A SELECTIVE DEPOLARISATION-INDUCED INCREASE IN EXCITATORY AMINO ACID NEUROTRANSMITTER RELEASE IN RAT MEDIAL PREFRONTAL CORTEX USING A MICRODIALYSIS MODEL OF TRAUMATIC BRAIN INJURY

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Abstract. This study describes a microdialysis model that investigates the biochemical response of the brain to non-fatal impact trauma. A controlled cortical impact (mild and severe) was performed to the left medial prefrontal cortex (mPfc) in the isoflurane-anaesthesised rat. This was followed by intracerebral microdialysis whereby a microdialysis probe was implanted into the site of the injury. Changes in dialysate glutamate, aspartate and GABA levels were investigated immediately (*i.e.* 25 min) and 265 min following a local mild and severe impact to the brain. In addition, the effect of local perfusion with a depolarizing concentration of KCl (100 mM, 20 min) was also investigated 165 min after impact.

Dialysate levels measured 25 min after impact (n=14) showed an impact-dependent increase in glutamate (6 and 8-fold), aspartate (4 and 5-fold) and GABA (3 and 6-fold) following mild and severe impact respectively compared to non-impact controls. Dialysate levels measured 265 min after mild (n=12) and severe (n=13) impact had stabilized and continued to show a local 5-fold (mild) and 4-fold (severe) increase in local glutamate, a 6-fold (mild) and 3-fold (severe) increase in aspartate and a (3-fold (mild) and 5-fold (severe)) increase in GABA levels compared to control. Intra-mPfc KCl (n=14) increased local dialysate glutamate levels (4-fold following mild impact and 3-fold following severe impact) and aspartate levels (2-fold after both mild and

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severe impact) while GABA levels did not differ from non-impacted controls following either a mild or severe impact.

The present findings show that microdialysis in intact brain can be combined with the controlled cortical impact model to reveal selective impact-dependent and prolonged increases in local dialysate amino acid neurotransmitter levels. Furthermore, we reveal that 165 min following either a mild or severe impact to the left mPfc KCl-stimulated glutamate and aspartate release is abnormally increased while GABA release is not different compared to non-impacted controls. This finding may in part explain the excitotoxicity that contributes to diffuse posttraumatic lesions associated with secondary injury.

Key words: glutamate, GABA, KCl, HPLC, rat, basal, stimulated levels.

1. INTRODUCTION

Traumatic brain injury is the main cause of death in children and young adults living in the industrialized world. Current estimates suggest that in the U.S. between 2.5 million and 6.5 million individuals are living with the consequences of traumatic brain injury, much of it caused by motor vehicle accidents. Studies in human subjects have shown that brain trauma induces an immediate and profound increase in local extracellular concentrations of excitatory amino acids such as glutamate. However, major discrepancies have been noted with respect to the magnitude of the posttraumatic event. Baker et al. (1993) have reported that in humans, glutamate concentrations are elevated up to 28-times the normal CSF concentration up to 3 days post-injury whereas Palmer et al. (1994) have reported a 2 to 8-fold increase in CSF aspartate, glutamate and glycine up to four days post injury. This excess glutamate is believed to initiate a chain of events at pathological concentrations. An excitotoxic cascade results in critical concentrations of calcium in the cell and is one of the most important factors in the premature death of neurons. In man, the majority of brain trauma is directed towards the forehead – the medial prefrontal cortex (mPfc) in the rat. Here the main excitatory amino acid neurotransmitters glutamate and aspartate are derived from the dendrites and axon collateral terminals of corticofugal outputs (Carr and Sesack, 2000) while the inhibitory amino acid neurotransmitter GABA is derived from a separate population of local interneurons (Hendry et al., 1987).

