve was calculated by graphing the optical density versus ATP concentrations in the standard blocks, normalized to the readings for the enzyme block used on the same slide (thus accounting for potential differences in activity across enzyme blocks). The CA1 regions in digital images of tissue sections (between –3.3 and –3.8 mm from bregma) were identified using templates from the atlas of Paxinos and Watson (1986). Optical densities within these CA1 regions were obtained and tissue ATP concentrations were calculated based on the ATP standard curve.

Standards—The ATP standards were made using denatured fresh brain homogenates. Fresh brain tissue was incubated at room temperature for 30 minutes to remove endogenous ATP and then denatured in a laboratory grade microwave. The tissue was weighed and homogenized with ATP (0.5–4 µmol/g) dissolved in 5% sterile gelatin. The brain paste was packed into an embedding capsule and frozen at –70 °C. The frozen standards were freed from the capsule, mounted onto labeled cryostat chucks and stored at –70 °C until use. Ten standard curves were generated throughout the study and the mean value for each standard was used to generate a standard curve ($R^2=0.97$). One of the standard blocks served as an internal standard for the brain tissue samples. A 20 µm section of the internal standard was included on each sample slide.

Enzyme block—All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The enzyme block was made in 0.2M/0.1M HEPES-Arsenate buffer. The buffer was heated to 50 °C and 1% polyvinylpyrrolidone, 2% gelatin and 1% glycerol were added. The solution was cooled to 30 °C and then 220 mg of powdered firefly tails were added to 10 mL of the buffer. This mixture was centrifuged at 13,000 rpm for 2 min and 20 µL of 1M MgCl₂ was mixed in. The final solution was poured into small plastic molds and frozen in a –70 °C freezer.

Analysis—The normalizing standard was imaged with each tissue sample slide to account for enzyme activity variability. If the normalizing standard values were over 20% from the standard curve, the case was repeated with another set of brain sections. Six sets of tissue sections with acceptable normalizing standard values were used to calculate the mean ATP value and reported as µmol/g wet tissue weight.

4.6. Quantitative Assessment of Anatomical Damage

Seven days after surgery, 20 animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and euthanized by transcardial perfusion with 0.1 M phosphate buffered saline

(PBS, pH 7.4) followed by a 4% paraformaldehyde in PBS. Brains were post-fixed in the same paraformaldehyde solution for 2 h and then placed in a solution of 30% sucrose in PBS overnight. Frozen brains were sectioned coronally (40 μ m), mounted on gelatinized slides, and stained in cresyl violet. Surviving neuronal cells in the dorsal CA1 region of the hippocampus were counted at $\times 400$ magnification at two anatomically standardized levels (−3.3 and −3.8 mm from bregma). Cells with a clearly intact membrane and visible nucleus were identified for counting. Cell numbers in the CA1 sector of the dorsal hippocampus were systematically randomly sampled using a two-dimensional counting grid modified from Stereo Investigator (Version 3.0; MicroBrightField, Inc., Colchester, VT). A line was drawn centered on the middle of the pyramidal cell layer. The computer randomly placed the first counting frame (20 $\times$ 20 μ m) on the line drawn, and subsequent frames were systematically placed every 30 μ m.

4.7. Data Analysis

Data for all parameters measured were expressed as group means $\pm$ standard deviation. Group data was analyzed using analysis of variance, with repeated measures when appropriate, followed by post-hoc contrasts or pairwise comparisons (with Bonferroni adjustment for multiple comparisons). The ATP data for all 3 control groups (2, 6 and 24 h) were combined to have a more robust dataset to use for between group comparisons against injury groups at each time point. The relationship between LFPI severity and hippocampal cell loss at 7 days after LFPI+SI was determined using the Pearson's correlation statistic. Statistical significance was set at $p < 0.05$ for all comparisons. Statistical analyses were conducted using SYSTAT 9 software (SPSS, Inc., Chicago, IL).

Abbreviations

ATP, adenosine triphosphate
 CBF, cerebral blood flow
 CCI, cortical contusion injury
 CFPI, central fluid percussion injury
 EAA, excitatory amino acids
 FPI, fluid percussion injury
 LFPI, lateral fluid percussion injury
 LOC, loss of consciousness
 MABP, mean arterial blood pressure
 PBS, phosphate buffered saline
 SI, secondary ischemia
 TBI, traumatic brain injury

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Fig. 1.

Mean $\pm$ standard deviation of adenosine triphosphate (ATP) levels in the left CA1 region of the hippocampus as a function of injury condition and time after lateral (LFPI) or central fluid percussion injury (CFPI) alone or combined with secondary ischemia (SI). ATP levels were significantly decreased only at 2 h after LFPI or CFPI alone, whereas addition of SI after LFPI or CFPI decreased ATP levels at 2, 6 and 24 h compared to controls.