Animal models of brain trauma have generated data that has helped gain an insight into the events that occur during and after injury. In particular, a number of microdialysis models of traumatic brain injury in rats have shown increased dialysate concentrations of the excitatory amino acid

neurotransmitters following brain injury and these increases are generally related to the severity of injury. One of the most popular animal models of traumatic brain injury is the controlled cortical impact model (Dixon et al., 1991). This model utilises a pneumatic impactor that delivers an injury (generally through a craniotomy) to the intact dura. The head of the rat is restrained during the delivery of the impact. The controlled cortical impact model was chosen for investigation in the present study as the depth of penetration and velocity of impact can be accurately quantified and modified according to the experiment (Dixon et al., 1991) and for its ability to be combined with microdialysis in intact rat brain as reported by Tayag et al. (1996); Rose et al. (2002); Dixon et al. (1991); Stover et al. (2000) and Krishnappa et al. (1999).

The present study describes a model of brain injury that combines controlled cortical impact (Dixon et al., 1991, Gilchrist, 2004) with brain microdialysis (O'Connor, 2001). Towards this aim, we investigated the biochemical response of discrete sets of neurons within the left mPfc of an isoflurane anasethetised rat to non-fatal mild or severe impact.

2. MATERIALS AND METHODS

2.1 Animals

A total of 39 adult male Sprague Dawley rats (250-350 g) (Harlan Laboratories, United Kingdom) were used in this study. Prior to the experiment the animals were kept under regular lighting conditions (12 hr light/dark cycle) were given food and water ad libitum. Experimental protocols for ethical experiments on laboratory animals were approved by the local committee (license number B100/3366).

2.2 Surgery

On the day of the experiment rats are anaesthetised with isoflurane (4% - 2% in air delivered at 400 ml/min) in a vented anaesthesia chamber. Animals are placed in a stereotaxic frame (David Kopf Inc.) and the head adjusted until the skull between the bregma and lambda is level. An incisor bar hook and blunt ear bars are used to stabilise the rat's head. Anaesthesia is maintained throughout the experiment using a mask fitted over the nose of the animal, which allows the rat to breathe free. Body temperature is continuously monitored and maintained at 37.5 ºC using a temperature controlled heating pad (CMA 150 Carnegie Medicin, Sweden). Using sterile techniques, a

sagittal incision of the scalp is made along the midline from the level of the eyes to the occipital protuberance so that the frontal bones are exposed. A craniotomy 4 mm in diameter is drilled through the skull above the mPfc and the dura above it is exposed.

3. CONTROLLED CORTICAL IMPACT

Cortical contusion injury is performed at the left mPfc using a pneumatically driven vertical impactor (Figure 1). The device consists of a pneumatic cylinder mounted on an adjustable crossbar, which is positioned above the left mPfc to provide an impact by a 3.5 mm rounded impactor tip. Air pressure is set at 4 bar. The depth of penetration is determined by zeroing the piston to the cortical surface, withdrawing it and then lowering it to the required impact deformation. Animals are injured directly to the brain through a 4 mm craniotomy to the intact dura. To characterise the injury response, one of two injury levels is produced; mild (0.87 mm deformation) or severe (2.62 mm deformation). The velocity of impact (1.2 m/s) is verified using the storage oscilloscope.

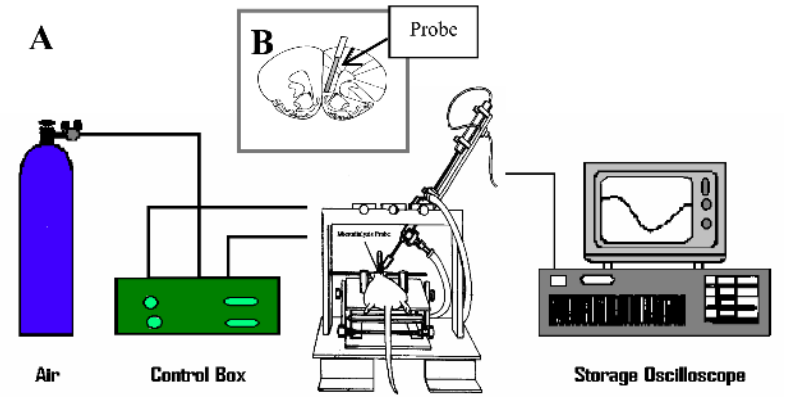
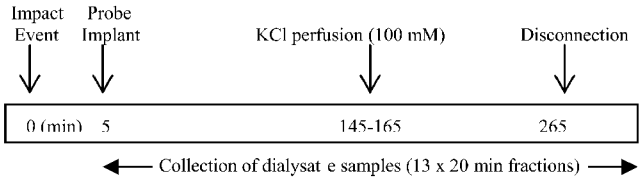


Figure 1. (**A**) Schematic representation of the experimental set-up employed in the present study. Pressurised air is linked to the pneumatic piston loaded on the controlled cortical impact rig with isoflurane-anaesthetized rat in situ (adapted from Cherian et al., 1994). An impactor tip is attached to the lower piston rod. Insert (**B**) Transverse section through the rat brain showing implantation of a 4 mm microdialysis probe in the left medial prefrontal cortex (mPfc). The left mPfc is the site of impact, local KCl perfusion and collection of dialysate samples.

A. Mild or Severe Impact



B. Non-Impacted Control

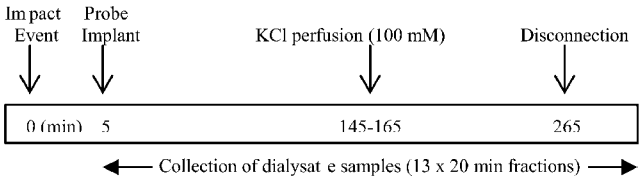


Figure 2. Schematic illustration of the experimental procedure employed in the present study. **A**. A mild or severe impact is applied to the left medial prefrontal cortex (mPfc). Within 5 min of injury a microdialysis probe is inserted into the mPfc and 13 x 20 min dialysate samples are collected for HPLC analysis. **B**. Animals in the control group are subjected to identical surgery and microdialysis probe implantation procedures except for the omission of a brain injury.

3.1 Microdialysis

As shown in Figure 2 immediately following impact to the left mPfc the piston is removed from the rig and a microdialysis probe (4 mm long, outer diameter 0.5 mm, CMA/12; CMA Microdialysis, Stockholm, Sweden) is carefully implanted into the left mPfc (co-ordinates (mm) from bregma: AP + 2.7, ML + 1.4, DV – 6.5 from bone; incisor bar: - 3.3). Samples are collected every 20 min, 265 min (4.3 hrs) post-injury with the samples stored on ice at –70°C until analysis by high pressure liquid chromatography (HPLC). The microdialysis probe is perfused with sterile Ringer solution (Baxter, UK; composition (mmol/L): Na^+ 147; K^+ 4; Ca^{2+} 2; Cl^- 156, pH 6) at a rate of 2 µl/min for 20 min prior to implantation. Dialysate sample collection is adjusted to allow for dead space in the probe and exit tubing. The flow rate of the perfusion medium through the probe is maintained at a constant flow by a microperfusion pump (CMA 100, Carnegie Medicin AB, Sweden) during implantation and for the duration of the microdialysis experiment.

3.2 Experimental Design

Rats were randomly divided into three groups namely those subjected to a (1) mild (n=12) or (2) severe impact (n=13) and (3) a control group (n=14). Animals in the control group underwent identical surgery and microdialysis probe implantation procedures except for the omission of a brain injury.

3.3 Neurotransmitter Analysis; HPLC

Each perfusate sample (40 μl) is divided into two aliquots with 10 μl used for glutamate/aspartate, 10 μl for GABA and 20 μl for back-ups. The dialysate samples are placed in separate refrigerated microsampler/injectors (CMA 200/240; Carnegie Medicin, Sweden) and are automatically injected onto high pressure liquid chromatography columns (HPLC) for detection of glutamate/aspartate and GABA.