*: $p < 0.05$ vs. controls, **: $p < 0.01$ vs. controls.

Fig. 2.

Mean $\pm$ standard deviation of adenosine triphosphate (ATP) levels in the right CA1 region of the hippocampus as a function of injury condition and time after lateral (LFPI) or central fluid percussion injury (CFPI) alone or combined with secondary ischemia (SI). LFPI alone and LFPI+SI did not affect ATP levels in the contralateral CA1. CFPI alone significantly decreased ATP levels in the right CA1 at 2 h, but not at 6 or 24 h. The combination of CFPI+SI prolonged the duration of the significant ATP depression to at least 24 h.

*: $p < 0.05$ vs. controls, **: $p < 0.01$ vs. controls.

Fig. 3.

Mean $\pm$ standard deviation number of surviving CA1 neurons in left and right hippocampus 7 days after lateral (LFPI) or central fluid percussion injury (CFPI) alone or combined with secondary ischemia (SI). CA1 neuronal counts did not differ in the left or right hemisphere of LFPI or CFPI alone groups. Significant loss of CA1 neurons occurred in the left, but not right, hippocampus in the LFPI+SI group and bilaterally in the CFPI+SI group.

***: $p < 0.001$ vs. contralateral (right) CA1 of LFPI group.

Fig. 4.

Photomicrographs of cresyl violet stained sections illustrating the extent of neuronal cell loss in left and right CA1 regions 7 days after injury. Higher power photos to the left and right of each row were taken in CA1 regions indicated by open arrows in the center, low power photomicrographs. (A) No CA1 cell loss was observed after secondary ischemia (SI) alone (from preliminary studies). (B) When bilateral SI was induced after lateral fluid percussion injury, cell loss was only observed in the CA1 ipsilateral to the primary injury (area between filled arrows). (C) Bilateral CA1 cell loss (areas between arrow heads) occurred after central fluid percussion injury combined with SI.

Scale bar = 250 μ m.

Table 1

Mean $\pm$ standard deviation (seconds) for loss of consciousness and apnea (in parentheses) after lateral (LFPI) or central fluid percussion injury (CFPI) alone or in combination with secondary ischemia (SI). (n = 4–5 per group).

	LFPI	LFPI+SI	CFPI	CFPI+SI
2 h	216.8 $\pm$ 17.4 (10.0 $\pm$ 2.9)	215.2 $\pm$ 53.0 (10.6 $\pm$ 4.8)	221.0 $\pm$ 46.6 (15.5 $\pm$ 3.5)	223.8 $\pm$ 51.0 (17.3 $\pm$ 15.4)
6 h	212.4 $\pm$ 45.2 (13.0 $\pm$ 8.9)	181.6 $\pm$ 38.5 (13.0 $\pm$ 4.6)	216.3 $\pm$ 20.4 (18.8 $\pm$ 4.3)	203.3 $\pm$ 72.8 (17.0 $\pm$ 8.9)
24 h	216.4 $\pm$ 30.3 (10.4 $\pm$ 5.0)	212.4 $\pm$ 35.0 (18.4 $\pm$ 8.0)	256.5 $\pm$ 21.1 (23.8 $\pm$ 15.1)	232.5 $\pm$ 61.1 (22.3 $\pm$ 10.3)
7 days	223.8 $\pm$ 25.2 (15.0 $\pm$ 5.4)	200.5 $\pm$ 42.1 (10.5 $\pm$ 5.3)	231.3 $\pm$ 36.5 (15.3 $\pm$ 7.1)	222.0 $\pm$ 34.9 (10.3 $\pm$ 2.2)

Table 2
Mean $\pm$ standard deviation for pH, PaCO₂ (mmHg) and PaO₂ (mmHg) measures taken pre-, during, and post-secondary ischemia (SI) in groups with lateral (LFPI) or central fluid percussion injury (CFPI).

	LFPI+SI (n=19)			CFPI+SI (n=16)		
	Pre	During	Post	Pre	During	Post
pH	7.30 $\pm$ 0.05	7.44 $\pm$ 0.05***	7.30 $\pm$ 0.04	7.33 $\pm$ 0.03	7.43 $\pm$ 0.05***	7.31 $\pm$ 0.03
PaCO ₂	44.8 $\pm$ 6.8	26.9 $\pm$ 4.7***	44.0 $\pm$ 5.8	44.9 $\pm$ 5.4	27.3 $\pm$ 4.3***	42.1 $\pm$ 5.7
PaO ₂	422.1 $\pm$ 45.0	423.6 $\pm$ 85.3	475.9 $\pm$ 52.6	451.8 $\pm$ 51.3	447.6 $\pm$ 69.3	466.8 $\pm$ 77.4

*** p<0.001 compared to pre- or post-SI.