3.4 Glutamate and Aspartate Analysis

Glutamate is determined by precolumn derivatisation with o-phthaldialdehyde/ mercaptoethanol reagent followed by separation by reverse phase HPLC and fluorimetric detection. A CMA 280 fluorescence detector (Carnegie Medicin, Sweden) with excitation wavelength set at 315 nm and emission cut off filter set at 395 nm is used. The limit of detection is 0.5 pmol/sample.

3.5 GABA Analysis

The GABA assay is based on precolumn derivatisation with *o*-phthaldialdehyde/ *t*-butylthiol reagent and separated by reverse phase HPLC on a Nucleosil 3,C18 column electrochemical detector (BAS LC-4C; Bioanalytical Systems, West Lafayette, IN, USA) under isocratic conditions. The limit of detection is 20 fmol/ sample.

3.6 Verification of Probe Placement

At the completion of the experiment the animals are euthanased with an overdose of isoflurane, the brain removed and placed in a 4% formaldehyde solution. Probe placement is verified by cryostat sectioning (10 μm) on a Leitz freezing microtome (Wetzlar, Germany) followed by microscopic examination to qualitatively confirm the morphologic extent of injury. Rats displaying improper probe implantation are omitted from the study.

3.7 Statistical Analysis

Data is calculated as the mean ± SEM. Investigated variables are compared for significant differences using one-way analysis of variance (ANOVA).

4. RESULTS

4.1 Impact Velocity

There was no difference in the mean impact velocity for mild and severe impact to the mPfc as verified using a storage oscilloscope. Thus the mean impact velocity (mean ± SEM) for mild impact was 1.13 ± 0.01 m/s (n=4) and for severe impact was 1.06 ± 0.01 m/s (n=6).

4.2 Dialysate Amino Acid Levels

Dialysate glutamate, aspartate and GABA levels in non-impacted control rats, decreased gradually over the duration of the experiment. Glutamate levels decreased by 75% and stabilised at a concentration of 0.50 ± 0.18 μM 120 min following probe implantation. Aspartate levels decreased by 76% and stabilised at a concentration of 0.16 ± 0.03 μM 60 min following probe implantation, while GABA levels decreased by 85% and stabilised at a concentration of 33.6 ± 10.5 nM 80 min following probe implantation.

Table 1. Local dialysate glutamate, aspartate and GABA levels measured in the left medial prefrontal cortex (mPfc) 25 min and 265 min after mild or severe impact. Non-impacted animals acted as controls. Each data point represents the mean + SEM raw dialysate level from 7-14 animals. (* = $p < 0.05$ v's Control, * * = $p < 0.01$ v's Control, * * * = $p < 0.001$ v's Control).

Group	Glutamate (μM)	Aspartate (μM)	GABA (nM)	Glutamate (μM)	Aspartate (μM)	GABA (nM)
	25 min after impact			265 min after impact		
Control	2.04 ± 0.58	0.71 ± 0.22	222.12 ± 60.37	0.19 ± 0.04	0.07 ± 0.02	11.19 ± 2.05
Mild	12.49 ± 2.72 * *	2.9 ± 0.54 * *	607.71 ± 176.7 *	1.03 ± 0.16	0.42 ± 0.08 * *	30.69 ± 12.69 * * *
Severe	16.88 ± 1.86 * * *	3.60 ± 0.42 * * *	1189.77 ± 131.61 * * *	0.7 ± 0.11 * *	0.22 ± 0.04 *	54.36 ± 8.89 * * *

4.3 Impact Levels

As shown in Table 1 a local impact-dependent increase in dialysate glutamate (6 (mild) and 8-fold (severe)), aspartate (4 and 5-fold) and GABA (3 and 6-fold) levels was observed 25 min after impact compared to non-impacted controls. This increase was still present 245-265 min after impact, glutamate (5 (mild) and 4-fold (severe)), aspartate (6 and 3-fold) and GABA (3 and 5-fold).

4.4 Stimulated Levels

As shown in Figure 3 local perfusion with KCl (100 mM, 20 min) increased dialysate glutamate (4-fold (mild) to 3-fold (severe) and aspartate levels (2-fold following both mild and severe impact). In contrast, GABA levels did not statistically differ from non-impacted controls following either a mild or severe impact.

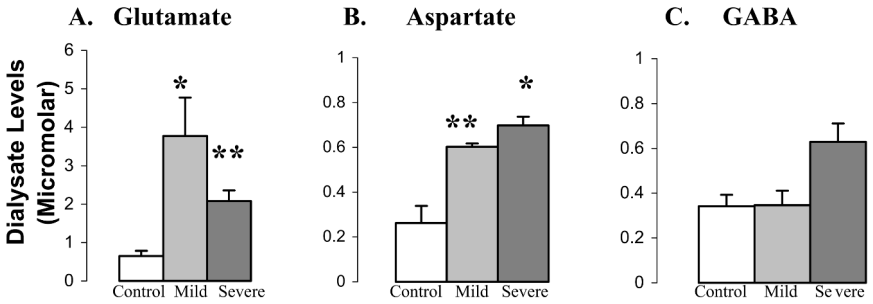


Figure 3. KCl (100 mM, 20 min) stimulated dialysate A. glutamate, B. aspartate and C. GABA levels measured 145-165 min following mild and severe impact to the left medial prefrontal cortex (mPfc). Non-impacted animals acted as controls. Each data point represents the mean ± SEM raw dialysate level from 6-13 animals. For glutamate (μM); control 0.65 ± 0.13; mild 3.77 ± 0.9; severe 2.08 ± 0.27. Aspartate (μM); control 0.26 ± 0.07; mild 0.6 ± 0.01 severe 0.7 ± 0.04. GABA (nM); control 340.84 ± 51.05; mild 346.31 ± 65.97; severe 628.31 ± 83.72. * = $p < 0.05$; * * = $p < 0.01$ (v's non-impacted control).

5. DISCUSSION

Head injury is a spontaneous, unpredictable event and while no single animal model is entirely successful in reproducing the complete spectrum of pathological changes observed after traumatic brain injury the controlled cortical impact model is superior to devices that are driven by gravity or a free falling guided weight because deformation parameters can be controlled

with pneumatically driven devices (time, velocity, and depth of impact). In addition, the rigid impact model can be employed to mimic the whole spectrum of focal-type damage including contusions with haemorrhages and/or subdural haematoma formation. Furthermore, when combined with microdialysis the quantifiable nature of the single mechanical input used to produce the injury allows correlations to be made between the amount of deformation and the resultant pathology and functional changes (Lighthall et al., 1989).

Therapeutic innovations in the field of brain injury rely on advances in the understanding of the underlying pathophysiological cascade that causes neuronal damage. Indeed, the pathogenesis of traumatic brain injury is incompletely understood, in large part because patients often present with a variety of lesions of varying severity and distribution. However, it is widely accepted that there are two main stages in the development of brain damage after injury to the head; primary (mechanical) and secondary (delayed non-mechanical). The direct, primary event may be due to traumatic brain injury – blunt or penetrating, a neoplasm or a cerebrovascular accident – ischaemic stroke or haemorrhage and it is generally thought to be an irreversible event that warrants preventative measures. The concept of secondary injury was initially proposed at the end of the 19th century and was proven to be of significance in the 1960s and 1970s. It is produced by complicating processes that are initiated at the moment of injury, although the symptoms do not present clinically for a period of hours to days after injury. Ischaemia, axonal injury, focal haematomas, contusions and oedema all contribute to the progressive loss of brain cells involved in the process of delayed damage to the brain tissue. This delayed pathophysiological cascade is now believed to result from a combination of pathological factors including monumental rises in neurotransmitter release, notably the excitatory amino acid glutamate which causes excitotoxicity.

The findings in the present study show that immediately following mild and severe unilateral injury to the mPfc a local impact-dependent increase in dialysate glutamate (6 (mild) and 8-fold (severe)), aspartate (4 and 5-fold) and GABA (3 and 6-fold) levels is observed compared to non-impacted controls and this increase in still present 265 min after impact. Thus, we report an acute and prolonged increase in extracellular excitatory and inhibitory amino acid neurotransmitter levels in the mPfc after impact. Nilsson et al. (1990) combined microdialysis with the weight-drop model of traumatic brain injury in the rat and reported an increase in dialysate glutamate (8-fold (mild -1.5 mm deformation), 13-fold (severe - 2.5 mm deformation)) and aspartate levels (6-fold (mild), 17-fold (severe)) following injury to the parietal cortex. Maximal levels were observed in the first 10 min after impact and returned to stable basal levels within 20-30 min after

injury. Using a separate model Faden et al, (1989) combined fluid percussion injury to the left parietal cortex of the rat with microdialysis in the hippocampus and reported an injury-related increase in dialysate glutamate – (3-fold (mild) to 9 fold (severe)) and aspartate (3-fold (mild) to 18-fold (severe)) compared to controls. Maximal levels were observed in the first 10 min after trauma with elevations sustained for more than 60 min in the severely injured group. However, injury related increases are not solely confined to excitatory amino acid neurotransmitters. GABA levels were reported to rise from a non-detectable basal level to 0.6–1.2 μM following injury to the parietal cortex in the rat (Nilsson et al., 1990). In humans, Palmer et al. (1994) reported CSF GABA levels to be 56 to 317-fold higher following injury compared to controls. Taken together, these findings strengthen the evidence that the extracellular levels of both excitatory and inhibitory amino acid neurotransmitters are increased following injury.

The mechanisms by which excitatory amino acids contribute to secondary tissue injury remain speculative, although increases in intracellular Na^+ and Ca^{2+} have been implicated in this delayed injury process (Choi, 1987). The release of glutamate from the contusion core is believed to be related to primary disruption of the cell membrane or vascular wall by the physical force of the impact, resulting in leakage from the metabolic pool in the cytosol or the blood stream (Maeda et al., 1998). In contrast, areas peripheral to the contusion may have a presynaptic mechanism of glutamate release, which consists in part, of a Ca^{2+}-dependent exocytotic release from depolarized nerve terminals. The glutamate released in the contusion core may diffuse towards a peripheral direction and act on the postsynaptic receptors, causing neuronal depolarization. Such a diffusion-reaction process would induce additional release of glutamate from the depolarised nerve terminals, inducing a cascade of excitotoxicity. The present findings of an abnormally high KCl-induced increase in both dialysate glutamate (4-fold (mild) and 3-fold (severe)) and aspartate (2-fold (mild and severe)) but not GABA levels after impact compared to non-impacted controls supports this hypothesis and may reflect the fact that glutamate and aspartate axon terminals are only lightly myelinated compared to the more heavily myelinated GABA interneuron. Thus, excessive depolarisation induced increase in excitatory amino acid release may underlie the excitotoxic component that actively destroys populations of neurons and contributes to diffuse posttraumatic lesions associated with the secondary injury linked to brain injury and may at least in part explain why glutamate receptor antagonists alone fail to successfully improve neurological outcome in severely brain injured patients in the clinical setting (Laurer and McIntosh, 2001). Furthermore, on the basis of the present findings we suggest that in some clinical head trauma situations surgical removal of contused brain

tissue to prevent excessive depolarisation induced release may serve to protect the surrounding brain areas from glutamate-induced cytotoxicity.

In conclusion, we show that the controlled cortical impact model can be successfully combined with microdialysis in intact rat brain to reveal a selective impact-dependent and prolonged increase in local excitatory and inhibitory amino acid neurotransmitter levels. Furthermore, this microdialysis model differentiates the responsiveness of excitatory and inhibitory neuronal pools within the mPfc to reveal a selective increase in depolarisation-induced excitatory amino acid release following injury which may be useful for the investigation of new therapeutic strategies and pharmacological testing for an effective treatment for patients with head-injury.

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